

Myeloid HIF-1 Is Protective in *Helicobacter pylori*-Mediated Gastritis

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Helicobacter pylori infection triggers chronic inflammation of the gastric mucosa that may progress to gastric cancer. The hypoxia-inducible factors (HIFs) are the central mediators of cellular adaptation to low oxygen levels (hypoxia), but they have emerged recently as major transcriptional regulators of immunity and inflammation. No studies have investigated whether *H. pylori* affects HIF signaling in immune cells and a potential role for HIF in *H. pylori*-mediated gastritis. HIF-1 and HIF-2 expression was examined in human *H. pylori*-positive gastritis biopsies. Subsequent experiments were performed in naive and polarized bone marrow-derived macrophages from wild-type (WT) and myeloid HIF-1 α -null mice (HIF-1 ^{Δ myel}). WT and HIF-1 ^{Δ myel} mice were inoculated with *H. pylori* by oral gavage and sacrificed 6 mo postinfection. HIF-1 was specifically expressed in macrophages of human *H. pylori*-positive gastritis biopsies. Macrophage HIF-1 strongly contributed to the induction of proinflammatory genes (IL-6, IL-1 β) and inducible NO synthase in response to *H. pylori*. HIF-2 expression and markers of M2 macrophage differentiation were decreased in response to *H. pylori*. HIF-1 ^{Δ myel} mice inoculated with *H. pylori* for 6 mo presented with a similar bacterial colonization than WT mice but, surprisingly, a global increase of inflammation, leading to a worsening of the gastritis, measured by an increased epithelial cell proliferation. In conclusion, myeloid HIF-1 is protective in *H. pylori*-mediated gastritis, pointing to the complex counterbalancing roles of innate immune and inflammatory phenotypes in driving this pathology. *The Journal of Immunology*, 2015, 194: 000–000.

Infection by *Helicobacter pylori* is the most common chronic bacterial infection of humans, affecting half the population worldwide. Persistent colonization of the gastric mucosa by *H. pylori* leads to chronic gastric inflammation, which may progress to peptic ulcer disease, gastric cancer, or MALT lymphoma (1). *H. pylori* infection is a major determinant initiating the sequence of events leading to gastric cancer, in combination with host and environmental factors. By recruiting immune cells, the bacterium triggers chronic inflammation of the gastric mucosa, and depletion of macrophages has been shown to reduce gastric

pathology in *H. pylori*-infected mice (2), emphasizing a key role for macrophages in this process.

Macrophages have multiple functions ranging from host defense, tissue repair, to inflammatory regulation (3), but their mechanistic role in *H. pylori* disease remains poorly understood. Macrophages exhibit remarkable developmental and functional plasticity, with classically activated macrophages (M1) representing one extreme—the proinflammatory state—and alternatively activated macrophages (M2) representing the opposite anti-inflammatory state. Generally, the M1–M2 switch observed during the transition from acute to chronic infection may provide the host protection against destructive uncontrolled inflammation (4). An enhanced degree of M1 macrophage polarization has been described in human *H. pylori*-associated atrophic gastritis (5).

The hypoxia-inducible factors (HIFs) are transcription factors involved in the adaptation to low levels of oxygen, composed of an O₂-regulated α -subunit and a constitutively expressed β -subunit. Hydroxylation of HIF-1 or HIF-2 by a family of prolyl hydroxylase promotes binding of the von Hippel-Lindau protein, leading to HIF ubiquitination and proteasomal degradation. Despite a high degree of sequence homology, HIF-1 and HIF-2 have been described to have nonoverlapping, and even sometimes opposing, roles (6, 7). HIF-1 is largely studied for its role during cancer development, modulating the transcription of genes involved in angiogenesis, cell survival, and invasiveness (8).

HIF factors have emerged as essential regulators of inflammation (9). The HIF-1 α subunit can be stabilized in normoxia by a number of cytokines, growth factors, and other circulating molecules (e.g., NO) by differential intracellular signaling mechanisms (10). HIF-1-deficient macrophages and neutrophils have a marked reduction in ATP pools, accompanied by impairment of cell aggregation, motility, and invasiveness (11). Moreover, HIF-1 deletion in the

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Abbreviations used in this article: BMDM, bone marrow-derived macrophage; Cag, cytotoxin-associated gene; HIF, hypoxia-inducible factor; HP, *H. pylori* infection; Nos2, inducible NO synthase; PAI, pathogenicity island; WT, wild-type.

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myeloid cells is associated with the downregulation of key cytokines and chemokines, such as TNF, IL-1, IL-6, and IL-8 (12–14). HIF-1 (15) and HIF-2 (16) also extend the lifespan of neutrophils by inhibiting apoptotic pathways. In addition, studies using RNA interference showed that HIF-1 α and HIF-2 α , more so than NF- κ B, are important in orchestrating the changes in macrophage receptor and cytokine gene expression in response to hypoxia (17).

Given the evidence supporting a role for HIF-1 signaling in inflammatory conditions, a number of recent patents have described methods to inhibit HIF-1 signaling in the treatment of inflammatory disorders (18). However, we and other groups have shown that HIF-1 is induced in phagocytes by a certain number of bacteria even under normal levels of oxygen (12, 14, 19). The activation of the HIF-1 α pathway through deletion of von Hippel-Lindau protein or pharmacologic inducers supported myeloid cell production of defense factors and improved bactericidal capacity (14). Conversely, some pathogens interfere with HIF activity to improve their survival and proliferation (20, 21). Therefore, because of the dual role of HIF-1 in inflammation and bactericidal activity, there is an open question as to whether inhibiting myeloid HIF-1 would be beneficial or detrimental in chronic gastric inflammation triggered by *H. pylori* infection.

Materials and Methods

Cell culture, *H. pylori* strains, and growth conditions

SS1 (22) and B128 (23) *H. pylori* strains were grown on 10% blood agar under microaerophilic conditions. Bacterial lysates were obtained by passage through a French press, and protein concentrations were determined by a Bradford assay.

Bone marrow–derived macrophage infection

To isolate bone marrow–derived macrophages (BMDMs), the marrow of femurs and tibias of wild-type (WT) and HIF-1^{Δmyel} mice were collected. Cells were plated in DMEM supplemented with 10% heat-inactivated FBS and 30% conditioned medium (a 7-d supernatant of fibroblasts from cell line L-929 stably transfected with a M-CSF expression vector). Mature adherent bone marrow cells were harvested by gentle scraping after 7 d in culture. BMDMs were incubated with 5 μ g/ml bacterial extracts for 1–24 h. BMDMs were pretreated, or not, for 48 h with either 50 ng/ml IFN- γ to induce a M1 phenotype or 10 ng/ml IL-4 to induce a M2 phenotype. When specified, BMDMs were put on 0.5% oxygen pressure in a hypoxic chamber (InVivo2 500; Ruskinn), 24 h before the incubation with bacterial extracts.

Neutrophil-killing assay

To isolate neutrophils from WT and HIF-1 α knockout mice, animals were injected with 1 ml 3% thioglycolate solution (BD Biosciences), and peritoneal neutrophils were collected via lavage with PBS after 4 h. The neutrophils were then counted, resuspended in RPMI 1640 with 2% FBS, and mixed with *H. pylori* (strain SS1) at a multiplicity of infection of 2 in 96-well plates. Plates were centrifuged at 1600 rpm to facilitate contact between the neutrophils and bacteria and incubated for 1 h at 37°C. Subsequently, samples from each well were serially diluted in water to lyse neutrophils, and the final dilutions (1:1000) were plated on BBL trypticase soy agar plates with 5% sheep blood (BD Biosciences). *H. pylori* colonies were counted after a 5-d incubation period at 37°C with 10% CO₂.

Macrophage-killing assay

BMDMs were seeded at a density of 8×10^5 cells/well in 24-well plates the day prior to the assay. Bacteria were added at a multiplicity of infection of 10 in 350 μ l assay media (RPMI 1640, 2% FBS). Plates were then centrifuged at 1600 rpm for 5 min to facilitate contact between the bacteria and the macrophages. Cells and bacteria were then incubated for 30 min at 37°C, 5% CO₂, before gentamicin (100 μ g/ml) was added to each well to kill extracellular bacteria. The cells were rinsed with PBS to remove extracellular dead bacteria, and 350 μ l fresh media was added to each well. After an additional 2.5 h, cells were lysed via addition of Triton X-100 (0.025% final), and dilutions were plated to quantify bacteria.

Reverse transcription and real-time quantitative PCR

After cells were harvested, total RNA was extracted from cells using TRIzol (Life Technologies), according to the instructions provided by the manu-

facturer. Reverse transcription was performed with 100 ng total RNA, as described earlier. Quantitative PCR was performed with 1 μ l 1/10 dilution of reverse-transcribed total RNA dilution, 10 μ M each primer, and 2 mM MgCl₂ in 1 \times LightCycler DNA Master SYBR Green 1 mix using a Light-Cycler apparatus (Roche Applied Science). All samples were normalized to the threshold cycle value for cyclophilin. Sequences of the primers used are presented in Table I.

Mice infection

Animal studies described in this work were reviewed and approved (Agreement P2.CP.151.10.) by the Président du Comité d’Ethique pour l’Expérimentation Animale Paris Descartes. HIF-1^{Δmyel} mice in a C57BL/6 background have been provided by R. Johnson (University of Cambridge). Cell-specific inactivation of *Hif1a* in the myeloid lineage was achieved by cross-breeding LysM-Cre^{+/-} transgenic mice (24) with HIF-1 α ^{fl/fl} mice (25). In all experiments, littermates from the same breeding pair were used as controls. Mice were inoculated once by oral gavage with 100 μ l suspension of 10⁹ CFUs/ml *H. pylori* strain SS1 (WT, $n = 14$; HIF-1^{Δmyel}, $n = 15$). Control groups of mice (WT, $n = 7$; HIF-1^{Δmyel}, $n = 11$) were given peptone trypsin broth alone. The mice were killed 6 mo postinfection. Stomachs were isolated and divided into three homogenous segments, each containing antrum and body, and used for histopathologic analyses, determination of colonization, and quantitative PCR analyses.

Assessment of infection

Serum samples were obtained at 3 and 6 mo postinfection. Infection status was tested for *H. pylori* Ag-specific IgG Ab, as previously described by ELISA technique (26). The presence of *H. pylori* bacteria was also histologically confirmed by PCR and by Warthin–Starry staining.

Human biopsies

Gastric samples were obtained from patients referred at the digestive endoscopy unit of Ambroise Paré Hospital. All gastric biopsies were performed for diagnostic purpose. The diagnosis was based on histology performed on these samples. The presence of *H. pylori* infection (HP) was confirmed by staining with Giemsa and/or immunohistochemistry. None of the patients had any clinical evidence of atheromatous disease complication.

Patients were informed that samples could also be used for research analysis, according to French ethical laws. Collection of human tissues was declared to the French Ministry of Research (DC 2009-933-404 and DC 2009-933-406) and Commission Nationale de l’Informatique et des Libertés.

Histology and immunohistochemistry

Gastric tissue samples from noninfected and infected mice were fixed in formalin and embedded in paraffin wax. Serial 4- μ m sections were cut and stained with H&E, periodic acid–Schiff, and Alcian blue using standard procedures. Tissue sections were examined for histopathologic lesions in a blinded fashion. The severity of gastritis in all groups of mice was evaluated semiquantitatively. Gastritis scores were determined according to the density of lymphocytes and plasma cells according to the Sydney classification and as previously described in the mouse model (27). This scoring is based on the standard histologic criteria used in human pathology (28). Briefly, this grading involves an evaluation of the recruitment of inflammatory cells (polymorphonuclear and mononuclear cells) in both antrum and corpus classified into four grades, as follows: none, mild, moderate, or severe.

Mouse gastric biopsies were subjected to proteinase Ag retrieval, blocking endogenous peroxidases, and immunohistochemistry with primary Ab against F4/80 (ab6640; Abcam, Cambridge, U.K.) diluted 1:200. After incubation with biotinylated secondary Ab, F4/80 was revealed with diaminobenzidine method.

For Ki67 immunofluorescence, tissues were subjected to a heat-induced epitope retrieval, incubated with rabbit polyclonal anti-Ki67 Ab (ab15580; Abcam, Cambridge, U.K.) diluted 1:100, and revealed with secondary Ab conjugated to Alexa 488.

Human gastric tissue sections were subjected to a heat-induced epitope retrieval. Sections were incubated with both rabbit polyclonal anti-HIF1 α Ab (NB100-449; Novus Biologicals, Littleton, CO) diluted 1:50 and mouse monoclonal anti-human CD68 (clone KP1; Dako, Glostrup, Denmark) diluted 1:100 in PBS containing 3% BSA, 0.1% Tween 20, and 1% goat serum at 4°C overnight. The sections were washed in PBS and then incubated with secondary Ab conjugated, respectively, to Alexa 488 and Alexa 568 (Invitrogen, Paisley, U.K.) diluted 1:400.

HIF-2 α was detected using rabbit polyclonal Ab (NB100-122; Novus Biologicals, Littleton, CO) with TSA Fluorescence Systems Tyramide Signal Amplification (NEL701; PerkinElmer), according to manufacturer’s protocol.

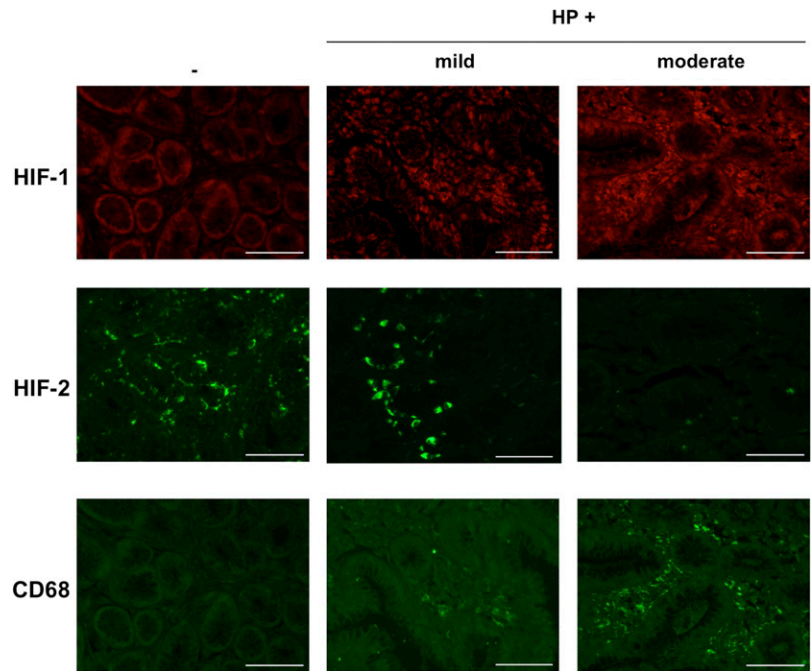


FIGURE 1. Immunostaining for HIF-1 α , HIF-2 α , and CD68 on sections of human normal (–) gastric biopsies and mild or moderate *H. pylori* positive (HP⁺) gastritis. One representative picture for each group ($n = 5$ per group). Scale bars, 50 μ m. (HCX PL Fluotar L 40 \times /0.6 PH2, Leica DMI-3000B microscope, Leica DFC 310FX digital camera, LAS V3.8 Core software.)

Statistical analysis

Data were analyzed in Graphpad Prism 5, and the significance of experimental differences was evaluated by one-way ANOVA, followed by Newman-Keuls posthoc testing. Values in the figures are expressed as mean \pm SEM. Statistical significance is indicated by symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

HIF-1, but not HIF-2, is stabilized in macrophages of human H. pylori-positive gastritis biopsies

Expression of HIF-1 and HIF-2 was investigated by immunohistochemistry performed on biopsies of control patients with normal gastric mucosa ($n = 5$) and of gastritis patients with *H. pylori* infection (HP⁺; $n = 5$). Although HIF-1 was not expressed in normal gastric biopsies, HIF-1 staining was greatly enhanced in HP⁺ biopsies, with a higher expression in moderate than in mild gastritis. HIF-1 expression was therefore correlated to the severity of the gastritis. In particular, HIF-1 was abundantly expressed in macrophages of human *H. pylori*-associated gastritis biopsies (Fig. 1), as shown by a costaining of HIF-1 with CD68, a specific marker of macrophages. Conversely, HIF-2 was not stabilized in the macrophages of *H. pylori*-associated gastritis biopsies. Noteworthy, epithelial HIF-2 expression appeared to gradually decrease with the severity of the *H. pylori*-associated gastritis.

Differential regulation of HIF-1 and HIF-2 by H. pylori in BMDMs

The species *H. pylori* comprises a large diversity of strains. Many *H. pylori* strains encode the cytotoxin-associated gene (Cag) pathogenicity island (PAI), which expresses a type IV secretion system, a syringe-like structure that injects virulence factors including the CagA effector protein into gastric epithelial cells (29). We examined the ability of a Cag-PAI⁺ *H. pylori* strain (B128) (22) and a Cag-PAI inactive *H. pylori* strain (SS1) (23) to stabilize HIF-1 *in vivo*. BMDMs from WT C57BL/6 mice were incubated with bacterial extracts of B128 and SS1 for 24 h. Both strains were able to induce the transcription of HIF-1 (Fig. 2A), suggesting that the increase of HIF-1 mRNA levels in BMDMs in response to *H. pylori* occurs independently of Cag-PAI function. The moderate increase in HIF-1 α transcriptional activation, however, correlated to a strong induction of

HIF-1 α at the protein level, suggestive of both transcriptional and posttranscriptional regulation (Fig. 2B). The increase in HIF-1 expression was also observed with live B128 and SS1 *H. pylori* strains

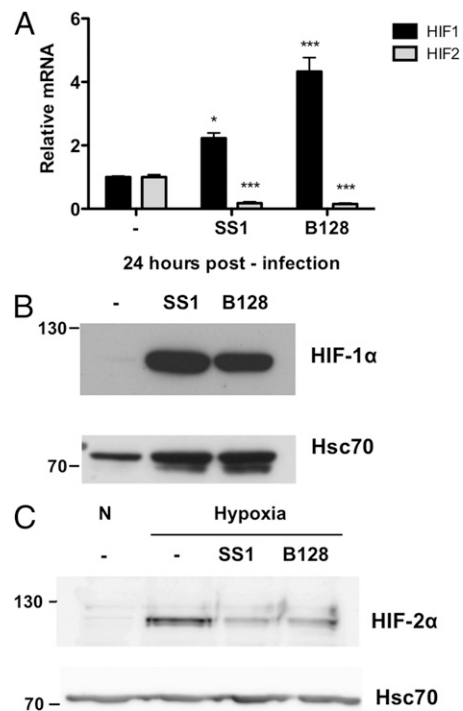


FIGURE 2. Regulation of HIF-1 in BMDMs infected with SS1 or B128 *H. pylori* strains. **(A)** Quantitative RT-PCR for *Hif1a* and *Hif2a* in WT BMDMs incubated with 5 μ g/ml SS1 or B128 bacterial extracts for 24 h. One representative experiment (in triplicate) of three independent experiments. **(B)** Western blot for HIF-1 in WT BMDMs incubated with 5 μ g/ml SS1 or B128 bacterial extracts for 24 h. One representative experiment of three independent experiments. **(C)** HIF-2 expression in WT BMDMs challenged or not with 0.5% oxygen pressure 24 h before incubation with 5 μ g/ml SS1 or B128 bacterial extracts for 24 h. One representative experiment of three independent experiments. * $p < 0.05$, *** $p < 0.001$.

Table I. Sequence of the primers

Primers	Sequence
mHIF-1-F	5'-TGAGCTTGCTCATCGTTGC-3'
mHIF-1-R	5'-CCATCTGTGCCTTCATCTCA-3'
mHIF-2-F	5'-TGAGTTGGCTCATGAGTTGC-3'
mHIF-2-R	5'-TGCTGATGTTTCCGACAG-3'
mIL-1 β -F	5'-GCCCATCCTCTGTGACTCAT-3'
mIL-1 β -R	5'-AGCCACAGGTATTTGTGTCG-3'
mIL-6-F	5'-CTTCAACCAAGAGGTAAGATTTA-3'
mIL-6-R	5'-GAGAGCATGGAATTGGGTAGGAAG-3'
miNOS-F	5'-CTGCTGGTGGTGACAAGCACATTT-3'
miNOS-R	5'-ATGTCATGAGCAAAGGCGAGAAC-3'
mFibronectin-F	5'-TGCCCTCGGGAATGGAAG-3'
mFibronectin-R	5'-ATGGTAGGTCCTCCATCGTCATA-3'
mArginase-F	5'-AGCTCCAAGCCAAGTCCTTAGA-3'
mArginase-R	5'-CCTCCTCGAGGCTGCTCTT-3'

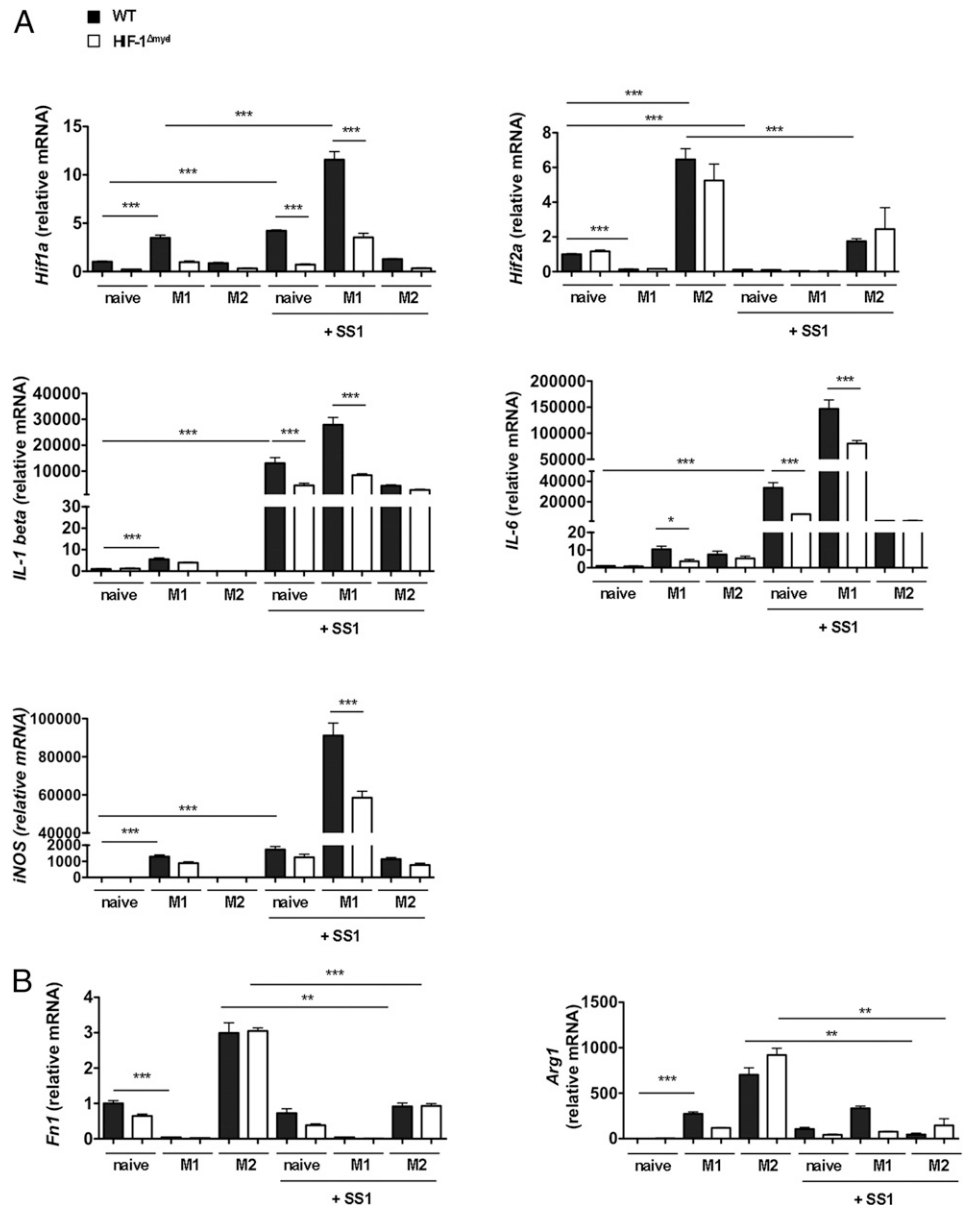
(Supplemental Fig. 1) both at the mRNA and protein levels. Unexpectedly, HIF-2 mRNA levels drastically decreased in response to infection with both *H. pylori* strains. This decrease was also observed at the protein level, when the cells were preincubated for 24 h under

hypoxia (Fig. 2C). For subsequent experiments, we limited our study to SS1, which is the mouse-adapted *H. pylori* strain (23).

HIF-1 contributes to the proinflammatory response induced by H. pylori

To assess the functional consequences of HIF-1 α activation by *H. pylori*, we first measured the expression of proinflammatory genes in naive BMDMs from WT mice and HIF-1 Δ myel mice (list of primers used in Table I). Targeted deletion of the HIF-1 α gene was achieved via crosses onto a background of Cre expression driven by the lysozyme M promoter (lysM-cre), thus allowing for specific deletion of the transcription factor in the myeloid lineage, that is, monocytes/macrophages and neutrophils (11). We first confirmed the decrease in HIF-1 expression in BMDMs of HIF-1 Δ myel mice compared with WT BMDMs and validated the lack of compensatory mechanism by HIF-2 (Fig. 3A). We examined the mRNA induction for the genes encoding IL-1 β , IL-6, and inducible NO synthase (Nos2), whose increased expression levels have been previously reported in *H. pylori*-induced gastritis in humans and mice (30–34). We found that the expression of HIF-1 α , Nos2, IL-1 β , and IL-6 was strongly in-

FIGURE 3. HIF-1 dependency of SS1-induced proinflammatory cytokine mRNA expression by BMDMs. Quantitative RT-PCR for (A) *Hif1a*, *Hif21a*, *Nos2*, *IL-1 β* , and *IL-6* and (B) *Fnl* and *Arg1* on WT and HIF-1 Δ myel BMDMs stimulated with 5 μ g/ml SS1 bacterial extracts for 24 h. BMDMs were pretreated, or not (naive), for 48 h with either 50 ng/ml IFN- γ (M1) or 10 ng/ml IL-4 (M2). Pool of three repeated experiments performed in triplicate. * p < 0.05, ** p < 0.01, *** p < 0.001.



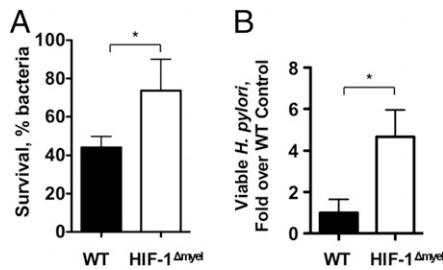


FIGURE 4. Killing of SS1 by WT and HIF-1 α -null neutrophils or macrophages. **(A)** WT and HIF-1^{Δmyel} neutrophils were inoculated with *H. pylori* (SS1) at a multiplicity of infection equal to 2 for 1 h before lysis and plating on agar plates. **(B)** WT and HIF-1^{Δmyel} BMDMs were inoculated with *H. pylori* (SS1) at a multiplicity of infection of 10 for 2.5 h after antibiotic treatment. Pool of three repeated experiments performed in triplicate. **p* < 0.05.

creased upon exposure of macrophages to SS1, but that this response was significantly attenuated in HIF-1 α -null macrophages (Fig. 3A).

In response to IFN- γ or IL-4, macrophages undergo M1 (classical) or M2 (alternative) activation, respectively (4). We next polarized WT and HIF-1^{Δmyel} BMDMs (Supplemental Fig. 2A, 2B), incubated them with bacterial extracts of SS1 for 24 h, and examined the relative level of HIF-1 and HIF-2, IL-1 β , IL-6, and Nos2 in each cell type (Fig. 3). We found that HIF-1 α was up-regulated at the mRNA (Fig. 3) and protein (Supplemental Fig. 2C) level in response to stimuli that induce M1 polarization but was not altered under conditions in which macrophages undergo M2 polarization (Fig. 3A). Deletion of HIF-1 affected IL-1 β and IL-6 mRNA (Fig. 3A) and protein (Supplemental Fig. 3) levels as well as Nos2 expression in M1 macrophages, but not in M2 macrophage. HIF-2 expression was strongly attenuated in M1 macrophages and upregulated in M2-polarized macrophages (Fig. 3A).

We next asked whether *H. pylori* could interfere with macrophage polarization. Two murine M2 markers, fibronectin (Fn1) and arginase (Arg1), were drastically decreased upon *H. pylori* infection in M2 macrophages (Fig. 3B). This decrease appeared to be independent of HIF-1. Altogether, these results suggest that *H. pylori*

induced M1 polarization by stabilizing HIF-1, and prevent a switch to M2 macrophages through a HIF-1-independent mechanism.

HIF-1 contributes to the bactericidal activity against H. pylori

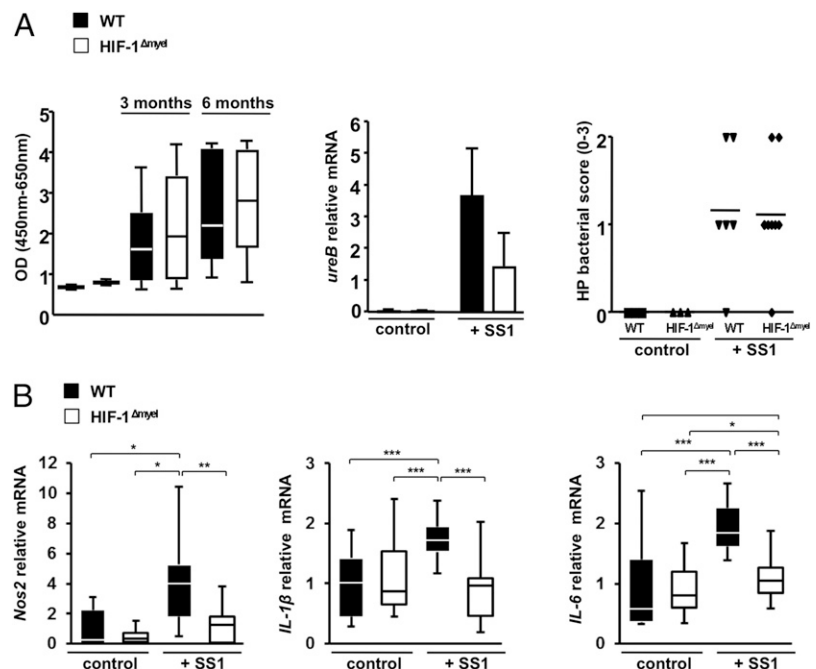
A hallmark feature of *H. pylori* gastritis and persistent infection is the pronounced accumulation of phagocytes, particularly neutrophils, in the gastric mucosa, and the ability of *H. pylori* to resist neutrophil killing (35). We compared the bactericidal activity of WT and HIF-1 α -null neutrophils and macrophages against *H. pylori*, adapting an intracellular killing assay we had developed for studies of respiratory tract and skin pathogens (14) (Supplemental Fig. 4A). HIF-1 has been shown to be important for anoxia-induced neutrophil survival (15). In this study, the viability of *H. pylori*-infected WT and HIF-1 α -null neutrophils, under normoxia, was not significantly different (Supplemental Fig. 4B). HIF-1 α -null neutrophils demonstrated a 66% increase in the number of surviving *H. pylori* SS1 compared with WT neutrophils (Fig. 4A). HIF-1 α -null macrophages had ~5-fold more surviving intracellular *H. pylori* SS1 compared with WT macrophages (Fig. 4B). Therefore, HIF-1 α -deficient neutrophils and macrophages exhibit a killing defect against the pathogen.

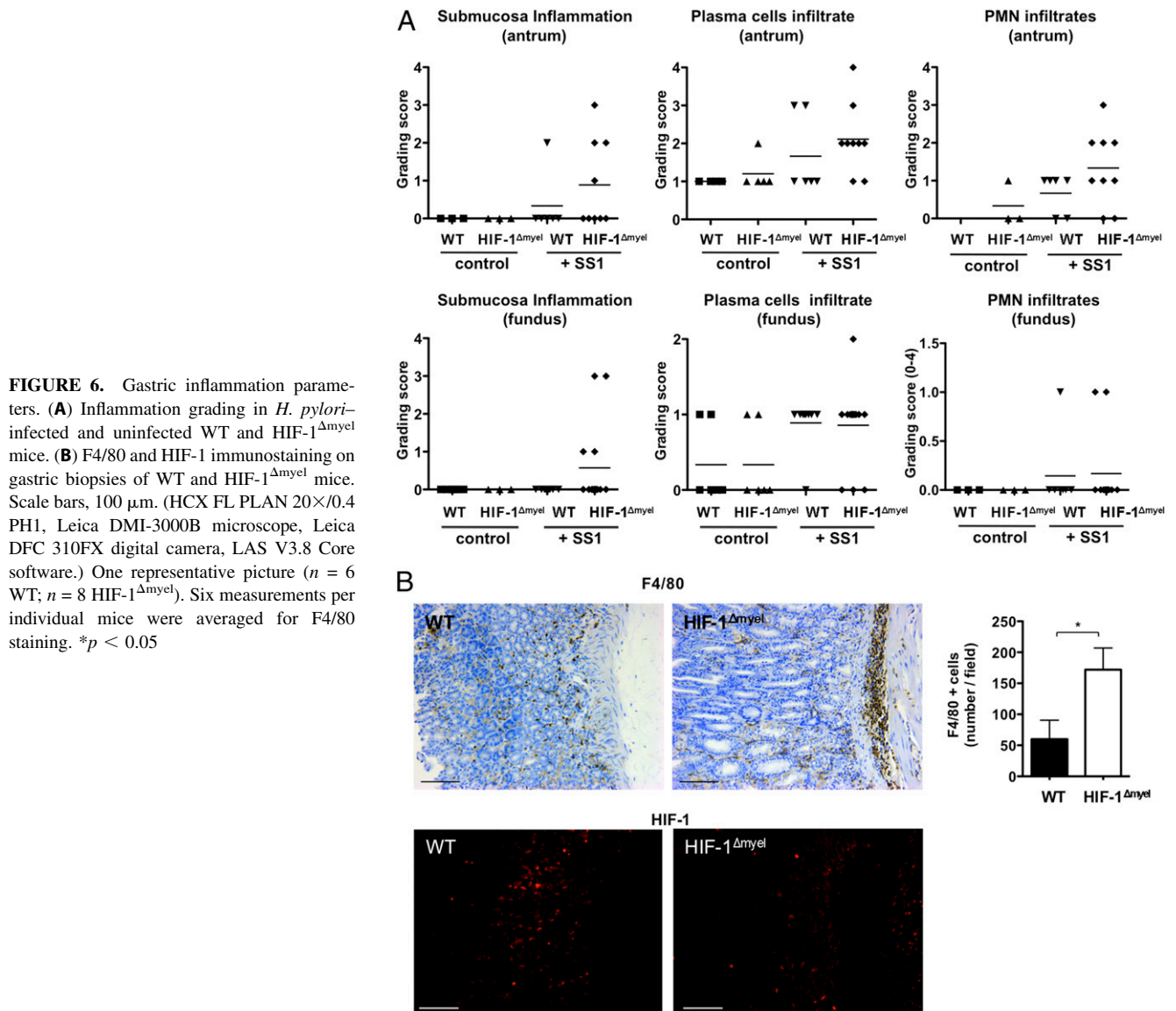
No change in bacteria upon deletion of myeloid HIF-1 in a murine model of SS1-induced gastritis

Next, we used a murine model of chronic *H. pylori* infection to characterize the role of myeloid-HIF-1 in the induction of gastritis. WT and HIF-1^{Δmyel} mice (on a C57BL/6 background) were inoculated with SS1 by oral gavage, as previously described (32). Control and infected mice were sacrificed 6 mo postinfection.

We first assessed the level of infection by serologic analyses. In the SS1-inoculated mice, the *H. pylori* Ag-specific Ab responses increased 3 and 6 mo postinfection (Fig. 5A), but no significant difference was observed between WT and HIF-1^{Δmyel} mice. We next measured the magnitude of *H. pylori* colonization by a well-established approach of quantitative PCR to determining the levels of the *H. pylori* urease B on gastric biopsies. Urease B mRNA levels were strongly increased upon infection, but did not differ significantly between WT and HIF-1^{Δmyel} mice. This was correlated with a similar *H. pylori* colonization score by direct visualization on a Warthin–Starry staining between WT and HIF-1^{Δmyel} mice (Fig. 6A).

FIGURE 5. No change in bacterial loading, but decrease of proinflammatory genes upon deletion of myeloid HIF-1 in a murine model of SS1-induced gastritis. **(A)** Anti-*H. pylori* in the sera of WT and HIF-1^{Δmyel} mice infected with *H. pylori* SS1 detected by ELISA at 0, 3, and 6 mo postinfection (left). Quantitative RT-PCR for *ureB* in gastric biopsies of WT and HIF-1^{Δmyel} mice 6 mo postinfection (middle). *H. pylori* bacterial score performed blindly on a Warthin–Starry staining on gastric biopsies of WT and HIF-1^{Δmyel} mice 6 mo postinfection (right). **(B)** Quantitative RT-PCR analysis of *Nos2*, *IL-1 β* , and *IL-6* mRNA level in gastric extracts of WT and HIF-1^{Δmyel} mice. *n* = 7–13 mice per group. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.





Decrease of proinflammatory genes upon deletion of myeloid HIF-1 in a murine model of SSI-induced gastritis

We also evaluated the effect of the deletion of myeloid HIF-1 on the expression of proinflammatory genes in the gastric mucosal micro-environment. A significant increase in Nos2, IL-1β, and IL-6 mRNA was observed in the gastric biopsies of infected WT mice compared with control mice. Specific deletion of HIF-1 in the myeloid lineage

blunted these increases, suggesting that, at this stage of the gastritis, these inflammatory mediators were mainly produced by the myeloid lineage and were under the control of HIF-1 (Fig. 5B).

Increase of the inflammation score in HIF-1^{Δmyel} mice

A blinded analysis to provide inflammation scores was performed by a pathologist (Fig. 6A). Surprisingly, an increase in the sub-

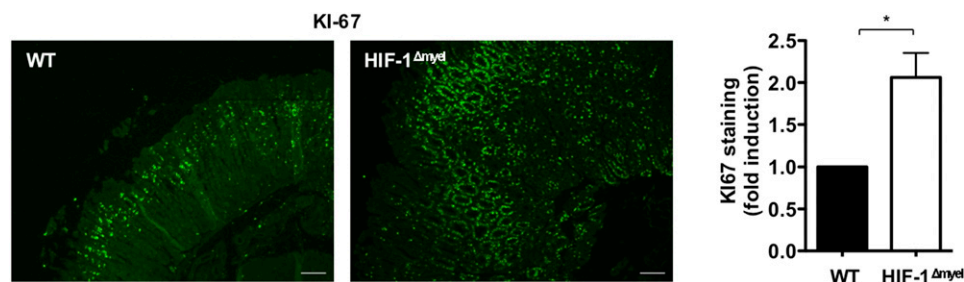


FIGURE 7. Increase in epithelial cell proliferation in HIF-1^{Δmyel} mice. KI67 staining on gastric biopsies of WT and HIF-1^{Δmyel} mice. Scale bars, 100 μm. HCX FL PLAN 20×/0.4 PH1, Leica DMI-3000B microscope, Leica DFC 310FX digital camera, LAS V3.8 Core software. In the gastritis area, the KI67 staining was quantified (normalized per the number of positive cells in the Hoechst staining) and represented as a fold change (knockout versus WT). Representative picture (left) of five stainings. **p* < 0.05

mucosa inflammation was observed in the antrum and fundus of the gastric tissue sections of HIF-1^{Δmyel}-infected mice compared with WT mice. The number of plasma cells and polymorphonuclear cell infiltrates was also increased in the gastric tissue sections (antrum) of HIF-1^{Δmyel}-infected mice compared with WT mice.

In accordance with the calculated inflammation score, the number of macrophages, as visualized by a F4/80 staining, was higher in the gastric biopsies of HIF-1^{Δmyel} mice (Fig. 6B). HIF-1 immunohistochemistry revealed that HIF-1 seems to be mainly expressed by macrophages and that myeloid-specific HIF-1-ablation did not affect epithelial HIF signaling (Fig. 6B).

Increase of epithelial cell proliferation in HIF-1^{Δmyel} mice

The severity of the chronic gastritis is linked to the index of epithelial cell proliferation (36, 37). To determine the degree of severity of the gastritis, we performed a KI67 immunostaining on gastric biopsies of WT and HIF-1^{Δmyel} mice. In correspondence with the increase in inflammation in the HIF-1^{Δmyel} mice (Fig. 6), epithelial cell proliferation (measured by the ratio of the number of KI67-positive cells in HIF-1^{Δmyel} versus WT mice) was 2-fold higher in the antrum of HIF-1^{Δmyel} versus WT mice (Fig. 7). HIF-1^{Δmyel} mice developed more severe gastritis than WT mice, suggesting a protective role of myeloid HIF-1.

Discussion

Chronic inflammatory conditions, such as arthritis (38), asthma (39), and psoriasis (40), have been characterized to exhibit increased HIF-1 signaling. Given this paradigm, therapeutics for the inhibition of HIF-1 signaling have been considered for the treatment of a broad spectrum of chronic inflammatory disease states (18). Our study shows for the first time, to our knowledge, that HIF-1 is stabilized in the macrophages of human *H. pylori*-positive gastritis, which on its surface suggests that myeloid HIF-1 could perhaps be a therapeutic target. Chronic gastritis is characterized by mononuclear cell infiltrates, composed mainly of lymphocytes and macrophages, and has been correlated with increased levels of Nos2, and of the proinflammatory cytokines IL-6, IL-1β, and TNF-α in the gastric mucosa (30–34). Depletion of macrophages by drug-loaded liposomes ameliorated the gastritis induced by *H. pylori* infection (2), demonstrating the importance of macrophages in the innate immune response to *H. pylori*-associated gastritis.

All *H. pylori* isolates, irrespective of their cag PAI phenotype, induce a chronic gastritis in the host (41). We showed in this work that both the Cag⁺ B128 and the SS1 *H. pylori* strain, which lacks a functional cag-PAI, are able to upregulate HIF-1 in the macrophage. HIF-1 activation may be a convergent signaling point by *H. pylori* isolates for the induction of the inflammatory response in macrophages. Recent data showed that *H. pylori* induces HIF-1 in human gastric epithelial cells through a reactive oxygen species-dependent mechanism (42). However, HIF-1 stabilization in the innate immune cells and a putative role in these cells had not been investigated.

We found that *H. pylori* not only induced a prolonged M1 polarization by stabilizing HIF-1, but also prevented a switch to M2 macrophages, which play a critical role in the resolution of inflammation. The mechanism by which M2 markers are decreased remains to be investigated. Interestingly, we found that PPARγ, known to be involved in the M1 to M2 switch (43), was drastically decreased upon SS1 infection (data not shown). The decrease in HIF-2 expression by *H. pylori* may be a consequence of the decrease in M2 polarization (7). Imtiyaz et al. (44) showed that HIF-2 regulates some proinflammatory cytokines (IL-1β, IL-6, and IL-12) during acute and tumor inflammation. However, as

HIF-2 expression was decreased by *H. pylori*, the inflammatory response mediated by *H. pylori* is unlikely dependent on HIF-2.

We found in vitro and in vivo that HIF-1 contributes to the production of Nos2, IL-1β, and IL-6. However, the net effect of the deletion of HIF-1 in the myeloid lineage was a more severe gastritis (Fig. 6), characterized by an increase of the inflammatory score and proliferation index. The increase in inflammatory cell influx may be a downstream consequence of the decrease in killing ability and cytokine production in the HIF-1^{Δmyel} mice (Fig. 6A). Together these opposing processes may result in similar levels of colonizing *H. pylori* during chronic infection. Although HIF-1-deficient myeloid cells produce less cytokines, the production of superoxides by the oxidative burst was the same in both WT and HIF-1^{-/-} macrophages (14). Therefore, even if not experimentally proven in this work, the increase in the number of inflammatory cells in the HIF-1^{Δmyel} mice may be responsible for the worsening of the gastritis, through the production of reactive oxygen species. The increase in epithelial cell proliferation in *H. pylori* gastritis is regarded in the literature as one of the earliest mucosal modifications in the development of gastric adenocarcinoma. This proliferation is also a key factor in the initiation of carcinogenesis, enhancing the effects of mutagens by limiting the time for DNA repair (36, 37).

In conclusion, whereas targeting HIF-1 has been proven to be beneficial in sterile chronic inflammation, myeloid HIF-1 is protective in *H. pylori*-mediated gastritis, pointing to the complex counterbalancing roles of innate immune and inflammatory phenotypes in driving this pathology.

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Disclosures

The authors have no financial conflicts of interest.

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