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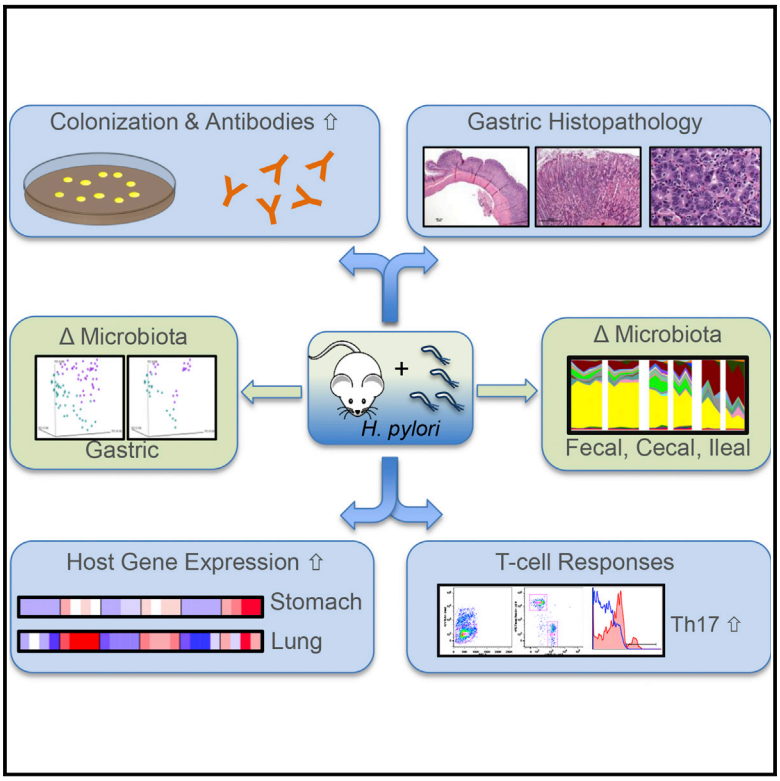
Gastric *Helicobacter pylori* Infection Affects Local and Distant Microbial Populations and Host Responses

Graphical Abstract



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In Brief

Kienesberger et al. utilize a mouse model to study *H. pylori* infections over 6 months. They report that *H. pylori* significantly affects the population structure of the gastric and intestinal microbiota. The infection alters gastric immune and inflammatory responses and causes distant effects via altered hormones and immunity.

Highlights

- *H. pylori* stably colonizes the mouse stomach over 6 months
- Infected hosts recognize *H. pylori* antigens
- *H. pylori* alters the population structure of the gastric and intestinal microbiota
- *H. pylori* influences both gastric and pulmonary inflammatory gene expression

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Gastric *Helicobacter pylori* Infection Affects Local and Distant Microbial Populations and Host Responses

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SUMMARY

Helicobacter pylori **is a late-in-life human pathogen with potential early-life benefits. Although *H. pylori* is disappearing from the human population, little is known about the influence of *H. pylori* on the host's microbiota and immunity. Studying the interactions of *H. pylori* with murine hosts over 6 months, we found stable colonization accompanied by gastric histologic and antibody responses. Analysis of gastric and pulmonary tissues revealed increased expression of multiple immune response genes, conserved across mice and over time in the stomach and more transiently in the lungs. Moreover, *H. pylori* infection led to significantly different population structures in both the gastric and intestinal microbiota. These studies indicate that *H. pylori* influences the microbiota and host immune responses not only locally in the stomach, but distantly as well, affecting important target organs.**

INTRODUCTION

Starting at birth, humans are extensively exposed to a wide variety of microbial cells, which colonize the inner and outer surfaces of our body and subsequently shape our immune system and physiology (Dominguez-Bello et al., 2010; Hooper et al., 2012). The gastro-intestinal tract is one of the most densely populated sites in the human body and accordingly plays a major role in the development of the immune system (Goodnow et al., 2005; Kabat et al., 2014). Later in life, disruption of this microbial community may lead to disease consequences (Buffie et al., 2015; Högenauer et al., 2006); conversely, restoration of impacted microbial community could be an efficient approach for their reduction (Reid et al., 2011; van Nood et al., 2013).

Despite its acidic environment, the human stomach is home to a diverse microbial community (Bik et al., 2006; Maldonado-Contreras et al., 2011). Depending on the gastric milieu, the composition and functions of the microbial community can vary (Manson et al., 2008; Martinsen et al., 2005). A prominent member of the gastric microbiota is *Helicobacter pylori*; in colonized humans, its relative abundance usually is high but can vary (Bik et al., 2006). *H. pylori* is typically acquired early in life (Goodman et al., 1996; Kumagai et al., 1998), often transmitted from mothers (Pérez-Pérez et al., 2003; Thomas et al., 1999), and persistently colonizes the gastric mucosa (Oertli et al., 2013). Unless antibiotic therapy is used, colonization generally persists for decades or throughout the entire host lifespan (Kosunen et al., 1997). *H. pylori* carriage is associated with illnesses, such as peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue (MALT) lymphoma (Ernst and Gold, 2000). For decades, *H. pylori* research has focused on understanding the pathogenesis of these diseases (Amieva et al., 2003; Mahdavi et al., 2002; Odenbreit et al., 2000; Robinson et al., 2008).

H. pylori prevalence in populations in developed countries is diminishing due to changing hygienic standards and antibiotic treatments. Despite the significance of *H. pylori* as a pathogen, concerns have been raised about the consequences of the loss of an organism that has colonized humans for >100,000 years (Moodley et al., 2012). Inverse associations of *H. pylori* with diseases, including early-onset asthma (Arnold et al., 2011a; Chen and Blaser, 2007, 2008), gastro-intestinal (Cohen et al., 2012; Higgins et al., 2011; Rothenbacher et al., 2000) and systemic (Perry et al., 2010) infections, and Barrett esophagus and its consequences (Corley et al., 2008; Rubenstein et al., 2014; Thrift et al., 2012) have been observed. In mice, the age at which *H. pylori* is acquired is critical in influencing outcomes; challenged adult mice developed pre-neoplastic lesions, but neonatal mice were protected against severe pathology by immune tolerance (Arnold et al., 2011b). The fact that this tolerance subsequently protected against the development of asthma (Arnold et al., 2011a) highlights the importance of cross-talk between particular bacterial colonizers and the host's immune system. During the first years of life, the development and maturation of the mammalian immune system is strongly

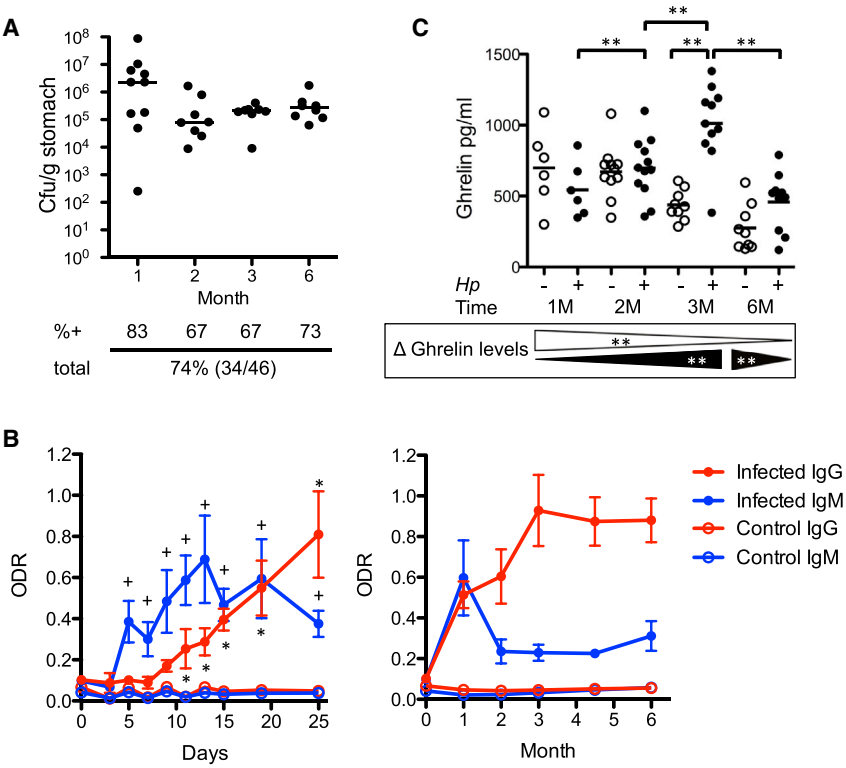

Figure 1. Bacterial Colonization and Host Antibody and Ghrelin Responses of C57Bl/6 Mice Infected with *H. pylori* Strain PMSS1

(A) Colony-forming units (Cfus) per gram stomach in animals sacrificed at the indicated times post-infection. Bars indicate the medians; %+ indicates percent of mice positive for *H. pylori,* as determined by culture for each time point of sacrifice; and total percentage and number of (positive/total number) of mice are shown in bottom line.

(B) Antibody responses. Early (first 25 days following challenge) and long-term (over 6 months) anti-*H. pylori* IgM and IgG responses of control and infected mice expressed in optical density ratios (ODRs). (+) or (*) indicates when IgM or IgG levels, respectively, were significantly higher compared to baseline; Mann-Whitney U test; +,*$p < 0.05$;

(C) Ghrelin responses. Levels of total plasma ghrelin measured over the 6-month challenge (horizontal bars represent mean values). Bottom strip: Δ Ghrelin represents changes of ghrelin over time for controls (open triangles) and infected mice (black triangles); Kruskal-Wallace followed by Mann-Whitney U test; **$p < 0.001$.

See also Figure S1.

dependent on the micro-organisms to which it is exposed, and simultaneously, the immune system shapes the composition of our residential bacterial community (Hooper and Macpherson, 2010); the two phenomena are cross-linked.

Studies to explore the potential effects of *H. pylori* using the C57BL/6 mouse model and the functional CagA *H. pylori* strain PMSS1 highlight the importance of the developmental stage of the mouse at time of infection (Arnold et al., 2011a, 2011b; Oertli and Müller, 2012; Oertli et al., 2012, 2013). However, the most-relevant time windows are not yet known, nor are the direct or indirect influences of *H. pylori* on gastric and gut microbial communities and immunity. The goal of the current study was to understand the effect of gastric *H. pylori* colonization on local and systemic immune responses and whether it affects the composition of the gastro-intestinal microbiota.

RESULTS

C57Bl/6 Mice Were Stably Colonized with *H. pylori* PMSS1

We first examined whether experimental challenge with *H. pylori* strain PMSS1 would lead to productive infection resulting in stable colonization in cohort 1 (infected at 4 weeks of age) and cohort 2 (age 6 weeks) mice (Figure S1). Culture and PCR analyses of the two cohorts detected *H. pylori* infection in 44 of 46 challenged mice but in none of 40 non-challenged control mice, as expected. During the first month following challenge, the levels of colonization varied extensively (range 10^2 to 10^8 colony-forming units [CFUs] per gram stomach) but then stabilized (Figure 1A). In most challenged mice, *H. pylori* were detected by culture and confirmed by PCR or by PCR alone (Figures 1A and S1). The only two mice that were both culture and PCR negative had antibody responses to *H. pylori* and high gastric histologic inflammatory scores, with characteristic organisms visualized. Taken together, all 48 *H. pylori*-challenged mice were successfully infected and control mice were all culture and PCR negative (Figure S1). Over the 6-month study period, all mice, regardless of age at challenge, were stably *H. pylori* infected (median colonization 10^5–10^6 CFUs/g of stomach; Figure 1A), corresponding to ~10^5 CFUs per mouse stomach.

Challenged Mice Displayed High Levels of Antibodies against *H. pylori* Whole-Cell Lysates, but Not to CagA

Examining IgM and IgG responses to *H. pylori* (Figure 1B), control mice showed no responses, but the challenged mice had IgM responses detected as early as day 5. IgM levels declined by 1 month post-infection but remained significantly elevated compared to baseline and to controls. IgG levels rose significantly 13 days post-infection compared to the controls and remained elevated throughout the experiment (Figure 1B), with cohorts 1 and 2 essentially identical; however, no antibodies against CagA were detected at any time point in any mouse (data not shown). Nevertheless, these studies provide evidence of robust immunologic recognition of *H. pylori* beginning soon after challenge, independent of mouse age.

Ghrelin Was Elevated in *H. pylori*-Infected Mice

Because gastric *H. pylori* colonization in humans affects circulating metabolic hormones (Francois et al., 2011; Yap et al., 2015), we examined fasting ghrelin, leptin, insulin, and peptide

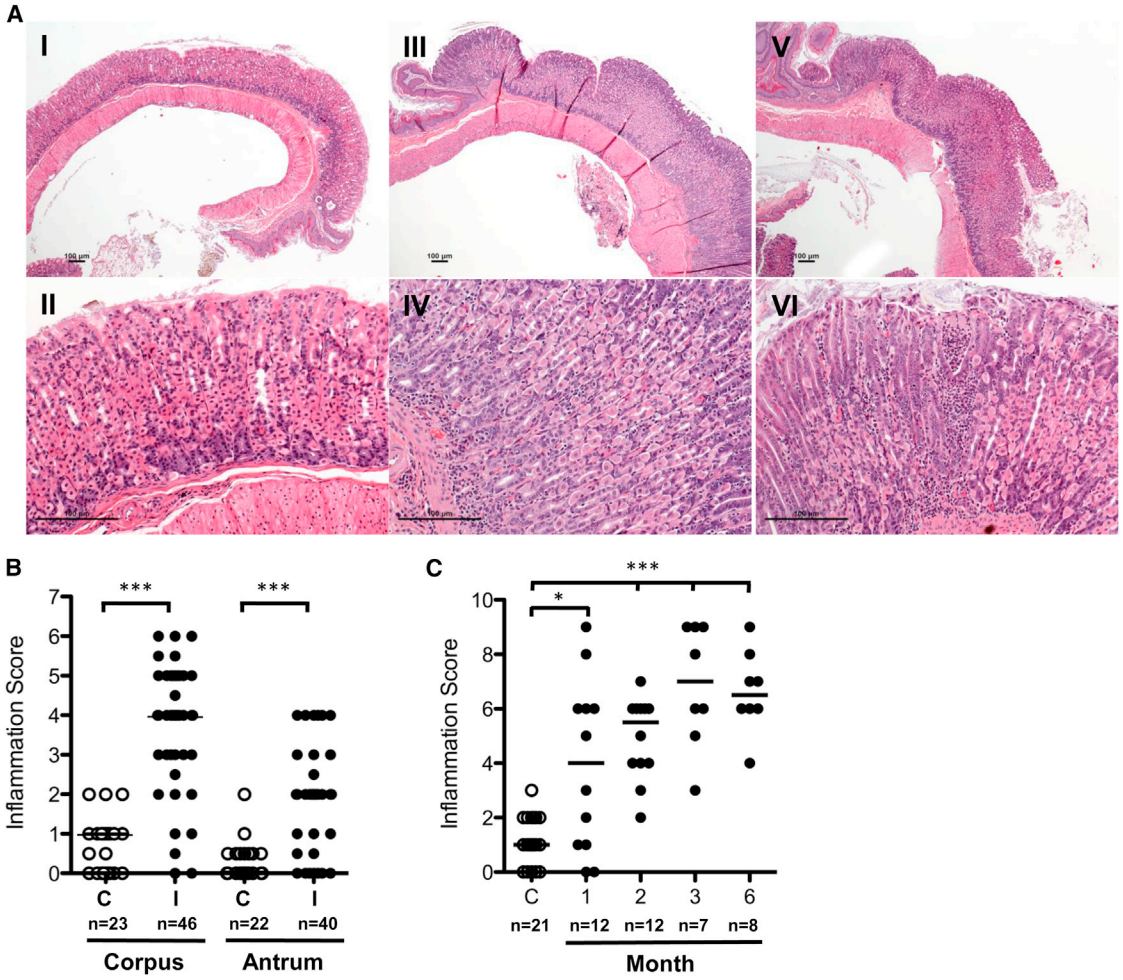

Figure 2. Histopathology and Gastric Inflammation Scores of Control and *H. pylori*-Infected C57Bl/6 Mice

(A) Histology. (I and II) Control mice with no inflammation in the glandular stomach are shown. (III and IV) Low- to medium-grade inflammation with abundant lymphocytes and sparse neutrophilic granulocytes in an infected mouse are shown; representative photomicrographs were obtained from a mouse sacrificed 6 months post-infection. (V and VI) Severe chronic active inflammation with abundant lymphocytes and neutrophilic granulocytes in an infected mouse is shown (3 months post-infection). Upper row (I, III, and V): 40× magnification; lower row (II, IV, and VI): 200× magnification. The scale bars represent 100 μm.

(B) Inflammation scores for antrum and corpus. C, control; I, infected. Active and chronic inflammation scores are combined.

(C) Total inflammation scores of control and *H. pylori*-infected mice by month. Active and chronic inflammation of corpus and antrum are combined. n, number of mice per group in which total inflammation score was determined. Bars indicate the medians.

Mann-Whitney U test; $^{**}p < 0.02$; $^{***}p < 0.0001$. See also Figure S2.

YY in plasma at sacrifice (Figure 1C). Ghrelin levels in control mice dropped significantly with age. In contrast, ghrelin levels of infected mice progressively rose until month 3 post-infection and diminished thereafter, with the same trend in both cohorts. No significant differences between the control and infected mice were detected for the other hormones tested (data not shown), indicating specific effects for ghrelin, the major metabolic hormone produced in the stomach.

Gastric Histopathology

To determine whether this experimental infection elicited specific tissue responses, we evaluated gastric pathology in all animals at sacrifice. In general, the control mice displayed no inflammation versus notable inflammatory changes in the infected mice, without significant differences between both cohorts (Figures 2A and S2). Compared to controls (Figures 2A, panels I and II, and S2A), tissues from infected mice showed gastric corpus infiltration with inflammatory cells and to a lesser extent in the antrum (Figures 2A, panels III–VI, S2B, and S2C). The inflammatory infiltrate, consisting of lymphocytes, plasma cells, and neutrophils in the lamina propria, was arranged around the gastric glands, extending in severe cases to the submucosa (Figures 2A, panels III–VI, and S2C) with abundant neutrophils in the gastric pits (Figure 2A, panel VI), with inflammation sometimes pronounced adjacent to the esophageal-gastric junction (Figure S2D). In other cases, lymphoid follicles were visible (Figure S2E) in the gastric antrum (Figure S2F). Higher magnification showed a mixed inflammatory infiltrate involving gastric glands, mirroring human

Table 1. Number of Immune Genes with Significant Changes in Expression

	Stomach			Lung		
	1 Month	3 Months	6 Months	1 Month	3 Months	6 Months
Total	109	158	317	29	10	0
Up	88	134	282	24	5	0
Down	21	24	35	5	5	0
Shared (months)	1 and 3	3 and 6	1, 3, and 6	1 and 3	3 and 6	1, 3, and 6
Total	64	142	64	3	0	0
Up	57	130	57	1	0	0
Down	7	12	7	2	0	0

Number of the 547 genes in the Nanostring (nCounter) Mouse Immunology Panel that were significantly differentially expressed, based on *H. pylori* colonization status; FDR-adjusted p value < 0.5; t test. Up and down refers to increased or decreased expression in tissue from mice colonized with *H. pylori* compared to controls, respectively. Shared represents genes with significant differential expression in the same direction at months 1 and 3, 3 and 6, or months 1, 3, and 6. See also Figure S4.

type B gastritis (Figures S2G and S2H). Rod-shaped bacteria, typical for *H. pylori,* were visible in several colonized animals (Figure S2I). Histopathological validation, according to the Sidney classification (Mainguet et al., 1993), showed that, as expected, scores were low in controls (Figures 2B and 2C), and infected mice had higher scores in both corpus and antrum (Figure 2B), trending toward more severe tissue injury at 3 and 6 months (Figure 2C).

H. pylori Modulates Immune Gene Expression in a Tissue-Specific Manner

To assess the extent to which *H. pylori* influences immune gene expression of local and distant tissues, we examined gastric and pulmonary genes related to host immune and inflammatory responses in mice infected at 4 weeks (cohort 1) with respect to uninfected mice. Unsupervised hierarchical cluster analysis revealed that the major separation of samples was based on tissue location, as expected (Figure S3), and that the secondary branch points generally separated samples based on *H. pylori* status, with stronger effects in the stomach than in the lung. *H. pylori* gastric infection led to more upregulation of immune response genes than downregulation (Table 1). The differences in gene expression in the stomach between infected and control mice progressively increased over the course of the infection, and several genes were consistently altered (Figures 3A and 3B; Data S1).

Genes altered in both the stomach and lung included those upregulated in both tissues and those regulated in opposing directions (Figures 3D–3F and S3B). Using ingenuity pathways analysis, upregulated pathways, including those for T cell activation (NFAT, iCOS, and TREM1) and pro-inflammatory molecules (nitric oxide [iNOS]), were more frequent than downregulated canonical pathways in the gastric samples from the *H. pylori*-infected mice, with alteration intensity generally increasing over time (Figure 3C). By 6 months post-challenge, 317 (58%) of the 547 investigated genes were significantly upregulated (Table 1), with >10% shared at all time points studied. Apoptosis pathways were significantly upregulated only at later time points. In the pulmonary samples, biological functions in response to *H. pylori* infection involved leukocyte development and migration, as well as T cell differentiation (Figure S3D). Altogether, the lung and stomach have distinct *H. pylori*-modulated immune expression profiles.

Pulmonary T Cell Infiltration in *H. pylori*-Infected Mice

To further assess systemic immunologic effects of the *H. pylori* infection, we examined splenic and pulmonary tissues by flow cytometric analysis of CD4+ cells and their RoRγT-, IL17a-positive (Th17), and FoxP3-positive (T-regulatory [T-reg]) subsets (Figures 4 and S4). Splenic cells from infected mice trended toward increased Th17 populations, but differences were not significant. However, in the pulmonary tissues, the *H. pylori*-infected mice showed higher levels of CD4+ RORγT-positive cells (Figure 4B) and CD4+ IL17-positive cells at least at month 1 after infection (Figure S4) compared to controls. CD4+ FoxP3-positive (T-reg) cells did not significantly differ (Figure 4C). These effects were more prominent in the cohort infected at a younger age (Figure S4). Although effects were modest and not completely consistent over time, differential presence of CD4+ RORγT-positive cells compared to controls might provide indication of non-local immunologic effects of gastric *H. pylori* infection, targeting the lungs.

H. pylori Influences the Gastric Microbial Community Structure

Assessing microbial diversity in the gastric samples, using the number of observed species and the Shannon index (Figure S5), there were no substantial changes over the experimental course or in relation to *H. pylori* status. Next, using UniFrac analysis, we addressed whether the community structure (β-diversity) of the two experimental cohorts varied (Figures 5 and S5); reflecting founding microbial population differences common to commercially obtained mice, the two control groups differed significantly (Figure 5A), and the control and treatment groups within each cohort were more similar to each other than to the corresponding group in the other cohort. Examining the cohorts individually, control and treatment groups separated as early as 1 month post-challenge (Figure 5B). For both cohorts, control and treatment samples moved monotonically over time and remained significantly ($p < 0.05$; ADONIS test) separated to the experiment's end (Figures 5B and S5). Thus, *H. pylori* infection clearly affected the gastric microbial population structure.

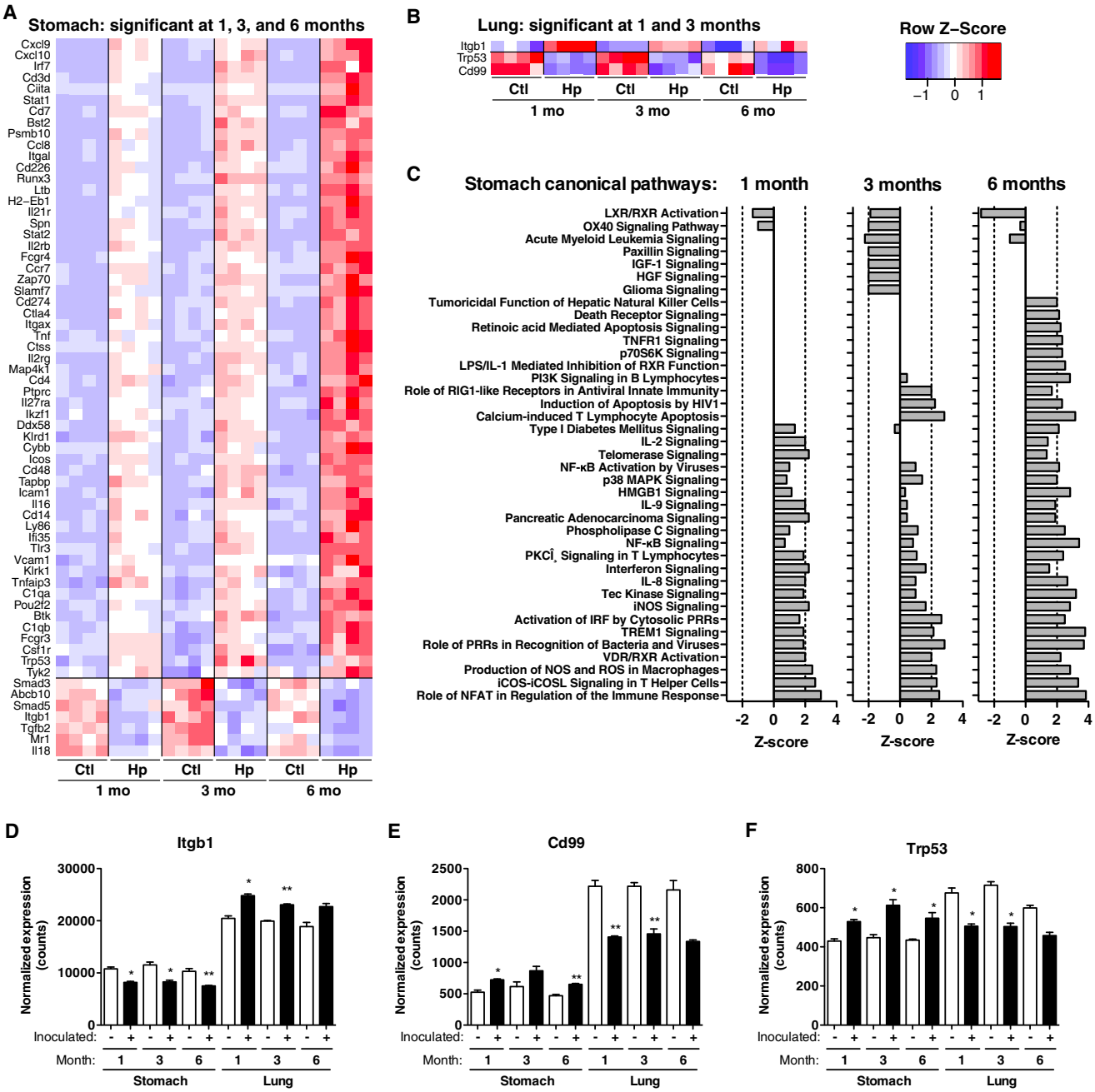

Figure 3. Immunologic Genes Consistently Altered by Gastric *H. pylori* Infection in Stomach and Lung

(A and B) Immunologic gene expression was measured by hybridization using Nanostring (nCounter) technology in (A) gastric and (B) pulmonary tissues at 1, 3, and 6 months after colonization in cohort 1 mice. Heatmaps show expression levels of genes significantly different (FDR-adjusted p value < 0.05; t test) at all three time points for the stomach and at two time points for the lung.

(C) Gastric canonical pathways, significantly induced or repressed by *H. pylori* infection.

(D–F) Relative expression levels (normalized counts) for genes significantly different in greater than or equal to two time points in each tissue. False-discovery-rate-adjusted p values: *p < 0.05; **p < 0.01; ***p < 0.001.

See also Figure S3.

Relative Microbial Abundances in the Mouse Stomach Are Influenced by the Presence of *H. pylori*

Although cohorts 1 and 2 were compositionally distinct, *H. pylori* presence had greater impact in the younger cohort (Figure S6A). In agreement with culture and PCR results, *H. pylori*-specific sequences were not detected in any of the control mice but were present in most of the infected mice over a range of three $\log_{10}$; relative abundance varied between 0.01% and 10%

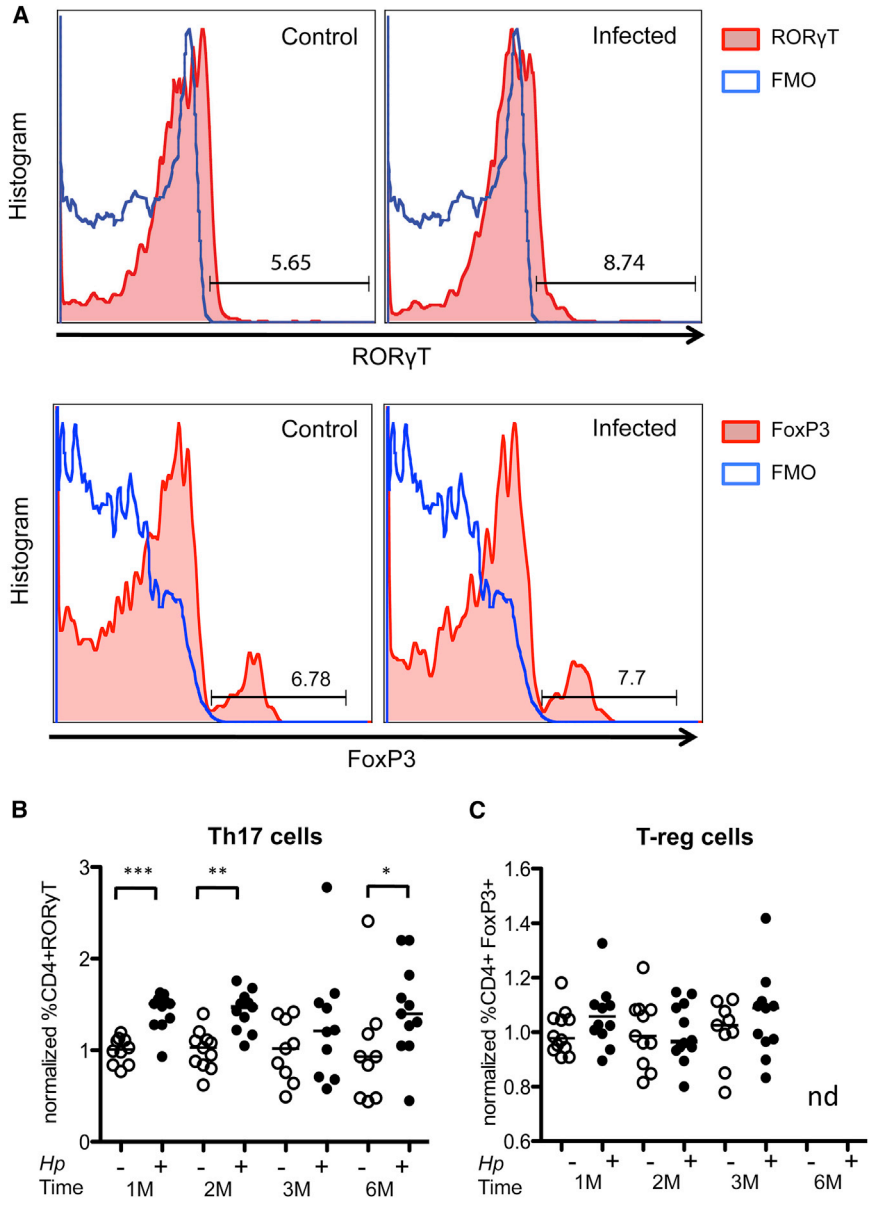

Figure 4. Increased Proportions of CD3+ CD4+ RORγT+ Cells in the Lungs of *H. pylori*-Infected Mice

(A) Gating strategy. Flow cytometry was performed on pulmonary specimens from control and *H. pylori*-infected mice. CD3, CD4, and CD8 surface stains and a viability stain were used to identify live T-helper cells. Nuclear staining for RORγT and FoxP3 was performed to identify Th17 and T-reg cells. A representative example of RORγT+ and FoxP3+ expression of CD4+ cells from control and infected mice is shown. Fluorescence minus one (FMO) is shown in blue in comparison to RORγT or FoxP3 shown in red.

(B and C) T cell proportions. Quantitation of the frequency of (B) CD4+ RORγT+ cells (Th17) and (C) CD4+ FoxP3+ cells (T-reg) is shown. Each mouse was normalized to the respective controls for each time point (average of controls for each month). Horizontal bars indicate group mean values. −, uninfected control; +, *H. pylori* infected; M, month. Statistical analysis was performed using the Mann-Whitney U test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

See also Figure S4.

(median 0.36%; Figure S6B) with levels similar in cohorts 1 and 2. Comparative analysis using the LEfSe algorithm (Segata et al., 2011) via the galaxy browser revealed numerous significant abundance differences from phylum to species level (Table S1) between control and infected mice. Taxa significantly and consistently affected by the *H. pylori* infection (greater than or equal to two time points in the same direction; Table S2) included six species belonging to five orders and six families (*Bacteroidaceae*, *Rikenellaceae*, *Lactobacillaceae*, *Lachnospiraceae*, *Erysipelotrichaceae*, and *RF39*; Table S3). To confirm our findings, we also examined taxa in a third cohort of experimentally infected mice (cohort 3), which were all confirmed to be *H. pylori* positive by culture and PCR (data not shown). In this experiment, the species and families identified as significantly different from control mice were those that also had been identified in cohorts 1 and 2 (Table S4). In summary, these studies clearly show that *H. pylori* has conserved effects on the mouse gastric microbiome.

H. pylori Influences Intestinal Microbial Community Structure

Next, we asked whether the presence of *H. pylori* in the stomach also affected downstream microbiota. Although alpha diversity increased in fecal samples as the mice aged, control and infected animals did not significantly differ (Figure S5). However, microbial community structure (β-diversity) significantly differed by intestinal locus, mouse cohort, and treatment (Figure 6A). Before *H. pylori* challenge (baseline), fecal samples from the subsequent control and infected mice form a single cluster. Over the course of the experiment, the fecal microbial populations in both cohorts shifted monotonically across PC1, but control and infected mice begin to significantly separate 3 months after infection ($p < 0.05$; ADONIS test; Figure 6B; Table S5) and the terminal cecal and ileal samples showed similar differences (Table S5). Thus, in mice, *H. pylori* challenge not only influences the population structure of the stomach but also more distally, with differences increasing over time.

Relative Microbial Abundance in the Mouse Gut Is Influenced by the Presence of *H. pylori* in the Stomach

H. pylori was not detected in the mouse feces by highly sensitive PCR (data not shown), nor did high-throughput sequencing detect *Helicobacter*-specific sequences in the fecal, ileal, and

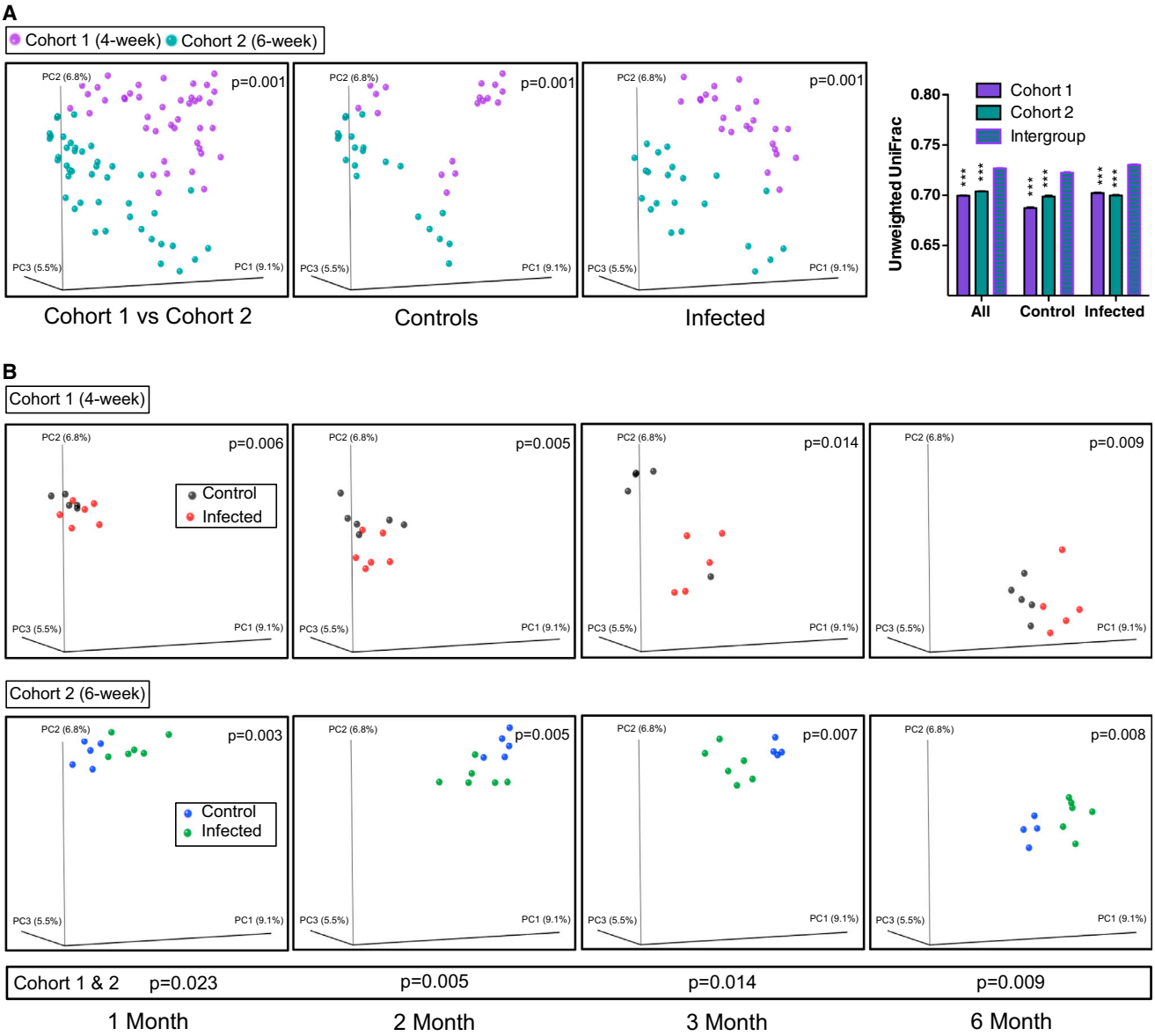

Figure 5. The Effect of *H. pylori* Colonization on Gastric Microbial Community Structure

Principal coordinates analysis (PCoA) of the unweighted UniFrac distances computed from 16S rRNA sequences from gastric samples at an even sampling (500) depth.

(A) Comparison of cohort 1 (infected at 4 weeks) and cohort 2 (infected at 6 weeks), combined and separated by control or infected status, respectively. Right panel shows average unweighted UniFrac distances (±SEM) comparing intragroup variation for the early or late cohort to the intergroup variation (early versus late) for all mice, control mice, and infected mice. *** indicates that intragroup distance is significantly different from the intergroup distance; $p < 0.005$; t test.

(B) Cohort 1 and cohort 2, viewed separately, over time (months 1, 2, 3, and 6). Each gastric sample is represented as a colored circle; p values (ADONIS test) are based on unweighted UniFrac distances Significance: p values < 0.05.

See also Figure S6 and Table S5.

cecal samples, indicating that *H. pylori* effects on the intestinal microbiota was indirect. Using the LEfSe algorithm, cohorts 1 and 2 were compositionally distinct (Figure S6B), but within each cohort, controls and infected mice differed (Table S6). We identified three species belonging to three families (*Bacteroidaceae*, *Turicibacteraceae*, and unclassified *Clostridiales*) that were persistently altered in the *H. pylori*-colonized mice (Tables S6 and S7); *Turicibacter sp* also was decreased in the infected mice from cohort 3. Analyzing ileal and cecal samples independently, we identified three persistently altered ileal *Firmicutes* species (*Lactobacillus* other, *Turicibacter*, and *Allobaculum* species) and one cecal *Firmicutes* species (*Turicibacter* species) and one *Tenericutes* species (*Anaeroplasma* species); in cohort 3 mice, features were similar (Table S4). In

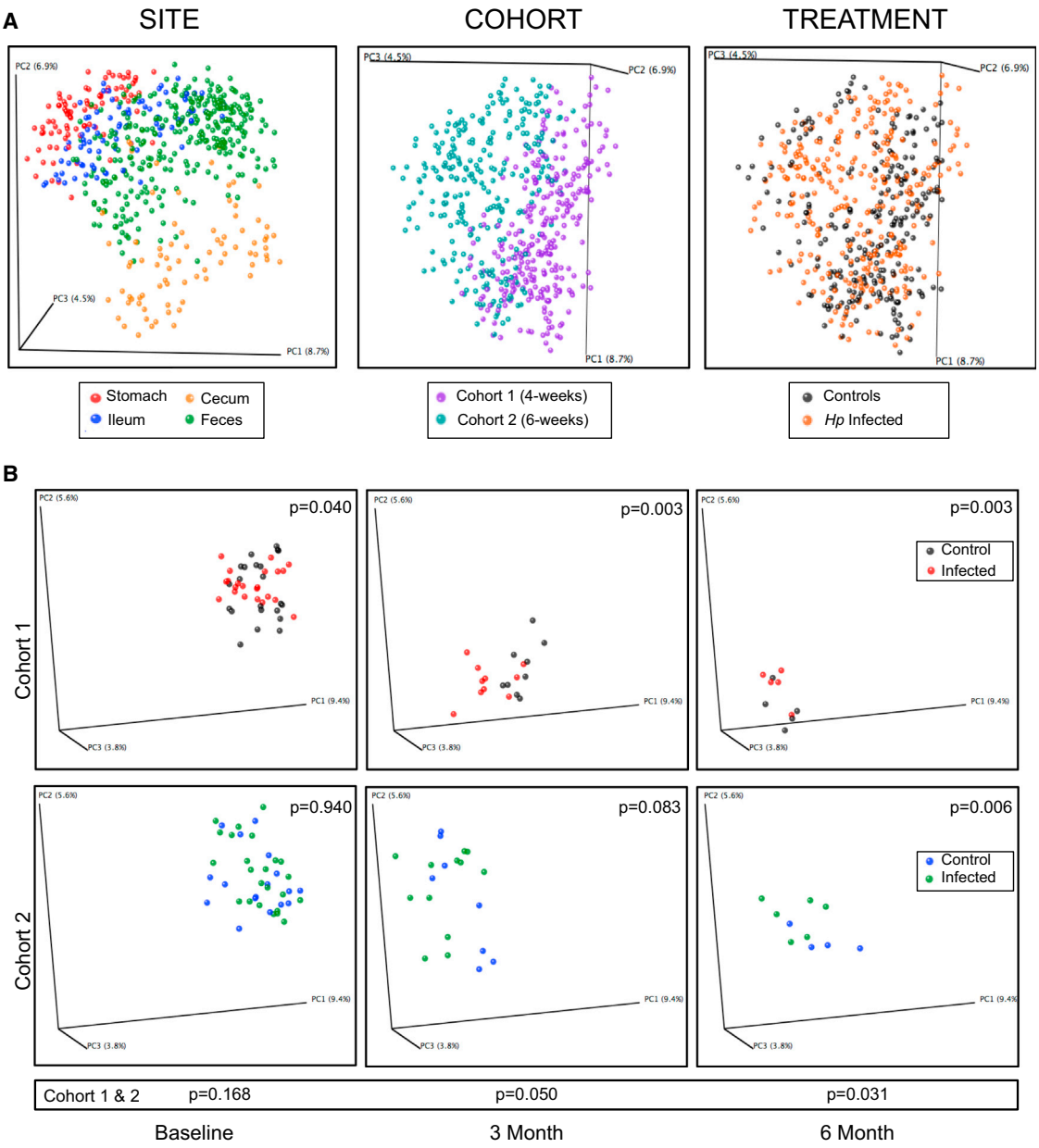

Figure 6. The Effect of *H. pylori* Colonization on Gastro-intestinal Microbial Community Structures

PCoA of the unweighted UniFrac distances computed from 16S rRNA sequences at even sampling depth. (A) All samples were grouped for site, age at *H. pylori* challenge (cohort), or treatment status and (B) fecal samples over time (baseline and months 3 and 6) of cohort 1 and cohort 2, respectively. Plots are organized as in the legend to Figure 5. See also Figure S6 and Table S5.

summary, gastric *H. pylori* infection and persistence affected intestinal microbial composition with conserved stable differences emerging.

DISCUSSION

Although much of the world's population is asymptomatically colonized with *H. pylori* (Blaser and Atherton, 2004), risk for peptic ulcer disease and for gastric adenocarcinoma and lymphoma is increased (Kusters et al., 2006). Disease outcomes depend on *H. pylori* strains, host genotype, and environmental factors (e.g., salt intake; Castaño-Rodríguez et al., 2014). *H. pylori* is both recognized by and directs host immune responses (Arnold et al., 2012; Lewis et al., 2011; Oertli et al., 2012; Robinson et al., 2007), which could have further effects on colonizing microbiota and host physiology. Both epidemiologic and experimental data provide evidence that *H. pylori* colonization early in life may protect against early-onset asthma (Arnold et al., 2011a, 2011b; Chen and Blaser, 2007, 2008) and infections (Perry et al., 2010; Rothenbacher et al., 2000), and

there is substantial clinical and epidemiologic evidence for a protective role in esophageal disease (Corley et al., 2008; Rubenstein et al., 2014; Thrift et al., 2012). Given the organism's complex relationship with human biology, both promoting and preventing disease, animal models focusing on host and microbiota responses to *H. pylori* are useful.

The CagA+ *H. pylori* strain PMSS1 (Arnold et al., 2011b), the pre-mouse parental strain of SS1 (Lee et al., 1997), colonizes mice efficiently and induces differential immune responses and disease outcomes, based on mouse age (Arnold et al., 2011a). Low-level gastric *H. pylori* load in neonatal mice may reflect antimicrobial functions of mother's milk (Minoura et al., 2005) but in any event appears to yield tolerogenic rather than immunogenic responses (Arnold et al., 2012). We excluded possible effects of nursing by challenging post-weaned (4-week-old) mice and sexually mature 6-week-old mice with *H. pylori*. For both cohorts, we observed similar infection levels with long-term stability and with anti-*H. pylori* IgM and IgG levels confirming continuing immunological recognition. As in other mouse experiments, we did not detect responses to CagA in any of the infected mice, despite the initial CagA+ functional status of PMSS1 (data not shown). It is intriguing to speculate that the lack of immunological responses to CagA may reflect cumulative effects, including low *cagA*-expression in the non-acidic mouse stomach (Karita et al., 1996) and/or functional loss in vivo (Arnold et al., 2011b; Barrozo et al., 2013), in which *cagY* variation eliminates CagA delivery to host cells (Barrozo et al., 2013).

Early in the experiment, infection levels varied substantially. Because the same strain was used, differences may have been stochastic or reflect host (and/or microbiota) characteristics; subsequent convergence to a narrower range may reflect conserved pressures and underscores the value of using inbred mice and a single diet. Histological evaluation of the mice, also confirming stable infection, showed corpus-dominant inflammation, mirroring typical human responses with mixed mononuclear and neutrophilic infiltrates around the gastric glands (Stolte and Meining, 2001). The intensity of the interaction also is indicated by the extent to which *H. pylori* influenced immune and inflammatory gastric gene expression (Table 1). Many of the genes upregulated only late play roles in apoptosis and oncogenesis, and the increased expression of most Toll-like receptors (TLR) reflects a re-programming of innate immunity in response to *H. pylori* persistence. Little is known about the interplay of *H. pylori* with TLR3 (Pachathundikandi et al., 2015), but TLR3 and LPS-recognizing CD14 were both upregulated throughout the study period. TLR3 recognizes dsRNA, but bacterial LPS also induces its expression (Pan et al., 2011). Dendritic cells, which play a crucial role in *H. pylori* recognition (Oertli and Müller, 2012; Oertli et al., 2012; Shiu and Blanchard, 2013), may modulate the evolving responses to *H. pylori* components.

As expected, in the absence of a local inducing agent, genes classically involved in pathogen recognition were not affected in the lung. Smad3 and Smad5 were downregulated in the stomach by *H. pylori* infection but were overexpressed at multiple time points in the lung. Expression of the SMAD proteins, influenced by cytokines of the transforming growth factor (TGF)-β family, play crucial roles in T-helper cell differentiation. Smad3 negatively regulates Th17 cell development through FoxP3 (Lu et al., 2010; Tone et al., 2008). In agreement with prior studies (Futagami et al., 2006; Lee et al., 2012; Lundgren et al., 2005), gastric T-reg and Th17 cell response-related genes (e.g., STAT3, Ccr2, and Tgfb) were differentially expressed in response to *H. pylori* (Data S1). However, we also observed modestly higher levels of Th17 cells and a trend toward more T-reg cells in pulmonary tissues of *H. pylori*-infected mice, with effects enhanced in the cohort infected at a younger age. These findings provide evidence that *H. pylori* gastric infection might influence populations of specific immune cells in peripheral tissues, consistent with prior studies (Arnold et al., 2011a). Throughout the experiment, we observed downregulation of IL18, important for T-reg cell development in neonatal mice (Oertli et al., 2012), which we speculate may affect later immune responses. In adult mice, *H. pylori* infection usually yields Th1/Th17-dominated responses (Shi et al., 2010) but also triggers T-reg cell development, important for immune tolerance and facilitating *H. pylori* persistence (Arnold et al., 2011b). Th17 and T-reg cells then also can migrate to and influence distant tissues (Lim et al., 2008), with responses affected by independent factors, including host age and genotype, and immune system development. Although it remains formally possible that during oral dosing the nasopharyngeal mucosa was damaged exposing immune cells to *H. pylori,* we doubt that such a scenario would occur in every mouse. We further speculate that only a chronic infection and not an acute injury can result in prolonged T cell activation lasting several months. The differences in Th17 responses between the infected and control groups were not uniform throughout the experiment, raising the question of whether this is related to the cohort age differences or biases in flow cytometry analyses. Alternatively, this phenomenon could be due to the complex immunological cross-regulation in the lung. Although only few genes in the lung were affected consistently over time, early in the infection, there were significant changes in genes, including Tgfbr1, IL4R, IL6R, and Smad3. Expression of these genes could potentially influence Th17 cell numbers or suppress IL17 production (see Data S1; Gagliani et al., 2015; Lu et al., 2010; Tone et al., 2008).

The stomach is the major site for production of ghrelin, centrally involved in metabolic homeostasis (De Vriese and Delporte, 2007; Kojima et al., 1999). The differential ghrelin physiology in control and *H. pylori*-infected mice that we observed, consistent with prior studies (Bercik et al., 2009), provides further evidence of the systemic effects of gastric *H. pylori.* The anti-inflammatory properties of ghrelin, inhibiting Th17 cell differentiation (Xu et al., 2015), might partially explain why, when levels are high (3 months), pulmonary Th17 populations are not significantly elevated. This hormonal interaction may provide *H. pylori* with additional means for evading host immune responses. Nevertheless, our findings confirm that *H. pylori* infection is a dynamic process, involving different cytokines, receptors, and signaling molecules over an on-going temporal dimension.

Despite the relatively low *H. pylori* abundance in mice compared to humans, physiology and immunity were substantially affected. Because gastro-intestinal tract gene expression is influenced by other micro-organisms, we asked whether *H. pylori* or the induced host response affects species

composition locally in the stomach or distally in the intestine. Although founder effects were important drivers of the variation in bacterial community composition (Figure 6A), similar shifts in the *H. pylori*-positive mice, across all three cohorts, indicate conserved effects. The observed early population structure shifts could reflect changes in niche physiology (e.g., altered gastric pH and ghrelin production), whereas later shifts might be driven by the cumulative and enhanced changes in the immune and inflammatory responses to *H. pylori* having systemic manifestations. Experimental approaches involving manipulation of ghrelin, gastric pH, or gastric immune responses, in isolation, could provide insight about which *H. pylori*-induced perturbation has the greatest effect on the microbiota. We identified taxa that were influenced by the presence of *H. pylori*. Although changes in the gastric microbiota were expected, we also identified taxa affected in the intestines in all three cohorts. These include members of the families *Turicibacteraceae*, *Erysipelotrichaceae*, and *Desulfobirionaceae*, species of which have also been linked to host immune effects (Bisson-Boutelliez et al., 2010; Devkota et al., 2012; Lukens et al., 2014; Presley et al., 2010). The potential link between changes in microbial composition due to the presence of *H. pylori* and the possible effects on allergic diseases requires further investigation (Fujimura and Lynch, 2015).

In conclusion, this mouse model provides evidence that gastric *H. pylori* infection affects local histologic, physiologic, immune, and microbiologic features and indicates systemic effects on the distal intestinal microbiota and in the lung. The gastric niche is not isolated, and future studies are needed to extend our understanding of *H. pylori* colonization of humans to optimize and individualize health strategies.

EXPERIMENTAL PROCEDURES

Animal Experiments

All animal experiments were performed according to an IACUC-approved protocol, with mice housed in specific pathogen-free conditions and maintained on a 12 hr light/dark cycle. Female C57BL/6 mice (Jackson Labs) were obtained right after weaning at 3 or at 5 weeks of age, adapted to the animal facility, and then orally infected at either 4 (cohort 1) or 6 weeks (cohorts 2 and 3) of age. Within each cohort, there was an infected and a control group. Mice were gavaged twice (2 days apart) either with 0.5 ml sterile Brucella broth or with *H. pylori* resuspended in Brucella broth (1×10^9 CFUs ml^{-1}). At months 1, 2, 3, and 6 (cohort 3 only at month 2) after infection, mice of each cohort were fasted overnight and humanely euthanized with CO_2 narcosis for specimen collection. An overview of the description of the mouse model is shown in Figure S1.

Strain Recovery and Histopathology

Following sacrifice, for each mouse (including controls), one longitudinal quarter of the stomach was collected for strain recovery and one for histopathology (see Supplemental Experimental Procedures).

Measurement of *H. pylori*-Specific Antibodies and CagA Antibodies in Mice by ELISA

Blood was obtained from living animals by sub-mandibular bleeding or at sacrifice, blood cells and serum were separated by centrifugation (300 *g* for 10 min at room temperature), and the serum was frozen. Anti-*H. pylori* immunoglobulin G (IgG) and M (IgM) levels in the mouse plasma were determined, using a modification of a method with high sensitivity and specificity for human *H. pylori* positivity, as described (Dubois et al., 1994). The protocol was adapted to mice by using mouse-specific IgG and IgM secondary antibodies (see Supplemental Experimental Procedures).

Isolation of Leukocytes from Lungs and Spleens

Spleens and lungs were dissected at sacrifice; for the lungs, the right lobe was minced with a razor blade and incubated in RPMI supplemented with 10% FCS and 0.5 mg/ml collagenase IA and incubated for 30 min at 37°C. Lungs and spleens were homogenized between frosted slides in RPMI and then passed through a 40-μm nylon mesh filter (BD Biosciences). Cells were pelleted at 300 *g* for 5 min and red blood cells lysed by addition of ACK (ammonium-chloride-potassium) lysis buffer (Life Technologies) for 7 min at room temperature, washed with RPMI, and re-suspended in Dulbecco PBS for staining.

Lymphocyte Staining and Analysis of Lymphocyte Subsets by Flow Cytometry

Cells were incubated with LIVE/DEAD Fixable Blue dead cell stain (Life Technologies) for 10 min at 4°C to identify live cells. Splenic and pulmonary leukocytes were identified using the following antibodies: CD3-APC-Cy7 (BD Biosciences); CD4-PE-Alexa Fluor 610 (Invitrogen); CD8-V500 (BD Biosciences); RORγt-PE (eBioscience); Foxp3-PE-Cy7 (eBioscience); and IL17a-Alexa Flour 700 (BioLegend). For staining of nuclear markers, cells were first incubated with surface antibodies (each at 1:50 in FACS buffer) along with FC block (anti-mouse CD16/CD32; eBioscience) at 1:200 for 30 min at 4°C, fixed and permeabilized with fixation/permeabilization buffer (eBioscience), and subsequently incubated with the nuclear antibodies in permeabilization buffer (eBioscience) for 30 min at 4°C. Prior to IL17 staining, isolated lymphocytes were cultured for 4 hr at 37°C in presence of PMA, ionomycin (Sigma), and GolgiPlug (BD Biosciences). All cells were acquired on a LSRII (Becton Dickinson) and analyzed using FlowJo software (Tree Star), with graphics and statistical tests performed using the GraphPad Prism software. To determine statistical significance between groups, we used the Mann-Whitney test. The applied gating strategy is shown in Figure S4. The proportion of CD4+RoRγt+, CD4+FoxP3+, and CD4+IL17a+ cells were expressed as percentages of total CD4+ cells. For each time point, the value observed in each mouse then was normalized using the mean value from control mice of each cohort and month, respectively, as denominator.

Nanostring and Hormone Measurements

At months 1, 3, and 6, immune gene expression of cohort 1 mice was measured by the nCounter GX Mouse Immunology Panel (NanoString). Hormones were measured in both cohorts at all time points using the Millipore Mouse Gut Hormone Panel (Millipore) using a Luminex 200 (Millipore) analyzer (see Supplemental Experimental Procedures).

DNA Library Preparation, Sequencing, and Sequence Analysis

DNA extraction was done using the Power Soil DNA isolation kit (Mo Bio Laboratories), the V4 region of the 16S rRNA gene was amplified, and barcoded fusion primers were attached through PCR, as described (Caporaso et al., 2012). Of the 563 samples, the average depth of coverage was 3,504 ± 3.449 sequences per sample. Six samples that had coverage below 500 reads were eliminated from the downstream analysis. Taxonomy was assigned using the open reference method in QIIME (Caporaso et al., 2010), with an RDP confidence interval of 50%, and the sequences clustered at 97% identity from the Green Genes 2013 May database release were used as a reference (McDonald et al., 2012; see Supplemental Experimental Procedures).

Statistical Analysis

Statistical analysis of colonization, antibody and ghrelin response, and histology scoring data was done with GraphPad Prism 5 using Mann-Whitney U tests or for multiple group comparisons and Kruskal-Wallace tests followed by Mann-Whitney U test.

ACCESSION NUMBERS

The accession number for the microbial 16S rRNA data reported in this paper is SRA: SRP063216.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, seven tables, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.017.

AUTHOR CONTRIBUTIONS

Conceptualization and Methodology, S.K. and M.J.B.; Investigation, S.K., G.I.P.-P., A.L., G.G., and X.-S.Z.; Formal Analysis, S.K., L.M.C., and J.C.; Writing – Original Draft, S.K., L.M.C., and M.J.B.; Writing – Review & Editing, S.K., G.G., E.L.Z., and M.J.B.; Funding Acquisition, M.J.B.; Resources, E.L.Z. and M.J.B; Supervision, M.J.B.

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