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Heme Oxygenase-1 Dysregulates Macrophage Polarization and the Immune Response to *Helicobacter pylori*

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Abstract

Helicobacter pylori incites a futile inflammatory response, which is the key feature of its immunopathogenesis. This leads to the ability of this bacterial pathogen to survive in the stomach and cause peptic ulcers and gastric cancer. Myeloid cells recruited to the gastric mucosa during *Helicobacter pylori* infection have been directly implicated in the modulation of host defense against the bacterium and gastric inflammation. Heme oxygenase-1 (HO-1) is an inducible enzyme that exhibits anti-inflammatory functions. Our aim was to analyze the induction and role of HO-1 in macrophages during *H. pylori* infection. We now show that phosphorylation of the *H. pylori* virulence factor cytotoxin associated gene A (CagA) in macrophages results in expression of *hmox-1*, the gene encoding HO-1, through p38/nuclear factor (erythroid-derived 2)-like 2 signaling. Blocking phagocytosis prevented CagA phosphorylation and HO-1 induction. The expression of HO-1 was also increased in gastric mononuclear cells of human patients and macrophages of mice infected with *cagA+ H. pylori* strains. Genetic ablation of *hmox-1* in *H. pylori*-infected mice increased histologic gastritis, which was associated with enhanced M1/Th1/Th17 responses, decreased Mreg response, and reduced *H. pylori* colonization. Gastric macrophages of *H. pylori*-infected mice and macrophages infected in vitro with this bacterium showed an M1/Mreg mixed polarization type; deletion of *hmox-1* or inhibition of HO-1 in macrophages caused an increased M1 and a decreased of Mreg phenotype. These data highlight a

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mechanism by which *H. pylori* impairs the immune response and favors its own survival via activation of macrophage HO-1.

Introduction

Helicobacter pylori infects half of the world's population and is the causative agent of chronic gastritis, peptic ulcer disease and gastric mucosa-associated lymphoid tissue lymphoma. Long-term infection is a major risk factor for the development of gastric cancer, the second leading cause of cancer deaths worldwide. *H. pylori* expresses several virulence factors that impact disease outcome. Most of the *H. pylori* strains that provoke neoplastic transformation possess the cytotoxin-associated gene (cag) pathogenicity island (1), which carries genes encoding a type 4 secretion system (T4SS) and the virulence factor CagA (2, 3). When injected into the cytoplasm of gastric epithelial cells (3), CagA is sequentially phosphorylated on tyrosine residues by c-Src and Abl kinases (4) and then causes signaling events in host cells (5-7).

Besides this interaction with gastric epithelial cells, *H. pylori* has an impact on the recruitment and differentiation of lymphoid cells in the gastric mucosa. Thus, *H. pylori* infection results in a mixed Th1/Th17-dominant T cell response, which contributes to the establishment of chronic gastritis (8, 9). It has been also demonstrated that the *H. pylori*-induced Treg response plays a role in failure of specific immunity, thus favoring the persistence of the bacterium in its ecological niche (10). Moreover, *H. pylori* interacts with myeloid cells either directly, when bacteria cross the epithelial barrier and reach the lamina propria (11), or indirectly, through the release of bacterial products (12).

Macrophages play an essential role in host defense against bacterial infection and in the regulation of inflammatory processes, including during *H. pylori* infection (13). In response to various signals from the extracellular milieu, macrophages can be polarized into different populations of activated cells exhibiting different phenotype, receptor, and cytokine secretion patterns (14). Classically activated macrophages, also called M1 macrophages, interact with Th1 lymphocytes and exhibit microbicidal activity by producing oxygen radicals and NO, the latter through enhanced expression of inducible NO synthase (iNOS). In contrast, IL-4-stimulated wound-healing macrophages (M2 cells) contribute to the production of the extracellular matrix and exhibit indirect regulatory effects on the immune response. Regulatory macrophages (Mreg, also called type II-activated macrophages) synthesize high levels of IL-10 that limits inflammation, but predisposes the host to infections (15). It has been shown that gastric macrophages show features of the M1 profile during *H. pylori* infection (16). Nonetheless, we have found that gastric macrophages from *H. pylori*-infected mice exhibit activation of the arginase/ornithine decarboxylase metabolic pathway, a functional feature of M2 macrophages (17, 18), and an increase of M2 markers has been evidenced in the gastric mucosa from infected patients (19). Moreover, studies have associated macrophage production of IL-10, the typical Mreg cytokine, with infection by *H. pylori* (16, 19). Together, these data suggest that macrophage polarization during *H. pylori* infection is not a canonical process and results in a phenotypically mixed population of cells.

The direct effect of *H. pylori* on the molecular/cellular events that orchestrate macrophage polarization remains unknown. In the present work, we show that *H. pylori* induces macrophage *hmox-1*, the gene encoding heme oxygenase-1 (HO-1), a potent anti-inflammatory and antioxidant enzyme (20). This occurs by signaling events requiring CagA phosphorylation and the activation of p38 and nuclear factor (erythroid-derived 2)-like 2 (NRF-2). The activity of HO-1 in *H. pylori*-infected macrophages results in a switch of polarization toward a reduction of the M1 population and an increase of the Mreg profile, leading to a failure of innate and adaptive immune responses.

Materials and Methods

Reagents

The HO-1 inhibitor chromium mesoporphyrin (CrMP) was obtained from Frontier Scientific. The AP-1 inhibitor SR11302 (10 μM) was purchased from Santa Cruz Biotechnology. The following pharmacological compounds were obtained from Calbiochem: the NF-κB inhibitor Bay 11-7082 ((E)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile; 5 μM); the ERK1/2 inhibitor ERKi (3-(2-aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione, HCl; 20 μM); the JNK inhibitor SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one, 1,9-pyrazoloanthrone; 1 μM); the p38 inhibitor SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; 2 μM); the PI3K inhibitor LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; 10 μM); the c-Src inhibitor PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-d-3,4-pyrimidine); and cytochalasin D (10 μM), an inhibitor of actin polymerization.

Human tissues

Biopsies from gastric tissues were obtained from human subjects in Colombia as described (21), under protocols approved by the ethics committees of the local hospitals and of the Universidad del Valle in Cali, Colombia, as well as the Institutional Review Board at Vanderbilt University. The *cagA* status of *H. pylori* was determined from these tissues by PCR analysis performed on isolated colonies (21).

Bacteria, animals and infections

We used the *cagA*+ *H. pylori* strains 60190, 7.13, PMSS1, and G27. The *ureA*, *cagE*, *cagA*, *vacA*, and *flaA* isogenic mutants constructed in the strain 60190 (22, 23), and the strain G27 lacking the CagA phosphorylation domains ($cagA_{EPISA}$; 24) were also used.

C57BL/6×FVB $hmox\text{-}1^{+/-}$ mice were bred to generate wild-type (WT) and $hmox\text{-}1^{-/-}$ mice, as described (25, 26); $hmox\text{-}1^{+/-}$ breeder mice were provided by Anupam Agarwal (University of Alabama, Birmingham, AL). The genotypes were verified by PCR using primer sets for *hmox-1* and *neo* (Supplemental Table 1). Animals were used under protocol M/05/176 approved by the IACUC at Vanderbilt University. Mice were infected intragastrically 3 times, every two days, with 10^9 *H. pylori* PMSS1. Animals were sacrificed after two months. Colonization was assessed by qPCR using *H. pylori ureA* gene and mouse 18S rRNA primers (Supplemental Table 1) as described (18).

Purification of gastric macrophages

Macrophages were isolated from mouse stomach exactly as described (17, 27).

Cells, infections, and transfection

The murine macrophage cell line RAW 264.7 was maintained in DMEM containing 10% FBS, HEPES and sodium pyruvate. Peritoneal cells from WT or *hmox-1*$^{-/-}$ mice were collected after intraperitoneal injection of PBS. Cells were counted, plated and macrophages were purified by washing away nonadherent cells after 1 h of incubation. RAW 264.7 cells or peritoneal macrophages were stimulated with *H. pylori* at a multiplicity of infection of 100. All pharmacological inhibitors of signaling pathways were added 30 min prior to activation.

To determine the levels of adhesion and phagocytosis of *H. pylori*, RAW 264.7 cells were washed thoroughly five times with PBS after infection, incubated or not for one hour with 200 μg/ml gentamicin, and lysed in 0.1 % saponin for 30 min at 37°C. The number of bacteria in each lysate was determined by counting the CFUs after plating serial dilutions on blood agar plates.

RAW 264.7 cells in Opti-MEM I Reduced Serum Media (Invitrogen) were transfected using Lipofectamine 2000 with 100 nM ON-TARGET*plus* siRNAs (Dharmacon) directed against *hmox-1*, *nrf-2* or *lmnA*, or with 100 nM SignalSilence siRNAs (Cell Signaling) directed against murine *p38* or *erk1*. After 6 h, cells were washed, maintained 36 h in serum-containing antibiotic-free medium, and then stimulated.

Immunostaining

Immunohistochemistry was performed on human gastric tissues as described (18, 23) using a rabbit polyclonal anti-human/mouse HO-1 Ab (1:500; StressGen). Slides were reviewed and scored by a gastrointestinal pathologist (M.B.P.) who was blinded to the clinical status of the subjects. The percentage of mononuclear cells staining positively for HO-1 was determined in each patient by counting the cells with moderate or strong intensity staining on antral biopsies. Immunofluorescence for HO-1, iNOS, and F4/80 was performed on murine gastric tissues (18) using the Abs described in Supplemental Table 2.

Luminex assay

Gastric tissues were lysed in CelLytic™ MT Reagent (Sigma) containing the Protease Inhibitor Cocktail (Set III, Calbiochem) and protein concentrations were determined using the BCA Protein Assay (Pierce). Samples were assayed using a magnetic bead-based protein detection assay for IL-17 using a Millipore FlexMap 3D Luminex machine.

Flow cytometry

Immune cells were isolated from the total glandular stomach by enzymatic digestion (27). Cells were stained for HO-1 and for F4/80 using the Abs described in Supplemental Table 2. Stained cells were analyzed with an LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Inc.).

Analysis of mRNA levels

RNA purification, reverse transcription, and real-time PCR were performed as described (23) using the primers listed in Supplemental Table 1.

Western Blot analysis

RAW 264.7 cells were lysed using RIPA buffer or NE-PER Nuclear Protein Extraction Kit (Pierce) containing the Protease Inhibitor Cocktail (Set III, Calbiochem) and the Phosphatase Inhibitor Cocktail (Set I, Calbiochem). Protein concentrations were determined using the BCA Protein Assay (Pierce). Western blotting was performed using 10 μg of protein per lane. Primary and secondary Abs are listed in Supplemental Table 2. Densitometric analysis of Western blots was performed with ImageJ 1.45s software (rsbweb.nih.gov/ij/).

Statistics

All the data shown represent the mean ± SEM. Student's t test or ANOVA with the Newman-Keuls test were used to determine significant differences between two groups or to analyze significant differences among multiple test groups, respectively. In the case of the staining for HO-1 in human subjects, nonparametric testing was conducted with the Kruskal-Wallis test followed by Dunn's Multiple Comparisons test.

Results

H. pylori stimulates hmox-1 expression in macrophages

There was a significant increase in *hmox-1* mRNA in macrophages infected with *H. pylori* strains 7.13, 60190, or PMSS1 compared to uninfected cells (Figs. 1A). However, the level of *hmox-1* mRNA was 5.6 ± 0.7-fold and 4.3 ± 0.9-fold more elevated in macrophages infected with *H. pylori* 60190 and PMSS1, respectively, than in those stimulated with the strain 7.13 (Fig. 1A). We also demonstrated that *hmox-1* mRNA expression was upregulated in peritoneal macrophages isolated from C57BL/6 mice and infected ex vivo with *H. pylori* 60190 (Fig. 1A). HO-1 protein expression was also rapidly induced in RAW 264.7 cells infected with *H. pylori* 60190, peaking 6 h post-inoculation (Fig. 1B). Interestingly, we found that *H. pylori*-induced *hmox-1* mRNA expression was significantly inhibited when the bacteria were separated from the macrophages using a 0.22 μm filter support (Fig. 1C). Further, we observed that *hmox-1* mRNA expression (Fig. 1D) and the phagocytosis of *H. pylori* by macrophages (Fig. 1E) were both reduced in infected macrophages treated with cytochalasin D that prevents phagocytosis of *H. pylori* (28). Lastly, we found that *H. pylori* 7.13, which induced *hmox-1* relatively poorly, was significantly less phagocytized by RAW 264.7 cells than the strains 60190 or PMSS1 (Fig. 1E). It should be noted that there was complete killing of *H. pylori* when the macrophages cocultured with bacteria in the presence of cytochalasin D were treated with gentamicin (Fig. 1E), validating that these bacteria were extracellular. These results suggest that *H. pylori* phagocytosis is required to induce HO-1 in macrophages.

H. pylori-induced HO-1 in macrophages requires CagA phosphorylation

We then assessed which bacterial factor was implicated in *hmox-1* expression. There was a significant reduction of *hmox-1* mRNA levels in RAW 264.7 cells infected with *H. pylori* *cagA*$^{-}$ compared to macrophages infected with the WT strain or with the *flaA*, *cagE*, *ureA*, or *vacA* mutants (Fig. 2A). This difference between the *cagA* and *cagE* mutants suggests that CagA, but not the T4SS, is involved in *hmox-1* expression. We then assessed the effect of phospho-CagA (p-CagA) in HO-1 induction. We first observed that CagA was rapidly phosphorylated in infected macrophages (Fig. 2B); importantly, the phosphorylation of CagA was also observed when macrophages were infected with a *H. pylori* strain with deletion of *cagE*, thus lacking a functional T4SS; this demonstrates that CagA is phosphorylated in macrophages independently of the T4SS. Moreover, we found that the levels of CagA and p-CagA were greater in macrophages infected with the strains 60190 or PMSS1 than with strain 7.13 (Fig. 2C), which correlated with the level of phagocytosis depicted in Fig. 1E. Further, the levels of intracellular p-CagA and CagA were reduced when macrophages infected with the HO-1-inducing *H. pylori* strain 60190 were pretreated with cytochalasin D (Fig. 2D), proving that phagocytosis is an essential step for CagA phosphorylation in macrophages. Moreover, the reduction in phosphorylation of CagA when RAW 264.7 cells infected with strain 60190 were pre-treated with the *c*-Src inhibitor PP1 (Fig. 2E) correlated with a marked attenuation in the expression of *hmox-1* (Fig. 2F). Lastly, the *hmox-1* gene was significantly less expressed in macrophages stimulated with a *cagA*$_{\mathrm{EPISA}}$ mutant strain than with WT *H. pylori* (Fig. 2G), demonstrating the involvement of p-CagA in inducible transcription of *hmox-1*.

Induction of HO-1 by H. pylori is mediated by p38 and NRF-2

As shown in Fig. 3A, the specific inhibition of p38 by SB203580 resulted in a significant reduction of *H. pylori*-induced *hmox-1* mRNA expression, whereas inhibitors of ERK1/2, JNK, PI3K, NF-κB, or AP-1 had no effect. None of these pharmacologic inhibitors had a significant effect on *hmox-1* expression in uninfected cells (data not shown). The data with the p38 inhibitor was confirmed by the use of siRNA directed against p38 (Fig. 3B), which significantly inhibited *hmox-1* mRNA expression in *H. pylori*-stimulated macrophages (Fig. 3C); in contrast, the *erk1* siRNA (Fig. 3B) had no effect on *hmox-1* induction (Fig. 3C). Then, because we found that HO-1 induction was mediated by p-CagA and by p38, we determined whether p38 activation was dependent on CagA phosphorylation. Fig. 3D depicts that the phosphorylation of p38 on Thr^{180}/Tyr^{182} was decreased in macrophages *i*) pre-treated with PP1 and infected with *H. pylori* 60190 or *ii*) infected with the *cagA* mutant strain, when compared to RAW 264.7 cells infected with *H. pylori* 60190. Together, these results show that p-CagA signals in macrophages to activate p38. In accordance with the level of phagocytosis (Fig. 1E) and of CagA phosphorylation (Fig. 2C) with the various *H. pylori* strains, we found that p38 was less activated in macrophages infected with *H. pylori* 7.13 than with the strain 60190 (Fig. 3E). It has been reported that NRF-2 is a transcription factor activated by p38 that may transactivate the *hmox-1* gene (29); consistent with this, we found that blocking of NRF-2 expression using siRNA (Fig. 3F) resulted in a significant reduction of *H. pylori*-induced *hmox-1* mRNA expression (Fig. 3G).

HO-1 is induced in gastric macrophages during H. pylori infection

To demonstrate the in vivo relevance of our findings, we evaluated the presence of HO-1 in mononuclear cells of gastric tissues of infected patients in which the *cagA* status of the infecting *H. pylori* strains was known (21). Tissues from subjects infected with *cagA*+ *H. pylori* strains exhibited more staining in mononuclear cells than tissues from controls or patients infected with *cagA*– strains (Figs. 4A and 4B); in particular, strong staining of cells with the appearance of tissue macrophages was detected. Moreover, we observed that HO-1 levels were increased in C57BL/6 mice infected for 2 months with *H. pylori* PMSS1 that retains a functional T4SS in vivo (30), when compared to uninfected mice (Fig. 5A and Supplemental Fig. 1), and that HO-1 staining co-localized to cells that were positive for the macrophage marker F4/80 (Fig. 5A and Supplemental Fig. 1). To confirm this observation, we isolated gastric immune cells and analyzed F4/80 and HO-1 expression by flow cytometry. A representative flow cytometric dot plot (Fig. 5B) and analysis performed from multiple animals (Fig. 5C) demonstrate a significantly increased percentage of F4/80+/HO-1+ cells in infected mice compared to control animals. Further, the expression levels of HO-1 in gastric macrophages were also enhanced in the isolated gastric macrophages from *H. pylori*-infected mice (Figs. 5D and 5E).

Genetic ablation of HO-1 exacerbates gastritis and restores immunity to H. pylori

To further investigate the role of macrophage HO-1 in the pathophysiology of *H. pylori* infection, we infected WT and *hmox-1*-deficient mice for 2 months with strain PMSS1. There was a significant increase in gastric inflammation in infected *hmox-1*$^{-/-}$ mice compared to WT animals, as demonstrated by histologic gastritis scores (Fig. 6A) and representative histologic sections (Fig. 6B). We also found that the mRNA expression of the genes encoding the M1 markers iNOS, TNF-α and IL-12p40 was increased, and conversely, the mRNA level of the prototype Mreg cytokine IL-10 was decreased, in gastric macrophages isolated from *hmox-1*$^{-/-}$ mice, when compared to those from WT animals (Fig. 6C); in accordance with this, iNOS protein immunolocalizing to gastric macrophages was more induced in the gastric tissue of infected *hmox-1*$^{-/-}$ mice than WT animals (Fig. 6D). In addition, there were more transcripts of the genes encoding IFN-γ and IL-17 (Fig. 6E), the prototype cytokines of Th1 and Th17 responses, and more IL-17 protein (Fig. 6F) in gastric tissues from infected *hmox-1*$^{-/-}$ mice compared to infected WT animals. Consistent with the increased M1, Th1, and Th17 immune response in the *hmox-1*$^{-/-}$ mice, gastric colonization by *H. pylori* was significantly reduced with *hmox-1* deletion (Fig. 6G). These data establish that HO-1 downregulates gastric inflammation and favors *H. pylori* survival.

H. pylori-induced HO-1 regulates macrophage polarization

Because our studies indicated that HO-1 induction in gastric macrophages during *H. pylori* infection is associated with decreased iNOS and M1 cytokine expression and increased IL-10 expression (Fig. 6C) in WT mice, we reasoned that HO-1 may directly affect macrophage polarization. To test this hypothesis, we infected resident peritoneal macrophages from WT and *hmox-1*$^{-/-}$ mice with *H. pylori* for 24 h ex vivo, and analyzed mRNA expression of polarization markers. The genes encoding the M1 markers iNOS, TNF-α, IL-12p40, and IL-1β, and the Mreg markers IL-10, LIGHT, and CCL1 were

significantly induced by *H. pylori* in WT macrophages (Fig. 7A and Supplemental Fig. 2); among the eight M2 marker genes tested, only CCL17 was significantly induced during the infection of WT macrophages (Fig. 7A and Supplemental Fig. 2). These results suggest that *H. pylori*-infected macrophages exhibit a predominantly mixed M1/Mreg phenotype. Remarkably, the expression levels of iNOS, TNF-α, IL-12p40, and CXCL10 (M1 populations) were significantly increased in infected macrophages from *hmox-1*-deficient mice when compared to WT macrophages (Fig. 7A and Supplemental Fig. 2). Inversely, the M2 (CCL17) and Mreg (IL-10, LIGHT, and CCL1) genes were less expressed in infected *hmox-1*$^{-/-}$ macrophages than in WT cells (Fig. 7A and Supplemental Fig. 2). In accordance with these data, we found that significantly more NO and less IL-10 were released by infected macrophages from *hmox-1*$^{-/-}$ mice than from WT mice (Fig. 7B).

To further investigate the role of HO-1 on the modulation of the expression of the genes encoding M1 and Mreg markers, we used siRNA directed against *hmox-1* (Fig. 8A) or the HO-1 inhibitor CrMP to block the expression and the activity of HO-1 in RAW 264.7 cells, respectively. We observed that knockdown or pharmacological inhibition of HO-1 resulted in increased expression of iNOS and in a concomitant decrease in expression of IL-10 in *H. pylori*-infected macrophages (Figs. 8B and 8C). Collectively these data support the contention that macrophage HO-1 downregulates M1 polarization and favors an Mreg phenotype during *H. pylori* infection.

Discussion

Both innate and adaptive immunity play a cardinal role in controlling bacterial burden of *H. pylori* within the gastric mucosa (9, 18, 31). Nonetheless, the bacterium has elaborated numerous strategies to prevent the efficiency of the host immune response to survive in its ecological niche (32). In this context, we have identified a specific process by which *H. pylori* downregulates the inflammatory response of macrophages. The induction of HO-1 by *H. pylori* in murine macrophages through a p-CagA/p38/NRF-2-dependent pathway favors the polarization of macrophages towards an Mreg phenotype. Our finding has direct significance in vivo, since we have also demonstrated that HO-1 is induced in gastric macrophages of *H. pylori*-infected C57BL/6 mice. Lastly, this work also establishes that *H. pylori*-induced macrophage HO-1 restricts gastritis and favors colonization. In the same way, we have previously shown that the experimental induction of HO-1 in the gastric tissue by a treatment with hemin before *H. pylori* infection decreases the level of acute gastric inflammation (23).

The induction of HO-1 in macrophages is mostly known as a cellular response to oxidative or nitrosative stress (33). However, bacterial endotoxins (34) or invasive pathogens, such as *Mycobacterium tuberculosis* (35) or *Leishmania mexicana* (36), can also induce HO-1. The present work shows for the first time that *H. pylori* stimulates *hmox-1* expression in macrophages. It has been reported that these cells can be activated by numerous factors released by *H. pylori*, such as urease (12), Hsp60 (37), or LPS (38). Others have shown that contact between macrophages and *H. pylori* is required to stimulate the production of IL-18 by the human macrophage cell line THP-1 (39) and that phagocytosis contributes to maximal activation of dendritic cells (28). Accordingly, we found that separating *H. pylori*

from macrophages or the inhibition of phagocytosis resulted in a failure of *hmox-1* expression. Further, our experiments have established that CagA reaches the cytoplasm of macrophages after phagocytosis independently of the T4SS, is phosphorylated by c-Src, and induces HO-1 in macrophages. The phosphorylation of CagA in the murine macrophage cell line J774 has been reported (40). However, a cleaved form of CagA was evidenced in J774 cells infected for 4 h and 6 h (40), while we found intact CagA protein after a 1 h or 3 h infection. The difference in infection time may explain this difference. Interestingly, we found that the *H. pylori* strain 7.13 is less phagocytized by macrophages than the strain PMSS1 and 60190; in accordance with this, the protein CagA from the strain 7.13 is less phosphorylated and this results in less induction of *hmox-1*. Because we found that the phosphorylation of CagA in macrophages is not dependent on the presence of a T4SS, it should be noted that the ability of various strains of *H. pylori* to express and inject CagA in gastric epithelial cells is not relevant to what occurs in mononuclear cells.

While CagA has been implicated in cellular events leading to macrophage apoptosis (41), we have now discovered that p-CagA also signals in macrophages to stimulate the inducible transcription of *hmox-1* through the p38-NRF-2 pathway. The implication of this transduction pathway in *hmox-1* expression has been reported in macrophages stimulated with IL-10 (29), α-lipoic acid (42), or cobalt protoporphyrin (43); further, the genetic ablation of NRF-2 completely suppressed *hmox-1* transcription in peritoneal macrophages stimulated with diesel exhaust particles (44). Our results are consistent with the fact that the kinase p38 is rapidly activated in gastric epithelial cells by a molecular mechanism involving CagA (45, 46), and in monocytes/macrophages infected with *H. pylori* (47) or stimulated with purified *H. pylori* products including VacA or HP0175, a peptidyl prolyl cis-, trans-isomerase (48, 49). The ability of these other *H. pylori* components to activate p38 may explain why in our experiments the complete inhibition of CagA phosphorylation did not entirely suppress p38 phosphorylation and *hmox-1* expression.

Although the polarization of macrophages is usually initiated by cytokines and bacterial endotoxins, mediators of the innate immune response may also regulate the differentiation of myeloid cells (50, 51). Here we demonstrate that *H. pylori*-induced HO-1 is a regulator of macrophage polarization by tipping the M1/Mreg balance in favor of an Mreg phenotype. In support of the contention that HO-1 orchestrates the Mreg switching, it has been reported that *hmox-1* is one of the genes significantly upregulated in bone marrow-derived macrophages polarized into Mregs when compared to an M1 population (15) and that HO-1 is induced by M-CSF in IL-10-producing macrophages (52). Additionally, the transfer of a functional *hmox-1* cDNA using adenoviral delivery has been shown to enhance IL-10 production from alveolar macrophages that attenuates LPS-induced acute lung injury in mice (53) .

Various immunological mechanisms, such as impaired NO production (18, 54) and recruitment of regulatory T cells (10), may explain the persistence of *H. pylori* within the gastric mucosa. The Mreg population is known to dampen the immune response, which results in the decrease of inflammation (55) and/or in the progression of infectious diseases (56, 57). Moreover, these macrophages are efficient antigen-presenting cells inducing T-cell responses that are dominated by the production of anti-inflammatory cytokines (58).

Accordingly, we found that the genetic deletion of *hmox-1* leads to increased gastritis and decreased colonization in *H. pylori*-infected mice. Moreover, HO-1 products have been shown to regulate the expression of bacterial virulence factors, such as the dormancy regulon of *M. tuberculosis* (35). HO-1 might thus have a direct effect on *H. pylori* growth/virulence, and this deserves further investigation.

This work reveals another mechanism by which the *H. pylori* virulence factor CagA contributes to *H. pylori* pathogenesis, by causing signaling in macrophages that induces HO-1. This directly shapes the inflammatory response and favors the immune evasion of this pathogen. Conversely, we have shown that *H. pylori* inhibits HO-1 in gastric epithelial cells in vitro, as well as in the stomach of mice or humans infected with *cagA+ H. pylori* strains (23); we have also demonstrated that HO-1 inhibits *H. pylori*-induced c-Src activation and consequently CagA phosphorylation in gastric epithelial cells (59). Hence, the *H. pylori*-induced downregulation of HO-1 in epithelial cells can be a mechanism by which this pathogen facilitates phosphorylation of CagA and p-CagA-dependent neoplastic transformation. Therefore, the activation of HO-1 in macrophages and the inhibition of HO-1 in gastric epithelial cells are cellular mechanisms that both favor *H. pylori* persistence and pathogenesis. In this context, we propose that a specific crosstalk exists between *H. pylori* and host HO-1. This cell-dependent dichotomous regulation of HO-1 expression orchestrated by CagA represents an example of a successful adaptation of a pathogenic bacterium in its ecological niche.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

cag	cytotoxin-associated gene
CrMP	chromium mesoporphyrin
HO-1	heme oxygenase-1
iNOS	inducible NO synthase
NRF-2	nuclear factor (erythroid-derived 2)-like 2
T4SS	type IV secretion system
WT	wild-type

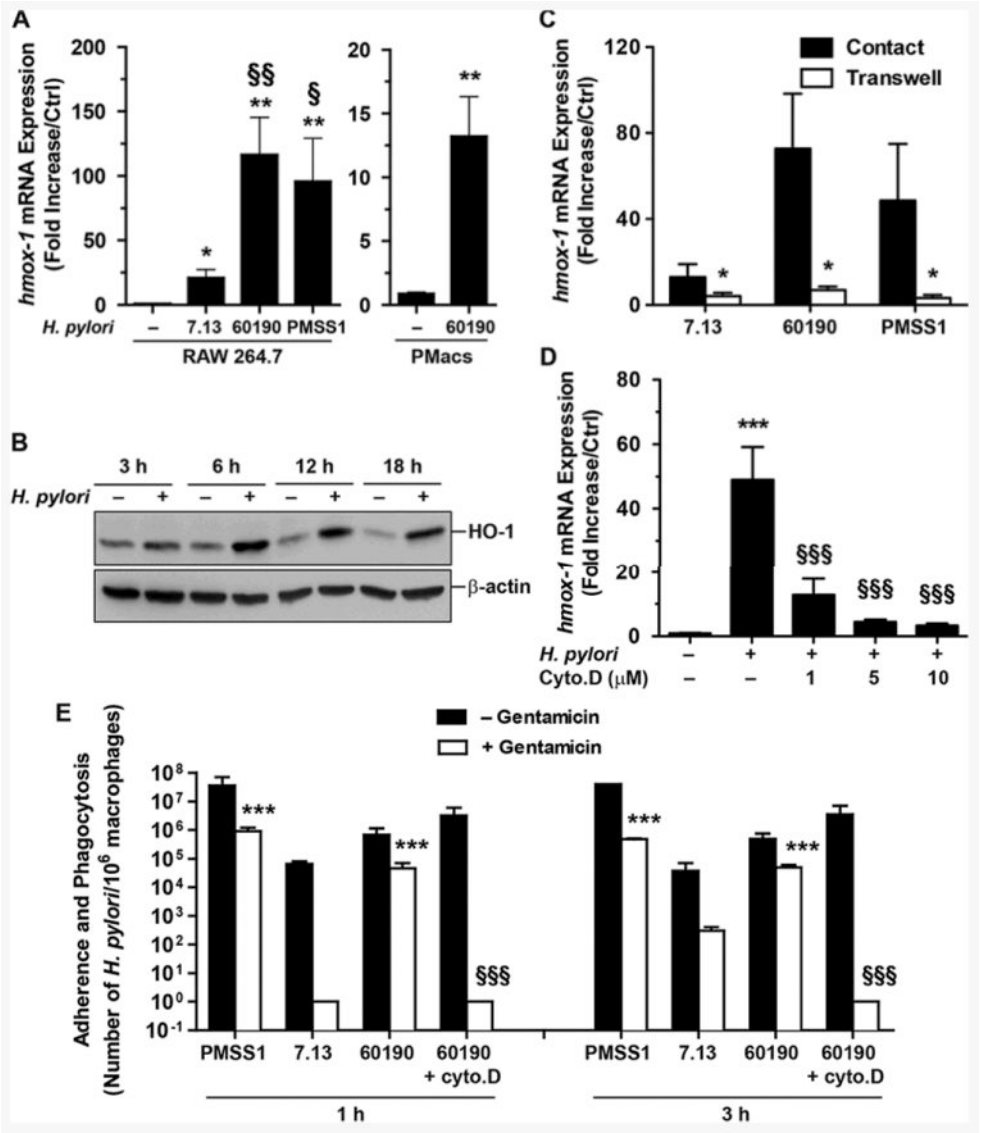

Figure 1.

Effect of *H. pylori* on HO-1 induction in macrophages. *A*, *hmox-1* RNA expression in RAW 264.7 cells and in murine peritoneal macrophages (PMacs) infected for 6 h with *H. pylori*. **P* < 0.05, ***P* < 0.01 vs. Ctrl; §*P* < 0.05, §§*P* < 0.01 vs. cells infected with strain 7.13; *n* = 5 for RAW 264.7 cells and *n* = 3 for PMacs. *B*, Levels of HO-1 in macrophages infected with *H. pylori* 60190. Data representative of 4 independent experiments. *C*, Induction of *hmox-1* by *H. pylori* in contact with the cells or separated from macrophages by Transwell filter supports. **P* < 0.05 vs. contact. *D*, Effect of cytochalasin D (Cyto.D) on *hmox-1* transcript levels in RAW 264.7 cells. ****P* < 0.001 vs. uninfected cells; §§§*P* < 0.001 vs. cells infected with the strain 60190; *n* = 3. *E*, Determination of *H. pylori* adherence plus phagocytosis (– Gentamicin) and phagocytosis (+ Gentamicin) by macrophages. Gentamicin added to *H. pylori* without macrophages killed 100% of the bacteria (data not shown). ****P* < 0.001 compared to the number of *H. pylori* 7.13 bacteria phagocytized by RAW 264.7 cells (+ Gentamicin); §§§*P* < 0.001 vs. the level of phagocytosis of *H. pylori* 60190; *n* = 3.

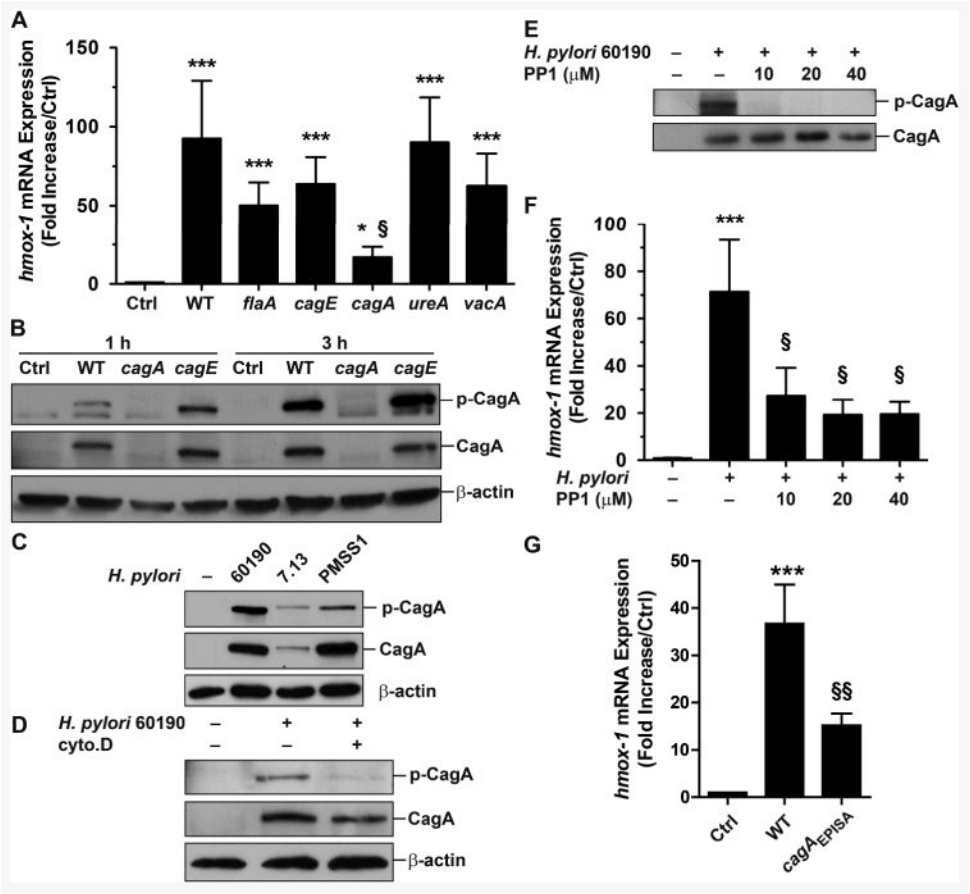

Figure 2.

Effect of *H. pylori* virulence factors on *hmox-1* expression. *A*, RAW 264.7 cells were infected for 6 h with WT *H. pylori* 60190 or with various isogenic mutants. The expression of *hmox-1* was analyzed by real-time PCR. **P* < 0.05, ****P* < 0.001 vs. Ctrl; §*P* < 0.05 vs. cells infected with WT; *n* = 5. *B*, Analysis of CagA phosphorylation in RAW 264.7 cells infected with *H. pylori* 60190 or with the *cagA* or *cagE* mutant strains. Data representative of 4 independent experiments for each. C, CagA phosphorylation in cells infected with 60190, 7.13, or PMSS1 for 3 h; data are representative of 3 experiments. *D*, RAW 264.7 cells pretreated with cytochalasin D (Cyto.D) were infected 2 h with *H. pylori* 60190; after a 1 h gentamicin treatment, CagA delivery and phosphorylation was analyzed. Data representative of 3 independent experiments. *E* and *F*, Effect of increasing concentrations of the c-Src inhibitor PP1 on CagA phosphorylation (*E*) and on *hmox-1* expression (*F*) in macrophages. ****P* < 0.001 vs. Ctrl; §*P* < 0.05 vs. *H. pylori*-infected macrophages; *n* = 3. *G*, *hmox-1* RNA expression in RAW 264.7 cells infected for 6 h with *H. pylori* G27 or the $cagA_{EPISA}$ mutant. ****P* < 0.001 vs. Ctrl; §§*P* < 0.05 vs. macrophages infected with the WT strain; *n* = 5.

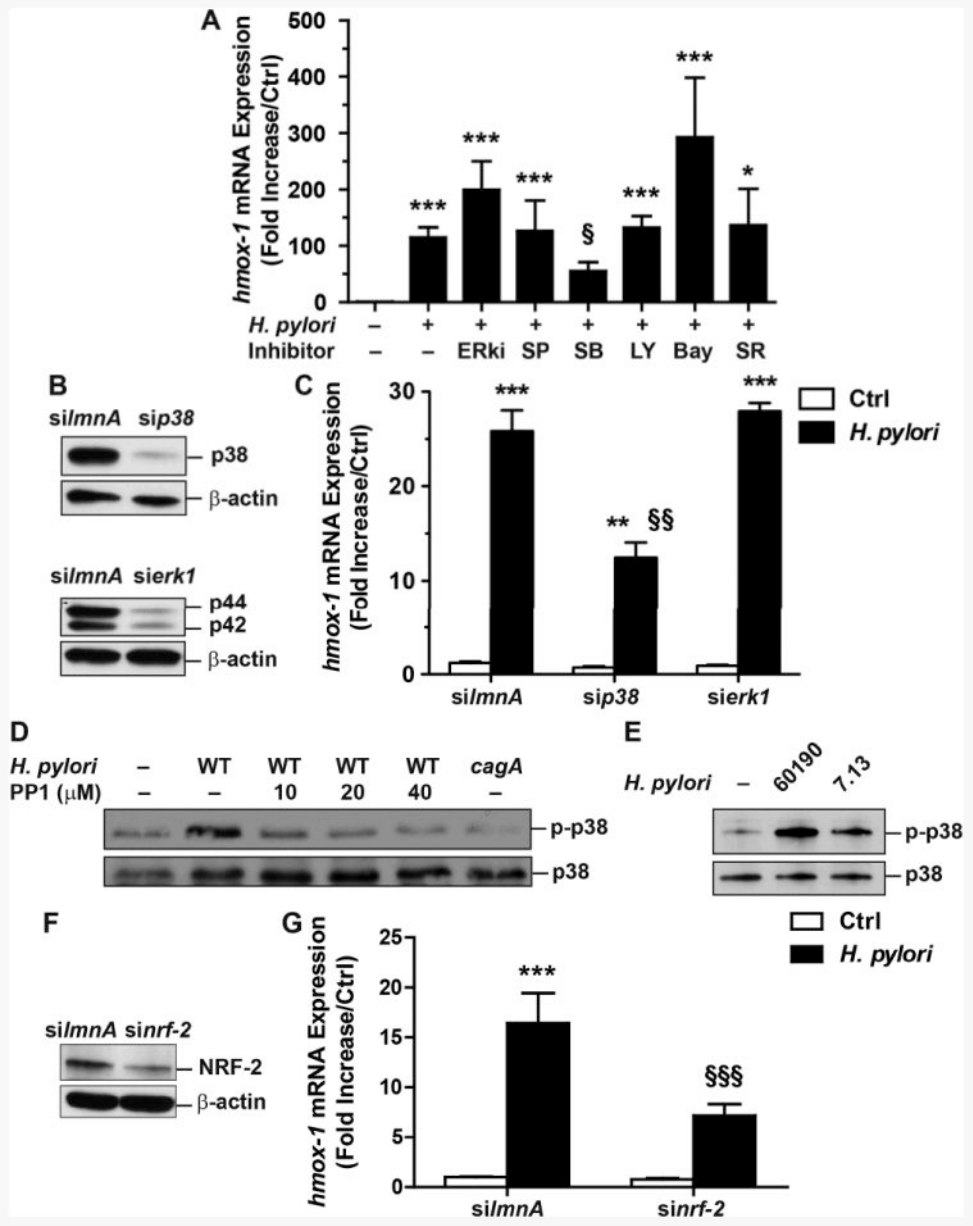

Figure 3.

Molecular regulation of *hmox-1* transcription in macrophages. *A*, *hmox-1* mRNA expression in RAW 264.7 cells pre-treated with ERKi, SP600125 (SP), SB203580 (SB), LY294002 (LY), Bay11-7082 (Bay), or SR11302 (SR) and infected with *H. pylori* for 6 h. ***$P <$ 0.001 vs. Ctrl; §$P < 0.05$ vs. infected cells; $n = 5$. *B*, Western blots showing the effect of knockdown of p38 and p42/p44 in RAW 264.7 cells transfected with *lmnA*, *p38*, or *erk1* siRNAs. *C*, Levels of *hmox-1* mRNA in macrophages transfected with siRNAs directed against *lmnA*, *p38*, or *erk1* and then treated with *H. pylori* for 6 h. ***$P < 0.001$, **$P < 0.01$ vs. Ctrl; §§$P < 0.01$ vs. cells transfected with *lmnA* or *erk1* siRNA and infected with *H. pylori; n* = 5. *D*, Levels of p-p38 and p38 in macrophages pretreated with PP1 and infected with *H. pylori* or with the *cagA* mutant. Representative data of 3 independent experiments.

E, p38 phosphorylation in RAW 264.7 cells infected with the strains 60190 or 7.13. *F*, Effect of *nrf-2* siRNA on knockdown of NRF-2 in RAW 264.7 cells. *G*, Levels of *hmox-1* mRNA in macrophages transfected with siRNAs directed against *lmnA* or *nrf-2* and then treated with *H. pylori* for 6 h. ***$P < 0.001$ vs. Ctrl; §§§$P < 0.001$ vs. cells transfected with *lmnA* and infected with *H. pylori; n* = 6.

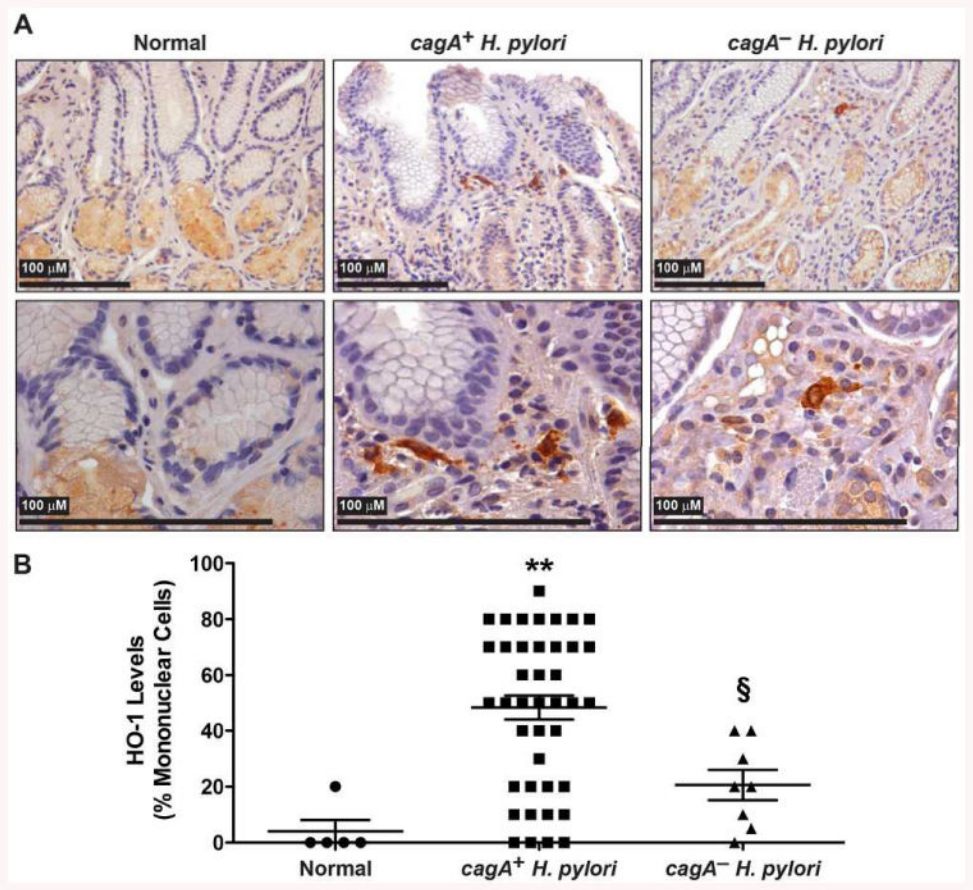

Figure 4.

Expression of HO-1 in patients infected with *H. pylori*. *A*, Representative HO-1 immunoperoxidase staining in gastric tissues. *B*, Quantification of staining score for HO-1 in mononuclear cells. $^{**}P < 0.01$ vs. uninfected patients; §$P < 0.05$ vs. individuals infected with *cagA+ H. pylori*. Each symbol is a different subject.

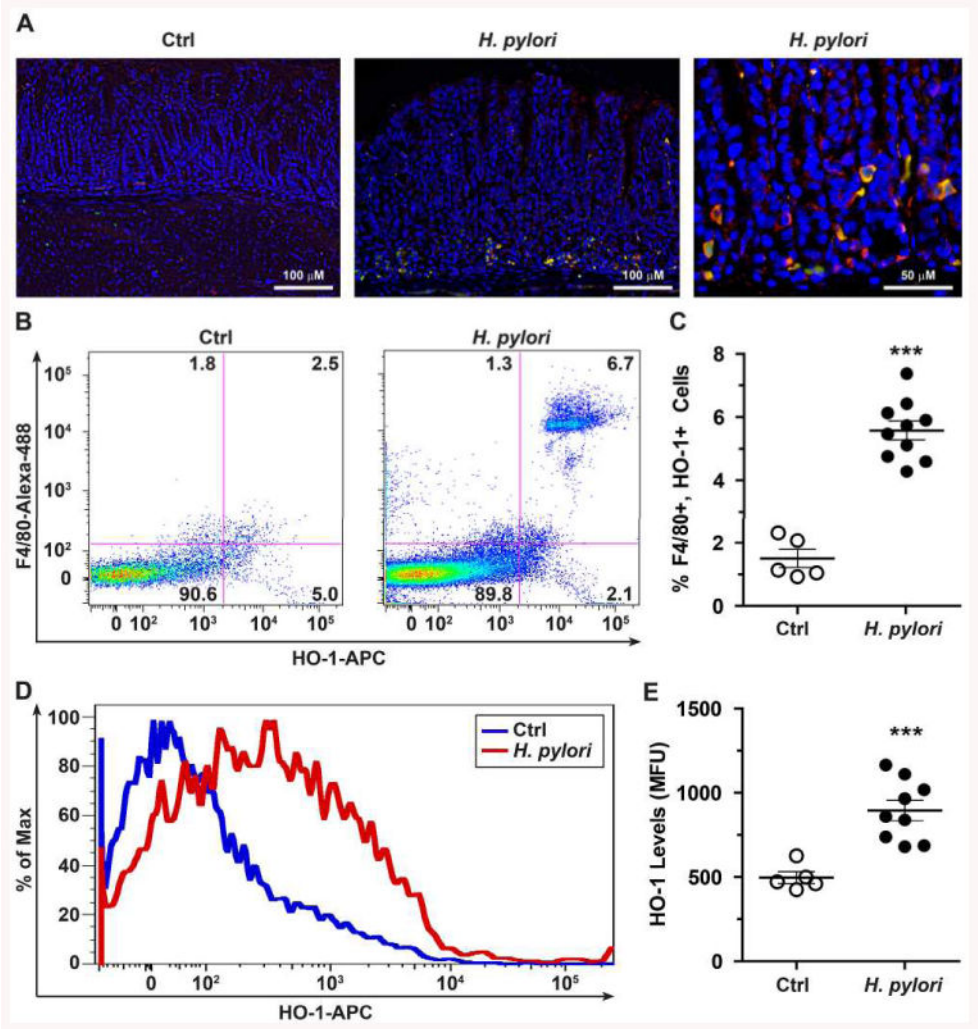

Figure 5.

Expression of HO-1 in gastric macrophages during *H. pylori* infection. *A*, Immunofluorescence performed in the gastric tissue of C57BL/6 mice infected or not with *H. pylori* PMSS1 for 2 months. The macrophage marker F4/80, HO-1, and nuclei were detected with TRITC (red), DyLight 488 (green), and DAPI (blue), respectively; merged images are shown, with cells double-positive for F4/80 and HO-1 depicted by yellow color. *B-E*, Gastric cells were isolated from mice and analyzed by flow cytometry for the expression of F4/80 and HO-1. Representative dot plots with percent of cells in each quadrant (*B*) and flow cytometric analysis of HO-1 levels in mean fluorescence units (*D*). The summary data are presented in (*C*) and (*E*). ***$P < 0.001$ vs Ctrl; each symbol represents a different mouse.

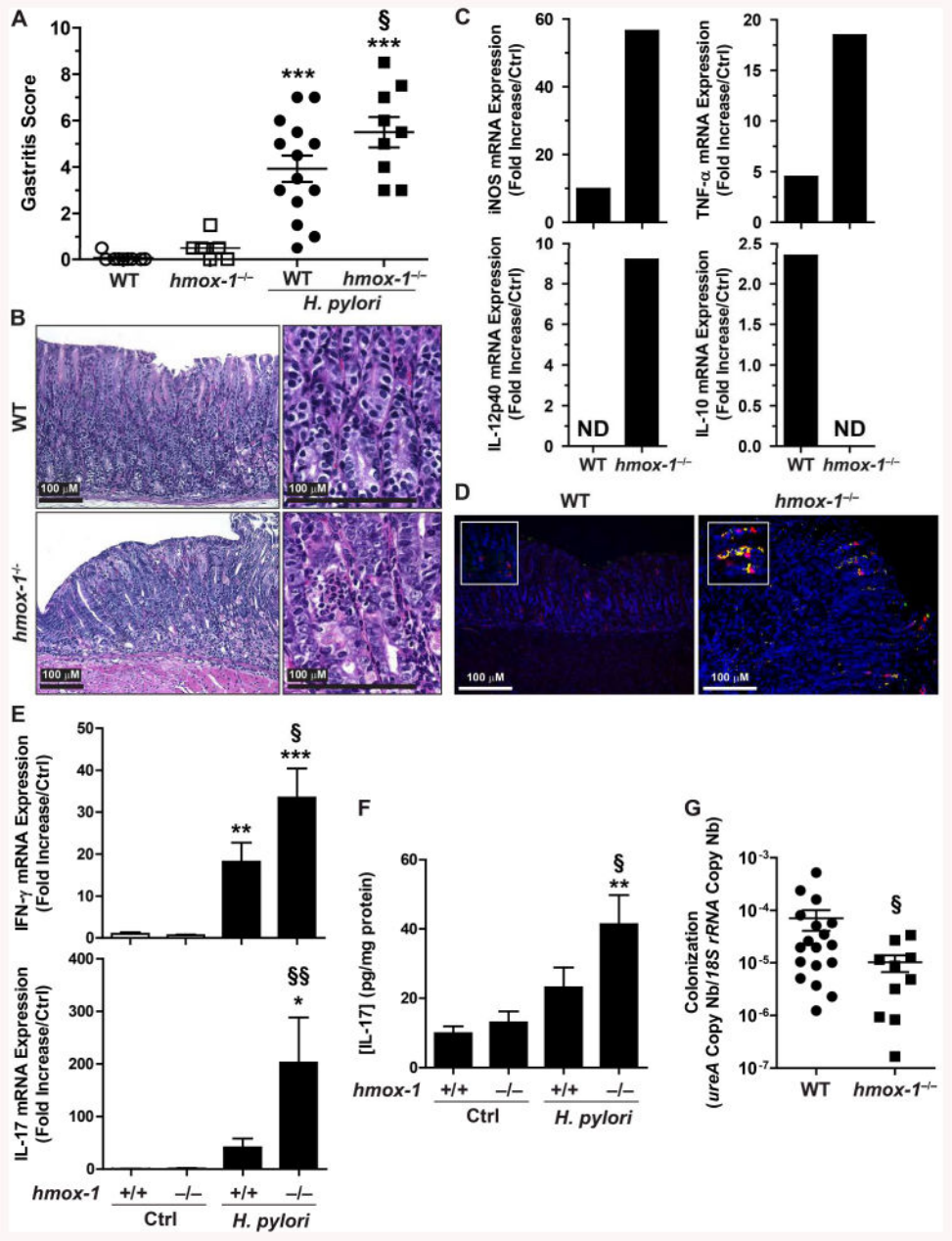

Figure 6.

Effect of *hmox-1* deletion on the outcome of *H. pylori* infection. WT and *hmox-1*$^{-/-}$ mice were infected with *H. pylori* PMSS1 for 2 months. *A* and *B*, Levels of gastritis. *C*, Expression of iNOS, TNF-α, IL-12p40, and IL-10 genes in gastric macrophages. Macrophages were purified from the gastric tissues of 3 WT mice, 5 *H. pylori*-infected WT mice, 3 *hmox-1*$^{-/-}$ mice, and 5 *hmox-1*$^{-/-}$ mice infected with *H. pylori*. The RNA from the gastric macrophages from each mouse was extracted and pooled in each group of mice before analysis by RT-qPCR. Values are expressed as fold increase compared to uninfected mice; ND, no PCR product detected. *D*, iNOS expression. Immunofluorescence for the macrophage marker F4/80 (red), iNOS (green), and nuclei (blue) in the gastric tissue of *H.*

pylori-infected mice. Merged images are shown, with the cells double-positive for iNOS and F4/80 evidenced by yellow color. *E*, Expression levels of IFN-γ and IL-17 mRNAs in gastric tissues. *F*, Concentration of IL-17 in the gastric tissues. *G*, Colonization of the stomach by *H. pylori*. For *A*, *D*, and *E*, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. uninfected animals; §*P* < 0.05, §§*P* < 0.01 vs. infected WT mice.

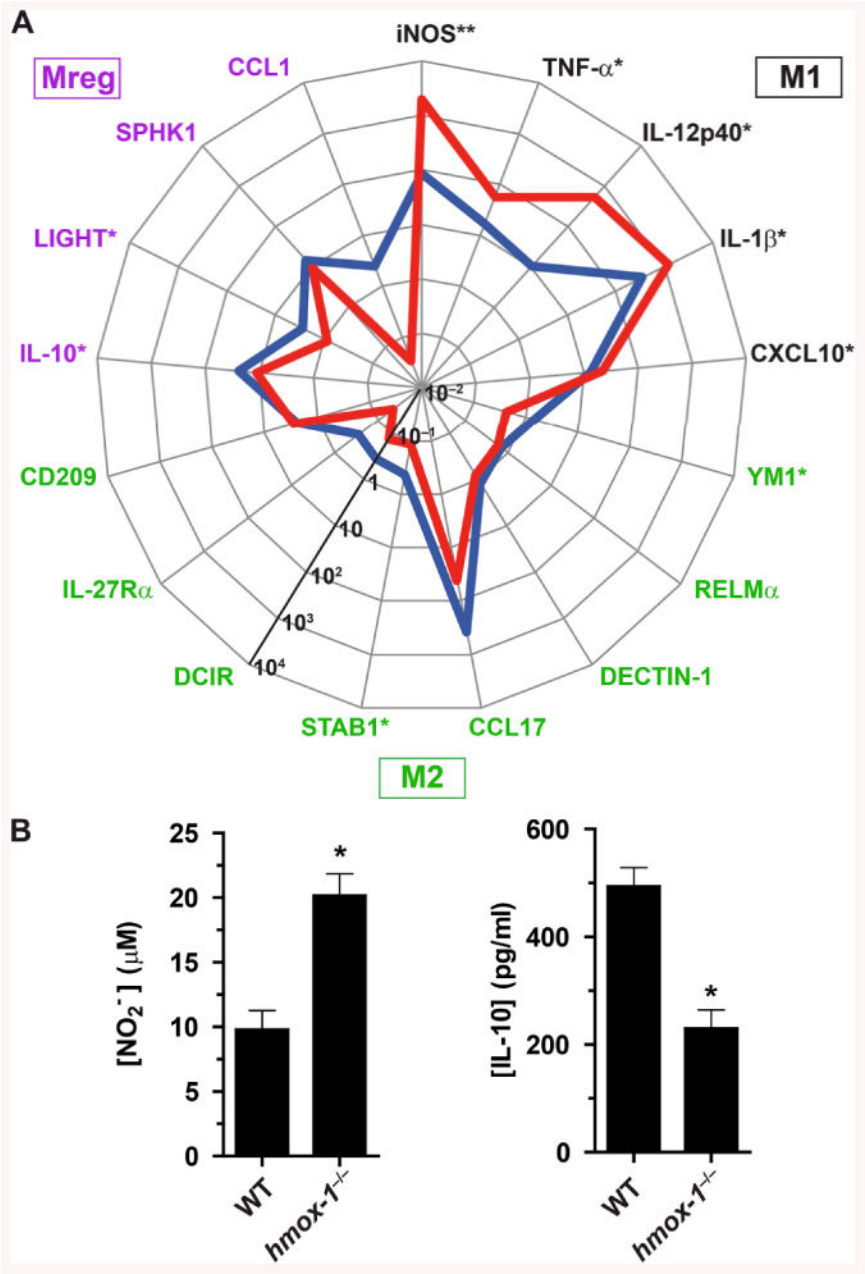

Figure 7.

Macrophage polarization in response to *H. pylori*. *A*, The mRNA levels of the genes encoding markers of the M1, M2, and Mreg populations were analyzed in peritoneal macrophages from WT (blue line) or *hmox-1*$^{-/-}$ (red line) mice infected with *H. pylori* 60190 for 24 h; $n = 6$ mice for each genotype. For each gene, asterisks denote significant differences between WT and *hmox-1*$^{-/-}$ mice (*$P < 0.05$, **$P < 0.01$). *B*, Concentrations of NO_2^- and IL-10 in the supernatant of peritoneal macrophages from WT and *hmox-1*$^{-/-}$ mice infected for 24 h with *H. pylori*. *$P < 0.05$ vs. WT; $n = 3$-6 mice.

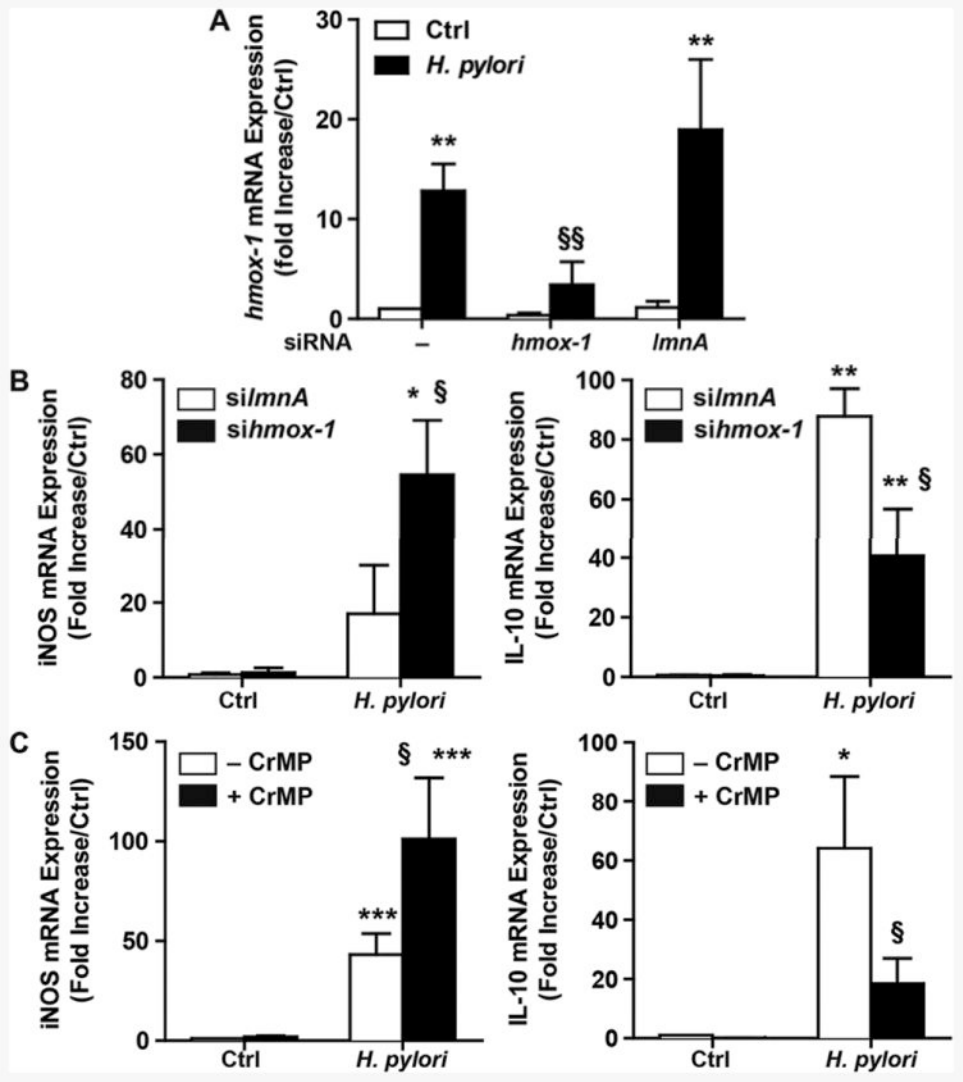

Figure 8.

Regulation of macrophage activation by HO-1. *A*, The expression of *hmox-1* was analyzed in RAW 264.7 cells that were transfected or not with siRNA against *hmox-1* or *lmnA* before infection with *H. pylori*. ***P* < 0.01 vs. uninfected macrophages; §§*P* < 0.01 vs. cells not transfected or transfected with si*lmnA* and infected with *H. pylori. B* and *C*, Levels of iNOS and IL-10 mRNA expression in RAW 264.7 transfected with siRNA against *lmnA* or *hmox-1* (*B*) or treated with CrMP (*C*), and infected with *H. pylori* for 24 h. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. Ctrl; §*P* < 0.05 vs. cells infected with *H. pylori* and transfected with *lmnA* (*B*) or not treated with CrMP (*C*); *n* = 3.