

Systems Modeling of the Role of Interleukin-21 in the Maintenance of Effector CD4⁺ T Cell Responses during Chronic *Helicobacter pylori* Infection

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ABSTRACT The development of gastritis during *Helicobacter pylori* infection is dependent on an activated adaptive immune response orchestrated by T helper (Th) cells. However, the relative contributions of the Th1 and Th17 subsets to gastritis and control of infection are still under investigation. To investigate the role of interleukin-21 (IL-21) in the gastric mucosa during *H. pylori* infection, we combined mathematical modeling of CD4⁺ T cell differentiation with *in vivo* mechanistic studies. We infected IL-21-deficient and wild-type mice with *H. pylori* strain SS1 and assessed colonization, gastric inflammation, cellular infiltration, and cytokine profiles. Chronically *H. pylori*-infected IL-21-deficient mice had higher *H. pylori* colonization, significantly less gastritis, and reduced expression of proinflammatory cytokines and chemokines compared to these parameters in infected wild-type littermates. These *in vivo* data were used to calibrate an *H. pylori* infection-dependent, CD4⁺ T cell-specific computational model, which then described the mechanism by which IL-21 activates the production of interferon gamma (IFN- γ) and IL-17 during chronic *H. pylori* infection. The model predicted activated expression of T-bet and ROR γ t and the phosphorylation of STAT3 and STAT1 and suggested a potential role of IL-21 in the modulation of IL-10. Driven by our modeling-derived predictions, we found reduced levels of CD4⁺ splenocyte-specific *tbx21* and *rorc* expression, reduced phosphorylation of STAT1 and STAT3, and an increase in CD4⁺ T cell-specific IL-10 expression in *H. pylori*-infected IL-21-deficient mice. Our results indicate that IL-21 regulates Th1 and Th17 effector responses during chronic *H. pylori* infection in a STAT1- and STAT3-dependent manner, therefore playing a major role controlling *H. pylori* infection and gastritis.

IMPORTANCE *Helicobacter pylori* is the dominant member of the gastric microbiota in more than 50% of the world's population. *H. pylori* colonization has been implicated in gastritis and gastric cancer, as infection with *H. pylori* is the single most common risk factor for gastric cancer. Current data suggest that, in addition to bacterial virulence factors, the magnitude and types of immune responses influence the outcome of colonization and chronic infection. This study uses a combined computational and experimental approach to investigate how IL-21, a proinflammatory T cell-derived cytokine, maintains the chronic proinflammatory T cell immune response driving chronic gastritis during *H. pylori* infection. This research will also provide insight into a myriad of other infectious and immune disorders in which IL-21 is increasingly recognized to play a central role. The use of IL-21-related therapies may provide treatment options for individuals chronically colonized with *H. pylori* as an alternative to aggressive antibiotics.

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Helicobacter pylori is a Gram-negative microaerophilic bacterium and a dominant member of the gastric microbiota harbored by approximately 50% of the world's population. A hallmark of *H. pylori* infection is a gastric mucosal inflammatory response, termed superficial gastritis (1). The presence of *H. pylori* increases the risk for development of duodenal ulcer disease, gastric ulcer disease, noncardia gastric adenocarcinoma, and B-cell malignancies, such as gastric mucosa-associated lymphoid tumors (MALT lymphomas) and high-grade lymphomas (reviewed in references 2 and 3). Conversely, there is also increasing evidence

that *H. pylori* colonization protects against esophageal and cardiac pathologies (4–7), childhood asthma (8–10), and childhood allergies (9, 11). The gastritis associated with *H. pylori* infection reflects the recruitment and activation of immune cells representing both innate and adaptive immunity (12 reviewed in reference 13). Actual treatment for *H. pylori* involves an aggressive triple-antibiotic treatment that unbalances the gastric microbiota. Furthermore, recent studies suggest that *H. pylori* is finding strategies to bypass the treatment by developing resistance to clarithromycin (14). Other studies have pointed out that during chronic *H. pylori* in-

fection, the exacerbated immune response in the gastric lamina propria is driving more epithelial cell damage than the bacterium itself (15). Therefore, new strategies to treat chronic *H. pylori* infections are needed. *H. pylori* infection of humans and experimental infection of rodents typically results in a mixed T helper 1 (Th1)/Th17-mediated immune response (12, 16–26). The long-term chronic inflammatory response to *H. pylori* is believed to drive or initiate the pathways which lead to the adverse outcomes of colonization, including chronic gastritis, intestinal metaplasia, and gastric cancer. Our mouse model of *H. pylori* infection is set up to investigate this critical pathway during chronic infection, focusing on the outcome of gastritis.

T cells play a decisive role in initiating and shaping pathological and protective responses in tissues. Classical examples of T cell-mediated diseases are inflammatory bowel disease (IBD), type 1 diabetes, psoriasis, rheumatoid arthritis, and multiple sclerosis. Relevant to this study, interleukin-21 (IL-21) is a cytokine produced mostly by activated CD4⁺ T cells, especially Th17 cells, T follicular helper (Tfh) cells, and NKT cells. IL-21 induces proliferation and increases cell survival and cytokine synthesis in many immune cells (reviewed in reference 27). *H. pylori* also upregulates IL-21 during infection, correlating the IL-21 expression with levels of gastritis in the mouse model (28). Moreover, IL-21 was associated with *H. pylori* infection in a study of infected humans (29).

Immunoinformatic approaches cannot replace traditional experimentation; however, they can be used to synthesize, organize, and integrate diverse types of data and theoretical frameworks to help generate new knowledge and target *in vivo* experimentation. Indeed, computer simulations of immunological processes can predict novel experimental behaviors, correlations, and interactions between components of a complex system, such as the signaling pathways controlling the differentiation and function of Th cells (30, 31). A CD4⁺ T cell computational model was built, calibrated, and validated to investigate interactions of external cytokines and transcription factors within a CD4⁺ T cell in the absence of infection (32). Our initial CD4⁺ T cell modeling studies investigated the importance of the peroxisome proliferator-activated receptor gamma (PPAR γ) in regulating the plasticity between Th17 and inducible T regulatory cells (iTreg) (15). To not only observe intracellular events but also have a cellular understanding of the immune response toward *H. pylori*, we also used a published tissue-level model to study how CD4⁺ T cell subsets influenced the initiation, progression, and outcome of disease (15).

This study leverages our published CD4⁺ T cell model (32) and cellular *H. pylori* model (15) to establish a chronic-*H. pylori*-specific CD4⁺ T cell differentiation model that allowed us to investigate the role of IL-21 in the maintenance of the T cell-mediated gastric mucosal responses to chronic *H. pylori* infection. Thus, we combined computational modeling and mechanistic experimental studies in mice to dissect the effects of *H. pylori* infection on the intracellular pathways by which IL-21 modulates CD4⁺ T cell responses during chronic infection. Our *in silico* and *in vivo* data suggest that IL-21 is a key cytokine for maintenance of both the Th1 and Th17 response during *H. pylori* infection. Furthermore, we provide novel evidence that IL-21 is required for the development of gastritis and control of the *H. pylori* bacterial burden, as well as for the modulation of T cell-derived IL-10 and phosphorylation of both STAT1 and STAT3. These data together represent key knowledge that could help in the development of novel IL-21-

centered immunotherapeutics for controlling infectious and immune-mediated diseases.

RESULTS

IL-21 deficiency leads to increased *H. pylori* colonization in the mouse model. The first step to evaluate the role of IL-21 during *H. pylori* infection *in vivo* was to determine any effects on *H. pylori* burden in the mouse stomach. IL-21-deficient (IL-21^{-/-}) mice and their wild-type littermates were infected with *H. pylori* strain SS1. At time points up to 3 months postinfection, mice were sacrificed and *H. pylori* colonization was measured by culture using serial dilution colony counting. At 1 month postinfection, there was no significant difference in the levels of colonization of IL-21^{-/-} mice and wild-type mice (data not shown). However, at later time points, as chronicity developed, IL-21^{-/-} mice had significantly higher levels of *H. pylori* colonization than their wild-type littermates on the B6;129 background at both 2 months (Fig. 1A) and 3 months postinfection (Fig. 1B). We also observed this increase in bacterial burden in infected IL-21^{-/-} mice from the C57BL/6 background (see Fig. S3A in the supplemental material), suggesting that IL-21 modulates the immune response, resulting in greater *H. pylori* clearance in the gastric mucosa of wild-type mice. In order to localize the presence of *H. pylori* bacteria in the gastric tissue, a modified Steiner stain was performed on sections from the gastric tissue at 3 months postinfection. When inflammation was present in the *H. pylori*-infected wild-type mice (Fig. 1C), bacteria localized to the mucus on the lumen side of the tissue and were rarely observed deeper in the tissue. In contrast, in the *H. pylori*-infected IL-21^{-/-} mice, where there was minimal inflammation, as well as in areas of the stomach in wild-type mice where inflammation was low, bacteria were present both in the mucus and in the glands and were also deeper in the tissue (Fig. 1C).

IL-21 deficiency protects *H. pylori*-infected mice from chronic gastritis. We next sought to evaluate the level of gastritis following *H. pylori* infection, and tissues were scored for inflammation. Scoring the tissue for acute and chronic inflammation in both the antrum and the corpus provided a quantitative method for assessing the presence of the neutrophils (acute inflammation) and lymphocytes (chronic inflammation). *H. pylori*-infected IL-21^{-/-} mice had significantly reduced inflammation compared to the levels in *H. pylori*-infected wild-type mice on both the B6;129 (Fig. 2A) and C57BL/6 background (see Fig. S3B in the supplemental material). Representative photomicrographs demonstrated the lack of inflammation present in the IL-21^{-/-} mice compared to that in their wild-type littermates at 3 months postinfection on the B6;129 (Fig. 2B) and C57BL/6 backgrounds (Fig. S3C). These data suggest that IL-21 plays a role in controlling chronic *H. pylori* colonization and makes a significant contribution to the generation of chronic gastritis.

To evaluate specifically the effects of IL-21 deficiency on cell migration to the stomach, we immunophenotyped isolated gastric lamina propria cells by flow cytometry. The most striking finding was that IL-21 deficiency significantly affected the numbers of CD4⁺ T cells in the *H. pylori*-infected stomachs. In addition, reduced numbers of CD8⁺ T cells, B lymphocytes, and neutrophils were found in the gastric mucosa of *H. pylori*-infected IL-21^{-/-} mice compared to the numbers of these cells in *H. pylori*-infected wild-type littermates (Fig. 2C).

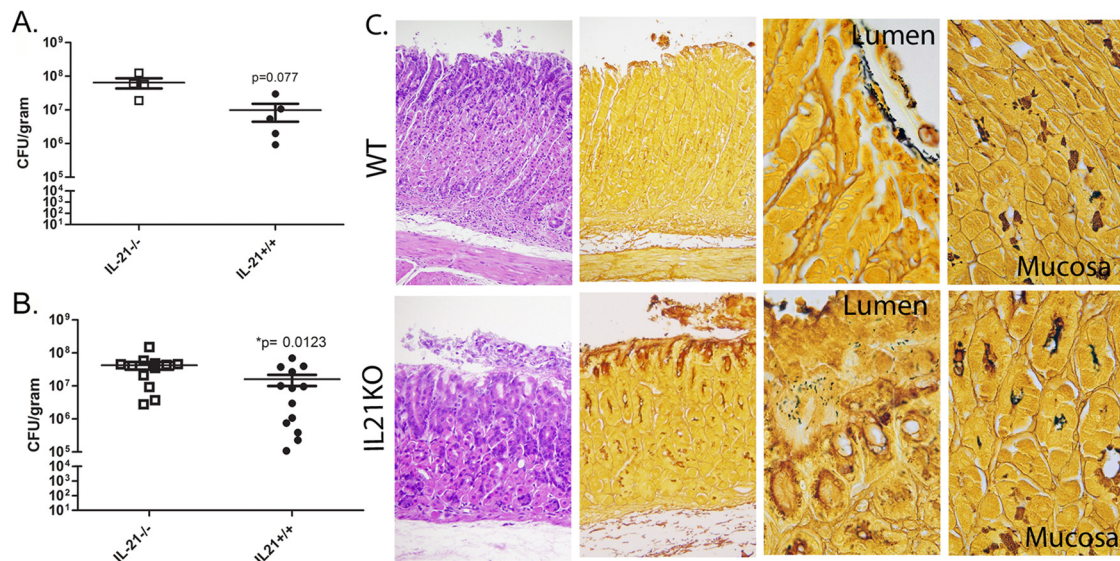


FIG 1 IL-21 is required to control *H. pylori* infection in the mouse model. IL-21^{-/-} mice and wild-type littermates were infected with *H. pylori* strain SS1 for up to 3 months. (A and B) Levels of colonization were measured by plating serial dilutions of stomach homogenates. The CFU counts were then calibrated to the weight of the tissue and are presented in the graphs as log(CFU/gram) at 2 months (A) and 3 months (B) postinfection. Bars and error bars represent means \pm standard errors of the means (SEM). Results are representative of 3 independent experiments. (C) Steiner and hematoxylin and eosin stains were performed in gastric mucosa sections of *H. pylori*-infected wild-type (WT) and IL-21^{-/-} mice (from left to right, sections are at $\times 200$, $\times 200$, $\times 1,000$, and $\times 1,000$). Sections are representative of those from 10 wild-type and 10 IL-21^{-/-} mice at 3 months postinfection.

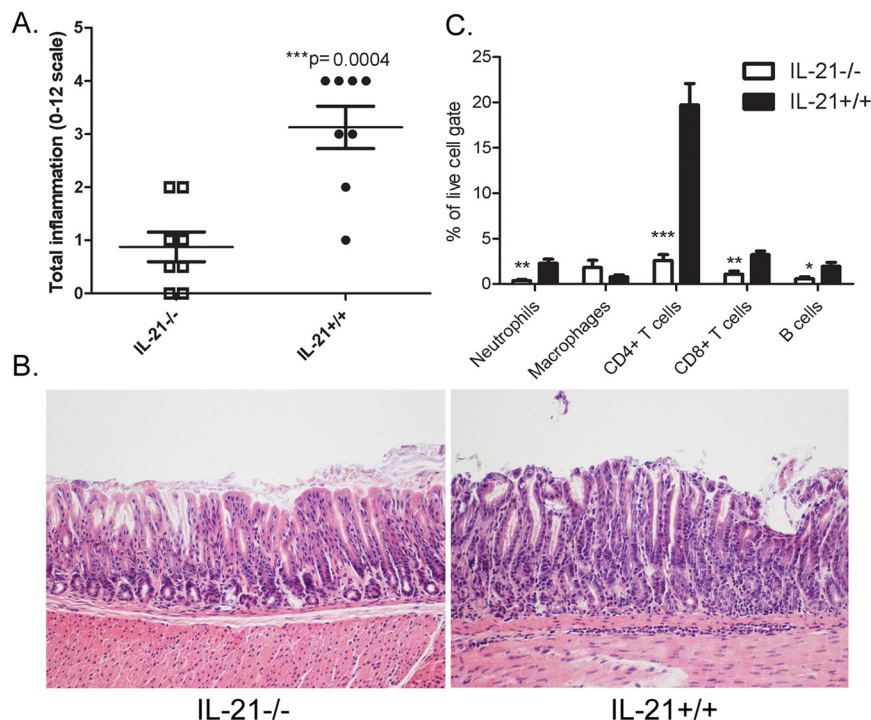


FIG 2 Inflammation is reduced in *H. pylori*-infected IL-21^{-/-} mice compared to the levels in their *H. pylori*-infected wild-type littermates. Levels of acute and chronic inflammation were scored for stomach tissue (in the corpus and antrum) at 3 months postinfection. (A) Total inflammation was scored on a scale of 0 to 12 (bars represent means, and error bars represent upper and lower interquartile ranges). (B) Representative sections of the gastric mucosa from 3 months postinfection are presented ($\times 200$). (C) Flow cytometric analysis was performed on dissociated stomach tissue at 3 months postinfection ($n = 8$ per genotype). Percentages of neutrophils (Gr1⁺ CD11c⁺), macrophages (CD11b⁺ Gr1⁻), CD4⁺ CD3⁺ T cells, CD8⁺ CD3⁺ T cells, and B cells (B220⁺) were calculated in the live-cell gate from the *H. pylori*-infected mice. Bars and error bars represent means \pm SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Results are representative of 3 independent experiments.

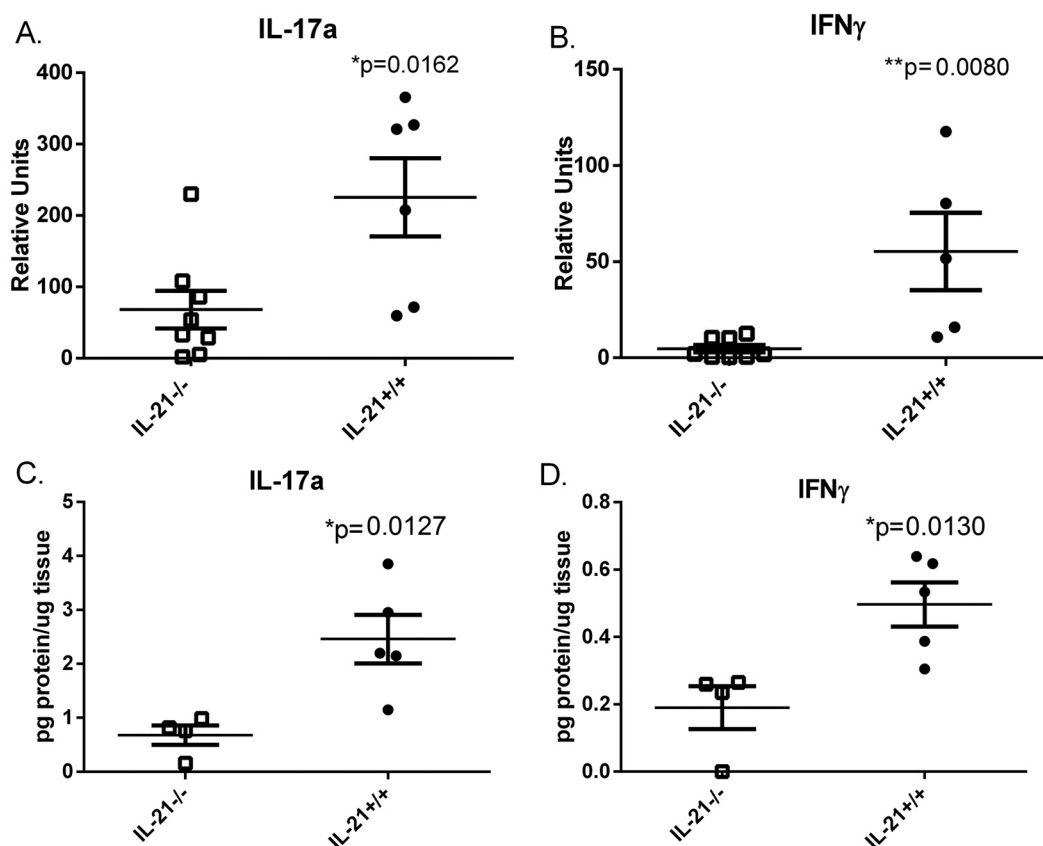


FIG 3 Th17 and Th1 responses are reduced in *H. pylori*-infected IL-21^{-/-} mice compared to the levels in *H. pylori*-infected wild-type mice. (A and B) Real-time RT-PCR was performed on stomach tissue of *H. pylori*-infected mice. Relative units of IL-17A (A) and IFN-γ (B) were measured at 3 months postinfection. Relative units are normalized using the relative expression calibrated to that in uninfected wild-type mice, with GAPDH as the endogenous control. Results are representative of 3 independent experiments. Bars and error bars represent means ± SEM. *, $P \leq 0.05$; **, $P \leq 0.01$. (C and D) Gastric protein levels were measured in the stomach tissue using a Milliplex assay at 2 and 3 months postinfection. Protein levels are reported as picogram of protein per microgram of total tissue. IL-21^{-/-} mice express reduced protein levels of IL-17A (C) and IFN-γ (D) during chronic infection. Graphs are representative of 2 independent experiments. Bars and error bars represent means ± SEM. *, $P \leq 0.05$.

Chemokine and cytokine expression are abrogated in IL-21-deficient mice. To investigate how the levels of chemokines and other inflammatory cytokines were affected by the IL-21 deficiency, a multiplex protein assay was performed at 2 and 3 months postinfection. Our data demonstrate that many interferon gamma (IFN-γ)-induced chemokines, including RANTES, IFN-inducible protein-10 (IP-10), and macrophage inflammatory protein-1β (MIP-1β), were present at significantly lower levels in the *H. pylori*-infected IL-21^{-/-} mice than in their *H. pylori*-infected wild-type littermates (see Fig. S4A in the supplemental material). Moreover, the levels of the IL-17-induced chemokine KC (a mouse homologue of IL-8) were significantly lower in the *H. pylori*-infected IL-21^{-/-} mice than in their *H. pylori*-infected wild-type littermates (Fig. S4B). The levels of the proinflammatory cytokines TNF-α and IL-1β, which can enhance inflammation or induce further effector T cell differentiation, were significantly lower in the *H. pylori*-infected IL-21^{-/-} mice than in their *H. pylori*-infected wild-type littermates both at the protein (Fig. S4C) and RNA levels (Fig. S4D).

IL-21 deficiency leads to abrogated Th17 and Th1 effector responses. IL-21 plays a role in the maintenance of the Th17 responses (33). To investigate the role of IL-21 in the production of

mucosal T cell-derived cytokines in the context of *H. pylori* infection, real-time reverse transcription (RT)-PCR was performed on RNA isolated from the stomachs of *H. pylori*-infected IL-21^{-/-} mice and their *H. pylori*-infected wild-type littermates. The expression of both IL-17 (Fig. 3A) and IFN-γ (Fig. 3B) was significantly reduced in the *H. pylori*-infected IL-21^{-/-} mice compared to their expression in *H. pylori*-infected wild-type littermates by 3 months postinfection. We found similar results with our C57BL/6 mice (Fig. S3D). These significant differences were confirmed by using a protein-based assay on stomach lysates (Fig. 3C and D).

IL-21 contributes to Th17 differentiation and modulates Th1 responses upon infection with *H. Pylori*. CD4⁺ T cell responses are believed to drive chronic inflammation and contribute to long-term damage associated with adverse outcomes of chronic *H. pylori* infection. Based on our experimental data, IL-21 plays a role in maintaining the activation of CD4⁺ T cells, since *H. pylori*-infected IL-21^{-/-} mice have significantly reduced inflammation in their gastric mucosa compared to that in wild-type littermates, with the most striking reduction in CD4⁺ T cells. To gain a more comprehensive mechanistic understanding of why the lack of IL-21 has such an impact on the development and maintenance of

CD4⁺ T cells and their function in the gastric mucosa of *H. pylori*-infected mice, we used our cytokine data to leverage a computational and mathematical model that simulates CD4⁺ T cell differentiation. Specifically, the original CD4⁺ T cell differentiation model (32) was calibrated with real-time RT-PCR data from wild-type or IL-21^{-/-} mice infected with *H. pylori* for 3 months (Fig. 3; see also Text S1 in the supplemental material) to represent the influence of the cytokine environment that a CD4⁺ T cell encounters during infection. To determine the effect of the loss of IL-21 in CD4⁺ T cell differentiation and function, we engineered and modeled an IL-21-deficient system (Text S1). Modulation of transcription factors is critical for Th cell differentiation and cytokine production (reviewed in reference 34). To broadly investigate the contribution of specific molecules to IL-21, sensitivity analysis was run on internal CD4⁺ T cell-specific IL-21. The simulation results showed a positive correlation for Th17-related molecules, such as phosphorylated STAT3 (P-STAT3), IL-17, and ROR γ t (Fig. 4A). T-bet, IFN- γ , and phosphorylated STAT1 (P-STAT1) were also found to be positively correlated with IL-21 (Fig. 4A). Interestingly, the FOXP3 and IL-10 results from the sensitivity analysis showed a negative correlation to IL-21 (Fig. 4A). *In silico* experimentation indicated that there is a dramatic downregulation of ROR γ t, IL-17, and phosphorylation of STAT3 during Th17 differentiation when IL-21 production is deleted (Fig. 4B). Our results also demonstrated a downregulation of IFN- γ and T-bet following Th1 differentiation in the IL-21-deficient model compared to their expression in the wild-type model (Fig. 4C). Next, we sought to determine the effect of an *in silico* upregulation of IL-21 in Th17-differentiated CD4⁺ T cells and found that P-STAT3, IL-17, and ROR γ t were upregulated with increasing doses of IL-21 (Fig. 4D). These effects were abrogated in the IL-21-deficient system (Fig. 4E). Of note, when evaluating Th1 and Th17 populations in our *H. pylori* tissue-level model, we also found reduced levels of Th1 and Th17 populations in the IL-21-deficient model compared to the levels in the wild-type model during the chronic stage of infection (Fig. S6). These *in silico* results provided evidence that IL-21 plays a key role in CD4⁺ T cell modulation during *H. pylori*-induced Th1/Th17 CD4⁺ T cell responses at chronic time points.

Experimental validation of model predictions: IL-21 modulates the expression of Th1 and Th17 transcription factors. Guided by the model predictions, we sought next to investigate whether IL-21 affects the gene expression of CD4⁺ T lymphocyte transcription factors *tbx21* and *rorc* (which translate to T-bet and ROR γ t) *in vivo*. CD4⁺ T cells were isolated from spleens of *H. pylori*-infected IL-21^{-/-} mice and wild-type littermates at 3 months postinfection, and real-time RT-PCR was performed. As predicted, the expression of *tbx21* and *rorc* was significantly reduced in the CD4⁺ cells from *H. pylori*-infected IL-21^{-/-} mice compared to their expression in CD4⁺ cells from *H. pylori*-infected wild-type littermates (Fig. 5A).

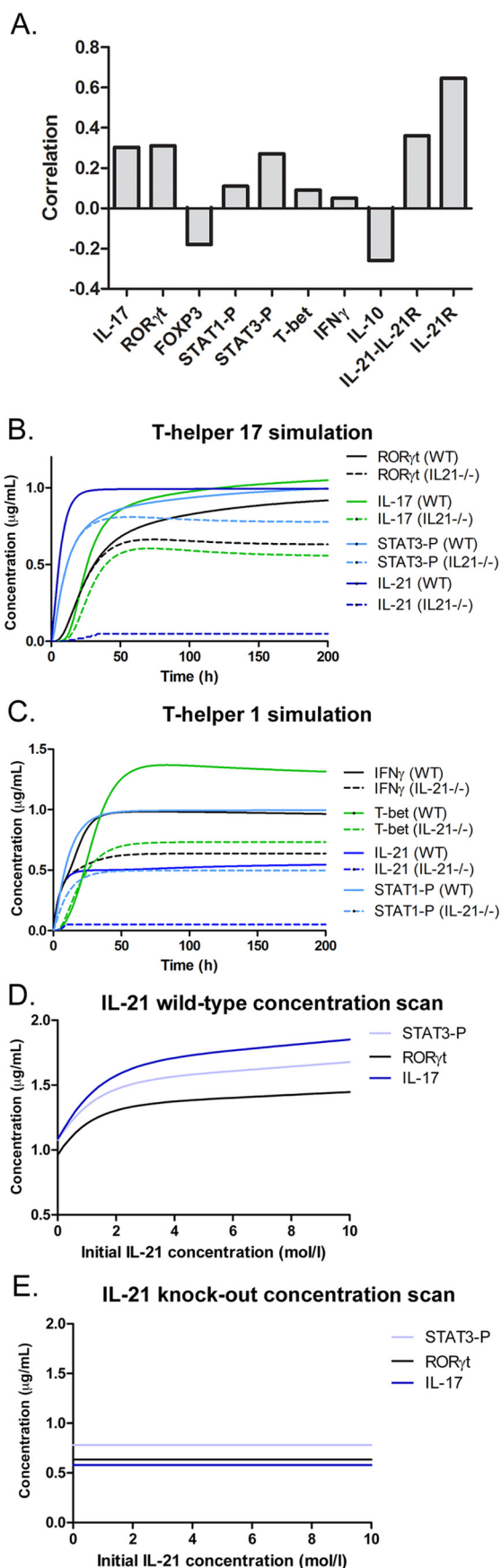
Our computational model also predicted that IL-21 correlates positively with phosphorylation of STAT1 and STAT3. Indeed, the *in silico* IL-21^{-/-} model showed a downregulation of P-STAT3 in Th17 cells (Fig. 4B) and P-STAT1 in Th1 cells (Fig. 4C). To test this prediction *in vivo*, the levels of P-STAT1 and P-STAT3 were measured in CD4⁺ splenocytes from *H. pylori*-infected IL-21^{-/-} and *H. pylori*-infected wild-type mice at 3 months postinfection. The data indicate that, while total phosphorylation of STAT1 and STAT3 is low when the cells are not

restimulated *ex vivo*, the mean fluorescence intensities of the P-STAT1 and P-STAT3 staining are significantly lower in the CD4⁺ splenocytes from IL-21^{-/-} mice than in those from wild-type mice (Fig. 5B).

Expression of markers of T cell regulation (FOXP3 and IL-10) in IL-21-deficient mice. Since we observed a negative correlation of IL-10 and FOXP3 in relation to IL-21 (Fig. 4A), we investigated whether there was a change in IL-10 and FOXP3 expression in iTreg cells in the gastric mucosa, since the major expression of FOXP3 comes from regulatory T cells. To do so, we performed computational simulations in the CD4⁺ T cell differentiation model and evaluated the level of IL-10 in Th17 and the level of FOXP3 in iTreg cells. We hypothesized that an increase in Treg cells may explain the decrease in gastritis found in IL-21^{-/-} mice. However, *in silico* results differentiating the mathematical model with transforming growth factor- β (TGF- β) and IL-2 showed no difference between wild-type and IL-21^{-/-} mice in the expression of FOXP3 within the Treg cell subset (Fig. 6A). Furthermore, our mathematical model predicted a dramatic upregulation of IL-10 in Th17 cells in the IL-21-deficient system compared to the response in the wild-type system when induced with IL-6 and TGF- β together (Fig. 6B). To validate these predictions, we measured *foxp3* transcript levels in CD4⁺ T cells isolated from the spleens of *H. pylori*-infected IL-21^{-/-} mice and wild-type littermates at 3 months postinfection. The experimental results showed no significant difference in the CD4⁺ T lymphocytes, validating the model's prediction and suggesting that the reduction in Th1 and Th17 effector responses is not mediated through an increase in iTreg cells (Fig. 6C). We similarly measured IL-10 expression in CD4⁺ T lymphocytes isolated from the spleens of *H. pylori*-infected IL-21^{-/-} mice and wild-type mice. As the model predicted, IL-10 expression was significantly higher in CD4⁺ T lymphocytes from *H. pylori*-infected IL-21^{-/-} mice than in those from *H. pylori*-infected wild-type littermates (Fig. 6D).

DISCUSSION

Since the identification of Th17 cells almost a decade ago, we have gained a better understanding of how CD4⁺ T helper cells help control bacterial colonization beyond providing B cell help and activating macrophage function. Th17 cells play a central role in controlling many bacterial infections through the activation of chemokine pathways and antimicrobial responses. They activate these pathways through the production of IL-17A, IL-17F, and IL-22. In addition, IL-21, which is produced by Th17 cells, plays a role in amplifying the Th17 cell effector response (35). In this study, we find not only that IL-21 is required for the maintenance of IL-17 production in the gastric mucosa following *H. pylori* infection but also that IL-21 plays an important role in the development and maintenance of the IFN- γ response. As a result, a deficiency in IL-21 protected *H. pylori*-infected mice from chronic gastritis, at the expense of increased bacterial burden. Since IFN- γ and IL-17 can induce chemokine expression, the lack of Th1 and Th17 responses in the gastric mucosa of the *H. pylori*-infected IL-21^{-/-} mice leads to reduced chemokine expression and fewer neutrophils. Since neutrophils are likely controlling the bacterial burden, IL-21^{-/-} mice have more bacterial colonization and, interestingly, Steiner stains indicated that the bacteria in the IL-21^{-/-} mice are typically localized not only in the mucus layer but also deeper in the tissues (i.e., in glands) (Fig. 1C). Despite the increased bacterial burden and deeper localization of *H. pylori* in



the tissue, in our mouse model, the infected IL-21 $^{-/-}$ mice did not show any signs of discomfort or distress. In wild-type mice, where inflammation occurs in a localized manner, the *H. pylori* bacteria localized to the mucus layer, but the bacteria were not observed in the gland. It is worth noting that there are areas in wild-type mice where inflammation is low, and in those areas, *H. pylori* can also be found deeper in the tissue.

Immunoinformatic approaches are increasingly useful tools to provide insight into potential trends through integrating current datasets and knowledge and detecting behavioral responses at the systems level. In this specific study, after observing the significantly higher bacterial burden in the stomachs of IL-21 $^{-/-}$ mice (Fig. 1), we found that these mice in fact have less leukocytic infiltration, including very few CD4 $^{+}$ T cells in the gastric lamina propria (Fig. 2). One potential explanation is that IL-21 impairs the ability of CD4 $^{+}$ T cells to mount a proper inflammatory response and affects a specific T cell product that would maintain or promote CD4 $^{+}$ T cell recruitment in the gastric lamina propria. This would explain the lack of inflammation in the gastric lamina propria. The ability of the innate immune compartment to clear out the bacteria could then be less effective without the proper effector response driven by CD4 $^{+}$ T cells. As a matter of fact, since CD4 $^{+}$ T cells were our focus, we leveraged the existing CD4 $^{+}$ T cell differentiation model (32) by recalibrating the model with *H. pylori*-specific data (Fig. 3) to understand what intracellular events were occurring within the CD4 $^{+}$ T cell compartment, in order to target experimental studies with predicted hypotheses. Indeed, by using a modeling approach that simulates the time period of CD4 $^{+}$ T cell differentiation and calibrating with chronic *H. pylori* data, we could determine which are the pathways most affected by the presence or lack of IL-21. Moreover, we could characterize cross talk between pathways within the CD4 $^{+}$ T cell differentiation model at the chronic stages of *H. pylori* infection. The results of previous studies suggest that IL-21 is a key cytokine for Th17 cell maintenance. Indeed, our *in silico* results demonstrate that differences between the wild-type and the IL-21 knock-out models are not noticeable at the first stages of differentiation. However, when Th17 is fully differentiated, IL-21 is required for the upregulation of IL-17 and ROR γ t and phosphorylation of STAT3. In fact, the results of the tissue-level model show a clear difference in Th1 and Th17 populations when comparing the IL-21-deficient system with the wild-type system. Furthermore, our computational simulations show a dramatic downregulation of IL-17 and ROR γ t and phosphorylation of STAT3 in a fully differentiated Th17 cell. IL-21 also plays an important role in Th1 responses, as illustrated by the smaller amounts of P-STAT1 and IFN- γ and reduced T-bet expression. This computational approach allowed us to target experimental studies and be able to confirm and validate all these findings in our *in vivo* model of *H. pylori* infection.

FIG 4 The CD4 $^{+}$ T cell computational model predicts modulation of differentiation and maintenance by IL-21 *in silico*. (A) Sensitivity analysis of IL-21 over Th1- and Th17-related molecules showing positive or negative correlation to IL-21. (B) Effect of IL-21 deficiency on modulation of ROR γ t, IL-17, IL-21, and STAT3 in the computed Th17 system. (C) Effect of IL-21 deficiency on modulation of T-bet and IFN- γ in the computed Th1 system. (D and E) Upregulation of ROR γ t, IL-17, and STAT3-P in a Th17 state with increasing concentrations of IL-21 in the wild-type system (D) but not in the IL-21 $^{-/-}$ system (E) during a time course of 200 hours.

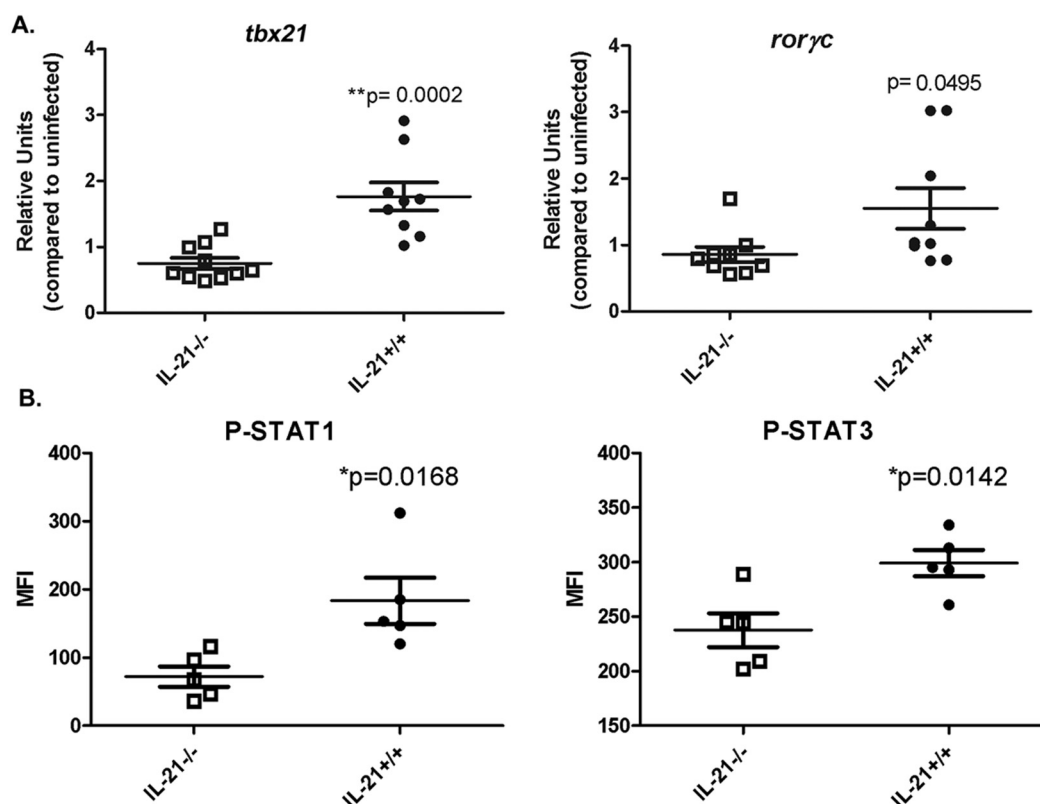


FIG 5 Transcription factors associated with Th1 and Th17 are affected by IL-21 deficiency in the mouse model. (A) CD4 lymphocytes isolated from *H. pylori*-infected IL-21^{-/-} mice express reduced levels of *tbx21* and *rorc*. Gene expression levels of transcription factors *tbx21* and *rorc* were measured in CD4⁺ T cells isolated from the spleens of *H. pylori*-infected IL-21^{-/-} and wild-type mice. Relative units are normalized using the relative expression calibrated to expression in CD4⁺ splenocytes from uninfected mice, with GAPDH as the endogenous control. (B) The levels of phospho-STAT1 and phospho-STAT3 in unstimulated cells from infected mice were measured by flow cytometry, and the mean fluorescence intensities (MFI) of the CD4⁺ splenocytes are reported. Bars and error bars represent means \pm SEM. *, $P \leq 0.05$; **, $P \leq 0.01$.

In this regard, IL-10 has long been known to play an important role in the regulation of Th1 cell responses to pathogens (36). It has also been shown that Th1 effector cells are themselves coproducers of IL-10 and IFN- γ , since they use IL-10 as a self-regulation mechanism (37, 38), and that TGF- β and IL-6 can induce a CD4⁺ T cell subset that coproduces IL-17 and IL-10 (39). These coproducers are considered self-regulatory Th17 cells. Our modeling results predict that IL-21 might be involved in controlling the balance between regulatory and effector responses during *H. pylori* infection through an IL-10 mechanism. Indeed, the CD4⁺ T cell computational model predicted an upregulation of Th17-specific IL-10 in IL-21^{-/-} mice, supporting the notion that IL-21 negatively modulates the expression of IL-10 in Th17 cells. Taken together, these findings indicate that IL-21 has emerged as a central molecule in CD4⁺ T cell differentiation, which promotes effector responses in Th1 and Th17 cells through P-STAT1 and P-STAT3, respectively, and also downregulates the gene expression of *il-10* in Th17 cells (Fig. 7A). In this study, our modeling approaches were predictive of the *in vivo* outcomes (Fig. 7B).

While several studies have investigated the role of Th17 versus Th1 cells during control of *H. pylori* infection and the development of gastritis, there does not appear to be a clear indication that one Th subset plays a more important role than the other. Murine studies have shown that Th1 responses are associated with increased gastritis, since IFN- γ ^{-/-} mice have decreased levels of

gastric inflammation (40). These studies have also shown that an insufficient Th1 response is associated with increased bacterial colonization (40, 41). However, there is also evidence that adoptive transfer into SCID mice of CD4⁺ T cells from T-bet^{-/-} mice, which do not exhibit IFN- γ production and Th1 differentiation, still results in gastritis (42), leaving the door open for a role for Th17 cells or other effector immune cell subsets in gastritis. Some studies have suggested that, in the absence of IL-17, there is a decrease in inflammation and an increase in Th1 effector responses, which then drive down bacterial infection. On the other hand, the results of our previously published study suggest that, in the absence of IL-17RA (and IL-17 signaling), the Th1 response is not affected and, while there is a decrease in acute inflammation driven by neutrophils, the loss of IL-17 signaling leads to increased bacterial burden and increased chronic inflammation, especially B lymphocyte density (28). The increased bacterial burden and chronic inflammation were accompanied by an increase in IL-21 expression as well. We hypothesize that IL-21 may drive the chronic inflammation in the IL-17RA^{-/-} mice infected with *H. pylori*. Our studies in IL-21^{-/-} mice infected with *H. pylori* presented here demonstrate that IL-21 is required for both Th1 and Th17 responses to be maintained during chronic infection.

A hypothesis for why both Th1 and Th17 responses are suppressed in IL-21^{-/-} mice is that there may be an increase in Treg cells in the absence of IL-21 signaling. Whereas sensitivity analysis

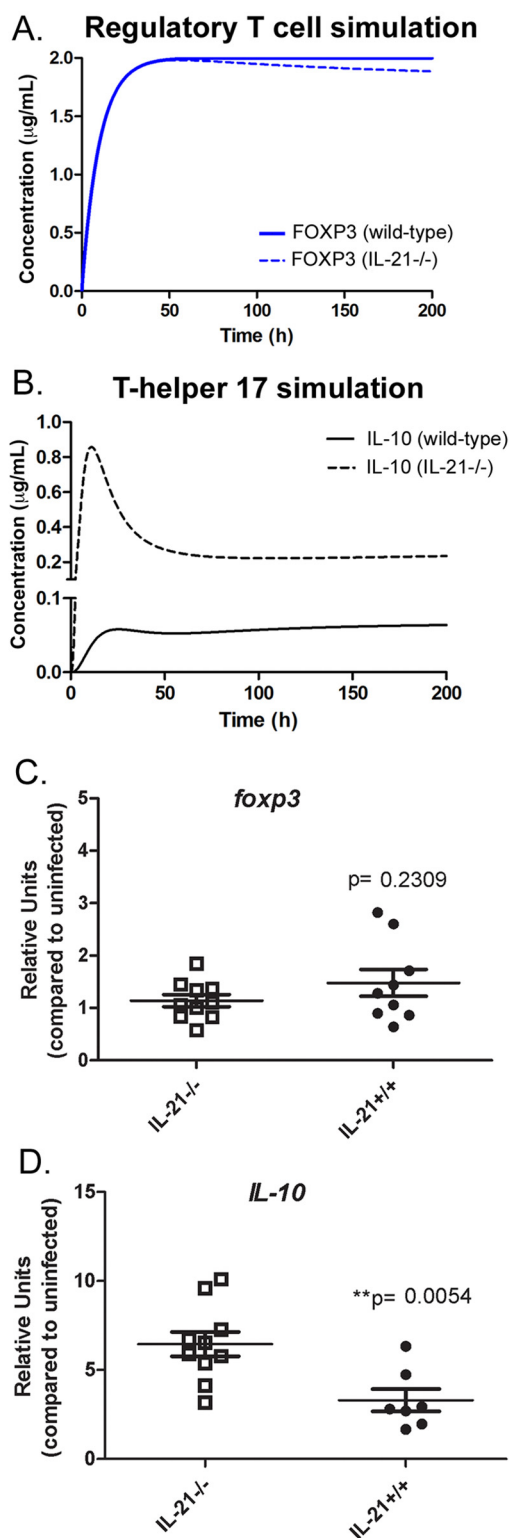


FIG 6 IL-21 modulates the expression of IL-10 during *Helicobacter pylori* infection. (A and B) The CD4 $^{+}$ T cell model predicted no differences in the expression of FOXP3 in wild-type and IL-21 $^{-/-}$ mice (A) and a higher production of IL-10 in IL-21 $^{-/-}$ mice (B). (C and D) To validate those predictions, the levels of mRNA in CD4 $^{+}$ T cells of spleens of *H. pylori*-infected mice were assessed for *foxp3* transcripts (C) and *il-10* transcripts (D). Bars and error bars represent means \pm SEM.

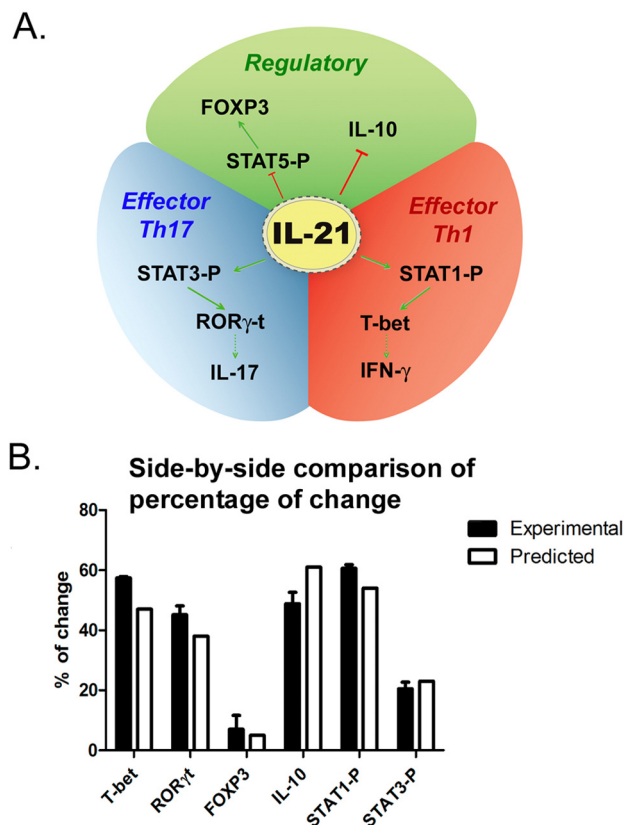


FIG 7 Predictive value of the CD4 $^{+}$ T cell differentiation model. (A) Cartoon representation of IL-21 interaction within the CD4 $^{+}$ T cell subset. (B) Side-by-side comparison assessing the differences between the model's predicted percentages of change between the wild-type and the IL-21 $^{-/-}$ systems and the experimental results for wild-type and IL-21 $^{-/-}$ mice. Bars and error bars represent means \pm SEM.

performed with our CD4 $^{+}$ T cell computational model indicated that FOXP3 could potentially be negatively correlated to IL-21, the additional time course, loss-of-function, and *in silico* experiments and our data suggest that the number of Tregs is not affected by the deficiency of IL-21. Indeed, the suppression of Th1 and Th17 responses is a more direct effect on the CD4 $^{+}$ T cells as a consequence of the IL-21 deficiency. Our data indicate that at 3 months postinfection, there is no increase of Treg cells in the stomachs or in the spleens of the *H. pylori*-infected IL-21 $^{-/-}$ mice compared to the Treg cell numbers in the stomachs or spleens of their wild-type littermates, but this is not surprising, since *H. pylori* IL-21 $^{-/-}$ mice have little inflammation in their stomachs and there is an overall decrease in T cells in the stomachs of the IL-21 $^{-/-}$ mice. Even in the periphery (spleen), the levels of *foxp3* expression are not significantly different in the *H. pylori*-infected IL-21 $^{-/-}$ mice and their *H. pylori*-infected wild-type littermates. IL-21 may still affect the efficiency of the Treg response during infection, but our data indicate that it does not affect the levels of *foxp3* expression. However, we cannot rule out the possibility that Tregs contribute to the higher levels of CD4 $^{+}$ -derived IL-10 in the IL-21 $^{-/-}$ mice.

IL-21 has been implicated in expanding both Th1 and Th17 cells in our study with *H. pylori*, but it is also a key modulator in intestinal CD4 $^{+}$ T cell populations (43). These studies suggest

IL-21 as a promising therapeutic target for treatment of T cell-mediated diseases, such as inflammatory bowel disease (IBD). IL-21 is upregulated in mouse models of IBD (trinitrobenzene sulfonic acid [TNBS]-induced relapsing colitis and dextran sulfate sodium [DSS]-induced colitis) and during chronic bacterial infections like *H. pylori* infection (reviewed in reference 27). In models of experimental colitis, IL-21^{-/-} mice were largely protected against both DSS-induced colitis and TNBS-induced relapsing colitis (44). In these models, the IL-21^{-/-} mice were unable to upregulate Th17-associated molecules during gut inflammation (44). Blockade of IL-21 with IL-21R.Fc inhibits disease progression in a lupus-prone mouse model (45) and ameliorates disease in a mouse model of rheumatoid arthritis (46). Recent findings in systemic lupus erythematosus (SLE) patients demonstrate that IL-21 expression correlates with alterations of T cell and B cell subsets and suggest that targeting IL-21 could provide beneficial effects on both T cell and B cell alterations (47). In our IL-21^{-/-} model, B cell responses are also affected. We observed that *H. pylori*-infected IL-21^{-/-} mice lack an *H. pylori*-specific IgG1 or IgG2a antibody response (measured in the serum; see Fig. S8 in the supplemental material), but B cells have never been reported to play a pathogenic role during *H. pylori* gastritis. Of note, an anti-IL-21 receptor monoclonal antibody is being tested in a phase I clinical trial (14), positioning IL-21 as a promising, host-targeted therapeutic that could potentially replace the current aggressive triple-antibiotic treatment in the context of *H. pylori* infection.

In summary, we find that IL-21-deficient mice are protected from *H. pylori*-induced gastritis, similar to the protection observed in models of chemically induced colitis. Protection from *H. pylori*-induced gastritis was associated with marked decreases in IL-17 and IFN- γ in infected IL-21-deficient mice compared to their levels in wild-type mice. The results of our combined approach, utilizing mathematical modeling and *in vivo* *H. pylori* infections in the mouse model, indicate that IL-21 has a role in sustaining both Th1 and Th17 effector cell responses through the induction of phosphorylation of STAT1 and STAT3 and induced expression of *tbx21* and *rorc*. These data suggest that chronic maintenance of the T cell-mediated inflammation during *H. pylori* infection requires IL-21. Hence, IL-21 may be an ideal target for the development of immunotherapeutics, although caution should be employed when Th1 and Th17 responses are necessary for controlling more virulent infections.

MATERIALS AND METHODS

Animals. Male and female interleukin-21^{+/-} mice (B6;129S5-*Il21*^{tm1Lex}/Mmucd, stock number MMRRC:011723-UCD, backcrossed to C57BL/6 mice, stock number MMRRC:032800-UCD) were obtained from the NIH Consortium (University of California, Davis) for the establishment of a breeding colony. *Helicobacter*-free IL-21^{-/-} and IL-21^{+/+} (wild-type) male littermates, 8 to 10 weeks old, were used in all experiments. The IL-21^{+/-} breeding pairs tested negative for intestinal *Helicobacter*. Feces from sentinel mice housed in the same room consistently tested negative for pinworms, mouse parvovirus, and several other murine pathogens. Mice were housed and maintained according to the requirements of the Vanderbilt University Institutional Animal Care and Use Committee (protocol M/11/055).

Culture of *H. pylori*. A mouse-passaged derivative of *H. pylori* strain SS1 was used in these experiments. Bacteria were grown on Trypticase soy agar (TSA) plates containing 5% sheep blood. Alternatively, bacteria were grown in Brucella broth containing 10% heat-inactivated fetal bovine serum (FBS) and 10 μ g/ml vancomycin. Plate cultures were grown at 37°C

either in room air supplemented with 5% CO₂ or under microaerobic conditions generated by a CampyPak plus* hydrogen-plus-CO₂ system with integral palladium catalyst (BD). Liquid cultures for infection were grown under microaerobic conditions with shaking at 150 rpm.

Infection of mice with *H. pylori*. One day prior to infection of mice, *H. pylori* strain SS1 was inoculated into liquid medium and was cultured for 18 h under microaerobic conditions as described above. Mice were orogastrically inoculated with a suspension of 5×10^8 CFU *H. pylori* (in 0.5 ml of brucella broth) twice over 5 days.

Processing of mouse stomachs. Mouse stomachs were processed as previously described (48). In brief, after rinsing in phosphate-buffered saline (PBS), the stomach tissue was cut into longitudinal strips that were used for bacterial culture, RNA analysis, histology, and/or protein quantification. For histological analyses, sections were stained with hematoxylin and eosin to assess inflammation (scoring described below). Moreover, a Steiner stain was performed to localize the *H. pylori* bacteria within the gastric tissue, as previously described (49).

Mathematical modeling. To assert the dynamics of IL-21-related pathways in CD4⁺ T cells, an ordinary differential equation (ODE)-based CD4⁺ T cell differentiation model was used in its wild-type and IL-21^{-/-} setup. Briefly, the model was calibrated with *H. pylori*-specific experimental data (Fig. 3), and time courses were performed with a Th1-, Th17-, or iTreg-specific initialization to assess the role of IL-21 in these phenotypes. More information about the calibration and simulation process can be found in Text S1 in the supplemental material.

Culture of *H. pylori* from mouse stomach. Gastric tissue was homogenized using a Tissue-Tearor (Biospec Products, Inc.) in brucella broth with 10% FBS. Serial dilutions of the homogenate were plated as previously described (49). After 5 to 7 days of culture under microaerobic conditions, *H. pylori* colonies were counted.

Stomach inflammation scoring. Acute and chronic inflammation in the gastric antrum and corpus were graded on a scale from 0 to 3 (50–52). Acute inflammation was graded by a blinded pathologist (M.K.W. or M.B.P.) based on density of neutrophils, and chronic inflammation was graded based on the density of lamina propria (LP) mononuclear cell infiltration independent of lymphoid follicles. Total gastric inflammation was calculated as the sum of acute and chronic inflammation scores for the corpus and the antrum, allowing quantification of total inflammation on a scale from 0 to 12.

RNA extraction and real-time RT-PCR. Total RNA was isolated from the stomach using the TRIZOL isolation protocol (Invitrogen) with slight modifications as previously described (49). CD4⁺ T cell RNA was isolated using Qiagen's RNeasy kit as directed by the manufacturer. RNA was reverse transcribed using the high-capacity cDNA reverse-transcription kit (Life Technologies). For real-time reverse transcription-PCR (RT-PCR), we used the relative gene expression method (50). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the normalizer, and tissue from uninfected mouse stomachs (of the same genotype) served as the calibrator sample, as previously described (49).

Flow cytometric analysis. To analyze gastric cellular infiltrates, whole mouse glandular stomachs were harvested and processed (with dispase and collagenase) using the gentleMACS dissociator (Miltenyi Biotec) as previously described (49). The gastric cells were stained with anti-CD4, anti-CD8, anti-CD3, anti-Gr1, anti-CD11b, and anti-B220 antibodies (BD Biosciences) as previously described (49). Samples were collected and analyzed on a BD LSR II flow cytometer (BD Biosciences).

Analysis of cytokine and chemokine protein levels in gastric tissues. Freshly excised glandular stomach tissues were rinsed in PBS and homogenized in CelLytic MT mammalian tissue lysis/extraction buffer (Sigma). Twenty-five cytokine analytes were measured in tissue lysates using the Milliplex MAP mouse cytokine/chemokine magnetic bead panel kit according to the manufacturer's instructions (Millipore). Standards were also prepared for all 25 cytokine analytes according to the manufacturer's instructions. Protein concentrations were measured using the DC protein

assay kit (Bio-Rad Laboratories). The concentration of each cytokine is presented as picograms of protein per microgram of tissue.

CD4⁺ T cell isolations. CD4⁺ T cells were isolated from the spleens of *H. pylori*-infected IL-21^{-/-} mice and *H. pylori*-infected wild-type littermates between 2 and 3 months postinfection. Spleens were harvested, and after red blood cell lysis, the cells were magnetically labeled with CD4 microbeads (clone L3T4; Miltenyi Biotec). CD4⁺ cells were positively selected using the positive selection program in sensitive mode on the autoMACS machine (Miltenyi Biotec) according to the manufacturer's protocol. The resulting population was 92 to 96% CD4⁺ by flow cytometry analysis.

STAT1 and STAT3 phosphorylation assays. The levels of STAT phosphorylation were measured by flow cytometry. Briefly, 1 million splenocytes were either unstimulated or stimulated with 10 ng/ml recombinant IL-6 or recombinant IL-21 (Peprotech) for 15 min. After fixation with a final concentration of 1.5% paraformaldehyde, the cells were permeabilized with cold methanol. The cells were stained with either anti-phospho-STAT3 (tyr705, D3A7) antibody (Cell Signaling Technology) or anti-phospho-STAT1 (tyr 701, D4A7) antibody for 45 min. After several washes, anti-rabbit IgG-Alexa Fluor 647 was added for 30 min. Cells were washed three times and analyzed by flow cytometry.

Statistical analysis. Four to seven mice per group per time point were used for all of the studies. Colonization, inflammation, Luminex assays, and cytokine real-time RT-PCR were all performed as distinct experiments at least three times. To compare results obtained with different groups of mice, statistical analysis was performed using one-way analysis of variance, followed by a Student-Neuman-Keuls *post hoc* test. For analyses of bacterial numbers and cell numbers, the data were normalized by log transformation prior to statistical analysis. For histology scores, the Mann-Whitney *U* test was applied to compare results between wild-type and IL-21^{-/-} mice.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01243-14/-/DCSupplemental>.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 2.9 MB.
Figure S2, TIF file, 5.6 MB.
Figure S3, TIF file, 12.5 MB.
Figure S4, TIF file, 5.2 MB.
Figure S5, TIF file, 15.6 MB.
Figure S6, TIF file, 5.3 MB.
Figure S7, PDF file, 0.1 MB.
Figure S8, TIF file, 0.1 MB.
Table S1, XLSX file, 0.1 MB.

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