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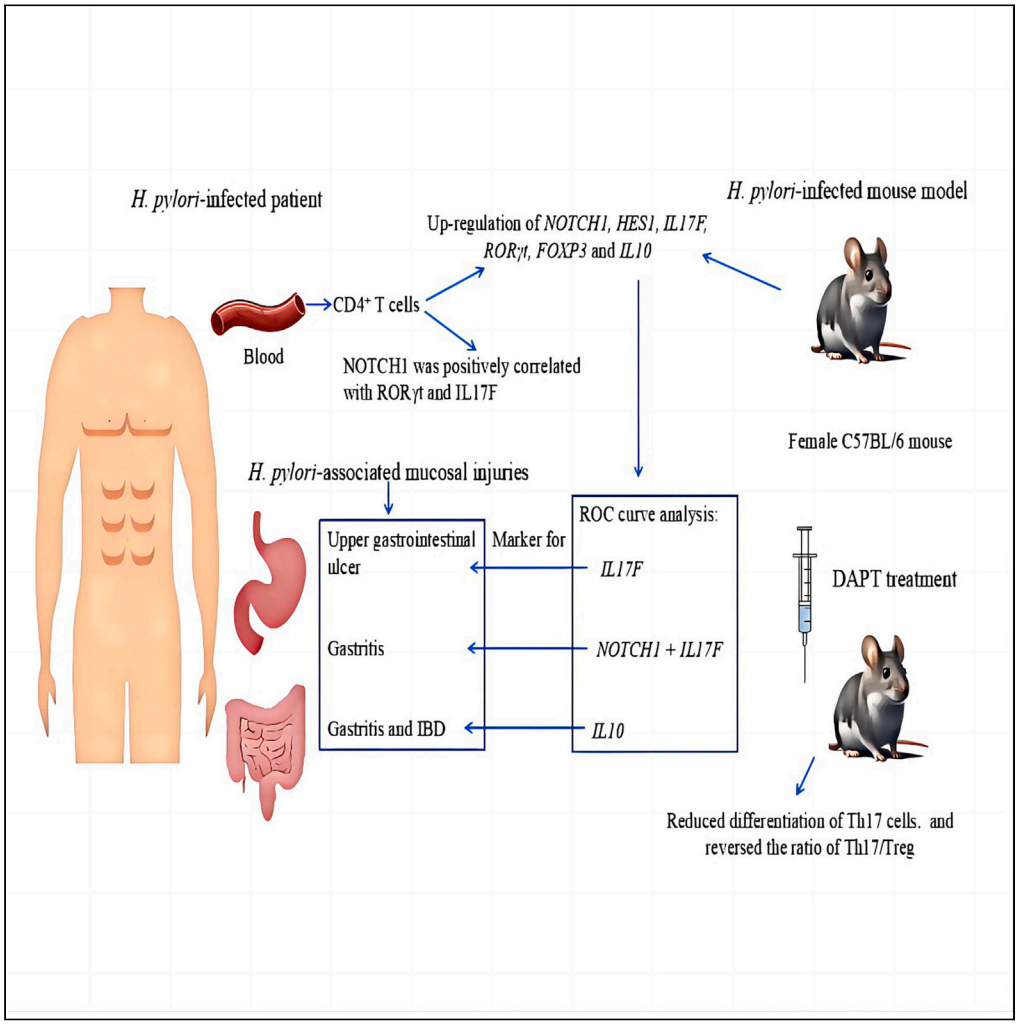

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Highlights

NOTCH1, *HES1*, *IL17F*, *FOXP3*, and IL10 mRNA was elevated during *H. pylori* infection

NOTCH1 was positively correlated with *RORγt* and *IL17F* during *H. pylori* infection

H. pylori-induced gastritis was distinguished by *NOTCH1* and *IL17F* combiROC

H. pylori-induced gastritis and IBD was discriminated by *IL10* alone

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NOTCH1 is positively correlated with *IL17F* in *Helicobacter pylori* infection and a biomarker for mucosal injury

Xie Jinling,[1,2,3] Liu Guoan,[2,3] Chen Chuxi,[1] Liu Qiaoyuan,[1] Chen Yinzhong,[2] Chen Shihao,[2] Long Huaquan,[2] He Yunxuan,[1] Ning Yunshan,[1,*] and Li Yan[1,4,*]

SUMMARY

Our study previously showed the involvement of Notch1 in Th1 differentiation in *H. pylori*-infected patients. However, the role of Notch1 in Th17 or Treg differentiation during *H. pylori* infection and the potential diagnostic value of its associated genes remain unclear. Here, we found that *NOTCH1* was positively correlated with Th17-related genes *RORγt* ($r = 0.616$, $p < 0.001$) and *IL17F* ($r = 0.523$, $p < 0.01$), but not with Treg-related genes *FOXP3* and *IL10*. The mRNA levels of aforementioned genes were upregulated at different stages of mucosal injury except for upper gastrointestinal ulcers. A combiROC analysis of *NOTCH1* and *IL17F* discriminated *H. pylori*-infected gastritis from healthy controls with high accuracy (AUC of 0.952, sensitivity of 0.929, and specificity of 0.893). This study is the first to show that Notch1 is correlated with Th17-associated gene expression during *H. pylori* infection. Additionally, *NOTCH1* and *IL17F* are potential diagnostic markers.

INTRODUCTION

The long-term colonization of *H. pylori* in stomach mucosa often causes gastritis, peptic ulcer, and even gastric cancer (GC) or mucosa-associated lymphoid tissue (MALT), and WHO categorized *H. pylori* as a class I carcinogen in 1994. Although many studies have carried out more and more in-depth research on immunological mechanisms in *H. pylori* infection, the treatment of *H. pylori* infection is still a tough issue due to the main problems of refractory infection, drug resistance, and poor patient compliance. Therefore, it is urgent to better understand the mechanism of host immune defense during *H. pylori* infection and its potential value as biomarkers.

Previous studies have suggested that the immune response of Th17 cells attracts attention in *H. pylori*-associated diseases.[1,2] Th17 cells are a class subset of Th cells discovered in recent years that are different from Th1 and Th2 cells. Cytokines produced by Th17 cells, such as IL17A, IL17F, IL22, and IL21, play an important role in resisting pathogens, especially in bacterial infection as well as in chronic inflammatory disease.[3] Th17 cells control the colonization of *H. pylori* in the stomach mucosa and are correlated with the progression of gastritis[4] and peptic ulcer.[5] The role of Treg cells in the adaptive immune response to *H. pylori*-induced inflammation and bacterial persistence is complex.[6–8] Several studies have demonstrated that Treg cells exert anti-inflammatory effects during *H. pylori* infection.[9,10] Moreover, the development of *H. pylori*-associated chronic experimental colitis can be prevented via regulating Th17/Treg balance.[11] Downregulation of Th17 cells reduces the inflammation response of stomach mucosa and activates the protective function of Treg cells.[12] Therefore, Th17/*IL17* may be a therapeutic target for the control of *H. pylori*-associated diseases.

Recently, it has been demonstrated that Notch signaling is important for regulating naive $CD4^+$ T cells differentiation into Th1,[13] Th17,[14] and Treg.[15] Notch signaling is a conserved pathway that plays a crucial role in determining cells fate, including proliferation, differentiation, and apoptosis. There are four Notch receptors (NOTCH1, 2, 3, 4) and five mammalian ligands (DLL1, 3, 4, and Jagged1, 2) in mammalians. Once bound by Notch ligands, Notch intracellular domain (NICD), the active form of Notch receptor, is released into cytoplasm via γ-secretase. NICD translocases into the nucleus and binds to the CBF1 suppressor of hairless-Lag1(CSL) to activate relevant downstream target genes, such as HES1. Our previous study also confirmed that NOTCH1 was involved in the differentiation of $CD4^+$ T cells to Th1 subset in *H. pylori*-infected patients.[16] But it is unclear whether Notch signaling contributes to the differentiation of $CD4^+$ T cells into Th17 and Treg subtypes during *H. pylori* infection, and the diagnostic efficiency of relevant factors in *H. pylori*-induced mucosal injury remains to be identified.

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Table 1. Clinical characteristics and the WBC parameters in infected and healthy participants

Characteristic	*H. pylori*$^+$	*H. pylori*$^-$	*p*-value
Numbers	29	28	
Age (years), mean ± SD	59.34 ± 13.95	49.43 ± 15.25	*
Sex (*n* [%])			
male	19(33.33)	18(31.58)	ns
female	10(17.54)	10(17.54)	
White blood cells (E+09/L), median (IQR)	6.82 (5.95, 8.6)	7.38 (5.697, 8.248)	ns
Proportion of lymphocytes (%), median (IQR)	0.29 (0.257, 0.391)	0.35 (0.314, 0.407)	*
Lymphocytes (E+09/L), median (IQR)	2.04 (1.81, 2.382)	2.49 (2.131, 2.913)	**
Proportion of Granulocytes (%), median (IQR)	0.6 (0.517, 0.668)	0.54 (0.489, 0.583)	*
Granulocytes (E+09/L), median (IQR)	3.83 (3.284, 5.254)	3.82 (3.02, 4.894)	ns
Proportion of Monocytes (%), median (IQR)	0.06 (0.05, 0.067)	0.06 (0.055, 0.071)	ns
Monocytes (E+09/L), median (IQR)	0.4 (0.307, 0.569)	0.42 (0.355, 0.571)	ns

*$p < 0.05$, **$p < 0.01$, ns, not significant.

In this study, we found that the mRNA expression of *NOTCH1*, *HES1*, *RORγt*, *IL17F*, *FOXP3*, and *IL10* were all increased in CD4$^+$ T cells of *H. pylori*-infected patients and mice. Moreover, these genes showed similar trends in change at different stages of mucosal injury except for upper gastrointestinal ulcer. *NOTCH1* was positively correlated with *RORγt* and *IL17F* in *H. pylori*-infected patients. The combination of *NOTCH1* and *IL17F* receiver operating characteristic (ROC) curve analysis was favorable for discriminating *H. pylori*-infected gastritis patients from healthy controls, while *IL10* was effective in differentiating gastritis with inflammation bowel disease (IBD) patients. Overall, this study may provide insight into evaluating mucosal injury during *H. pylori* infection, and offer an important aid in medical diagnosis and treatment.

RESULTS

The absolute number and the proportion of lymphocytes in peripheral blood of *H. pylori*-infected patients were lower than those of healthy controls

The main characteristics of *H. pylori*-infected individuals and healthy controls were listed in Table 1. Peripheral blood leukocyte parameters were obtained using an automatic hematology analyzer. The analysis revealed that no significant difference was observed in sex, the absolute number of the total white blood cells, granulocytes, and monocytes, as well as the proportion of monocytes between two groups ($p > 0.05$). However, the age, the absolute number and the proportion of lymphocytes, and the proportion of granulocytes showed significant difference ($p < 0.05$). Specifically, there was a significant reduction in both the absolute number and proportion of lymphocytes in the peripheral blood of *H. pylori*-infected patients compared to healthy controls. Conversely, an increased proportion of granulocytes in the peripheral blood were observed in the *H. pylori*-infected group in comparison to the healthy controls. However, there was no statistically significant increase in the absolute number of granulocytes. Collectively, these findings suggest a more pronounced reduction in peripheral blood lymphocytes. In fact, one study highlighted the divergent cellular dynamics between peripheral blood and gastric infiltration. This disparity underscores the complex nature of the transition from *H. pylori* infection to chronic inflammation, which is characterized by an increased predominance of lymphocytes among gastric infiltrating cells.[17] Thus, our results in this study imply a heightened likelihood of lymphocyte infiltration into the gastric environment. However, more experiments are needed to support this hypothesis.

The age difference between *H. pylori*-infected individuals and healthy control had a negligible effect on gene expression

An appreciable divergence in age was observed between *H. pylori*-infected subjects and their healthy counterparts, warranting an exploration of the potential impact of age on target gene expression patterns during *H. pylori* infection. For a comprehensive statistical investigation, logistic regression was used as the statistical method to analyze the data comprehensively.

Preliminary univariate logistic regression analysis revealed an odds ratio (95% CI) of 1.049 (1.008–1.092) with a corresponding *p* value of 0.019. Subsequently, a more complex multivariate logistic regression analysis yielded an odds ratio (95% CI) of 1.036 (0.983–1.092) with a *p* value of 0.187. There was no statistical significance. These data confirmed that although age differences existed, they did not substantially impact the expression of genes associated with *H. pylori* infection. Therefore, it is reasonable to conclude that age-related variations in gene expression may be disregarded in this study.

NOTCH1 was positively correlated with *RORγt* and *IL17F* of Th17 cells in *H. pylori*-infected patients

To explore the relationship between *NOTCH1* signaling and the differentiation of Th17 cells during *H. pylori* infection, RT-qPCR was applied to compare the mRNA expression of *IL17F*, *RORγt*, *NOTCH1*, and *HES1* in CD4$^+$ T cells isolated from peripheral blood mononuclear cells

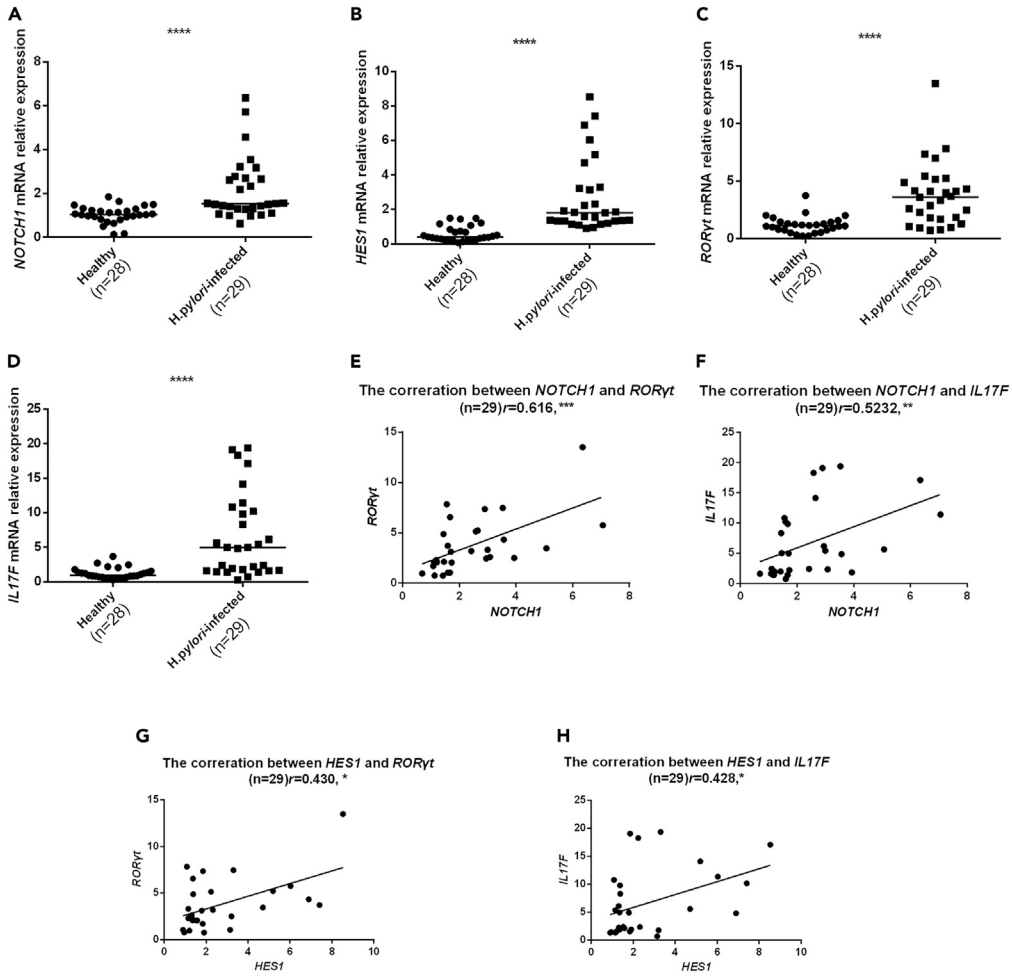

Figure 1. The expression and relationship of associated genes related to Notch signaling and Th17 cells

The mRNA expression of *NOTCH1*, *HES1*, *RORγt*, and *IL17F* in $CD4^+$ T cells isolated from PBMCs of *H. pylori*-infected group ($n = 29$) and healthy control group ($n = 28$) was measured via RT-qPCR. The results were normalized to β-*ACTIN* with the central value as the median. There were three biological replicates for each gene. The data were analyzed using the Mann-Whitney U test, and Spearman's correlation analysis was used for assessing the relationship between these genes.

(A–D) Respectively, showed the mRNA expression of *NOTCH1*, *HES1*, *RORγt*, and *IL17F*, was significantly higher in the *H. pylori*-infected group than that in the healthy control group.

(E–H) Showed the correlation between *NOTCH1* and *RORγt*, *NOTCH1* and *IL17F*, *HES1* and *RORγt*, *HES1* and *IL17F*, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

(PBMCs) between *H. pylori*-infected individuals and healthy controls. Human primer sequences are listed in Table 2. It was observed that the mRNA expression of *NOTCH1*, *HES1*, *RORγt*, and *IL17F* in *H. pylori*-infected subjects was significantly higher than that in control groups (Figures 1A-1D). Additionally, the levels of *IL17F* and *RORγt* were significantly positive correlation with *NOTCH1* (Figures 1E and 1F), and also with the *HES1* (Figures 1G and 1H). Collectively, these results demonstrate that *NOTCH1* activation is closely associated with the upregulation of key Th17 markers, *IL17F* and *RORγt*, during *H. pylori* infection.

NOTCH1 was not relevant with *FOXP3* and *IL10* of Treg cells in *H. pylori*-infected patients

To explore the correlation between NOTCH1 signaling and the differentiation of Treg cells during *H. pylori* infection, RT-qPCR was applied to compare the mRNA expression of *FOXP3* and *IL10* in $CD4^+$ T cells isolated from PBMCs between *H. pylori*-infected individuals and healthy controls. Human primer sequences are listed in Table 2. It was observed that the mRNA expression of *FOXP3* and *IL10* in *H. pylori*-infected subjects was significantly higher than that in control groups (Figures 2A and 2B). However, *NOTCH1* was not correlated with *FOXP3* or *IL10* (Figures 2C and 2D), nor was *HES1* correlated with *FOXP3* and *IL10* (data not shown). Collectively, these results demonstrate that although Treg cell-related genes are upregulated during *H. pylori* infection, *NOTCH1* does not appear to influence the expression of *FOXP3* and *IL10* of Treg cells.

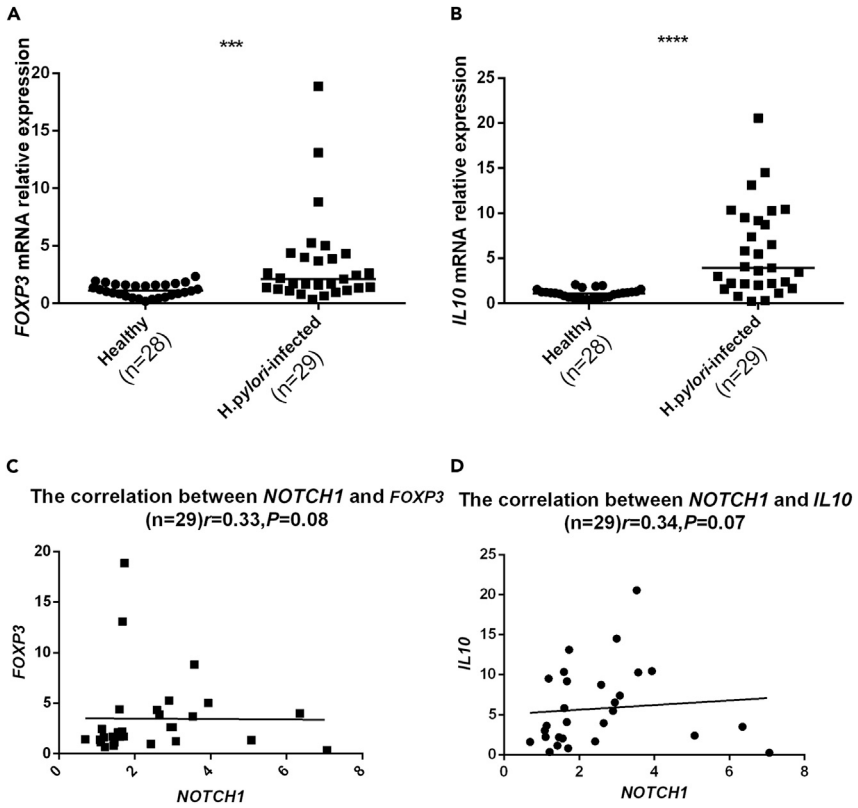

Figure 2. The expression and relationship of associated genes related to Treg cells and Notch signaling

The mRNA expression of *FOXP3* and *IL10* in CD4$^+$ T cells isolated from PBMCs of *H. pylori*-infected group (n = 29) and healthy control group (n = 28) was conducted by RT-qPCR. The results were performed by three independent replicates, and normalized to housekeeping gene β-*ACTIN*. The values were presented as medians. The data were analyzed by the Mann-Whitney U test, and Spearman's correlation analysis was used to evaluate the relationship between two genes.

(A and B) Respectively, showed the mRNA expression of *FOXP3* and *IL10*.

(C and D) Showed the correlation between *NOTCH1* and *FOXP3*, *NOTCH1* and *IL10*, respectively. ****$p < 0.0001$.

The mRNA expression of genes related to Notch signaling, Th17 cells, and Treg cells was upregulated in *H. pylori*-infected mouse models

To further verify the expression of related genes involved in this study, mouse models of *H. pylori* infection were established using C57BL/6 mice. CD4$^+$ T cells were isolated from the spleens of the mice, and the mRNA expression levels of each gene of interest were determined through RT-qPCR. Mouse primer sequences are listed in Table 3. The results showed a significant upregulation of mRNA expression levels of key genes involved in Notch signaling (*Notch1* and *Hes1*), Th17 cell differentiation (*Rorγt* and *Il17f*), and Treg cell function (*Foxp3* and *Il10*) in *H. pylori*-infected mouse models (Figure 3). Importantly, these findings were consistent with observed patterns of gene expression in *H. pylori*-infected patients, reinforcing the relevance of our findings.

Blockade of Notch signaling inhibited the differentiation of Th17 in splenic CD4$^+$ T cells from *H. pylori*-infected mice

To further confirm the relationship between Notch1 and Th17 and Treg cells, *H. pylori*-infected mice were intraperitoneally injected with DAPT to inhibit Notch signaling and the proportion of Th17 and Treg in splenic CD4$^+$ T cells were determined by flow cytometry. It was observed that *H. pylori* infection induced an increase in the percentage of Th17, but had no obvious effect on Treg and elevated the ratio of Th17/Treg; while DAPT treatment decreased the percentage of Th17 and Treg and reversed the ratio of Th17/Treg (Figure 4), suggesting that Notch signaling is associated with the differentiation of Th17 during *H. pylori* infection.

The mRNA expression of *NOTCH1*, *RORγt*, *IL17F*, *FOXP3*, and *IL10* was upregulated in CD4$^+$ T cells at different stages of mucosal injury except for upper gastrointestinal ulcer during *H. pylori* infection

To further explore the expression of *NOTCH1*, *RORγt*, *IL17F*, *FOXP3*, and *IL10* in CD4$^+$ T cells isolated from PBMCs of *H. pylori*-infected patients at different stages of *H. pylori*-associated mucosal injury, the patients were divided into four distinct groups based on their mucosal

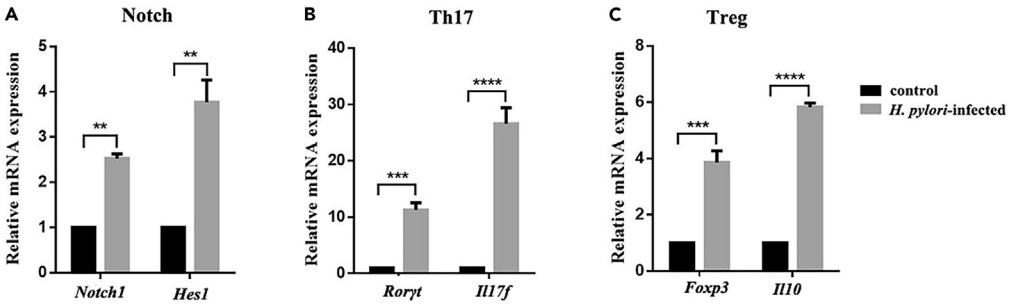

Figure 3. Genes expression in mouse models

The mRNA expression of related genes of Notch signaling (*Notch1* and *Hes1*), Th17 cell (*Rorγt* and *Il17f*), and Treg cell (*Foxp3* and *Il10*) in $CD4^+$ T cells isolated from spleen of *H. pylori*-infected mice were detected by RT-qPCR. *H. pylori*-infected mice were established by intragastric administration of *H. pylori* SS1 ($n = 3$) and PBS intragastric administration as control ($n = 3$). Results were expressed as mean ± SD of three independent replicates, normalized to the housekeeping gene β-*Actin*. The data were analyzed using the two-sample Student's t test to evaluate the difference between genes of two groups.

(A–C) Showed the mRNA expression of Notch signaling (*Notch1* and *Hes1*), Th17 cell (*Rorγt* and *Il17f*), and Treg cell (*Foxp3* and *Il10*), respectively. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

status as follows: healthy control group, gastritis group, upper gastrointestinal ulcer group (including conditions of gastric and duodenal ulcers), gastritis with IBD group. The mRNA expression of *NOTCH1*, *RORγt*, *IL17F*, *FOXP3*, and *IL10* was significantly higher in both the gastritis group and the gastritis with IBD group compared to the healthy control group (Figures 5A and 5C–5F). Furthermore, the expression of *HES1* was observed to be significantly elevated in the upper gastrointestinal ulcer group compared to the healthy group (Figure 5B). These results indicated that the mRNA expression of *NOTCH1*, *HES1*, *RORγt*, *IL17F*, *FOXP3*, and *IL10* was upregulated at different stages of mucosal injuries except in cases of upper gastrointestinal ulcers during *H. pylori* infection, highlighting the potential contribution of these genes in the immune response and pathogenesis of *H. pylori*-associated mucosal injury.

The combination of *NOTCH1* and *IL17F* ROC analysis was favorable to discriminate *H. pylori*-infected gastritis patients from healthy controls

To evaluate the diagnostic value of *NOTCH1*, *IL17F*, and *IL10* as potential biomarkers for distinguishing *H. pylori*-induced mucosal injury from healthy controls, the ROC analysis was conducted. Comparing patients with gastritis to healthy individuals, *NOTCH1* and *IL17F* showed higher diagnostic effectiveness than *IL10*. *NOTCH1* exhibited an AUC of 0.870, with a Youden index of 0.68 (95% CI: 0.74–1.00), sensitivity of 85.7%, specificity of 82.1%, and an optimal cut-off point of 1.38. *IL17F* was recorded an AUC of 0.949, with a Youden index of 0.75 (95% CI: 0.89–1.00), perfect sensitivity (100%), specificity of 75%, and a cut-off point of 1.36 (Figure 6A). However, individually, these biomarkers did not achieve ideal diagnostic accuracy. Notably, combining *NOTCH1* and *IL17F* significantly enhanced diagnostic performance, achieving an AUC of 0.952 with a Youden index of 0.82 (95% CI: 0.89–1.00), sensitivity of 92.9%, specificity of 89.3% (Figure 6D).

In patients with gastritis and IBD infected by *H. pylori*, levels of *NOTCH1*, *IL17F*, and *IL10* were significantly higher compared to healthy controls. Among them, *IL10* demonstrated the greatest diagnostic potential, perfectly distinguishing affected patients from healthy controls with an AUC, Youden index, sensitivity and specificity all equal to 1.0, and a cut-off point of 2.24 (Figure 6B). However, none of the assessed factors could perfectly differentiate patients with upper gastrointestinal ulcers from healthy individuals, as all AUC values were between 0.5 and 0.8. Interestingly, *IL17F* was most effective, differentiating patients with upper gastrointestinal ulcer from those with gastritis with an AUC of 0.804, Youden index of 0.66 (95% CI: 0.60–1.00), sensitivity of 87.5%, specificity of 78.6%, and a cut-off point of 2.39 (Figure 6C).

In summary, *NOTCH1*, *IL17F*, and *IL10* varied significantly in their ability to diagnose *H. pylori*-induced mucosal injury. The combination of *NOTCH1* and *IL17F* was particularly effective for identifying gastritis in infected patients, whereas *IL10* was most useful for diagnosing patients with gastritis and IBD. Additionally, *IL17F* proved to be the best marker for distinguishing between patients with upper gastrointestinal ulcers and those with gastritis.

DISCUSSION

Th17 cells have been recognized as key players in the context of *H. pylori* infection, with studies highlighting their potential dual roles as protective or pathogenic factors.[11,18,19] These cells secrete *IL17A* and *IL17F* cytokines, which are vital for mounting an immune response against *H. pylori*. Notably, there was a positive correlation between the upregulation of *IL17F* and *IL17A* in the gastric mucosa.[20] Additionally, *IL17F* has been implicated in promoting inflammatory reactions through the upregulation of various proinflammatory cytokines and chemokines.[21,22]

This study revealed an upregulation of *RORγt* and *IL17F* expression in $CD4^+$ T cells in *H. pylori*-infected patients and mice compared to the uninfected group, consistent with previous reports.[20] However, these results was different from the study conducted by Khatoon et al.,[23] in which a more diverse range of diseases, including gastric ulcer, duodenal ulcer, peptic ulcer, GC with intestinal and diffuse types, and functional dyspepsia. Moreover, their study did not incorporate disease subtypes in the comparison of *IL17F* mRNA expression between *H. pylori*-infected patients and non-infected subjects. Our findings will contribute to the expanding body of evidence regarding the involvement of Th17 cells in the immune response to *H. pylori* infection.

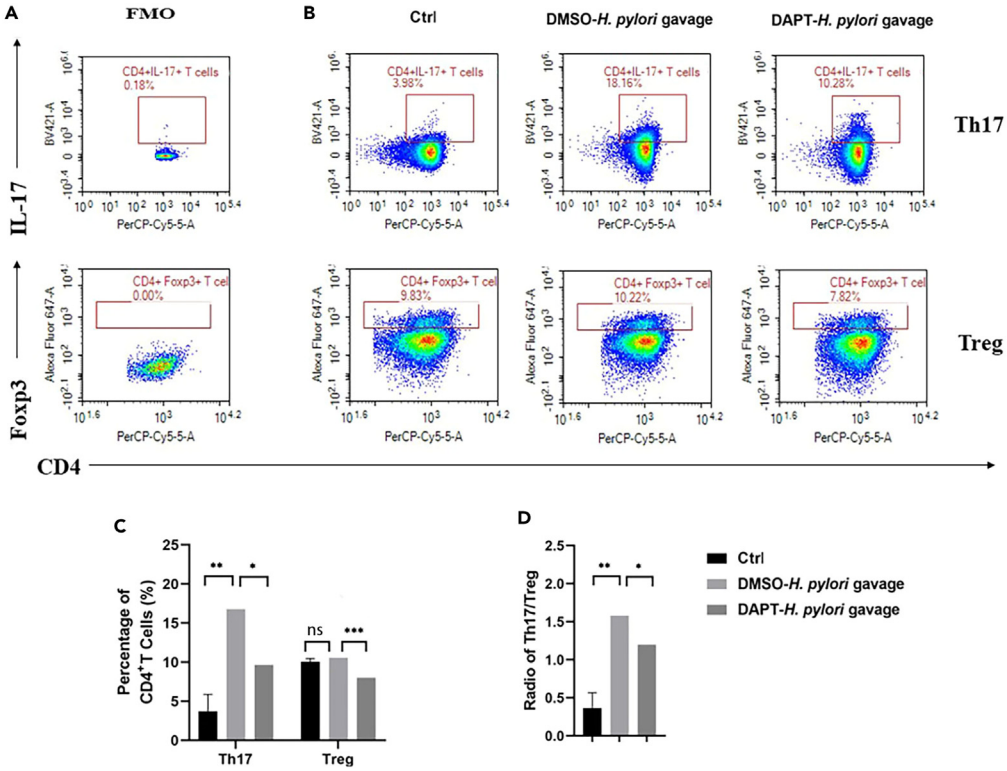

Figure 4. DAPT treatment inhibits the differentiation of Th17 in splenic CD4$^+$ T cells from *H. pylori*-infected mice

There were three mice in each experimental group. One week after the last *H. pylori* infection, *H. pylori*-infected mice were intraperitoneally administrated with DAPT (10 mg/kg) once every 48 h for 3 times. One week later, the mice were sacrificed. Splenic CD4$^+$ T cells were isolated by magnetic beads and flow cytometry was performed to analyze the differentiation of Th17 and Treg subtype. Ctrl: mice were gavaged with PBS and treated with DMSO. DMSO- *H. pylori* gavage: mice were gavaged with *H. pylori* and treated with DMSO. DAPT- *H. pylori* gavage: mice were gavaged with *H. pylori* and treated with DAPT. Three biological repeats were performed in each experimental group.

(A and B) Changes in the number of Th17 and Treg cells measured by flow cytometry in different treatments.

(C) Analysis of variability in the percentage of Th17 and Treg cells in different treatments.

(D) Analysis of changes in the ratio of Th17/Treg cells in different treatments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not statistically significant.

Notably, many studies have proved that Notch signaling plays a pivotal role in the differentiation of Th17 cells.[14,24] However, the role of Notch signaling in Th17 differentiation during *H. pylori* infection is unknown. In this study, our findings indicated a statistically significant positive correlation between the levels of *NOTCH1* and *HES1* and those of *RORγt* and *IL17F*. This interesting observation suggests that *NOTCH1* signaling may play a key role in Th17 differentiation during *H. pylori* infection. Indeed, these results represent an exciting discovery for our laboratory and we plan to integrate this study into our ongoing research.

In current study, the mRNA expression of *FOXP3* and *IL10* in CD4$^+$ T cells of *H. pylori*-infected subjects was significantly higher than that in control groups. This was consistent with a previous study on the relationship between *H. pylori* major virulence factor and Treg cells.[18] However, it differed from the study of Da Silva et al.[25] Their findings showed that there were no significant differences in the expression of *IL10* among chronic active gastritis patients, chronic inactive gastritis patients, or control cases, but the expression of *FOXP3* in *H. pylori*-infected patients was significantly lower than that in non-infected patients. This discrepancy may be the reason that Treg cells are associated with VacA alleles, virulence factor and oipA status of *H. pylori* and histological grade.[12,18] Notably, our study showed for the first time that *NOTCH1* was not relevant with *FOXP3* and *IL10* of Treg cells in *H. pylori*-infected patients. This may be due to the fact that samples were collected at the stage of disease progression and no association was captured, highlighting the complex dynamics of the immune response during *H. pylori* infection.

In this study, *H. pylori*-infected mice were constructed and the differentiation of Th17 cell was induced. Blockade of Notch signaling via DAPT *in vivo* inhibited the differentiation of Th17 subtypes and decreased the Th17/Treg ratio. These findings support the reliability of Th17-related gene expression observed in the human aforementioned, suggesting that the potential for developing Notch1 is a diagnostic target during *H. pylori* infection.

It has been reported that the interplay between Treg and Th17 cells regulates the immune response in *H. pylori*-infected gastric mucosa, and influences the persistence of *H. pylori* infection and mucosal injury.[12,26] Herein, we found that the mRNA levels of key genes of Notch signaling (*NOTCH1* and *HES1*), Th17 cells (*RORγt* and *IL17F*), and Treg cells (*FOXP3* and *IL10*) were upregulated in most stages of mucosal injury, except for upper gastrointestinal ulcers. This suggests that the dysregulation of Notch signaling and the associated Th17 and Treg

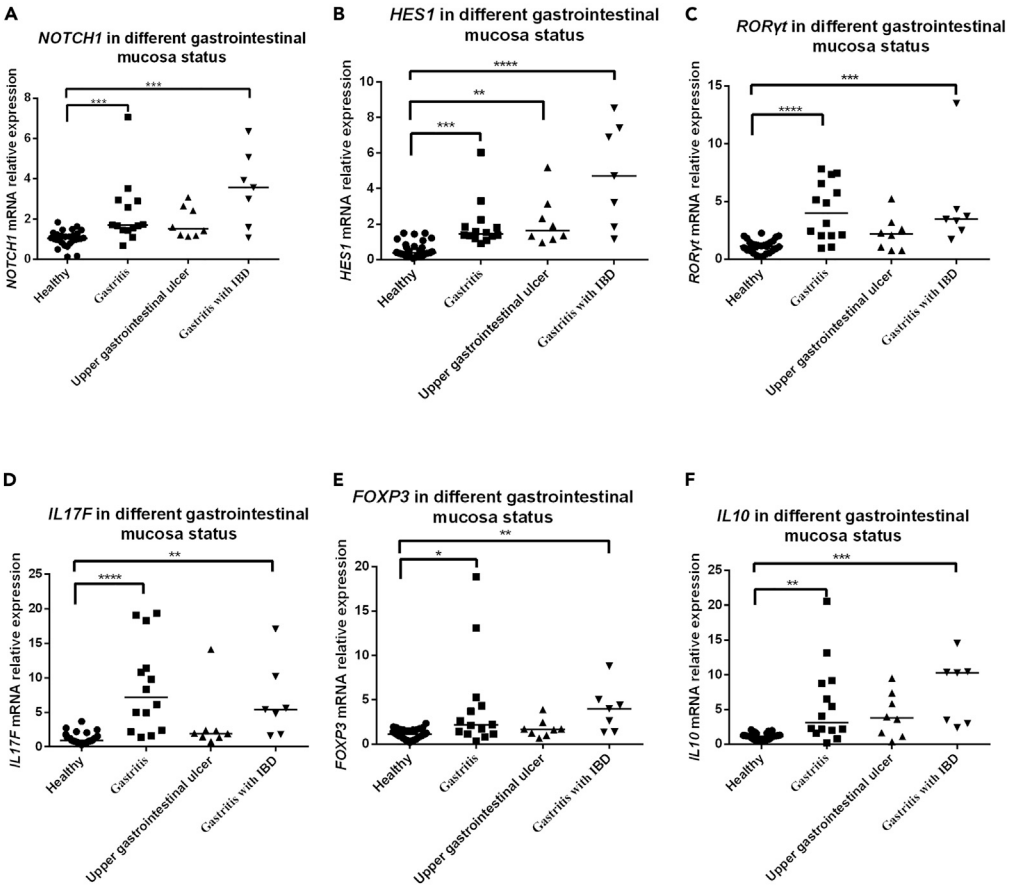

Figure 5. Genes expression at different stages of mucosal injury during *H. pylori* infection

The mRNA expression of genes associated with different stages of mucosal injury during *H. pylori* infection was compared by the Kruskal-Wallis test. $CD4^+$ T cells were isolated from PBMCs of patients with gastritis ($n = 14$), upper gastrointestinal ulcer ($n = 8$), gastritis with IBD ($n = 7$) and the control group ($n = 28$). Then the mRNA levels of related genes were assessed by RT-qPCR. The reference gene β-*ACTIN* was used for normalization of three times independent repeats. The values were expressed as medians.

(A) *NOTCH1*; (B) *HES1*; (C) *RORγt*; (D) *IL17F*; (E) *FOXP3*; (F) *IL10*. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

responses may be involved in the early stages of *H. pylori*-induced mucosal injury, but their role may diminish in later stages characterized by upper gastrointestinal ulcers. In addition, the expression of *FOXP3* and *IL10* for Treg cells was different from the result reported by Bagheri et al.,[5] who found that the number of Treg cells were inversely related to Th17 cells in mucosal tissues from patients with gastritis and peptic ulcer. This discrepancy may be due to individual differences between the two studies, or different expression in tissue and blood. On the other hand, the response of Th17 and Treg cells in gastritis and gastritis with IBD is consistent with the existing literature,[11,27,28] supporting the involvement of Th17 and Treg cells in *H. pylori*-induced gastrointestinal inflammation.

Given the pivotal roles of Notch signaling, Th17, and Treg cells in *H. pylori* infection, and the potential utility of related factors as markers for identifying *H. pylori*-induced mucosal injury remains uncertain, it is imperative to investigate their clinical relevance in diagnosis and treatment. Based on our current findings, we propose that *NOTCH1*, *IL17F*, and *IL10* may have the potential to diagnose different stages of *H. pylori*-induced mucosal injury. Then ROC analysis was used to explore the diagnostic value of *NOTCH1*, *IL7F*, and *IL10* as biomarkers. Our results demonstrated that the combination of *NOTCH1* and *IL17F* significantly enhanced diagnostic specificity and sensitivity in *H. pylori*-induced gastritis. There was also a significant increase in the Youden index, with the confidence interval ranging from 0.89 to 1.00. These results demonstrate that this biomarker combination may serve as an exemplary marker with robust potential for clinical application in disease prediction and monitoring treatment efficacy. However, further extensive data collection is required to substantiate this hypothesis. Moreover, *IL10* exhibited superior suitability for assessing gastritis with or without IBD in infected individuals, surpassing *NOTCH1* and *IL17F*, with exceptional accuracy reflected by high sensitivity, specificity, and AUC values (all equal to 1.0). This finding suggests that when the test value exceeds 2.24, it indicates mucosal injury within the gastrointestinal tract. Therefore, it is speculated that monitoring this marker before and after treatment provides critical insight into the therapeutic outcomes for patients with gastritis and IBD.

Furthermore, we observed that *IL17F* exhibited the highest diagnostic value among the three factors for distinguishing patients with upper gastrointestinal ulcers from those with gastritis. ROC curve analysis identified the optimal cut-off value of *IL17F* levels for predicting

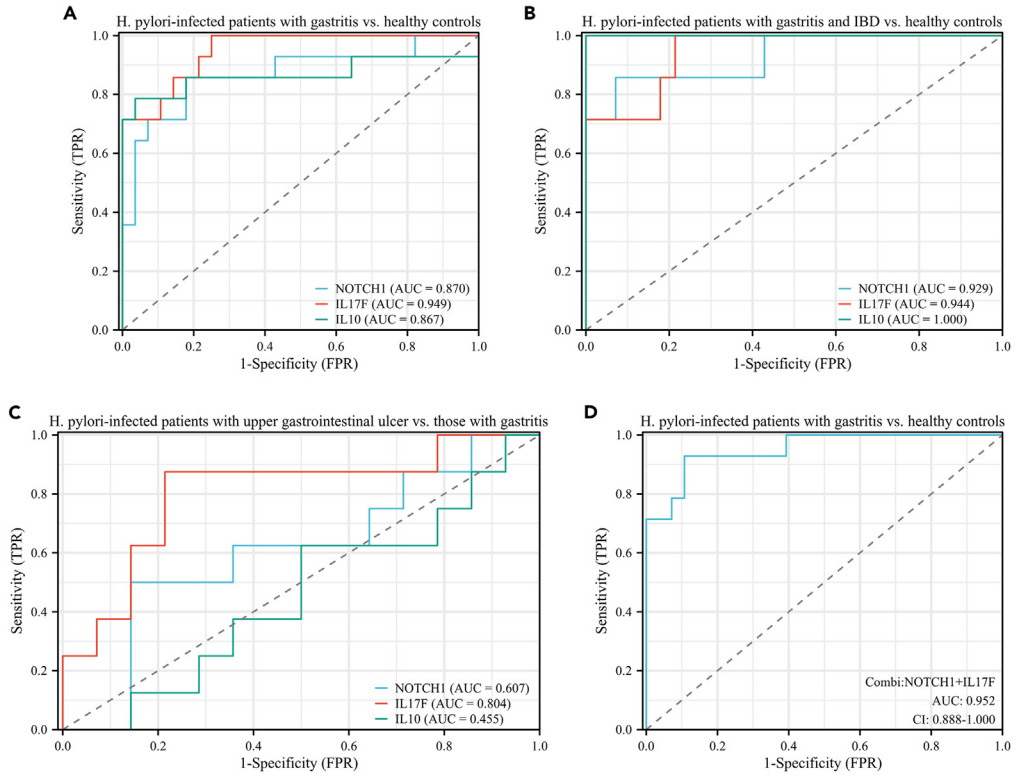

Figure 6. Analysis of the potential diagnostic value of genes

The ROC analysis was used to estimate the diagnostic value of *NOTCH1*, *IL17F*, and *IL10* in patients at various stages of mucosal injury during *H. pylori* infection.
(A–C) Represented the ROC analysis of *NOTCH1*, *IL17F*, and *IL10* in the groups of patients with gastritis vs. healthy controls, patients with gastritis and IBD vs. healthy controls, and patients with upper gastrointestinal ulcer vs. those with gastritis, respectively.
(D) The combiROC of *NOTCH1* and *IL17F* in patients with gastritis vs. healthy controls.

H. pylori-associated upper gastrointestinal ulcers. If the *IL17F* level exceeds this threshold, the patient is considered to be at high risk of developing upper gastrointestinal ulcer during the infection period. Consequently, appropriate therapeutic interventions are required to reduce the level of this biomarker in order to achieve favorable clinical outcomes, which will provide valuable guidance for clinical applications. In the future, modulation of *IL17F* levels in patients infected with *H. pylori* may represent a promising strategy to improve the prognosis of upper gastrointestinal ulcers in these individuals. Our result was similar to the study by Kumar et al.,[29] who demonstrated the discriminatory potential of *IL17A* as a standalone biomarker for distinguishing pediatric tuberculosis (TB) from non-TB cases. Additionally, other studies have explored the diagnostic value of relevant genes in various diseases. For instance, Chen et al.[30] investigated *IL10* as a biomarker for diagnosing vitreoretinal lymphoma, and Tantau et al.[31] examined the diagnostic utility of *IL17* and *IL10* as biomarkers in pancreatic adenocarcinoma and chronic pancreatitis.

To the best of our knowledge, this study was the first to investigate the clinical diagnostic potential of *NOTCH1*, *IL17F*, and *IL10* as biomarkers for different stages of *H. pylori*-induced mucosal injury. The implications of our findings are significant for the diagnosis and management of *H. pylori*-related diseases. By utilizing these biomarkers as potential indicators of mucosal injury, we lay the foundation for further research in this field and underscore the necessity for more sensitive and accurate diagnostic tools for *H. pylori* infection. Currently, the diagnosis of *H. pylori* infection associated mucosal injury relies on invasive techniques such as endoscopy and biopsy, which are expensive and uncomfortable for patients. In contrast, blood sampling offers a minimally invasive and more cost-effective approach that is generally more acceptable to patients, which may facilitate early detection and intervention of *H. pylori*-associated mucosal injury and ultimately improve the outcome of patients.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics

The studies involving human participants were reviewed and approved by the Medical Research Ethics Committee of Xinhui District People's Hospital, affiliated with Southern Medical University, Jiangmen, China. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethics Committee of the Laboratory Animal Center of Southern Medical University, Guangzhou, China.

Table 2. Primer sequences for Homo sapiens used in RT-qPCR experiments

Gene	Primer sequences for Homo sapiens 5′-3′	Reference
NOTCH1	F:5′-CACTGTGGGCGGGTCC-3′	Estrach et al.[39]
	R:5′-GTTGTATTGGTTCGGCACCAT-3′	
HES1	F:5′-CGTGTCTCCTCCTCCCATT-3′	PrimerBank
	R: 5′-GAGAGGTAGACGGGGGATTC-3′	
IL17F	F: 5′-TCGGAAAATCCCCAAAGTAGGAC-3′	Designed
	R:5′-ATTGATGATGCCAATGTCAAGC-3′	
RORγt	F: 5′-AAGGCACTTAGGGAGTGGGAGA-3′	Nogueira et al.[40]
	R: 5′-GGCAAATACGGTGGCATGG-3′	
FOXP3	F: 5′-GAGAAGGGCAGGGCACAAT-3′	Nogueira et al.[40]
	R: 5′-TGGGCCTGCATGGCAC-3′	
IL10	F: 5′-ACCTGCCTAACATGCTTCGAG-3′	Designed
	R: 5′-CCAGCTGATCCTTCATTTGAAAG-3′	
β-*ACTIN*	F:5′-CATGTACGTTGCTATCCAGGC-3′	Liu et al.[41]
	R:5′-CTCCTTAATGTCACGCACGAT-3′	

Limitations of the study

Several limitations should be acknowledged in this study. Firstly, the size of the dataset used is small. Thus, further research is needed to be validated with randomized trials and larger sample sizes in future studies to fully explore the clinical implications of our findings. It is crucial to investigate the expression of the three biomarkers before and after different treatment regimens to assess their diagnostic potential and therapeutic response. Additionally, longitudinal studies that track the dynamic changes in biomarker expression throughout the course of *H. pylori* infection and mucosal injury will provide valuable insights into their temporal profiles and clinical significance. Secondly, to obtain comprehensive data on the role of Notch1 signaling, it is important to assess *NOTCH1* levels after successful treatment of *H. pylori*-infected individuals, which will provide insight into the impact of treatment on *NOTCH1* expression and its potential as a prognostic marker. Moreover, in-depth studies are warranted to elucidate the mechanistic role of *NOTCH1* in Th17 differentiation and its interplay with other signaling pathways during *H. pylori* infection, thereby deepening our understanding of the relevant immunopathogenesis.

STAR★METHODS

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Table 3. Primer sequences for Mus musculus used in RT-qPCR experiments

Gene	Primer sequences for Mus musculus 5′-3′	Reference
Mice *Notch1*	F:5′-TATCGTGCGGCTTTTGGAT-3′	Designed
	R:5′-GATTGCCCAGGTAGCCATTG-3′	
Mice *Hes1*	F:5′-CCAGCCAGTGTCAACACGA-3′	Chiremba and Neufeld[42]
	R:5′-AATGCCGGGAGCTATCTTTCT-3′	
Mice *Rorγt*	F:5′-CCGCTGAGAGGGCTTCAC-3′	Ma et al.[43]
	R:5′-TGCAGGAGTAGGCCACATTACA-3′	
Mice *Il17f*	F:5′-TGCTACTGTTGATGTTGGGAC-3′	Dong et al.[44]
	R:5′-AATGCCCTGGTTTTGGTTGAA-3′	
Mice *Foxp3*	F:5′-CCCATCCCCAGGAGTCTTG-3′	Hou et al.[45]
	R:5′-ACCATGACTAGGGGCACTGTA-3′	
Mice *Il10*	F:5′-ATTTCCGATAAGGCTTGGCAA-3′	Designed
	R:5′-GCTGGACAACATACTGCTAACC-3′	
Mice β-*Actin*	F:5′-GCAGGAGTACGATGAGTCCG-3′	Chuvin et al.[46]
	R:5′-ACGCAGCTCAGTAACAGTCC-3′	

AUTHOR CONTRIBUTIONS

Conceptualization, L.Y., N.Y., and X.J.; methodology, X.J., L.G., C.C., L.Q., and H.Y.; formal analysis, X.J., L.G., and C.C.; investigation, L.G., C.Y., C.S., and L.H.; resources, L.Y., N.Y., X.J., and L.G.; data curation, L.Y., X.J., and C.C.; writing—original draft, X.J. and L.G.; writing—review & editing, L.Y. and N.Y.; visualization, X.J. and C.C.; supervision, L.Y., N.Y., and L.H.; funding acquisition, L.Y. and N.Y. All authors made significant contributions to the article and approved the final version for submission.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human CD4$^+$ T cells magnetic beads	Miltenyi Biotec, Palo Alto, CA, USA	#130-096-533
Mouse CD4 (L3T4) MicroBeads-MACS	Miltenyi, Bergisch Gladbach, Germany	#130-117-043
APC-Cy™7-anti-mouse CD3	BD Biosciences, San Diego, CA, USA	#557596
PerCP-Cy™5.5-anti-mouse CD4	BD Biosciences, San Diego, CA, USA	#550954
PE-Cy™7-anti-mouse CD25	BD Biosciences, San Diego, CA, USA	#552880
Brilliant Violet 421™-anti-mouse IL-17A	BD Biosciences, San Diego, CA, USA	#564170
Alexa Fluor® 647-anti-mouse Foxp3	BD Biosciences, San Diego, CA, USA	#563486
Biological samples		
Human blood samples	Xinhui District People's Hospital	N/A
Single cells from spleen of mice	Southern Medical University	N/A
Chemicals, peptides, and recombinant proteins		
DAPT	Selleck, Texas, Houston, USA	#S2215
DMSO	MP, California, USA	#196055
RNAiso Plus	Takara, Dalian, China	#9109
Critical commercial assays		
HiScript® III All-in-one RT SuperMix Perfect for qPCR kit	Vazyme, Nanjing, China	#R333
ChamQ Univesal SYBR qPCR Master Mix kit	Vazyme, Nanjing, China	#Q711
Color Reverse Transcription Kit (with gDNA Remover)	EZBioscience, Roseville, MN, USA	A0010CGQ
Color SYBR Green qPCR Mix	EZBioscience, Roseville, MN, USA	A0012-R2
Deposited data		
Clinical characteristics of *H. pylori*-infected patients data	This paper	Shared upon request by the Lead Contact
RT-qPCR data	This paper	Shared upon request by the Lead Contact
Flow cytometry analysis data	This paper	Shared upon request by the Lead Contact
Experimental models: Organisms/strains		
H. pylori-infected mouse models	Female C57BL/6 mice, 6-8 weeks old	From the Experimental Animal Center of Southern Medical University: SCXK (Guangdong) 2021-0041
Oligonucleotides		
Primer sequences for Homo sapiens, see Table 2	This paper	
Primer sequences for Mus musculus, see Table 3	This paper	

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
GraphPad Prism 6.0	La Jolla, CA, USA	https://www.graphpad.com
R language (Version 4.2.1)	R package: pROC [1.18.0], ggplot2 [3.3.6]	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and information should be directed to and will be fulfilled by lead contact, Li Yan, (luckyan@smu.edu.cn).

Materials availability

This study did not generate new unique materials.

Data and code availability

The datasets generated in this study is available upon request from Li Yan, (luckyan@smu.edu.cn). No original code is reported in this paper.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Experimental model

For this study, female C57BL/6 mice, 6-8 weeks old (weighing 14-16 g), were obtained from the Experimental Animal Center of Southern Medical University. The animal license number for this study was SCXK (Guangdong) 2021-0041, and the experimental protocols were approved by the Ethics Committee of the Laboratory Animal Center of Southern Medical University. *H. pylori*-infected mice were established by intragastric administration of *H. pylori* SS1 (n=3) and PBS intragastric administration as control (n=3). All experimental procedures were performed in strict accordance with the guidelines of the Guide for the Husbandry and Use of Laboratory Animals issued by the National Institute of Health of China. Stringent measures were implemented to minimize animal discomfort and stress throughout the study.

Study participants

The study participants were recruited from Xinhui District People's Hospital, which is affiliated with Southern Medical University in Jiangmen, Guangdong, China. Recruitment took place between May 2021 and February 2022. A total of 57 subjects were enrolled in this study. Of these, 29 *H. pylori*-infected patients and 28 healthy controls were 18 years old or older.Clinical information for all of these participants, including information on age and sex, is detailed in Table 1. In this study, participants were analyzed using biological sex only. No data were collected on participants' gender identity, ancestry, race, ethnicity, or socioeconomic status. The study protocol adhered to the guidelines set forth by the Medical Research Ethics Committee of Xinhui District People's Hospital, affiliated with Southern Medical University, Jiangmen, China. And ethical approval was obtained (Medical Research Ethics Committee Review 2020 No. 061). All subjects signed the informed consent form (No. 202012-01) and underwent C^{14} urea testing for *H. pylori* infection. Simultaneously, they were treated with sinus forceps under gastroscopy and detected with a rapid urease test. The patients were diagnosed with *H. pylori* infection when both results were positive. Exclude patients with serious chronic diseases and those who have been treated with antibiotics or proton pump inhibitors (PPIs) within 1-2 weeks prior to testing. All healthy controls were free from any active disease and infection through clinical examination.

The positive patients enrolled in this study presented with different gastrointestinal symptoms, including some bowel-related manifestations. Consequently, the clinicians performed targeted endoscopic examinations and mucosal biopsies based on the symptoms of patients. Endoscopic findings, histopathologic changes in the gastric mucosa and relevant indicators such as the extent and distribution of gastritis were evaluated in the classification process. Chronic gastritis grading was conducted using the updated Sydney System visual analog scoring method[32] and the Chinese Consensus Opinion on Chronic Gastritis, established in Shanghai in 2017.[33] The diagnosis of peptic ulceration adhered to the recognized Chinese standards for diagnosing and treating peptic ulcers.[34] Gastric ulcers and duodenal ulcers were considered as upper peptic ulcers within the context of this study. The Chinese Consensus Opinion on the Diagnosis and Treatment of inflammation bowel disease (IBD)[35] was used for the diagnosis of IBD. In this study, both ulcerative colitis and Crohn's disease were included.

METHOD DETAILS

Human blood sample preparation and $CD4^+$ T cells isolation

Venous blood (12 ml) was collected by venipuncture from *H. pylori*-infected individuals and healthy controls, and placed into tubes containing EDTA-K2 (BD Vacutainer, New Jersey, USA). Of the 12 mL, 10mL was collected for isolation of peripheral blood mononuclear cells (PBMCs), while the remaining 2mL was used for counting peripheral blood cells by using the Sysmex XN2000 Automatic Hematology Analyzer (Sysmex,

Tokyo, Japan). PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation (GE Healthcare Bio-Sciences AB, Hamburg, Germany) at 400 ×g for 30min at 20˚C. $CD4^+$ T cells were purified from PBMCs by human $CD4^+$ T cells magnetic beads (Miltenyi Biotec, Palo Alto, CA, USA). The purity for $CD4^+$ T cell isolation preparations was > 95% as determined by flow cytometry.

Establishing *H. pylori*-infected mouse models and isolating splenic $CD4^+$ T cells

To establish the animal models, the mice were housed under controlled conditions with a 12-hour light-dark cycle at a room temperature of 25 ± 1°C and relative humidity of 50 ± 10%. The animals were acclimated to their housing conditions for 1 week before the experiments. Then, C57BL/6 mice were given a gavage of *H. pylori* SS1 suspension following the standardized protocol previously described by Lee et al.[36] and Dey et al..[37] The control group received oral gavage with phosphate-buffered saline (PBS). The experimental group of mice received oral gavage with gastric antibiotic mixture for a period of 5 consecutive days. Following a 2-day interval, the mice were orally administered 0.1 mL of 50% alcohol to induce gastric mucosal damage. One day after the alcohol administration, the mice were given 0.2 mL of 2% $NaHCO_3$ by orally gavage. Subsequently, after a 2-hour interval, the mice received oral gavage with a solution containing 5×10^8 colony-forming units (CFU) of *H. pylori*, and this gavage procedure was repeated every 24 hours for a total duration of 2 weeks.

Within 3 weeks following the last gavage, the mice were euthanized via cervical dislocation. The spleens were aseptically removed and mechanically crushed. $CD4^+$ T cells were isolated from the splenocytes using the mouse $CD4^+$ T cell magnetic beads kit (Miltenyi Biotec, Palo Alto, CA, USA) according to the manufacturer's instructions. The purity for $CD4^+$ T cell isolation preparations was > 95% as determined by flow cytometry.

N-[N-(3,5-Difluorophenacetyl)-L-Alanyl]-S-phenylglycinet-butyl Ester (DAPT) treatment

One week after the last intragastric gavage of *H. pylori*, infected mice were administrated intraperitoneally with DAPT (10 mg/kg) once every 48 h for 3 times. DMSO was administrated as a control. There are three mice in each experimental group.

Flow cytometry analysis

Single cells were harvested by grinding spleen of mice, filtered with 70 μm cell strainer, and treated with Red Blood Cell Lysis buffer (Solarbio, Beijing, China). Splenic $CD4^+$ T cells were sorted by Mouse CD4 (L3T4) MicroBeads-MACS (Miltenyi, Bergisch Gladbach, Germany) with the purity of more than 90% determined by flow cytometry. Then harvested $CD4^+$ T cells were restimulated with Leukocyte Activation Cocktail containing BD GolgiPlug (BD Biosciences, San Diego, CA, USA) for 6 h and incubated with FcR blocking reagent for 15 min. Next, cells were surface stained using APC-Cy™7-anti-mouse CD3 (BD Biosciences, San Diego, CA, USA), PerCP-Cy™5.5-anti-mouse CD4 (BD Biosciences, San Diego, CA, USA) and PE-Cy™7-anti-mouse CD25 (BD Biosciences, San Diego, CA, USA) for 30 minutes on ice. After that, cells were fixed with Transcription Factor Buffer Set (BD Biosciences, San Diego, CA, USA) followed by intracellular staining for IL-17A and Foxp3 using intracellular antibodies Brilliant Violet 421™-anti-mouse IL-17A (BD Biosciences, San Diego, CA, USA) and Alexa Fluor® 647-anti-mouse Foxp3 (BD Biosciences, San Diego, CA, USA). Samples were run on Fortessa (BD Biosciences, New Jersey, USA) and analyzed with the FlowJo software.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from $CD4^+$ T cells using the RNAiso Plus kit (Takara, Dalian, China) and treated with RNase-free DNase (Solarbio, Beijing, China) to eliminate any residual genomic DNA. For human samples, the HiScript® III All-in-one RT SuperMix Perfect for qPCR kit (Vazyme, Nanjing, China) was employed for reverse transcription following the manufacturer's instructions. In the case of mouse samples, the Color Reverse Transcription Kit (with gDNA Remover) (EZBioscience, Roseville, MN, USA) was used.

Quantitative polymerase chain reaction (qPCR) was performed to measure the mRNA levels of *NOTCH1*, *HES1*, *RORγt*, *IL17F*, *FOXP3*, and *IL10*. For human samples, qPCR was carried out using the ChamQ Univesal SYBR qPCR Master Mix kit (Vazyme, Nanjing, China). In the case of mouse samples, the Color SYBR Green qPCR Mix (EZBioscience, Roseville, MN, USA) was utilized. For human samples, the qPCR reactions were conducted in an ABI-7500 Q-PCR System (Applied Biosystems, Foster, CA, USA) under the following conditions: initial denaturation at 95°C for 30 seconds, followed by 45 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 40 seconds. Mouse samples were subjected to an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 30 seconds.

Each experiment was independently repeated at least three times to ensure the reliability of the results. The primer sequences used for Homo sapiens and Mus musculus are provided in Tables 2 and 3, respectively. Of these, the primer sequence for the human *HES1* gene was obtained and validated from PrimerBank. The primer sequences for human *IL17F* and *IL10* genes, as well as for mouse *Notch1* and *Il10* genes, were previously designed and validated by our laboratory. References for the primer sequences of other genes are listed in the tables. The relative fold change in mRNA levels of the target genes was determined using the $2^{\wedge\text{-}\Delta\Delta Ct}$ method,[38] with β-*ACTIN* serving as an internal control gene for normalization purposes. Triplicate datasets were used to calculate averages and standard deviations (SDs). Specifically, the formula this study used to calculate ΔΔCt was as follows: ΔΔCt = (Ct target - Ct internal control) experimental group - (Ct target - Ct internal control) control group.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed by GraphPad Prism 6.0 and R language (Version 4.2.1). Categorical variables were presented as numbers and percentages, and the χ^2 test was performed. For normal distribution continuous variables, data were presented as mean $\pm$ SD and Student's t-test was performed. Otherwise, data were presented as median (1/4 quartile - 3/4 quartile) and Mann-Whitney U test was performed. For the non-parametric independent sample, Spearman correlation analysis was applied to assess the relationship between two genes. One-way analysis of variance (ANOVA) was performed to compare multiple factors within groups using Kruskal-Wallis test. Association test was performed using logistic regression. ROC curves analysis was utilized to predict diagnostic efficiency of related genes. The optimal cut-off point for each biomarker was determined using the Youden index, which maximizes the sum of sensitivity and specificity. For animal studies, the data were presented as the mean $\pm$ SD. All experiments were repeated at least three times for each treatment group. Comparisons between groups were assessed by two-sample Student's t-test. Differences were considered to be statistically significant when the *p*-value was < 0.05. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, not statistically significant.