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

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Helicobacter pylori infects half of the world’s population and is the causative agent of chronic gastritis, peptic ulcer disease, and gastric MALT 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 cag A (CagA) (2, 3). When 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 hmx-1 or inhibition of HO-1 in macrophages caused an increased M1 and a decrease of Mreg phenotype. These data highlight a mechanism by which H. pylori impairs the immune response and favors its own survival via activation of macrophage HO-1. The Journal of Immunology, 2014, 193: 000–000.

H. pylori infection is 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 H. 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 hmx-1, the gene encoding HO-1, through p38/NF (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 hmx-1 in H. pylori–infected mice increased histologic gastritis, which was associated with enhanced M1/Th1/Th17 responses, decreased regulatory macrophage (Mreg) response, and reduced H. pylori colonization. Macrophages of H. pylori–infected mice and macrophages infected in vitro with this bacterium showed an M1/Mreg mixed polarization type; deletion of hmx-1 or inhibition of HO-1 in macrophages caused an increased M1 and a decrease of Mreg phenotype. These data highlight a mechanism by which H. pylori impairs the immune response and favors its own survival via activation of macrophage HO-1.
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 this 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 NF (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 Cruz Biotechnology. The following pharmacological compounds were obtained from Calbiochem: the NF-κB inhibitor SB203580 (4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-1,9-cd]pyrazol-6[2H]-one, 1,9-pyrazoloanthrone; 1 μM); the PI3K inhibitor LY294002 (2-[4-morpholinyl]-8-phenyl-4H-1-benzo[π]pyran-4-one; 10 μM); the c-Src inhibitor PP1 (4-amino-5-[4-methylphenyl]-3-[3-butyryl]pyrazolo-n,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 previously (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, cagA, vacA, and flaA* isogenic mutants constructed in the strain 60190 (22, 23) and the strain G27 carrying the CagA phosphorylation domains (cagAbPxis) (24) were also used. C57BL/6 × PVB hmox-1<sup>−/−</sup> mice were bred to generate wild-type (WT) and hmox-1<sup>−/−</sup> mice, as described previously (25, 26); hmox-1<sup>−/−</sup> 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 I). Animals were used under protocol M05/176 approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Mice were infected intragastrically three times, every 2 d, with 10<sup>6</sup> *H. pylori* PMSS1. Animals were sacrificed after 2 mo. Colonization was assessed by quantitative PCR using *H. pylori ureA* gene and mouse 18S rRNA primers (Supplemental Table I) as described previously (18).

**Purification of gastric macrophages**

Macrophages were isolated from mouse stomach exactly as described previously (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<sup>−/−</sup> mice were collected after i.p. 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 before activation.

To determine the levels of adhesion and phagocytosis of *H. pylori*, we washed RAW 264.7 cells thoroughly five times with PBS postinfection, incubated or not for 1 h 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-TARGETplus siRNAs (Dharmacon) directed against hmox-1, nrf-2, or lmox, 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 previously (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, NOS, and F4/80 was performed on murine gastric tissues (18) using the Abs described in Supplemental Table II.

**Luminex assay**

Gastric tissues were lysed in CellLyte MT Reagent (Sigma-Aldrich) 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 II. Stained cells were analyzed with an LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

**Analysis of mRNA levels**

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

**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 protein/lane. Primary and secondary Abs are listed in Supplemental Table II. Densitometric analysis of Western blots was performed with ImageJ 1.45s software (http://rsbweb.nih.gov/ij/).

**Statistics**

All the data shown represent the mean ± SEM. Student 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 with uninfected cells (Fig. 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 \textit{H. pylori} 60190, peaking 6 h postinoculation (Fig. 1B). Interestingly, we found that \textit{H. pylori}–induced \textit{hmxox-1} mRNA expression was significantly inhibited when the bacteria were separated from the macrophages using a 0.22-\textmu m filter support (Fig. 1C). Further, we observed that \textit{hmxox-1} mRNA expression (Fig. 1D) and the phagocytosis of \textit{H. pylori} by macrophages (Fig. 1E) were both reduced in infected macrophages treated with cytochalasin D that prevents phagocytosis of \textit{H. pylori} (28). Lastly, we found that \textit{H. pylori} 7.13, which induced \textit{hmxox-1} relatively poorly, was significantly less phagocytized by RAW 264.7 cells than the strains 60190 or PMSS1 (Fig. 1E). Notably, there was complete killing of \textit{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 \textit{H. pylori} phagocytosis is required to induce HO-1 in macrophages.

\textit{H. pylori}–induced HO-1 in macrophages requires CagA phosphorylation

We then assessed which bacterial factor was implicated in \textit{hmxox-1} expression. There was a significant reduction of \textit{hmxox-1} mRNA levels in RAW 264.7 cells infected with \textit{H. pylori cagA} compared with macrophages infected with the WT strain or with the \textit{flaA}, \textit{cagE}, \textit{ureA}, or \textit{vacA} mutants (Fig. 2A). This difference between the \textit{cagA} and \textit{cagE} mutants suggests that CagA, but not the T4SS, is involved in \textit{hmxox-1} expression. We then assessed the effect of 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 \textit{H. pylori} strain with deletion of \textit{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 \textit{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 pretreated with the \textit{c-Src} inhibitor PP1 (Fig. 2E) correlated with a marked attenuation in the expression of \textit{hmxox-1} (Fig. 2F). Lastly, the \textit{hmxox-1} gene was significantly less expressed in macrophages stimulated with a \textit{cagAEPISA} mutant strain than with WT \textit{H. pylori} (Fig. 2G), demonstrating the involvement of p-CagA in inducible transcription of \textit{hmxox-1}.

\textit{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 \textit{H. pylori}–induced \textit{hmxox-1} mRNA expression, whereas inhibitors of ERK1/2, JNK, PI3K, NF-\textkappaB, or AP-1 had no effect. None of these pharmacologic inhibitors had a significant effect on \textit{hmxox-1} expression in unin-
strains was known (21). Tissues from subjects infected with *cagA* To demonstrate the in vivo relevance of our findings, we evaluated infection mRNA expression (Fig. 3G).

hmox-1 may transactivate the *H. pylori* we found that p38 was less activated in macrophages infected with CagA phosphorylation (Fig. 2C) with the various p38. In accordance with the level of phagocytosis (Fig. 1E) and of these results show that p-CagA signals in macrophages to activate

RAW 264.7 cells infected for 6 h with WT *H. pylori* RAW 264.7 cells infected with *H. pylori* or with the cagA or cagE mutant strains. Data representative of four independent experiments for each. (C) CagA phosphorylation in cells infected with 60190, after a 1-h gentamicin treatment, CagA delivery and phosphorylation was analyzed. Data are representative of three 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 versus Ctrl, p < 0.05 versus WT, *H. pylori*–infected macrophages. (G) hmox-1 RNA expression in RAW 264.7 cells infected for 6 h with *H. pylori* G27 or the cagAEPHA mutant. *n = 5, **p < 0.001 versus Ctrl, *p < 0.05 versus macrophages infected with the WT strain.

**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. *n = 5, *p < 0.05, ***p < 0.001 versus Ctrl, *p < 0.05 versus cells infected with WT. (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 four independent experiments for each. (C) CagA phosphorylation in cells infected with 60190, 7.13, or PMSS1 for 3 h; data are representative of three 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 are representative of three 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. *n = 3, ***p < 0.001 versus Ctrl, *p < 0.05 versus *H. pylori*–infected macrophages. (G) hmox-1 RNA expression in RAW 264.7 cells infected for 6 h with *H. pylori* G27 or the cagAEPHA mutant. *n = 5, **p < 0.001 versus Ctrl, *p < 0.05 versus macrophages infected with the WT strain.

HM-X1 is induced in gastric macrophages during *H. pylori* infection

To demonstrate the in vivo relevance of our findings, we evaluated the expression of HM-X1 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 (Fig. 4A, 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 mo with *H. pylori* PMSS1 that retains a functional T4SS in vivo (30), when compared with uninfected mice (Fig. 5A and Supplemental Fig. 1), and that HO-1 staining 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 with 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 (Fig. 5D, 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−/− mice for 2 mo with strain PMSS1. There was a significant increase in gastric inflammation in infected *hmox-1−/−* mice compared with 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 with 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 with 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*−/− mice when compared with 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 (Fig. 8B, 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 toward an Mreg phenotype. Our finding has direct significance in vivo, because 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 macrophages from each mouse was extracted and pooled in each group of mice before analysis by real-time quantitative PCR. Values are expressed as fold increase compared with uninfected mice. (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. (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*. (A, D, and E) *p < 0.05, **p < 0.01, ***p < 0.001 versus uninfected animals, †p < 0.05, ‡p < 0.01 versus infected WT mice. ND, no PCR product detected.
Concentrations of NO$_2$ to macrophage apoptosis (41), we have now discovered that populations were analyzed in peritoneal macrophages from WT (blue line) mRNA levels of the genes encoding markers of the M1, M2, and Mreg hmox-1 n 2

the presence of a T4SS, it should be noted that the ability of various hmox-1 protein CagA from the strain 7.13 is less phosphorylated, and than the strain PMSS1 and 60190; in accordance with this, the cells is not relevant to what occurs in mononuclear cells. H. pylori (28). Accordingly, we found that separating Macrophage polarization in response to hmox-1 differences between WT and 2

2

2

H. pylori i n f e c t e df o r4 a n d6h( 4 0 ) ,w h e n a sw ef o u n di n t a c tC a g A

However, a cleaved form of CagA was evidenced in J774 cells 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 and 6 h (40), whereas we found intact CagA protein after a 1- 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.

Although 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). In this study, 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 with an M1 population (15), and that HO-1 is induced by M-CSF in IL-10–producing macrophages (52). In addition, 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 APCs 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 cross talk exists between H. pylori and host HO-1. This cell-dependent dichotomous
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 versus uninfected macrophages; \( ^{\dagger}p < 0.01 \) versus cells not transfected or transfected with siRNAA 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. n = 3. \( *p < 0.05 \), \( **p < 0.01 \), \( ***p < 0.001 \) versus Ctrl, \( p < 0.05 \) versus cells infected with H. pylori and transfected with lmnA (B) or not treated with CrMP (C).

regulation of HO-1 expression orchestrated by CagA represents an example of a successful adaptation of a pathogenic bacterium in its ecological niche.

Disclosures
The authors have no financial conflicts of interest.

References