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HIF-1α-Induced GPR171 Expression Mediates CCL2 Secretion by Mast Cells to Promote Gastric Inflammation During *Helicobacter pylori* Infection

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ABSTRACT

Background: *Helicobacter pylori* (*H. pylori*) infection is one of the most important risk factors for chronic gastritis, gastric ulcers, and gastric cancer. Mast cells act as a crucial regulator in bacterial infection. The mechanisms underlying mast cell activation and their role in *H. pylori* infection remain poorly understood.

Materials and Methods: In gastric mucosal tissue, the number of mast cells, G-protein-coupled receptor 171 (GPR171) and CCL2 expression were detected by immunohistochemistry (IHC) or immunofluorescence between *H. pylori*-negative and *H. pylori*-positive patients. Mast cells were co-cultured with *H. pylori*, and transcriptome sequencing, RT-qPCR, and Western blotting (WB) were performed to identify receptors involved in mast cell activation. WB, chromatin immunoprecipitation (ChIP), and dual-luciferase reporter assays were conducted to investigate the molecular mechanism by which HIF-1α regulates GPR171 expression. Lentiviral knockdown, ELISA, WB, and IHC were used to evaluate the role of GPR171 during *H. pylori* infection. An in vivo mouse model of *H. pylori* infection was employed to assess the effects of GPR171 blockade on CCL2 expression and gastric mucosal inflammation.

Results: In the study, we found that mast cell numbers were greatly increased and correlated with the severity of inflammation in *H. pylori*-infected patients. We found a new receptor, GPR171, was upregulated and involved in mast cell activation upon *H. pylori* infection. Furthermore, *H. pylori* infection induced the expression of GPR171 by promoting the activation of hypoxia-inducible factor 1 alpha (HIF-1α), which directly bound to hypoxia response elements in the GPR171 promoter and regulated its transcriptional activity. Blockade or loss of GPR171 in mast cells partially inhibited CCL2 secretion via the ERK1/2 signaling pathway. In the human gastric mucosa, CCL2 derived from mast cells was associated with gastric inflammation during *H. pylori* infection. In vivo murine studies indicated that *H. pylori* infection significantly upregulated CCL2 expression, while GPR171 inhibition partially reduced CCL2 levels and alleviated gastric mucosal inflammation.

Conclusions: We provide a novel mechanism that *H. pylori* activates mast cells to promote gastric inflammation.

Hanmei Yuan and Yuetong Li share co-first authorship.

1 | Introduction

Helicobacter pylori, a gram-negative bacterium, colonizes the gastric mucosa of approximately half of the global population [1]. Long-term colonization by *H. pylori* increases the risk of gastritis, peptic ulcers, and gastric cancer [2, 3]. Innate and adaptive immune responses are closely engaged in the acute and chronic inflammation induced by *H. pylori* infection [4, 5]. A few studies have found that the number of mast cells, an innate immune cell, is obviously increased in *H. pylori*-infected patients, and these cells may act as a crucial regulator in *H. pylori*-induced gastritis [6, 7].

Mast cells reside mainly in the skin and in the mucosa of the gastrointestinal tract and airways, and they are well known for their crucial roles in allergies and asthma [8]. However, it has been demonstrated that mast cells are closely involved in pathogenic infections and inflammation because of their strategic location and capacity to release various mediators [9–11]. Upon stimulation, mast cells release preformed cytoplasmic granules in a few minutes and synthesize a variety of inflammatory mediators de novo [12, 13]. These effector molecules have a wide range of biological activities in response to pathogen infection [14–16]. It is well established that mast cell activation depends on the expression of various receptors [12, 17, 18]. The most studied receptor is FcεR1, which triggers mast cell activation via cross-linking of IgE in allergic diseases [19]. Mast cells are also activated via IgE-independent receptors, such as Toll-like receptors (TLRs), G-protein-coupled receptors (GPCRs) and chemokine receptors [20, 21]. A variety of substances can activate mast cells, including pathogens, endogenous peptides, and cytokines [22]. Degranulation of mast cells was observed in *H. pylori*-infected gastric biopsy samples by electron microscopy but not in uninfected patients [23], which suggested that *H. pylori* can activate mast cells either directly or indirectly. On the one hand, *H. pylori* infection can lead to the production of cytokines or chemokines that can activate mast cells through corresponding receptors such as ST2 (IL-33 receptor) [24]. On the other hand, our previous work and other studies have shown that *H. pylori* and its derivatives can directly induce mast cell degranulation and the release of proinflammatory cytokines [25, 26]. However, in response to *H. pylori* and its metabolites, the specific receptors involved in mast cell activation remain unclear.

Our transcriptome analysis revealed that GPR171 expression was significantly upregulated in mast cells upon *H. pylori* infection. GPR171 belongs to the GPCR superfamily and is homologous to P2Y receptors [27]. BigLEN is a neuropeptide composed of 16 amino acids and is abundantly expressed in the brain [28]. Early studies revealed that GPR171 could be activated by BigLEN to regulate feeding and mood-related behaviors [29–31]. A recent study indicates that GPR171 acts as an immune checkpoint receptor that contributes to the suppression of T-cell immunity [32]. GPR171 is also upregulated and is associated with tumor proliferation and progression in lung cancer [33]. According to a study, GPR171 can interact with bacterial metabolites, especially lipids [34]. These properties make it possible for GPR171 to participate in the cellular immune response to bacterial infection.

HIF is a heterodimeric transcription factor consisting of an α subunit and a β subunit. The α subunit is affected by oxygen concentration, while the β subunit in the nucleus is not affected by hypoxia. HIF-1α is rapidly degraded through the ubiquitin-proteasome pathway mediated by von Hippel–Lindau protein(pVHL)under normal oxygen conditions [35]. Under hypoxic conditions, the activity of proline hydroxylases (PHDs) is inhibited due to the lack of oxygen substrate, leading to reduced degradation of HIF-1α, which results in increased accumulation of HIF-1α and its transfer to the nucleus [36]. HIF-1α was initially identified for its ability to bind to the promoter sequence of erythropoietin to regulate its transcription, thereby significantly upregulating erythropoietin expression [37]. Nowadays, an increasing number of hypoxia-inducing genes regulated by HIF-1α have been discovered.

In the present study, the receptor GPR171 was upregulated and involved in mast cell activation upon *H. pylori* infection. GPR171 was identified as a novel hypoxia-induced gene, and HIF-1α upregulated GPR171 by binding to the hypoxia response element (HRE) in the GPR171 promoter, thereby regulating its transcription. *H. pylori*-induced mast cells to secrete CCL2 through the GPR171/ERK1/2 signaling pathway. CCL2 secretion mediated by GPR171 may contribute to gastric inflammation in *H. pylori* infection. These findings provide new insights into the mechanisms by which *H. pylori* activate mast cells to promote gastritis.

2 | Materials and Methods

2.1 | Patients and Specimens

Gastric biopsy specimens were collected from 116 adult patients who underwent upper esophagogastroduodenoscopy. Information on the degree and type of gastritis was obtained from the pathology department. The C-14 Urea Breath Test and serum antibody typing tests were used to determine *H. pylori* infection status. Informed consent was obtained from each patient. This study was approved by the Ethics Committee of The Eighth Affiliated Hospital of Sun Yat-sen University.

2.2 | Animals and Infection

SPF female C57BL/6J mice, 6–8 weeks old, were purchased from and housed at the Experimental Animal Center of Sun Yat-Sen University. MS21570, a GPR171 antagonist, was diluted in 10% DMSO in saline. Mice were injected with MS21570 (5 mg/kg, i.p.) or Vehicle (10% DMSO) prior to gavage [38]. The mice were fasted and then gavaged with 3×10^8 CFU of PMSS1 *H. pylori* or *Skirrow* broth, once a day for three days. Experimental procedures were approved by the Medicine Animal Care Committee of the Eighth Affiliated Hospital of Sun Yat-sen University.

2.3 | Determination of Gastric Mucosal Inflammation and *H. pylori* Colonization

The mice were euthanized at 4 weeks post-infection. One part of the gastric tissue was fixed for hematoxylin and eosin staining, and inflammation scores were evaluated by 2 independent

pathologists using selected criteria [39]. The remaining portion was used to extract DNA and RNA. Bacterial genomic DNA was extracted from mouse stomach tissue using the TIANamp Bacteria DNA Kit (TIANGEN, DP302). The colonization level of *H. pylori* in mice was determined by measuring *H. pylori*-specific 16S rDNA using a specific primer and probe (Table S1), following previously described methods [40, 41].

2.4 | Cell Culture

RBL-2H3 cells were obtained from Pricella Life Science & Technology Co. Ltd. (Wuhan, China). HMC-1 and RBL-2H3 cells were cultured in complete RPMI or MEM, consisting of the base medium, fetal bovine serum, and penicillin–streptomycin. All cells were maintained in a carbon dioxide incubator (21% oxygen and 5% CO_2), unless otherwise stated.

2.5 | Bacterial Culture and Mast Cell Stimulation In Vitro

H. pylori strain 11637 was cultured as previously described [42]. Briefly, bacteria were grown on brain heart infusion plates for 2 days at 37°C under microaerophilic conditions. RBL-2H3 cells (2×10^5 cells/mL) were seeded into 6-well plates for 24 h, and the culture medium was replaced with antibiotic-free medium. HMC-1 cells were cultured in antibiotic-free medium for 3 h. For the cell infection experiments, *H. pylori* was co-cultured with RBL-2H3 or HMC-1 cells at a multiplicity of infection (MOI) of 100 for 6 h, unless otherwise specified. BigLEN was co-cultured with RBL-2H3 cells for 3 h. When needed, the cells were pretreated with PX-478, U0126, and MS21570 for 2 h, followed by coincubation with *H. pylori*.

2.6 | Degranulation Assay

RBL-2H3 cells or HMC-1 cells were exposed to *H. pylori* or compound 48/80 in Tyrode's buffer for 60 min at 37°C. The supernatant was collected for subsequent analysis. The cells were washed twice with PBS and then solubilized with 0.1% Triton-X-100. The supernatants and cell lysates were incubated with p-nitrophenyl N-acetyl-β-D-glucosaminide (pNAG; Sigma-Aldrich) in 0.1 M sodium citrate buffer (pH 4.5). The absorbance was measured at 405 nm. The release of β-hexosaminidase was calculated as a percentage.

2.7 | RNA-Seq and Transcriptional Analysis

Total RNA from uninfected and *H. pylori*-infected RBL-2H3 cells was used for library construction and sequencing via the Illumina Sequencing Workflow (Novogene). Differential expression analysis between the *H. pylori* infection and control groups was performed using DESeq2 software (version 1.20.0). A threshold of adjusted $p \leq 0.05$ and $|\log_2 \text{ fold change}| \geq 1$ was applied to identify differentially expressed genes (DEGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted using ClusterProfiler (version 3.8.1). Gene Set Enrichment Analysis (GSEA) was performed using the following website: http://www.broadinstitute.org/gsea/index.jsp.

2.8 | Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from mast cells and gastric mucosal tissues using TRIzol (Invitrogen), and cDNA was synthesized using a kit. PCR amplification was performed using a system consisting of cDNA, SYBR Green PCR Master Mix, and primers on a LightCycler 480 II (Roche). The sequences of the primers used are listed in Table S1.

2.9 | Intracellular Reactive Oxygen Species (ROS) Detection

ROS levels were detected using a test kit (E-BC-K138-F, Elabscience, Wuhan, China). Briefly, RBL-2H3 cells were incubated with a fluorescent probe at 37°C for 30 min, followed by incubation with *H. pylori* for 3 h. After washing, fluorescence intensity was measured using a microplate reader (Spark, Tecan, Switzerland) with Ex/Em = 485 nm/525 nm.

2.10 | Cytokine Measurement by ELISA

The cell culture supernatants were centrifuged at 500 g for 20 min to remove debris, and the clarified supernatants were stored at −80°C until analysis. The levels of CCL2 were measured using ELISA kits (EK387, MULTI SCIENCES BIOTECH, China; and 1117392, Dakewe Biotech, China).

2.11 | Western Blot Analysis

The PBS-washed cells were lysed in RIPA buffer containing protease inhibitors on ice for 30 min. Loading buffer (5×) was added to the protein lysate supernatants, which were then heated at 95°C for 15 min. Cell lysates (30–50 μg) were loaded onto the SDS–PAGE gel with the same amount of protein per well. When separated in the gel by electrophoresis, the proteins were transferred to PVDF membranes (IPVH00010, Merck Millipore) at 280 mA for 2 h. After soaking in 5% BSA for 1 h, the membrane was incubated with a diluted primary antibody (1:1000) at 4°C for 12 to 14 h. After washing with TBST, the membranes were incubated with the corresponding secondary antibodies at room temperature for 1.5 h. Specific proteins were visualized with an imaging system (Chemi-Doc, Bio-Rad) in the presence of chemiluminescence substrate (34577, Invitrogen). ImageJ was used to analyze the grayscale values of the protein bands. The antibody and reagent information is listed in Table S2.

2.12 | Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed on HMC-1 cells with a SimpleChIP Enzymatic Chromatin IP Kit (9003, Cell Signaling Technology).

Briefly, approximately 30 million cells in 100 mm plates were treated with *H. pylori*, $CoCl_2$, or PBS for 6 h. After washing, the cells were cross-linked by formaldehyde and quenched by glycine. Nuclear pellets were sonicated 3 times for 20 s on wet ice. Sonicated chromatin was incubated with anti-HIF-1α, anti-histone H3, or normal rabbit IgG antibodies at 4°C overnight with rotation. The cross-linked chromatin was then reversed. Finally, the DNA was purified and then amplified with specific primers for HIF-1α binding sites within the GPR171 promoter. The binding sites in the GPR171 promoter are indicated in Table S3.

2.13 | Dual-Luciferase Reporter Assay

HRE reporter plasmids were customized (GeneChem Co. Ltd., Shanghai). The GPR171 promoter sequence and its mutant counterparts were cloned and inserted into the GV238 vector. Plasmids were transfected into HMC-1 cells with Lipofectamine 3000 (Thermo Fisher Scientific). After 48 h, the cells were treated with *H. pylori* (MOI = 100), 200 μM $CoCl_2$ (15862, Sigma) or PBS for 6 h. Luciferase activity was detected with a dual-luciferase reporter assay system (E1910, Promega). Briefly, after discarding the culture supernatant, the cells were lysed in PLB buffer for 15 min. Then, 100 μL of LARII was added to the lysates, and the firefly luciferase activity was immediately measured. After adding Stop&Glo Reagent, the Renilla luciferase activity was determined. The relative luciferase activity was normalized to Renilla luciferase activity.

2.14 | Immunohistochemistry (IHC)

Four-micron-thick paraffin-embedded gastric mucosa tissue sections were prepared. The sections were then incubated at 70°C for 1.5 h. Afterward, they were sequentially placed in xylene (10 min), xylene (10 min), absolute ethanol (5 min), absolute ethanol (5 min), 95% ethanol (5 min), 85% ethanol (5 min), and 75% ethanol (5 min). The slides were heated in 1× sodium citrate buffer in a microwave for 15 min. After allowing the sections to cool to room temperature, they were incubated with 3% H_2O_2 for 20 min. Subsequently, 5% BSA (bovine serum albumin) was added to completely cover the tissue, and the sections were incubated at room temperature for 1 h. The primary antibody dilution was added to each tissue circle, and the slides were placed in a humidified chamber for overnight incubation at 4°C. After washing, the corresponding secondary antibody dilution was added to the tissue circles, and the slides were incubated at room temperature for 1 h. The stained sections were then visualized under a microscope using a DAB kit. After DAB staining, sections were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene, and mounted with coverslips. CCL2 IHC scores were calculated by multiplying staining intensity with staining distribution. Staining intensity: 0 points for no staining, 1 point for weak, 2 points for moderate, and 3 points for strong. Staining distribution: 0%–5% is 0 points, 6%–25% is 1 point, 26%–50% is 2 points, 51%–75% is 3 points, and > 75% is 4 points [43].

2.15 | Immunofluorescence (IF)

Paraffin-embedded gastric mucosa tissue sections were deparaffinized, rehydrated, and blocked with 5% BSA at room temperature for 1 h. The primary antibody was applied to the tissue sections, and the sections were incubated at 4°C overnight. The washed slices were incubated with fluorescently labeled secondary antibodies at room temperature for 1 h. The nuclei were stained with DAPI for 8 min. Images were acquired via laser scanning confocal microscopy (Carl Zeiss).

2.16 | Generation of GPR171-Knockdown Cell Lines

Lentiviral vectors carrying GFP and either shRNA targeting GPR171 mRNA or a non-targeting control shRNA were customized (GenePharma, China). RBL-2H3 cells were transduced with lentiviral particles (MOI = 50) in the presence of Lipofectamine 3000. After 24 h, the medium was replaced with fresh growth medium. 48 h post-transduction, puromycin (1 μg/mL; MCE) was added to eliminate untransduced cells, and stable GPR171-knockdown cells were selected. The knockdown efficiency of GPR171 was confirmed by RT-qPCR and Western blot analysis.

2.17 | Statistical Analysis

Statistical analyses and data visualization were performed using GraphPad Prism 10 (GraphPad Software) or R version 4.2. Statistical significance between two groups was assessed using an unpaired two-tailed Student's *t*-test or a two-tailed Mann–Whitney *U* test. For comparisons among multiple groups, one-way or two-way ANOVA was used, as appropriate. Linear regression analysis was conducted to evaluate associations between variables. $p < 0.05$ was considered statistically significant.

3 | Results

3.1 | Mast Cells Correlate With the Severity of Inflammation During *H. pylori* Infection, and *H. pylori* Induces Mast Cell Activation

To investigate the relationship between *H. pylori* infection, mast cells, and gastric inflammation, we evaluated mast cell infiltration in human gastric mucosa using IHC (Figure 1A). We found that patients infected with *H. pylori* had greater mast cell infiltration compared to uninfected individuals (Figure 1B). Gastric inflammation was graded into three levels (mild, moderate, and severe) based on the inflammation score. In *H. pylori*-positive individuals, mast cell infiltration was significantly correlated with the severity of inflammation (Figure 1C). These results suggested that mast cells may participate in *H. pylori*-related gastritis.

To explore whether mast cells could be activated in response to *H. pylori*, we examined their degranulation and cytokine

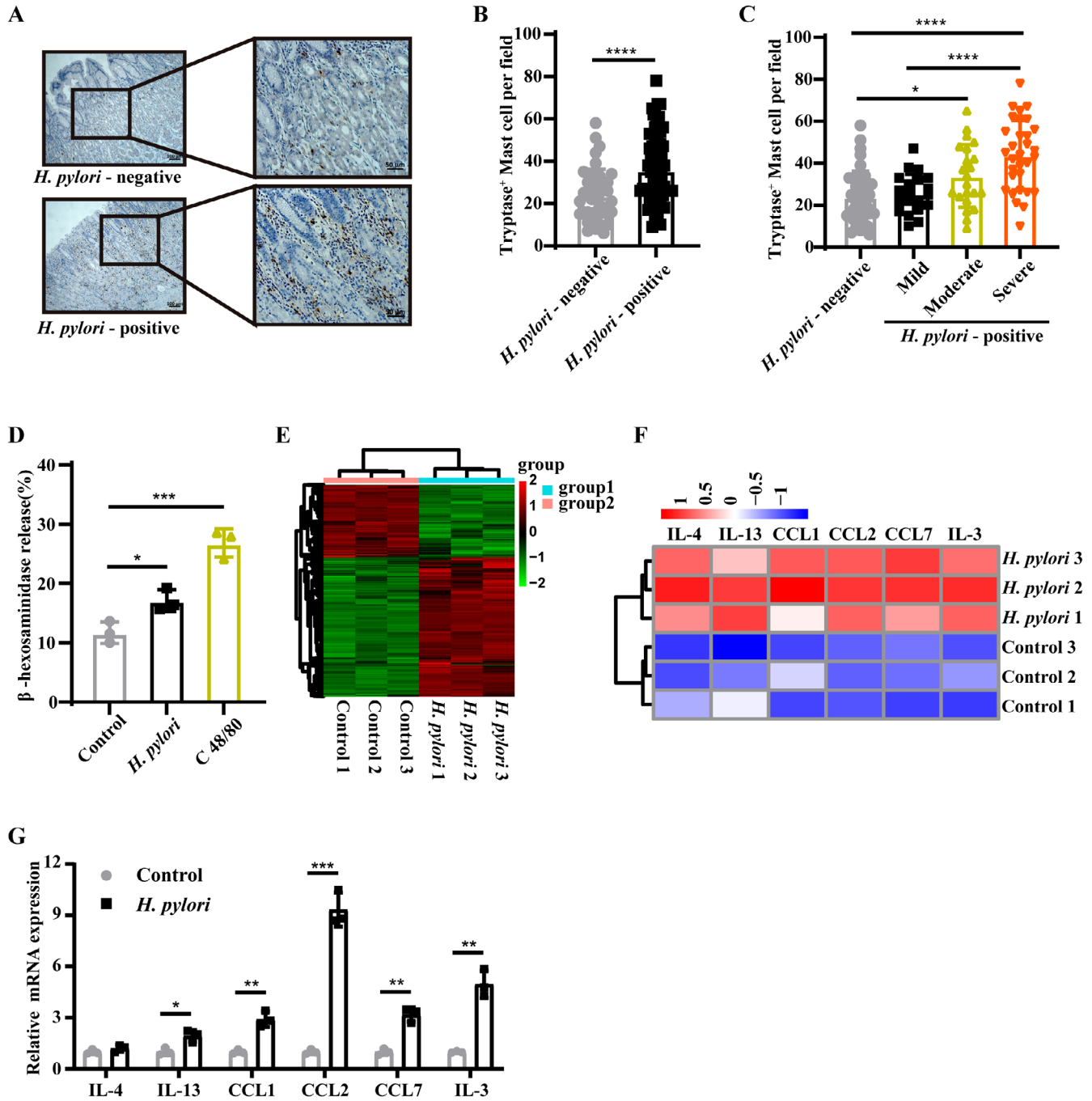

FIGURE 1 | The enrichment of mast cells in *H. pylori*-positive patients were correlated with inflammation, and *H. pylori* can activate mast cell in vitro. (A) Representative immunohistochemistry images of Tryptase-positive mast cells in human gastric tissues at 100× and 200× magnification. (B) Quantification of Tryptase-positive mast cells at 200× magnification in *H. pylori*-negative ($n = 42$) and *H. pylori*-positive patients ($n = 74$), symbols represent individual patients. Statistical significance was calculated by the Mann–Whitney test. (C) Quantification of mast cells at 200× magnification in *H. pylori*-negative ($n = 42$) and *H. pylori*-positive gastric tissue specimens with different levels of inflammation (Mild: $N = 21$, moderate: $N = 22$, severe: $N = 31$), p-values were determined by one-way ANOVA. (D) β-Hexosaminidase was released from RBL-2H3 cells into the supernatant in response to *H. pylori* infection, with compound 48/80 used as a positive control. (E) Differential genes of mast cells between *H. pylori* infection group and control group. $|\log_2 \text{fold change}| \geq 1$ and $p \leq 0.05$. (F) Heatmap representing increased cytokines based on RNA-seq data. (G) The mRNA levels of IL-4, IL-13, CCL1, CCL2, CCL7, and IL-3 in RBL-2H3 cells treated or untreated with *H. pylori* were determined by RT-qPCR. Statistical significance was calculated by Student's *t*-test. Data are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

secretion, key indicators of mast cell activation. We found that *H. pylori* increased the release of β-hexosaminidase into the supernatant of RBL-2H3 cells (Figure 1D). We then performed RNA sequencing (RNA-seq) to evaluate the effects of *H. pylori* on RBL-2H3 cells. Transcriptomic analysis revealed that *H. pylori* infection resulted in the upregulation of 1032 genes and the downregulation of 538 genes (Figure 1E). Six cytokines were found in the upregulated genes (Figure 1F). We validated the upregulation of IL-13, CCL1, CCL2, CCL7, and IL-3 by RT-qPCR upon *H. pylori* infection (Figure 1G). Among these genes, CCL2,

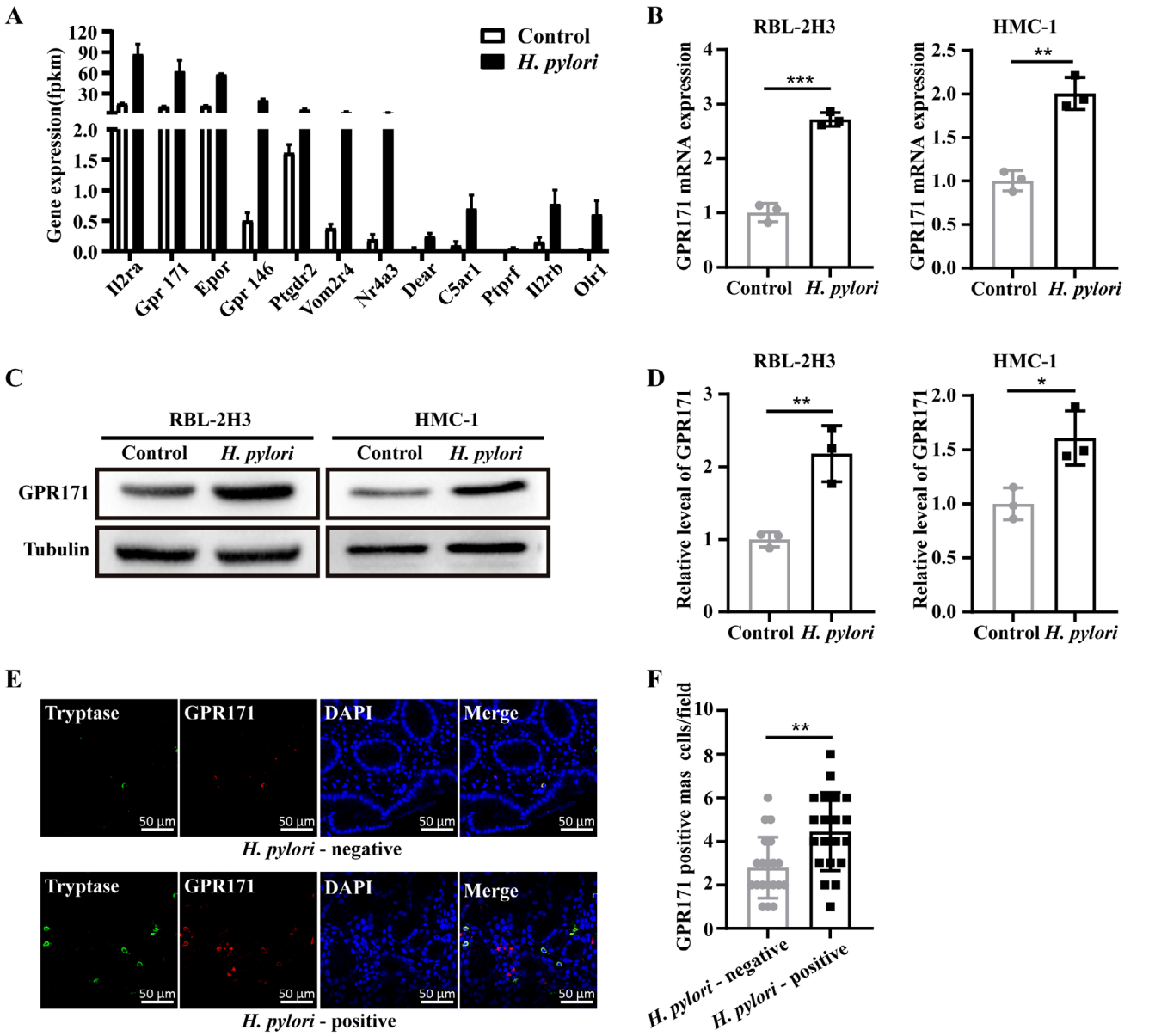

FIGURE 2 | GPR171 expression was increased in mast cells upon *H. pylori* infection in vitro. (A) The top 12 receptor genes upregulated in RBL-2H3 cells upon *H. pylori* infection based on RNA-seq data. Gene expression and abundance were measured in FPKM (reads per kilobase per million mapped reads). Log_2 fold change ≥ 2 and $p \leq 0.05$. (B) The expression of GPR171 in two mast cell lines stimulated with or without *H. pylori* were analyzed by RT-qPCR. (C, D) Immunoblot analysis of GPR171 expression in RBL-2H3 and HMC-1 cells. (E) Representative immunofluorescence images of Tryptase, GPR171 and DAPI in gastric mucosa tissues from individuals. Images were captured at 400× magnification. (F) GPR171-positive mast cells were counted in *H. pylori*-uninfected and *H. pylori*-infected patients, $n = 20$. Statistical significance was determined by Student's unpaired *t*-test (B, D) and the Mann–Whitney test (F). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

also known as MCP-1, exhibited the highest frequency of gene amplification and copy number changes. These data suggested that *H. pylori* could induce mast cell degranulation and secretion of cytokines.

3.2 | GPR171 Expression in Mast Cells Is Induced by *H. pylori* Infection In Vitro

H. pylori activated mast cells, which were closely related to surface receptors. Compared to uninfected cells, twelve receptor genes showed at least a 4-fold increase in expression in *H. pylori*-infected mast cells (Figure 2A). We focused on GPR171 because its expression is relatively high, and its function in mast cells is unknown. Our analysis of GPR171 mRNA expression at different time points during *H. pylori* infection revealed peak expression at 6 h, compared to 3 and 12 h (Figure S1). RT-qPCR and western blot analysis demonstrated that *H. pylori* infection induced an increase in GPR171 expression in both RBL-2H3 and HMC-1 cell lines (Figure 2B–D). These results confirmed that GPR171 expression was upregulated in mast cells upon *H. pylori* infection in vitro. Additionally, we examined GPR171 expression in gastric mucosal tissues using IF staining. The results showed

that GPR171 was expressed in mast cells, and the number of GPR171-positive mast cells was increased in *H. pylori*-infected individuals compared to uninfected controls (Figure 2E,F).

3.3 | The Upregulation of GPR171 Is Likely Mediated by HIF-1α

GO enrichment analysis indicated that cellular response to hypoxia and response to decreased oxygen levels had a strong enrichment in mast cells upon *H. pylori* infection (Figure S2A). We verified that *H. pylori* infection obviously induced intracellular ROS in RBL-2H3 cells (Figure S2B). KEGG and GSEA analyses revealed that HIF-1α signaling pathway was significantly enriched in *H. pylori*-infected mast cells (Figure 3A,B). In two mast cell lines, we then verified that HIF-1α mRNA levels were elevated under *H. pylori* stimulation (Figure 3C). HIF-1α expression at the protein level was also increased significantly in response to *H. pylori* infection (Figure 3D). Since a transcriptome analysis revealed that GPR171 was upregulated in a hypoxic environment [44] and activation of mast cells by *H. pylori* could lead to upregulation of ROS and HIF-1α expression, we hypothesized that GPR171 expression was related to HIF-1α activation. $CoCl_2$, a chemical inducer of HIF-1α, was used to enhance the stability and activate HIF-1α. Our results revealed that $CoCl_2$ increased GPR171 expression in a concentration-dependent manner in HMC-1 cells. For RBL-2H3 cells, when $CoCl_2$ concentration was 200 μM, the expression of GPR171 was most significantly upregulated (Figure 3E). Therefore, we selected 200 μM as the subsequent working concentration of $CoCl_2$. Consistent with the above findings, the increase in GPR171 expression was reduced in two mast cell lines pretreated with PX-478, a HIF-1α inhibitor (Figure 3F). Moreover, treatment with PX-478 attenuated the *H. pylori*-induced upregulation of GPR171 in RBL-2H3 and HMC-1 cells (Figure 3G). These results suggest that GPR171 expression is likely regulated by HIF-1α.

3.4 | The GPR171 Promoter Is a Transcriptional Target of HIF-1α

HIF-1α upregulates the expression of numerous genes by binding to the promoters of its target genes [45]. Most transcription factor binding site databases primarily focus on human data, while available datasets for *Rattus norvegicus* are both limited and unvalidated. Consequently, we opted to conduct the relevant experiments using human mast cell lines (HMC-1) as a practical alternative. We predicted HIF-1α binding sites in the GPR171 promoter using JASPAR software and identified two potential regions: HRE1 (1020–1029) and HRE2 (806–815) (Figure 4A). To determine whether HIF-1α could bind directly to putative HREs, we performed ChIP analysis. The results showed that HRE2, but not HRE1, robustly increased HIF-1α binding to the GPR171 promoter in HMC-1 cells treated with *H. pylori* or $CoCl_2$ (Figure 4B). To further investigate whether HIF-1α can activate GPR171 transcriptional activity, we constructed a GPR171 promoter-luciferase reporter construct and a corresponding HRE2-mut construct (Figure 4C). Mast cells infected with the HRE2-wt plasmid exhibited increased luciferase activity upon treatment with *H. pylori* or $CoCl_2$. However, *H. pylori* and $CoCl_2$ failed to induce luciferase expression when cells were transfected with the HRE2-mut plasmid or empty vector (Figure 4D). These findings demonstrate that HIF-1α upregulated GPR171 expression by directly binding to the GPR171 promoter to enhance its transcriptional activity.

3.5 | GPR171-Mediated CCL2 Production in Mast Cells Is Partially Dependent on ERK1/2 Activation

To further evaluate the role of GPR171 in mast cell activation by *H. pylori*, GPR171 expression was knocked down in RBL-2H3 cells using lentiviral shRNA. RT-qPCR and western blotting demonstrated that GPR171 expression was effectively knocked down (Figure 5A–C). To explore whether GPR171 affects mast cell activation, we assessed the impact of GPR171 knockdown on *H. pylori*-induced mast cell degranulation and secretion of cytokines. We found that GPR171 knockdown had no effect on mast cell degranulation (Figure 5D). We then assessed cytokine expression and found that GPR171 knockdown markedly reduced the levels of CCL2 and CCL7 in response to *H. pylori* infection (Figure 5E and Figure S3). Considering that CCL2 was the most upregulated cytokine among the six cytokines, it suggested that GPR171 may affect CCL2 secretion during the activation of mast cells by *H. pylori*. To further verify this effect at the protein level, we assessed CCL2 secretion in the supernatants of control and GPR171 knockdown cells following *H. pylori* stimulation and observed similar results (Figure 5F). Furthermore, BigLEN, the ligand of GPR171, induced upregulation of CCL2 expression in RBL-2H3 mast cells, further supporting the role of GPR171 in promoting CCL2 production (Figure S4).

GO enrichment analysis showed that ERK signaling, leukocyte activation, and cytokine production were strongly enriched in *H. pylori*-infected RBL-2H3 cells (Figure 5G). We verified that ERK1/2 phosphorylation was obviously increased in *H. pylori*-infected mast cells (Figure 5H). A similar result was observed in HMC-1 cells (Figure S5). To assess whether ERK1/2 mediated CCL2 secretion in mast cells upon *H. pylori* stimulation, cells were pretreated with the ERK1/2 inhibitor U0126. We verified that the expression of CCL2 was partially reduced when ERK1/2 phosphorylation was inhibited, as assessed by RT-qPCR and ELISA (Figure 5I,J). We found similar results in HMC-1 cells (Figure S6). Previous studies have shown that the BigLEN-GPR171 system influences ERK1/2 phosphorylation in Neuro2A cells [31]. We found that GPR171 knockdown impaired ERK1/2 phosphorylation during *H. pylori* infection (Figure 5K). Additionally, we found that the GPR171 antagonist (MS21570) partially downregulated ERK1/2 phosphorylation and CCL2 expression induced by *H. pylori* (Figure 5L–O). A similar result was observed in HMC-1 cells (Figure S7). These results suggested that GPR171 likely modulated CCL2 expression through the ERK1/2 signaling pathway during *H. pylori* infection.

3.6 | CCL2 Secretion Mediated by GPR171 May Contribute to Gastric Inflammation in *H. pylori* Infection

Because *H. pylori* can activate mast cells to secrete CCL2 in vitro, we assessed the expression and role of CCL2 in human

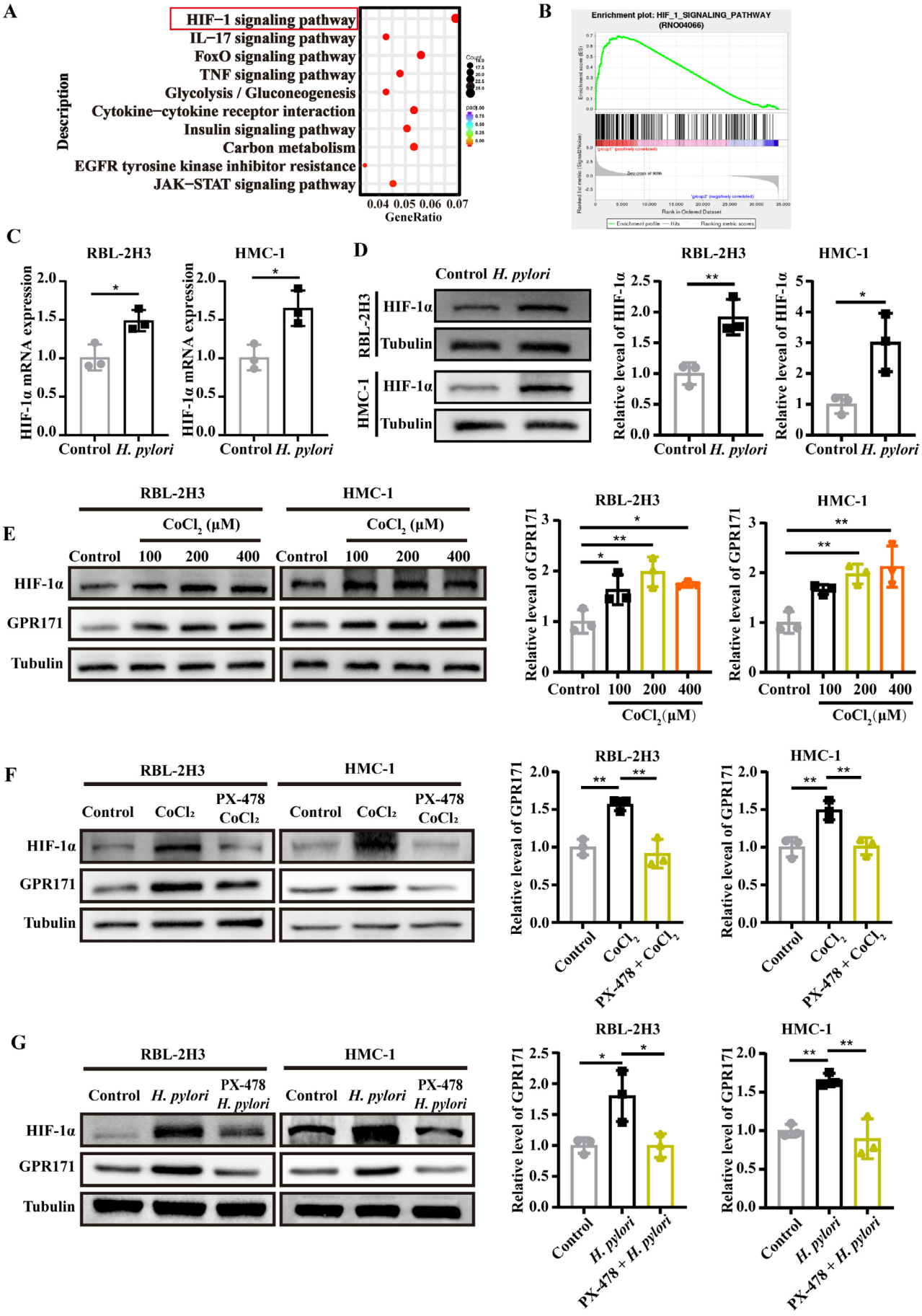

FIGURE 3 | Legend on next page.

FIGURE 3 | GPR171 expression is likely induced by HIF-1α. (A) Differentially upregulated genes based on RNA-seq data were applied to KEGG enrichment analysis. 10 significantly enriched signaling pathways were shown in the graph. (B) GSEA revealed that HIF-1α signaling was enriched in RBL-2H3 cells upon *H. pylori* infection. (C) The expression of HIF-1α mRNA was quantified by RT-qPCR in 2 mast cell lines that were or were not treated with *H. pylori*. (D) Immunoblot analysis and quantification of HIF-1α in 2 mast cell lines that were or were not treated with *H. pylori*. (E) Mast cells were treated with different concentrations of $CoCl_2$, and the protein levels of HIF-1α and GPR171 were assessed by immunoblotting. (F) Mast cells were pretreated with 25 μM PX-478 for 2 h prior to treatment with 200 μM $CoCl_2$, and HIF-1α and GPR171 expression was determined by immunoblot analysis. (G) Mast cells were pretreated with 25 μM PX-478 for 2 h, followed by treatment with *H. pylori*. HIF-1α and GPR171 expression levels were then assessed by immunoblot analysis. All the results were replicated in three independent experiments. Statistical significance was determined by Student's unpaired *t*-test (C, D) and one-way ANOVA (E, F, G), $^{*}p < 0.05$, $^{**}p < 0.01$.

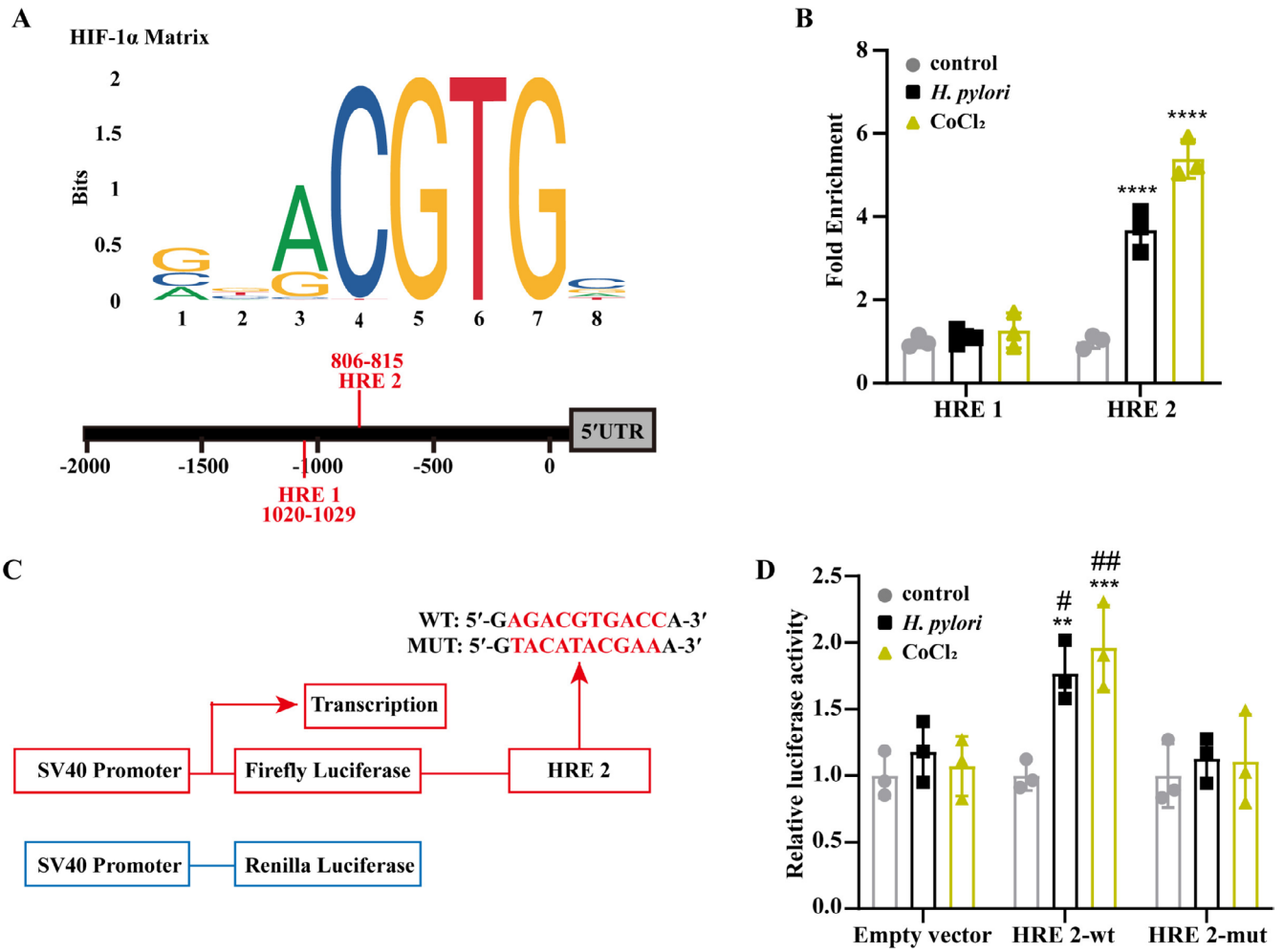

FIGURE 4 | HIF-1α regulates GPR171 expression by directly binding to specific HRE regions within the GPR171 promoter. (A) HREs in the GPR171 promoter were predicted by JASPAR. (B) HIF-1α binding sites in the GPR171 promoter were identified through ChIP assay. HMC-1 cells were exposed to *H. pylori*, $CoCl_2$ or PBS for 6 h, and chromatin bound to anti-HIF-1α antibody were obtained by immunoprecipitation. DNA isolated from chromatin was used as a template for PCR amplification with primers specific for the HIF-1α binding region within the GPR171 promoter. *p*-value was determined by two-way ANOVA, $^{****}p < 0.0001$. (C) Schematic diagram of the dual-luciferase reporting system. (D) Dual-luciferase reporter assay showing GPR171 promoter activity in HMC-1 cells exposed to *H. pylori* or $CoCl_2$ for 6 h. The luciferase activity was normalized to that of the corresponding Renilla luciferase. Statistical significance was determined by two-way ANOVA. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Empty vector. HREs, hypoxia response elements.

gastric mucosal tissue. IF staining revealed that there were CCL2-positive mast cells in the gastric mucosa (Figure 6A), indicating that mast cells in the gastric mucosa have the ability to secrete CCL2. We then evaluated the expression of CCL2 via immunohistochemical staining and found that *H. pylori* infection increased CCL2 expression in gastric tissue specimens (Figure 6B). High CCL2 expression levels were associated with severe gastric inflammation (Figure 6C,D). Moreover, linear regression analysis indicated a positive correlation between CCL2 expression and mast cell infiltration in *H. pylori*-infected patients (Figure 6E). These results suggest that mast cell–derived CCL2 in the gastric mucosa may be associated with inflammation.

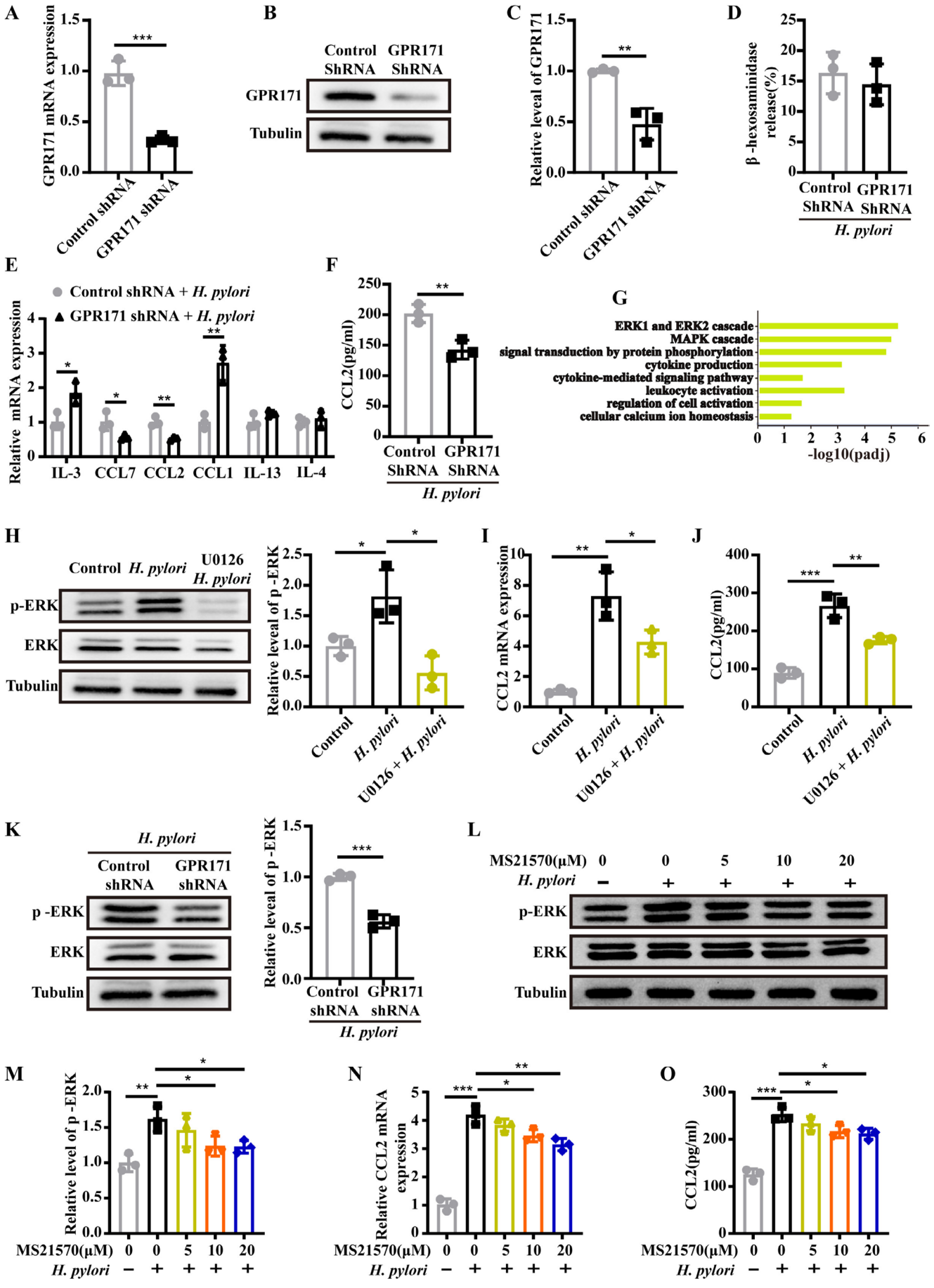

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FIGURE 5 | GPR171 partially mediates CCL2 production via the ERK1/2 pathway. (A–C) GPR171 expression was verified in GPR171-knockdown RBL-2H3 cells by RT-qPCR and western blotting. (D) Percentage of β-hexosaminidase released into the supernatants from control shRNA-treated and GPR171 shRNA-treated RBL-2H3 cells in response to *H. pylori* infection. (E) The mRNA levels of the 6 cytokines in control shRNA-treated and GPR171 shRNA-treated cells stimulated with *H. pylori*. (F) CCL2 levels in the supernatants of shRNA-treated and GPR171 shRNA-treated RBL-2H3 cells co-cultured with *H. pylori*. (G) GO enrichment of DEGs based on RNA-seq data. (H) Immunoblot analysis and quantification of ERK1/2 phosphorylation in RBL-2H3 cells that were or were not treated with *H. pylori*. p-ERK expression was normalized to total ERK. (I) RT-qPCR analysis of CCL2 mRNA expression in mast cells co-cultured with or without *H. pylori*. (J) CCL2 levels in supernatants were quantified by ELISA. Cells were pretreated with 5 μM U0126 as indicated (H–J). (K) Immunoblot analysis and quantification of p-ERK in control shRNA-treated and GPR171 shRNA-treated cells following *H. pylori* stimulation. (L, M) Western blot analysis of ERK1/2 phosphorylation expression in RBL-2H3 cells treated with different concentrations of MS21570 (0, 5, 10, and 20 μM) and co-cultured with or without *H. pylori*. (N, O) RT-qPCR and ELISA analysis of CCL2 expression in RBL-2H3 cells treated with different concentrations of MS21570 (0, 5, 10, and 20 μM) and co-cultured with or without *H. pylori*. MS21570: A blocker of GPR171. Statistical significance was determined by Student's unpaired *t*-test (A, C, D, E, F, K) and one-way ANOVA (H, I, J, M, N, O), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In vitro experiments show that *H. pylori* infection regulated CCL2 expression through GPR171, and CCL2 levels were positively correlated with the degree of gastric mucosal inflammation. To further validate whether GPR171 affects CCL2 expression and gastric mucosal inflammation in vivo, MS21570, a GPR171 antagonist, was administered intraperitoneally to mice prior to *H. pylori* PMSS1 infection. *H. pylori* infection in mice was successfully established, and inhibition of GPR171 had no significant impact on bacterial colonization (Figure S8). Analysis of gastric mucosal tissues revealed that *H. pylori* infection markedly increased CCL2 expression, as assessed by CCL2 mRNA levels and CCL2 IHC staining scores. In contrast, inhibition of GPR171 led to a partial reduction in CCL2 levels (Figure 6F–H). Additionally, H&E staining and inflammation scoring indicated that GPR171 inhibition partially alleviated *H. pylori*-induced gastric inflammation (Figure 6I,J). These results suggested that GPR171 may influence gastric mucosal inflammation through CCL2 in *H. pylori* infection.

4 | Discussion

In this study, mast cells were found to be increased in the gastric mucosa, and their accumulation was associated with inflammation. We identified a previously unrecognized regulatory mechanism and function of GPR171 in mast cells upon *H. pylori* infection. HIF-1α upregulated GPR171 expression by directly binding to the HRE in the GPR171 promoter. GPR171 mediated the production of CCL2 via ERK1/2 signaling. Mast cell-derived CCL2 in the gastric mucosa may contribute to the development of gastric inflammation. Inhibition of GPR171 in *H. pylori*-infected mice leads to a partial reduction in gastric mucosal CCL2 expression and a moderate attenuation of inflammation. These findings provide new insight into the mechanisms through which *H. pylori* activated mast cells to promote gastritis.

Our results, together with previous studies, confirmed that *H. pylori* and its virulence factors can activate mast cells [46, 47]. Mast cell activation depends on the diverse repertoire of receptors that they express. In our study, elevated GPR171 expression was observed in *H. pylori*-infected mast cells in vitro. This inducibility aligns with findings from another study, which demonstrated that GPR171 expression was upregulated in both T and NK cells upon stimulation. Additionally, GPR171 is highly expressed in murine mast cells, with its expression further enhanced following IgE sensitization and a 1-h antigen stimulation [32]. Although *H. pylori* infection induced GPR171 expression in RBL-2H3 and HMC-1 cell lines, the expression of GPR171 in gastric mast cells appeared to be independent of infection status. This discrepancy could be due to differences in cell types, experimental conditions, or the inherent nature of the gastric tissue environment in vivo, as cell culture experiments may not fully reflect the in vivo environment. Further studies are needed to explore the regulatory mechanisms of GPR171 in gastric mast cells during infection.

The regulation and role of GPR171 in mast cells remain largely unknown. In our study, *H. pylori* infection induced GPR171 and HIF-1α expression in mast cells. Pathogen infection, including *H. pylori*, can induce hypoxia and HIF-1α activation even under normoxic conditions [48–50]. We inferred that *H. pylori* infection promoted HIF-1α stabilization by inhibiting HIF-1α ubiquitination and protease degradation via ROS. HIF-1α plays various roles in different immune cells, including regulating cell metabolism and hypoxia-inducible gene expression [35–37]. To the best of our knowledge, this is the first study to reveal that GPR171 is the target of HIF-1α. A similar regulatory mechanism has been reported for GPR35 in cardiac myocytes [51].

GPR171 is a Gαi/o-coupled receptor that is sensitive to pertussis toxin (PTX) [29]. Loss of GPR171 has been shown to reduce ERK phosphorylation signaling in neurons upon BigLEN stimulation [31]. In contrast, BigLEN stimulation decreases OKT3-induced PLCγ1 and ERK phosphorylation in Jurkat cells [32]. Our results suggested that the loss or blockage of GPR171 partially mediated CCL2 secretion by downregulating ERK1/2 signaling in mast cells upon *H. pylori* infection. The production of CCL2 by mast cells can also be affected by other receptors under hypoxic conditions, such as calcium channels [52]. Previous studies have reported that mast cells can recognize bacteria via TLR receptors [53, 54]. Recently, several new receptors, such as MRGPRX2 and P2X7, were found to participate in mast cell activation during pathogen infection and inflammation [10, 55]. Here, we show that *H. pylori* can mediate mast cell activation through GPR171. In line with our results, a study suggested that neutrophil-activating protein derived from *H. pylori* can activate mast cells through PTX-sensitive GPCRs rather than through TLR2 [56]. We further show that GPR171 is involved in CCL2 expression in mast cells in response to *H. pylori* or BigLEN. Bacterial metabolites have been reported to interact with GPCRs, including

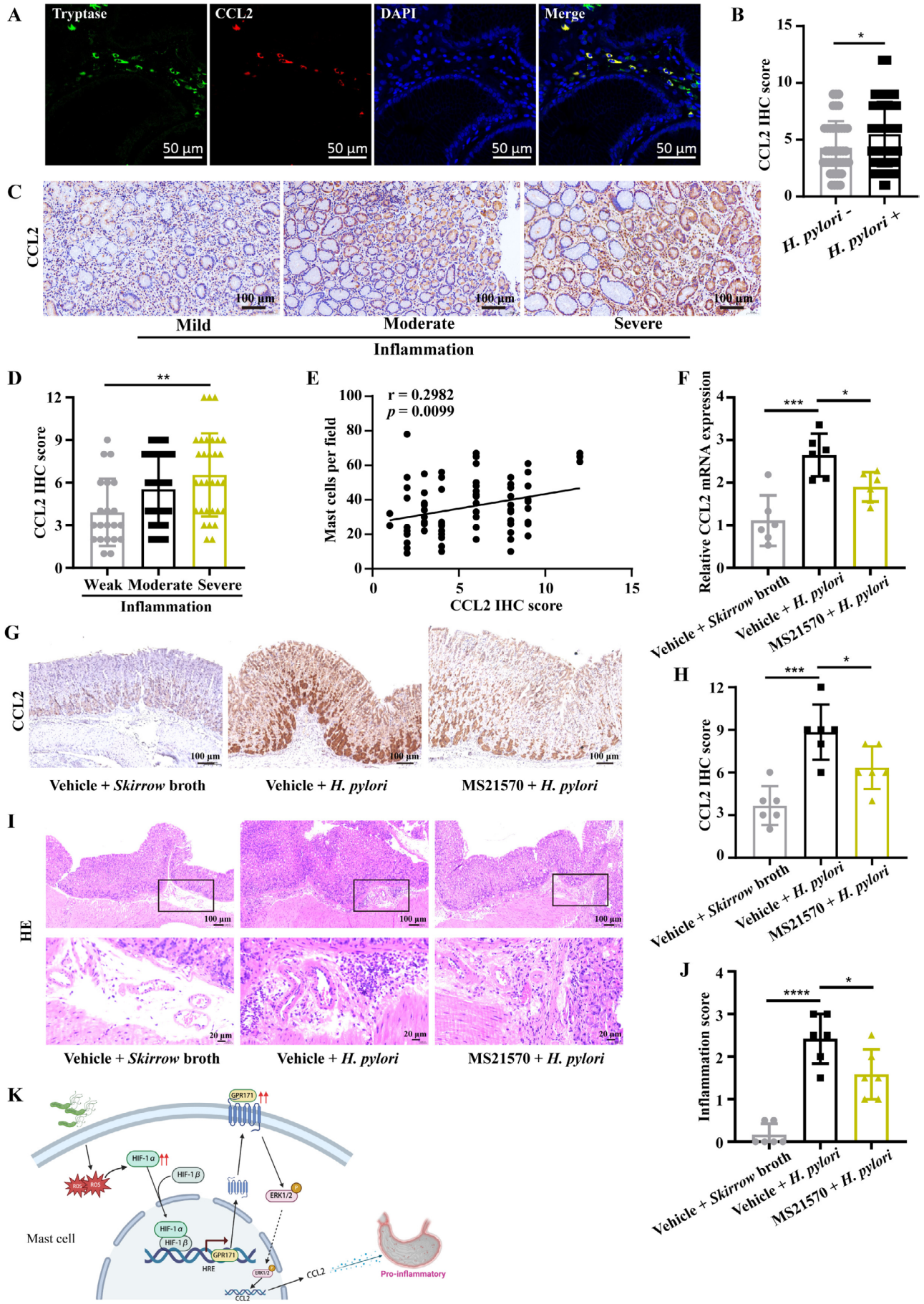

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FIGURE 6 | CCL2 secretion mediated by GPR171 may contribute to gastric inflammation during *H. pylori* infection. (A) Representative immunofluorescence images of Tryptase, CCL2, and DAPI in gastric mucosa tissues from *H. pylori*-infected patients. Images were captured at 400× magnification. (B) CCL2 expression was assessed by immunohistochemistry, and the CCL2 score was determined in *H. pylori*-negative ($n = 42$) and *H. pylori*-positive patients ($n = 74$), Statistical significance was determined by Mann–Whitney test. (C, D) Immunohistochemistry for CCL2 and quantification of the CCL2 score in the gastric tissues of *H. pylori*-infected patients with different levels of inflammation (mild: $N = 21$, moderate: $N = 22$, severe: $N = 31$). (E) Correlation between the CCL2 IHC score and mast cell number in *H. pylori*-positive patients ($n = 74$). (F) CCL2 mRNA expression in the gastric mucosa of mice in different treatment groups. (G, H) CCL2 IHC staining and the CCL2 score in different treatment groups. (I, J) HE staining and inflammation score quantification in the gastric tissue of mice from different treatment groups. Mice were treated with Vehicle + *Skirrow* broth, Vehicle + *H. pylori*, or MS21570 + *H. pylori*. *Skirrow* broth: A liquid culture medium for *H. pylori*, MS21570: A GPR171 antagonist. Each dot represents an individual mouse. $n = 6$. Statistical significance was determined by the Mann–Whitney test (B) and one-way ANOVA (D, F, H, J). Associations were assessed by linear regression (E). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (K) Model of *H. pylori* activating mast cell to exacerbate gastritis. IHC, immunohistochemistry.

GPR41, GPR43 and GPR171 [34, 57, 58]. However, due to the complexity of *H. pylori* and its metabolites, it is difficult to determine whether exogenous ligands for GPR171 exist.

Mast cells play an important role in pathogen infection by producing a wide range of cytokines [15]. In accordance with previous reports [23], we found that *H. pylori* infection led to significant infiltration of mast cells, especially in the gastric mucosa with severe inflammation. Early studies suggested that mast cells were associated with gastric epithelial cell apoptosis and neutrophil infiltration in *H. pylori*-infected patients [59]. Our results revealed that *H. pylori*-induced CCL2 production by GPR171-ERK1/2 signaling, and CCL2 derived from mast cells was positively correlated with the degree of inflammation. The in vivo mouse experiment showed that *H. pylori* infection significantly upregulated CCL2 expression. After blocking GPR171, CCL2 expression was partially downregulated, and gastric mucosal inflammation was alleviated, suggesting that GPR171 may mediate gastric mucosal inflammation in *H. pylori* infection by regulating CCL2 expression. Mast cells are highly heterogeneous, and different bacteria could induce different cytokine secretion profiles [21]. The lipopolysaccharide (LPS) of *Escherichia coli* activated mast cells to produce cytokines through TLR4 but failed to induce degranulation, while the peptidoglycan (PGN) derived from *Staphylococcus aureus* dependent on TLR2 induced both cytokine production and degranulation of mast cells [60, 61]. Moreover, the cytokine profiles induced by these stimuli are not identical. Our results suggested that mast cells may promote gastritis through mast cell–derived CCL2 during *H. pylori* infection. CCL2, a chemokine, contributes to inflammation by recruiting monocytes and T cells to sites of infection [62, 63].

Collectively, we propose a model involving *H. pylori* exacerbating inflammation by activating mast cells (Figure 6K). *H. pylori* infection activated mast cells and increased GPR171 expression via the ROS/HIF-1α pathway. GPR171 expression was upregulated by HIF-1α through direct binding to the HRE (AGACGTGACC) in the GPR171 promoter. Furthermore, GPR171 mediates CCL2 secretion through ERK1/2 phosphorylation upon *H. pylori* infection, which may contribute to gastric inflammation.

Author Contributions

Hanmei Yuan performed the experiments and drafted the manuscript. Yuetong Li contributed to the animal experiments and participated in data acquisition and analysis. Hui Wu and Bin Li assisted with data analysis and interpretation. Jin Zhang and Tingting Xia were involved in data acquisition and experimental procedures. Chao Wu conceived and designed the study and supervised its overall execution.

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Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of The Eighth Affiliated Hospital of Sun Yat-sen University (Approval No. ZB-KYIRB-AF/SC-06/01.0).

Consent

Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data supporting the findings of this study are included in the article and Supporting Information; Further inquiries can be directed to the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.