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Helicobacter pylori infection persists for the life of the host due to the failure of the immune response to eradicate the bacterium. Determining how *H. pylori* escapes the immune response in its gastric niche is clinically important. We have demonstrated in vitro that macrophage NO production can kill *H. pylori,* but induction of macrophage arginase II (Arg2) inhibits inducible NO synthase (iNOS) translation, causes apoptosis, and restricts bacterial killing. Using a chronic *H. pylori* infection model, we determined whether Arg2 impairs host defense in vivo. In C57BL/6 mice, expression of Arg2, but not arginase I, was abundant and localized to gastric macrophages. Arg2$^{-/-}$ mice had increased histologic gastritis and decreased bacterial colonization compared with wild-type (WT) mice. Increased gastritis scores correlated with decreased colonization in individual Arg2$^{-/-}$ mice but not in WT mice. When mice infected with *H. pylori* were compared, Arg2$^{-/-}$ mice had more gastric macrophages, more of these cells were iNOS$^+$, and these cells expressed higher levels of iNOS protein, as determined by flow cytometry and immunofluorescence microscopy. There was enhanced nitrotyrosine staining in infected Arg2$^{-/-}$ versus WT mice, indicating increased NO generation. Infected Arg2$^{-/-}$ mice exhibited decreased macrophage apoptosis, as well as enhanced IFN-γ, IL-17a, and IL-12p40 expression, and reduced IL-10 levels consistent with a more vigorous Th1/Th17 response. These studies demonstrate that Arg2 contributes to the immune evasion of *H. pylori* by limiting macrophage iNOS protein expression and NO production, mediating macrophage apoptosis, and restraining proinflammatory cytokine responses. *The Journal of Immunology*, 2011, 186: 3632–3641.

*H*elicobacter pylori is a Gram-negative, microaerophilic bacterium that selectively colonizes the human stomach. All infected individuals exhibit chronic active gastritis, and a substantial proportion of subjects develop peptic ulcer disease or gastric adenocarcinoma (1). *H. pylori* infects ∼50% of the world's population, and, more importantly, the associated gastric cancer is the second leading cause of cancer-related death worldwide (2). The infection is usually acquired in childhood and persists for the life of the host despite eliciting a seemingly vigorous immune response (3). Understanding the mechanisms by which *H. pylori* avoids being eliminated by the immune system is clinically relevant because antibiotic-based eradication regimens are expensive and not always effective, with success rates that can be <50% in some regions of the world (4).

Although *H. pylori* is typically considered to be noninvasive because most of the bacteria reside in the mucous layer of the stomach in contact with the epithelium, studies have demonstrated that the bacterium and its products can be in direct contact with lamina propria immune cells (5–7). Consequently, infection with *H. pylori* results in a large influx of immune cells that include neutrophils, macrophages, dendritic cells, and lymphocytes, and an associated innate and adaptive immune response (8). Although this has been shown to include both Th1 and Th17 components, one hallmark of the response is that there is also a downregulation of effective immunity that appears to involve recruitment of regulatory T cells (Tregs) and B cells (3). Vaccination studies, adoptive transfer of Th1-selected lymphocytes, and efforts to suppress Treg responses have been successful at reducing or clearing the infection in mice (9–12). These studies have provided evidence that the cellular immune response is not vigorous enough to lead to eradication of the infection. One important aspect that remains to be fully elucidated is the role of the innate immune response in the impairment of the host response. We propose that there may be an inability of effector cells to eliminate the infection when given the opportunity to do so.

One primordial mechanism for antimicrobial host defense is the generation of high levels of NO derived from the enzyme inducible NO synthase (iNOS) (13). Our laboratory and others have demonstrated that *H. pylori* induces the expression and activity of iNOS in macrophages both in vivo and in vitro (14–17). Further,

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we have reported that macrophages cocultured with *H. pylori* can kill the bacterium by an NO-dependent mechanism (15, 18). However, this killing is incomplete in vitro, and, moreover, there is clearly a failure of this mechanism in vivo despite the expression of iNOS in the infected mucosa (14, 17). This reasoning has led our laboratory to consider the possibility that iNOS-mediated host defense to *H. pylori* is suboptimal. We have reported that the generation of NO by macrophages in response to *H. pylori* is entirely dependent on the availability of its substrate, L-arginine, which enhances expression of iNOS protein, without altering the induction of mRNA expression (15, 18). Specifically, we found that addition of increasing levels of extracellular L-arginine results in a proportionate increase in NO production even at concentrations well above the circulating levels in humans and mice of 0.1 mM and above the K_m of the iNOS enzyme for L-arginine, which is in the range of 10 μM (19). In order for macrophages to produce bactericidal amounts of NO when cocultured with *H. pylori* in our model system, concentrations of L-arginine in the medium that exceeded 0.1 mM were needed. Importantly, infection of iNOS$^{-/-}$ mice with *H. pylori* results in similar levels of bacterial colonization as those in wild-type (WT) mice (20), further suggesting a defect in iNOS-dependent host defense.

Arginase enzymes are the endogenous antagonists to iNOS because they compete for the same L-arginine substrate by metabolizing it to urea and L-ornithine (21). The latter is used by ornithine decarboxylase to produce the polyamines putrescine, spermidine, and spermine. There are two isoforms of arginase: arginase I (Arg1) is abundant in liver and is important for the urea cycle, and arginase II (Arg2) is abundant in kidney and localizes to mitochondria (22). We have reported that Arg2, but not Arg1, is upregulated in *H. pylori*-stimulated macrophages (15). Induction of arginase activity by pathogens has been reported to modulate macrophage NO production in the case of both Arg1 and Arg2, which can restrict effective immunity (23–25). These effects have been attributed to substrate competition. However, we have recently reported that induction of macrophage Arg2 by *H. pylori* inhibits iNOS translation, NO production, and bacterial killing in vitro (15). Furthermore, in mice infected with *H. pylori* for 48 h, treatment with an arginase inhibitor resulted in increased iNOS protein levels and NO production in gastric macrophages (15). We have also reported that the downstream effects of arginase metabolism can be detrimental to iNOS-mediated host defense against *H. pylori*. The polyamine spermine inhibits L-arginine uptake in macrophages, thereby blocking NO production and bacterial killing (26). Moreover, back-conversion of spermine into spermidine by the enzyme spermine oxidase, which is also induced by *H. pylori*, releases hydrogen peroxide, which causes apoptosis (27, 28). Consequently, we have found that inhibition of arginase blocks *H. pylori*-induced macrophage apoptosis in our in vitro studies (29).

We hypothesized that Arg2 expression is detrimental to host defense against *H. pylori* in vivo by restricting macrophage NO production and inducing macrophage apoptosis. We now report that chronic infection of Arg2$^{-/-}$ mice with *H. pylori* results in decreased bacterial colonization and increased gastritis compared with that in infected WT mice. Importantly, we found that increased gastritis correlated with decreased bacterial colonization in individual Arg2$^{-/-}$ mice but not in WT mice. Moreover, we show that in Arg2$^{-/-}$ mice infected with *H. pylori*, there are more iNOS$^+$ gastric macrophages that express increased levels of iNOS, enhanced proinflammatory cytokine responses, and decreased levels of macrophage apoptosis. Our data suggest that upregulation of macrophage Arg2 is detrimental to the host response against *H. pylori* and demonstrate a mechanism by which *H. pylori* evades host immunity.

Materials and Methods

Reagents

All reagents used for RNA extraction were from Invitrogen (Carlsbad, CA). Real-time PCR reagents were from Bio-Rad (Hercules, CA). Isolation of DNA was performed using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Bacteria

H. pylori SS1, a mouse-adapted human strain, was grown on *Brucella* blood agar plates under microaerobic conditions as described (16, 28). Prior to infection, bacteria were grown in a *Brucella* broth culture for 16–20 h. Concentrations of bacteria were determined by OD at 600 nm (OD of 1 = 10^9 CFU/ml) (16).

Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee at Vanderbilt University (Nashville, TN). C57BL/6 Arg2$^{-/-}$ and WT male mice were used at 6–8 wk of age as described (15). Mice were gavaged with 5 $\times$ 10^8 *H. pylori* SS1 in 100 μl *Brucella* broth or with broth vehicle control alone. Some mice were sacrificed at 2 d postinoculation, and stomachs were excised and used for isolation of gastric macrophages, which was performed as described (14, 18). Other mice were sacrificed at 4 mo postinoculation, and colonization and gastritis were assessed. *H. pylori* levels were measured by quantitative DNA PCR for *H. pylori ureB*, standardized to mouse *18S rRNA* copy number (14). Histologic gastritis was quantified by a pathologist (M.B.P.) using a scale of 0–3 for acute inflammation and for chronic inflammation in the regions of the gastric antrum and body with the scores added together for a total score of 0–12 (14, 30).

Apoptosis detection by annexin V staining

Gastric macrophages were isolated and stained with annexin V conjugated to FITC and 7-aminoactinomycin D (7-AAD; BD Biosciences, San Jose, CA) as described (31, 32).

Real-time PCR

RNA was extracted using the RNeasy Mini Kit from Qiagen. PCR methods were performed as described (15, 18, 29, 33). Primer sequences used were as follows: IL-10, 5′-CCAAGCCTTATCGGAAATGA-3′ (forward) and 5′-TCACTCTTCACCTGCTCCAC-3′ (reverse); IL-12p40, 5′-GATTCAG-ACTCCAGGGGACA-3′ (forward) and 5′-CATCTTCTTCAGGCGTGT-CA-3′ (reverse); IL-17a, 5′-GCTCCAGAAGGCCCTCAGA-3′ (forward) and 5′-CTTTCCCTCCGCATTGACA-3′ (reverse); Foxp3, 5′-AGAGC-CCTCACAACCAGCTA-3′ (forward) and 5′-CCAGATGTTGTGGGTG-AGTG-3′ (reverse); IL-6, 5′-AGTTGCCTTCTTGGGACTGA-3′ (forward) and 5′-TCCACGATTTCCCAGAGAAC-3′ (reverse); and IL-23p19, 5′-CA-TGGGGCTATCAGGGAGTA-3′ (forward) and 5′-AATAATGTGCCCCG-TATCCA-3′ (reverse). Primer sequences for IFN-γ (33), Arg1 (29), Arg2 (15), β-actin (18), and iNOS (18) were as described.

Immunofluorescence staining for F4/80, Arg2, and iNOS, and immunoperoxidase staining for nitrotyrosine and cleaved caspase-3

Immunofluorescence staining for F4/80 and DAPI was performed as described (14). iNOS was detected with a rabbit polyclonal Ab (1:100 dilution; BD Biosciences, San Diego, CA). Arg2 was detected with a goat polyclonal Ab (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoperoxidase staining was performed as described (14). Nitrotyrosine was detected with a mouse monoclonal anti-vertebrate nitrotyrosine Ab (1:100 dilution; Millipore, Billerica, MA). Cleaved caspase-3 was detected with a rabbit polyclonal Ab (1:200 dilution; Cell Signaling Technology, Danvers, MA). Quantification of cleaved caspase-3 staining was performed by counting all positively-stained cells among the inflammatory infiltrate and dividing this number by the total number of inflammatory cells to calculate the percentage of positive cells. Cell counting was performed at $\times$600 magnification under oil immersion.

Gastric macrophages

Immune cells were isolated from the glandular stomach by enzymatic digestion as described (14, 18, 31). Briefly, mice were sacrificed, and the stomach was removed. The forestomach (nonglandular portion) was excised and discarded. The glandular portion of the stomach was washed, cut

into 2-mm pieces, and digested for 20 min with 1 mg/ml dispase, 0.25 mg/ml collagenase A, and 25 U/ml DNase (Roche Diagnostics, Indianapolis, IN) at 37°C while shaking. The suspension was passed through a 70-μm cell strainer (BD Biosciences, San Diego, CA) and cells harvested by centrifugation. Cells were stained for F4/80 and iNOS and analyzed by flow cytometry. F4/80$^+$ and F4/80$^+$ iNOS$^+$ cell counts were standardized to the weight in grams of the glandular stomach.

Statistical analysis

Quantitative data are shown as the mean ± SE. Statistical analysis was performed with Prism version 5.0c (GraphPad Software, San Diego, CA). Where two groups were compared, Student *t* test was used. Data with more than two groups were analyzed by ANOVA and the Student–Newman–Keuls post hoc multiple comparisons test. A *p* value <0.05 was considered statistically significant. The relationship between gastritis and colonization was determined using Spearman's correlation test; the correlation coefficient, *r*, is shown along with the *p* value.

Results

H. pylori *infection induces Arg2, not Arg1, in gastric lamina propria F4/80$^+$ macrophages*

Mice were infected for 4 mo, a time point at which we have demonstrated consistent development of chronic active gastritis

with strain SS1 (14). Arg2 mRNA expression was increased by >9–fold in *H. pylori*-infected WT mice when assessed by real-time PCR, whereas Arg1 was not induced (Fig. 1*A*, 1*B*). Furthermore, Arg1 was not upregulated in Arg2$^{-/-}$ mice (Fig. 1*B*). Additionally, iNOS mRNA expression increased in infected WT mice, and this was further enhanced in Arg2$^{-/-}$ mice (Fig. 1*C*).

To determine which cell type expressed Arg2 in the stomach, we immunostained formalin-fixed, paraffin-embedded gastric tissue sections from WT and Arg2$^{-/-}$ mice (Fig. 1*D*). This staining demonstrated increased Arg2 levels in *H. pylori*-infected tissues from WT mice that localized to F4/80$^+$ macrophages. Arg2 staining was not present in macrophages in the Arg2$^{-/-}$ tissues.

Chronically infected Arg2$^{-/-}$ mice have increased gastritis and decreased H. pylori *colonization*

To determine the role of Arg2 during *H. pylori* infection, we infected WT and Arg2$^{-/-}$ mice for 4 mo and assessed gastritis levels and bacterial colonization. With *H. pylori* infection, there was a significant increase in overall gastritis in WT mice that was further increased in the Arg2$^{-/-}$ mice (Fig. 2*A*). In addition to enhanced gastritis levels, we found a significant decrease in *H.*
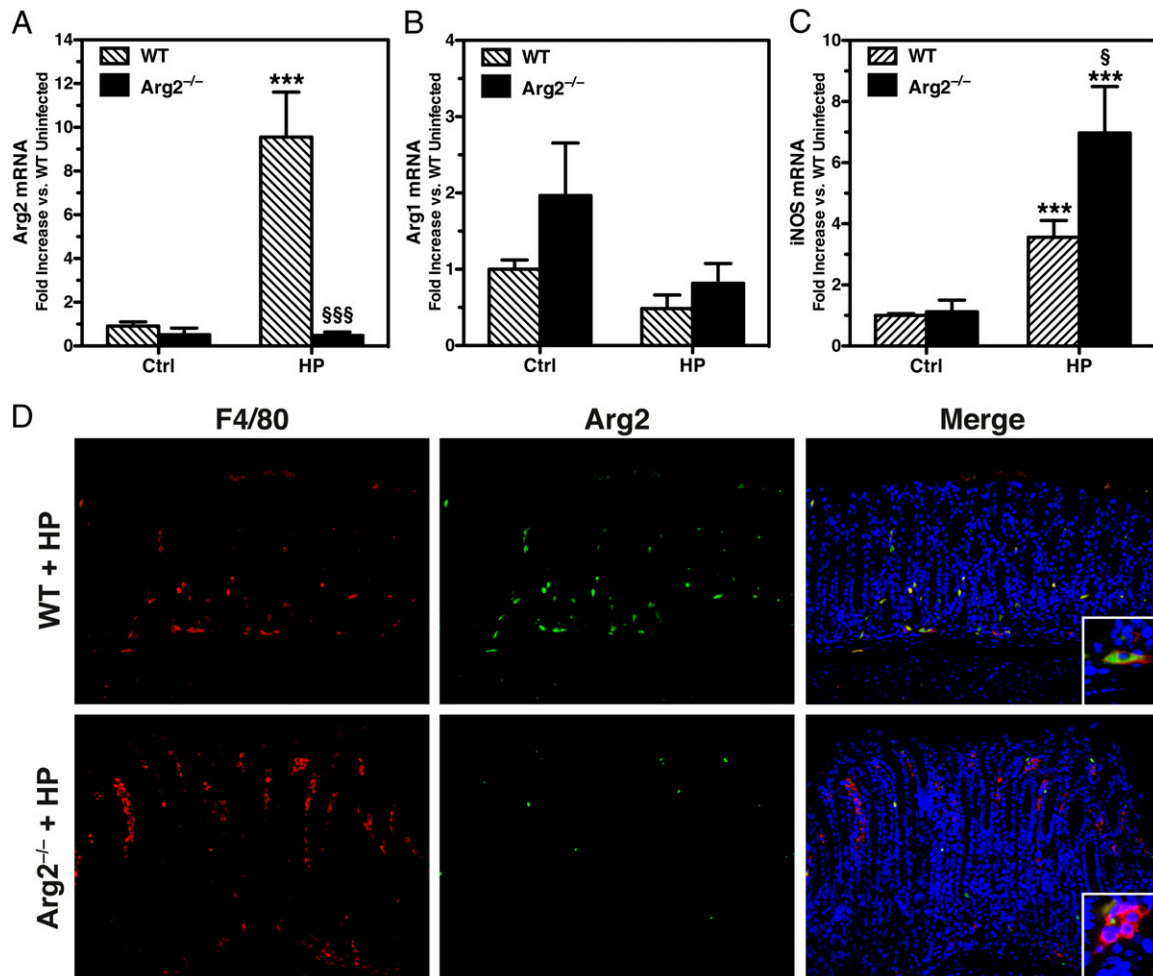
FIGURE 1. *H. pylori* infection induces iNOS and Arg2, but not Arg1, in gastric tissues. C57BL/6 WT and Arg2$^{-/-}$ mice were infected with *H. pylori* strain SS1. At 4 mo postinfection, mice were sacrificed, gastric tissues were removed, and RNA was extracted. *A–C*, Arg2 (*A*), Arg1 (*B*), and iNOS (*C*) mRNA levels were measured by real-time PCR from the antral portion of the stomach. All real-time PCR data were standardized to β-actin and presented as fold increase versus uninfected WT mice. Data are the mean ± SEM, *n* = 6 in the uninfected mice and *n* = 10 in each of the infected mice groups. ***$p <$ 0.001 compared with WT uninfected mice. $^{\S}p <$ 0.05, $^{\S\S\S}p <$ 0.001 compared with WT infected mice. *D*, Photomicrographs, displayed at original magnifications ×200 and ×600 for the *insets*, demonstrating Arg2 immunofluorescence staining in F4/80$^+$ macrophages. F4/80 was detected with tetramethyl rhodamine isothiocyanate (red), and Arg2 was detected with FITC. Data are representative of three to four mice per group.

FIGURE 2. Increased gastritis and decreased bacterial colonization in Arg2$^{-/-}$ mice. C57BL/6 WT and Arg2$^{-/-}$ mice were infected with *H. pylori* for 4 mo, sacrificed, and their stomachs were removed. *A*, H&E-stained slides were prepared from a strip of the glandular stomach containing both the antrum and body. Acute and chronic inflammation were each scored 0–3 in the antrum and body, and the scores were added for a total score of 0–12. **$p < 0.01$. *B*, DNA was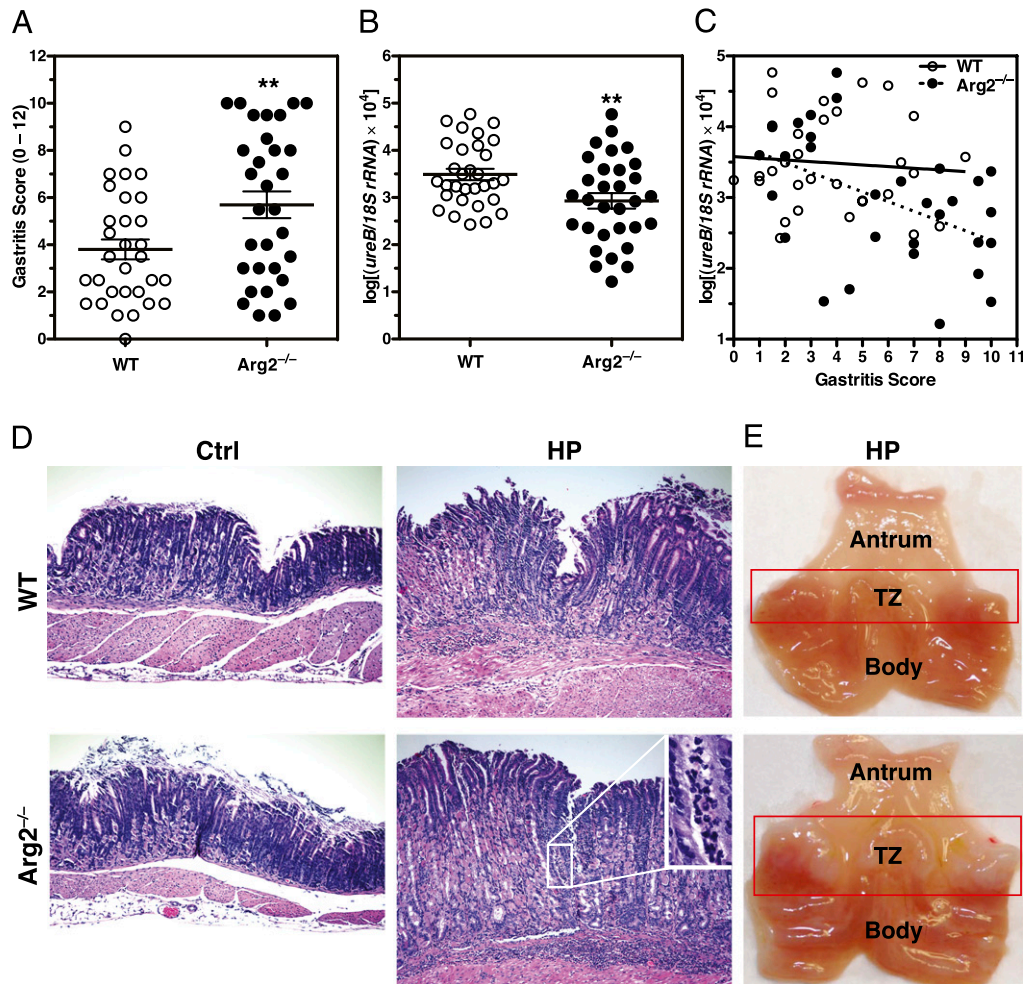 extracted from the body of the stomach, and bacterial colonization was quantified by real-time PCR for the *H. pylori* gene *ureB* normalized to *18S rRNA*. **$p < 0.01$. *C*, Gastritis scores and bacterial colonization were plotted for individual WT and Arg2$^{-/-}$ mice to determine if a correlation exists between gastritis and bacterial colonization. Linear regression lines are shown. For WT mice, Spearman's correlation coefficient $r = -0.088$ and $p = 0.636$. For Arg2$^{-/-}$ mice, Spearman's correlation coefficient $r = -0.491$ and $p = 0.006$. *D*, Representative H&E-stained sections are shown for both uninfected and infected WT and Arg2$^{-/-}$ mice. Photomicrographs are depicted at original magnification ×200 (original magnification ×600 for the *inset*). *E*, Photographs were taken to demonstrate the gross anatomy of the glandular portion of the stomach. The red rectangle highlights the transition zone (TZ) of the stomach.

pylori colonization levels in Arg2$^{-/-}$ mice (Fig. 2*B*). Notably, increased gastritis correlated significantly with decreased bacterial colonization in Arg2$^{-/-}$ mice ($p = 0.006$, $r = -0.491$), but there was no such effect in WT mice ($p = 0.636$, $r = -0.088$).

Representative photomicrographs of H&E-stained gastric sections are shown for uninfected and infected WT and Arg2$^{-/-}$ mice (Fig. 2*D*). These demonstrate that in the transition zone between the body and the antrum of the stomach where *H. pylori*-induced inflammation is typically most severe, there was more extensive acute and chronic inflammatory cell infiltration in Arg2$^{-/-}$ mice. There was no spontaneous inflammation in the uninfected Arg2$^{-/-}$ mice. Additionally, we observed gross thickening of the gastric mucosa in the transition zone of Arg2$^{-/-}$ mice that was indicative of increased gastric inflammation (Fig. 2*E*).

Chronic infection of H. pylori *induces proinflammatory cytokine production that is further enhanced in Arg2$^{-/-}$ mice*

To assess alterations in cytokine production during *H. pylori* infection and determine differences in the immune response, we analyzed mRNA expression of various cytokines in uninfected and

infected WT and Arg2$^{-/-}$ mice. IFN-γ, IL-12p40, and IL-17a were increased in infected WT mice and were further upregulated in Arg2$^{-/-}$ mice (Fig. 3*A*, 3*B*, 3*C*, respectively). IL-10 expression, a hallmark of the counterregulatory response to Th1 and Th17 responses in *H. pylori* infection (11, 34), was increased in infected WT but not in Arg2$^{-/-}$ mice (Fig. 3*D*). IL-23p19, IL-6, and Foxp3 were each modestly increased upon infection with *H. pylori*, but there was no difference between the WT and Arg2$^{-/-}$ mice (Supplemental Fig. 1).

Arg2$^{-/-}$ macrophages are more abundant, express more iNOS, and have increased nitrotyrosine staining compared with WT macrophages during H. pylori *infection*

We have previously demonstrated that *H. pylori* stimulation induces Arg2 expression in macrophages, and this expression attenuates iNOS translation in vitro (15). Furthermore, we have shown that Arg2$^{-/-}$ gastric macrophages isolated 48 h postinoculation with *H. pylori* express more iNOS protein and produce more NO compared with WT macrophages (15). Therefore, to determine if such an effect occurred during chronic infection with
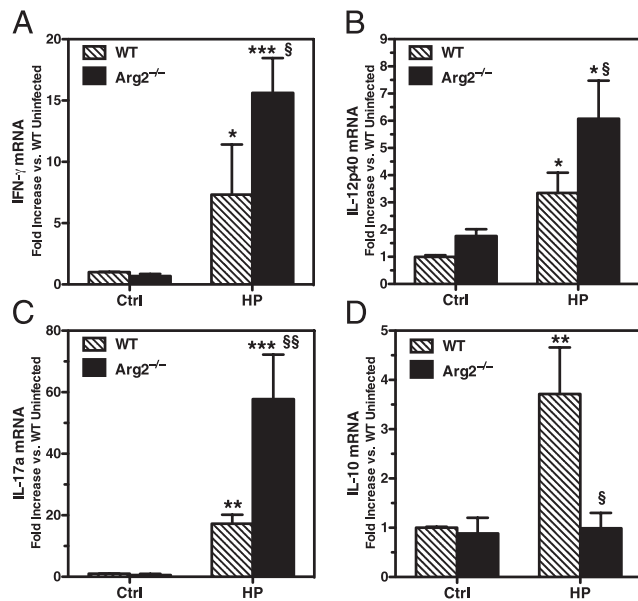
FIGURE 3. Chronic infection with *H. pylori* induces proinflammatory cytokine production that is further enhanced in Arg2$^{-/-}$ mice. mRNA was extracted from the gastric antrum of uninfected and infected WT and Arg2$^{-/-}$ mice at 4 mo postinoculation, converted to cDNA, and real-time PCR was performed for IFN-γ (*A*), IL-12p40 (*B*), IL-17a (*C*), and IL-10 (*D*). Data were standardized to β-actin and presented as fold increase versus uninfected WT mice. For uninfected mice, $n = 3$–6 per group, and for infected mice, $n = 5$–10 per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with uninfected WT mice. $^{§}p < 0.05$, $^{§§}p < 0.01$ compared with infected WT mice.

H. pylori, we analyzed levels of the macrophage surface marker F4/80 and iNOS protein expression by immunofluorescence in WT and Arg2$^{-/-}$ gastric tissues 4 mo postinoculation (Fig. 4), along with the appropriate isotype controls (Supplemental Fig. 2). Consistent with our recent report (14), there was increased macrophage staining in *H. pylori*-infected versus uninfected tissues in WT mice. The abundance of this F4/80 staining was significantly increased in the infected Arg2$^{-/-}$ mice. Similarly, with *H. pylori* infection, iNOS staining was increased in WT mice but substantially potentiated in the Arg2$^{-/-}$ mice (Fig. 4). When the merged images were assessed, the iNOS staining was found to localize predominantly to the F4/80$^+$ cells, and there were more iNOS$^+$ macrophages in the Arg2$^{-/-}$ mice (Fig. 4). This staining was present in the lamina propria, with trails of iNOS$^+$ macrophages migrating toward the lumen, as well as in the submucosal region.

To confirm our observation that there are more iNOS$^+$ macrophages in infected Arg2$^{-/-}$ mice compared with that in infected WT mice, we isolated gastric immune cells and analyzed F4/80 and iNOS expression by flow cytometry. In accordance with our immunofluorescence data, we found a significant increase in both the quantity of macrophages (Fig. 5*A*) and iNOS$^+$ macrophages (Fig. 5*B*) in infected Arg2$^{-/-}$ mice compared with that in WT mice. Representative flow cytometric dot plots are also shown demonstrating an increased percentage of F4/80$^+$ iNOS$^+$ cells in infected Arg2$^{-/-}$ mice versus WT mice (Fig. 5*C*). Importantly, the Arg2$^{-/-}$ gastric macrophages expressed more iNOS protein than that expressed by the cells from the WT mice (Fig. 5*D*, 5*E*).

Because we found that gastric macrophages isolated from *H. pylori*-infected Arg2$^{-/-}$ mice express higher iNOS protein levels than those of gastric macrophages from infected WT mice, we sought to determine if this resulted in increased NO production. To assess this, we used nitrotyrosine staining as a marker of NO

synthesis in the mucosa. Nitrotyrosine formation occurs when tyrosine residues react with peroxynitrite, which is formed by the reaction of NO with superoxide (O$_2^-$), and this has been used as a marker of the production of reactive nitrogen species (23, 35–37). We stained tissues from chronically infected WT and Arg2$^{-/-}$ mice and found that inflammatory cells in Arg2$^{-/-}$ mice had increased nitrotyrosine staining compared with that in WT mice (Fig. 5*F*). This staining was most intense in mononuclear immune cells of the lamina propria near the luminal surface, similar in location to the macrophages that we identified in Fig. 1*D* and Fig. 4.

H. pylori infection increases macrophage apoptosis that is abolished in Arg2$^{-/-}$ mice

To determine whether increased survival of macrophages could be a mechanism responsible for increased iNOS$^+$ macrophages in Arg2$^{-/-}$ mice, we isolated gastric macrophages and measured apoptosis by annexin V staining. We have previously shown that *H. pylori* induces macrophage apoptosis in vitro that is Arg2 dependent (29), therefore we sought to confirm this in vivo. To pursue this, we used a 48-h model of infection, as we have previously demonstrated that there is both maximum macrophage infiltration into the stomach and maximum apoptosis at this time point after inoculation with *H. pylori* (31). Additionally, we have demonstrated that Arg2 restricts NO production in gastric macrophages at this time point (15). Consistent with our in vitro data, we found that *H. pylori* infection induced macrophage apoptosis and that this was abolished in Arg2$^{-/-}$ macrophages (Fig. 6*A*). Representative flow cytometric dot plots demonstrating annexin V and 7-AAD staining in infected WT and Arg2$^{-/-}$ macrophages are shown in Fig. 6*B*.

We also sought to corroborate this finding in our chronic infection model by immunostaining tissues for cleaved caspase-3, a marker for apoptosis in *H. pylori* gastritis tissues (38). In infected WT mice, there was abundant staining in the mononuclear inflammatory cells with marked staining of cells with apoptotic bodies (Fig. 6*C*). In contrast, cleaved caspase-3 staining in Arg2$^{-/-}$ mice was less intense and less frequent, which correlates with our findings with the annexin V staining of isolated gastric macrophages in Fig. 6*A*. Additionally, quantification of this staining revealed a decrease in cleaved caspase-3 staining among the inflammatory cells in the Arg2$^{-/-}$ mice compared with that in WT mice (Fig. 6*D*).

Discussion

H. pylori infection induces a vigorous immune response, and in the murine model there is a rapid influx of macrophages (31, 39) and neutrophils (39) 48 h postinfection, followed by infiltration of lymphocytes 10 d postinfection (39). This produces a smoldering gastritis that persists as long as the bacteria reside in the gastric niche. Despite this robust immune response, the bacterium typically persists for the life of the host. It is generally assumed that the immune response is not vigorous enough to eliminate the infection, due to demonstration of clearance of the bacterium in adoptive transfer experiments and IL-10$^{-/-}$ mice, both of which exhibit enhanced gastric inflammation (9, 34). We sought to determine if the macrophage response is inhibited by *H. pylori*. In the current report, we demonstrate that *H. pylori* upregulates macrophage Arg2, thereby restricting iNOS protein levels and NO production and enhancing macrophage apoptosis. Consequently, this restricts host defense against *H. pylori*. This is the first report, to our knowledge, to demonstrate that macrophage Arg2 expression has a deleterious impact on the effectiveness of host immunity by impairing the inflammatory response in vivo.
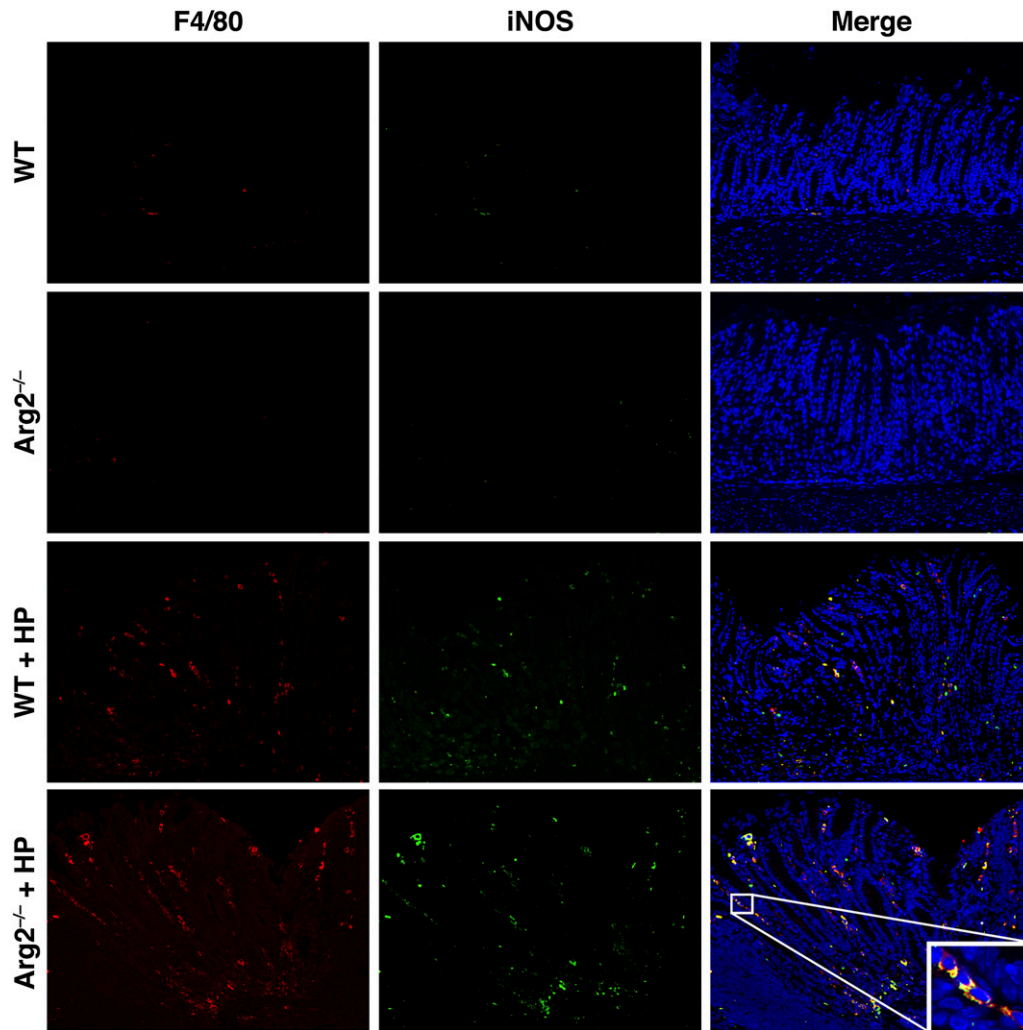
FIGURE 4. Arg2$^{-/-}$ mice have increased iNOS$^+$ macrophages during chronic infection with *H. pylori*. Representative immunofluorescence staining from uninfected and infected WT and Arg2$^{-/-}$ mice is shown. The macrophage marker F4/80 was detected with tetramethyl rhodamine isothiocyanate (red), iNOS was detected with FITC (green), and nuclei were stained with DAPI (blue); colocalization is shown in merged images by the yellow color. Photomicrographs are shown at original magnification ×200 (original magnification ×600 for the *inset*).

In this study, we have demonstrated that induction of Arg2 during chronic *H. pylori* infection restricts macrophage iNOS protein levels, limits the proinflammatory immune response, and increases bacterial colonization. These data confirm our recent studies showing that inhibition of macrophage arginase in vitro enhances iNOS translation and NO production and, consequently, causes more bacterial killing (15). We have now shown that infection with *H. pylori* causes upregulation of Arg2 that localizes to lamina propria and submucosal macrophages. We have previously reported that Arg2 expression is induced in RAW 264.7 cells and peritoneal macrophages stimulated ex vivo with *H. pylori* (15, 29). Furthermore, we have shown that Arg2 gene expression is upregulated in human gastric tissues infected with *H. pylori* (29). Simultaneous induction of both iNOS and arginase in macrophages is uncommon, as studies have demonstrated that induction of one usually leads to the inhibition of the other (40, 41). Nevertheless, several pathogens have devised strategies to upregulate arginase to suppress iNOS-dependent host defense. For example, downregulation of NO production by macrophages has been attributed to induction of Arg1 by the parasites *Leishmania major* (24) and *Toxoplasma gondii* (23) and the bacterium *Mycobacterium tuberculosis* (23) and to induction of Arg2 by the parasite *Trypanosoma brucei brucei* (42) and the bacteria *Chlamydia*

psittaci and *Chlamydia pneumoniae* (25). We now demonstrate that *H. pylori* upregulates Arg2 in vivo leading to an impaired macrophage immune response.

Our data suggest that the enhanced inflammation induced by *H. pylori* has no benefit for reducing bacterial colonization under normal circumstances. When we analyzed the gastritis scores and colonization levels in WT mice, we found that there was no correlation between these two parameters, and the linear regression line was almost flat. This was surprising because it is generally assumed that in *H. pylori* infection, enhanced inflammation will decrease bacterial colonization (9, 10, 34). In fact, persistence of the bacterial infection is primarily thought to be due to an immune response that is not vigorous enough (8). However, our data demonstrate that even in WT mice with very high levels of gastritis, there was no noticeable ability of this response to reduce bacterial colonization. These data suggest that there is a defect in the immune response against *H. pylori* and that effector mechanisms responsible for clearance of the bacterial infection are inhibited. In contrast, mice deficient in Arg2 showed a beneficial inverse correlation between gastritis and bacterial colonization, producing a negative linear regression line, thus demonstrating that mice with high levels of inflammation had less bacterial colonization. These data suggest that Arg2 induction contributes

FIGURE 5. Arg2$^{-/-}$ macrophages are more abundant, express more iNOS, and have increased nitrotyrosine staining compared with WT macrophages during *H. pylori* infection. Gastric cells were isolated from infected WT and Arg2$^{-/-}$ mice and analyzed by flow cytometry for the expression of the macrophage marker F4/80 and iNOS. *A* and *B*, The total number of (*A*) F4/80$^+$ cells (**$p < 0.01$ and ***$p < 0.001$ versus uninfected WT macrophages; $^\S p < 0.05$ versus infected WT macrophages; $n = 3$ for uninfected groups, and $n = 9$-10 for infected groups) and (*B*) F4/80$^+$ iNOS$^+$ cells (*$p < 0.05$) are shown. *C*, Representative flow cytometric dot plots are shown demonstrating iNOS and F4/80 staining in cells isolated from infected WT and Arg2$^{-/-}$ mice. *D*, In the F4/80$^+$ cells, iNOS mean fluorescence units (MFU) are shown to demonstrate the level of iNOS expression in macrophages. *$p < 0.05$. *E*, Representative histogram demonstrating iNOS fluorescence in macrophages from uninfected and infected WT and Arg2$^{-/-}$ mice. *F*, Immunoperoxidase staining for nitrotyrosine is shown for uninfected control mice and infected WT and Arg2$^{-/-}$ mice at original magnifications ×200 and ×600. Data are representative of two mice in the control group and four to six mice in the infected groups.
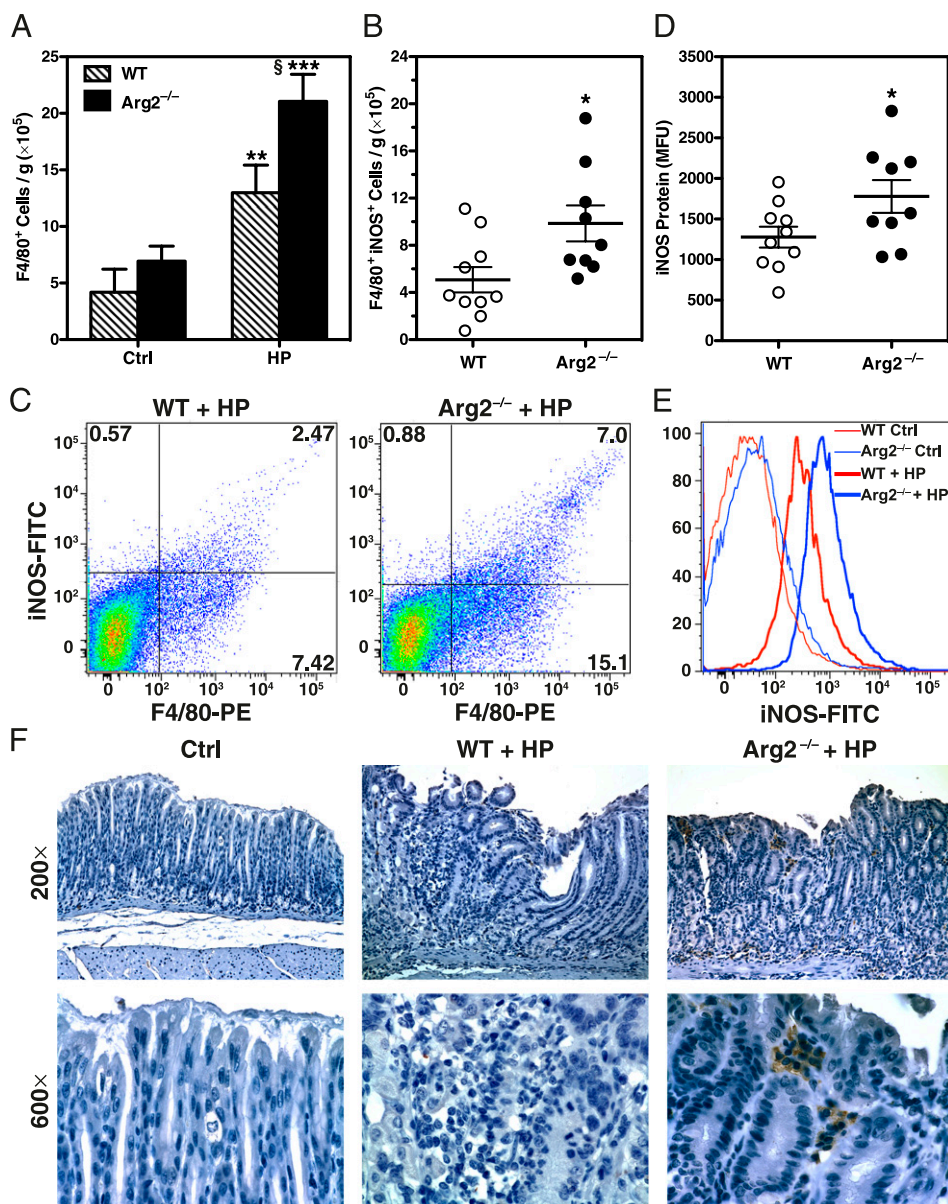
to the defective immune response in WT mice. However, in Arg2-deficient mice, the bacterial infection was decreased but not eliminated. Linear regression analysis of our Arg2$^{-/-}$ mice suggest that after removal of the Arg2 "block," the effectiveness of the immune response is restored, but enhancement of other responses may be needed to eliminate the infection completely, which correlates with what others have demonstrated with lymphocyte adoptive transfer experiments (9, 43).

We previously demonstrated that Arg2 localizes to mitochondria whereas iNOS resides in the cytoplasm (15). Although these two enzymes are physically separated, Arg2 is still able to restrict NO production by its inhibitory effect on iNOS translation; as such, NO production in response to *H. pylori* can be enhanced in vitro in macrophages by inhibition of arginase activity, knockdown of Arg2, or use of Arg2$^{-/-}$ cells (15). These previous findings are substantiated by our current in vivo data that gastric macrophages isolated from *H. pylori*-infected Arg2$^{-/-}$ mice express more iNOS protein than that expressed by WT macrophages. We also demonstrated enhanced nitrotyrosine staining in these cells, which is a signature of increased NO production. Our data indicate that

the competition for intracellular L-arginine is important for modulating host immunity against *H. pylori*.

It has been reported that Arg2$^{-/-}$ mice have higher levels of serum L-arginine than WT mice under basal conditions (44). Such an effect could enhance T cell responses because it has been reported that T cell responses in vitro are inhibited under conditions of limited L-arginine availability (45). Furthermore, it has been shown that Arg1-expressing macrophages can inhibit both the re-expression of TCRs and T cell proliferation in vitro (46) and T cell proliferation in vivo (47). We have considered the possibility that the effects we have described with our Arg2$^{-/-}$ mice may have been due to enhanced T cell responses that could ensue from increased L-arginine availability. However, when we supplemented WT mice with L-arginine in their drinking water, we found no reduction in *H. pylori* colonization when assessed at 4 mo postinoculation (data not shown). This lack of effect of L-arginine treatment may be due to the presence of Arg2, which impairs NO-dependent antimicrobial host defense. Our data suggest impairment of classical (M1) activation (48) of macrophages in *H. pylori* infection. However, the competing alternative (M2)
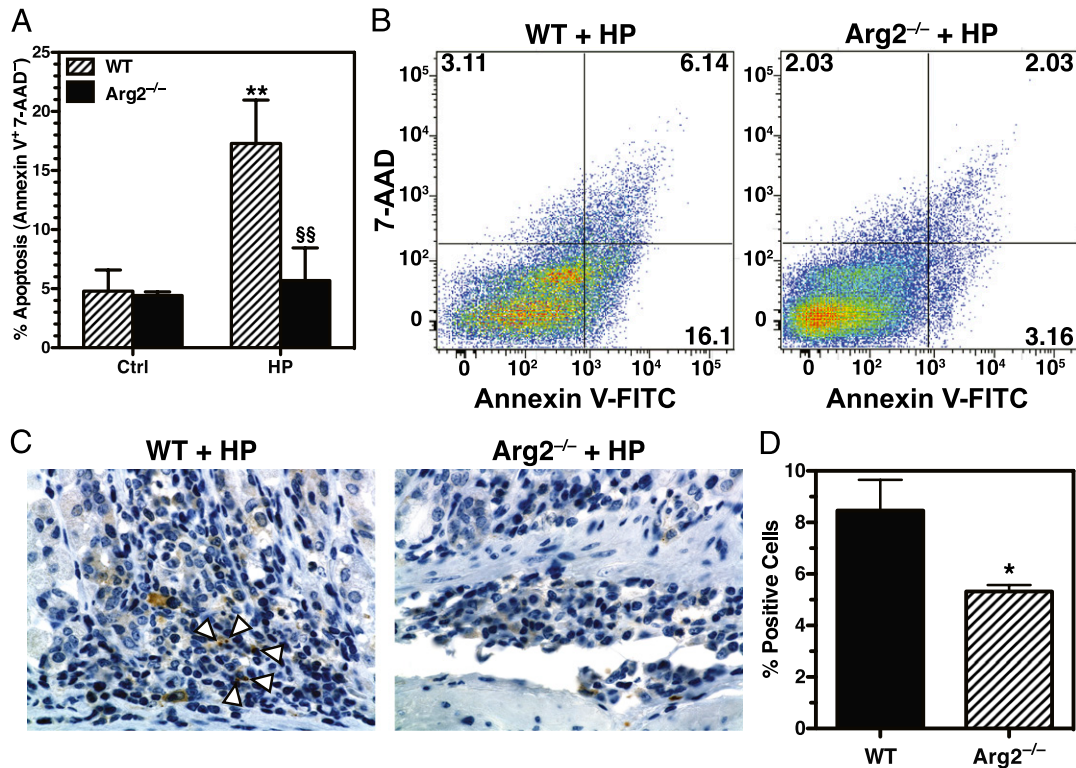
FIGURE 6. Arg2$^{-/-}$ macrophages undergo less apoptosis than WT macrophages during *H. pylori* infection. *A*, Gastric macrophages from WT and Arg2$^{-/-}$ mice were isolated 48 h after inoculation with *H. pylori* SS1 and stained for flow cytometry with annexin V–FITC and 7-AAD. The percentages of annexin-V$^+$ 7-AAD$^-$ cells are shown. $n = 4$ mice per group. **$p < 0.01$ compared with uninfected WT mice. §§$p < 0.01$ compared with infected WT mice. *B*, Representative flow cytometric dot plots are shown demonstrating staining for annexin V and 7-AAD in gastric macrophages isolated from infected WT and Arg2$^{-/-}$ mice. *C*, Photomicrographs (original magnification ×600) of slides from chronically infected WT and Arg2$^{-/-}$ mice stained for cleaved caspase-3 (brown immunoperoxidase stain). The arrowheads are used to highlight apoptotic bodies. Data are representative of seven to eight mice per group. *D*, Quantification of cleaved caspase-3 staining. The percentages of positively stained inflammatory cells are shown.

activation nomenclature has been used to refer to cells with induction of Arg1 rather than Arg2 (48). We have shown that upregulation of Arg1 does not occur in *H. pylori* infection, and intriguingly, Arg2 induction has recently been associated with M1 responses in a murine model of atherosclerosis (49). Another factor in the use of L-arginine in host defense is that its uptake into macrophages is required to allow generation of NO (18, 50). We have reported that while the L-arginine transporter, cationic amino acid transporter 2, is upregulated in gastric macrophages upon infection with *H. pylori*, L-arginine uptake is actually inhibited by the polyamine spermine, which is generated downstream of Arg2 (14). We are currently investigating the role of macrophage cationic amino acid transporter 2 during chronic *H. pylori* infection.

Additionally, we have found that induction of Arg2 enhances *H. pylori*-induced apoptosis in gastric macrophages, consistent with our previous in vitro findings (29). This apoptosis was associated with decreased abundance of macrophages in the gastric mucosa in WT mice compared with that in Arg2$^{-/-}$ mice. Another contributing factor to the increase in macrophages in the Arg2$^{-/-}$ mice may be increased stimulation of mononuclear cell infiltration, because enhanced Th1 and Th17 responses, as we have detected, have been correlated with increased inflammatory cells in the gastric mucosa (9, 10, 34). Taken together, our data suggest that the limited number of macrophages associated with Arg2 induction results in diminished gastric inflammation and proinflammatory cytokine production. Consequently, the inhibition of *H. pylori*-induced macrophage apoptosis that we achieved by using mice deficient in Arg2 caused an increase in the number of surviving infiltrating macrophages and an associated increase in

gross and histologic gastritis (increased neutrophils and lymphocytes) and proinflammatory cytokine production. Other reports have demonstrated that macrophages mediate gastric inflammation during *H. pylori* infection, as depletion of macrophages from mice with clodronate-loaded liposomes resulted in reduced levels of histologic gastritis (51). It should be noted that the reduction in macrophages had no effect on *H. pylori* colonization levels in that study (51), which is consistent with our current findings that WT mice with higher gastritis scores exhibited no reduction in colonization and further supports the concept that altered macrophage immune function is a hallmark of *H. pylori* infection, as loss of cells would not be expected to have an effect on host defense if the cells are already defective.

In summary, our data indicate that induction of Arg2 by *H. pylori* is a mechanism by which the pathogen escapes the host innate immune response and contributes to the immunopathogenesis of the infection. However, we also recognize that another possibility is that the Arg2 component of the innate immune response in macrophages may serve to protect the host from unrestrained inflammation, and as such Arg2 could prevent overabundant nitrosative stress and its associated mutagenic potential that would derive from unrestricted NO production (52). However, it should be noted that the increased nitrotyrosine staining in the Arg2$^{-/-}$ mice that we observed did not appear to involve epithelial cells in our model, indicating that Arg2 may be dispensable in protecting epithelial cells from nitrosative stress. Because we have reported that Arg2 is upregulated in *H. pylori* gastritis tissues from human subjects, insights into the importance of Arg2 could be gained from molecular epidemiology studies of Arg2 levels in human

subject groups, such as in persons from Latin America where regions of low versus high risk of gastric cancer have been described, despite similarly high prevalence rates of *H. pylori* (53–55). Studies related to this issue may be a promising area for future investigation.

Disclosures

The authors have no financial conflicts of interest.

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