Arginase, Nitric Oxide, and the Defective Immune Response to *Helicobacter pylori*

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To my beautiful wife Aneesah

and

lovely kids Na’imah and Ibrahim

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LIST OF ABBREVIATIONS

SCID.....Severe combined immunodeficiency

Treg.....Regulatory T cell

WT.....Wild-type

MALT.....Mucosa-associated lymphoid tissue

DC.....Dendritic cell

TLR.....Toll-like receptor

LPS.....Lipopolysaccharide

NO.....Nitric oxide

iNOS.....Inducible nitric oxide synthase

ODC.....ornithine decarboxylase

CAT2.....Cationic amino acid transferase 2

Arg1.....Arginase I

Arg2.....Arginase II

SMO.....Spermine oxidase

BEC.....S-(2-boronoethyl)-L-cysteine

cPTIO.....2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazole-1-oxyl-3-oxide

MOI.....Multiplicity of infection

NO_2^-.....Nitrite

eIF2α.....eukaryotic translation initiation factor 2α

7-AAD.....7-aminoactinomycin D

MRSA.....Methicillin-resistant *Staphylococcus aureus*

CHAPTER I

INTRODUCTION

Helicobacter pylori

H. pylori is a gram-negative, microaerophilic bacterium that selectively colonizes the human stomach. It is one of the most common infections of man and one of the most successful pathogens as it infects approximately 50% of the world's populations (1). *H. pylori* is the main cause of peptic ulceration, gastric lymphoma, and gastric adenocarcinoma (2). Importantly, the gastric cancer associated with *H. pylori* infection is the second leading cause of cancer-related death worldwide (3). Consequently, *H. pylori* was the first bacterium to be classified as a class 1 carcinogen in 1994 (4). Despite inducing gastritis in all infected individuals, only a small percentage of individuals infected with *H. pylori* ever develop peptic ulcer disease or gastric cancer, indicating that other factors may be involved (1). Infection with *H. pylori* occurs worldwide, however, developing countries have a higher prevalence than developed countries (5). The differences in prevalence have been attributed to the rate at which children acquire the infection. The infection persists for the life of the host despite eliciting a seemingly vigorous immune response (6). 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 less than 50% in some regions of the world (7).

Discovery of *H. pylori* and its association with gastric disease

Spiral-shaped bacteria were reported to colonize the human gastric mucus layer as early as 1893 (8); however, it was not until the groundbreaking findings of Barry J. Marshall and J. Robin Warren in the 1980's that infection with this 'unknown curved bacillus' was demonstrated to be the causative agent of the majority of cases of chronic gastritis and duodenal ulcers (9). Marshall and Warren were able to demonstrate the presence of these spiral-shaped, gram-negative bacteria in patients undergoing endoscopy (10); however, further analysis was hampered by the inability to culture the bacteria. Interestingly, they found a strong correlation between the presence of the bacteria and gastritis and a 100% correlation between presence of the bacteria and duodenal ulcers (10). Serendipitously, the investigators were eventually able to culture the organism and initially named it *Campylobacter pyloridis* (11). In the field of gastroenterology, there was much skepticism concerning a bacterial cause for gastritis and peptic ulcers, as there was a long-standing belief that stress along with certain lifestyle factors were the primary cause of duodenal ulcers. In order to prove his hypothesis, Marshall went so far as to drink a culture of the bacterium, document the resulting gastritis by endoscopy, and eliminate the infection with antibiotic treatment (9). To further test their hypothesis, Marshall and Warren undertook a double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pyloridis* (12). Therein, they demonstrated that treatment with bismuth subcitrate and the antibiotic tinidazole eradicated the infection in the majority of patients. Consequently, elimination of the infection resulted in the healing of peptic ulcers. This study, along with many others, provided the convincing evidence

necessary to begin treating patients with antibiotics supplemented with bismuth to eliminate *H. pylori*-induced gastritis and ulcers. Their discovery of the role of *H. pylori* in gastritis and peptic ulcer disease was so significant that they received the Nobel Prize in Medicine in 2005.

Immunology of *H. pylori infection*

Although the bacterium induces a seemingly vigorous chronic adaptive and innate immune response, *H. pylori* and the associated chronic inflammation persists for the life of the host and defense mechanisms are not effective in eradicating the organism (6). The reasons for the ineffective response are complex; in addition to its own defense factors, *H. pylori* induces multiple events that result in immune dysregulation (13). Much of the experiments delineating these processes have been performed using animal models of *H. pylori* infection. To date, several animal models have been developed using mice, rats, monkeys, Mongolian gerbils, guinea pigs, beagle dogs, cats, gnotobiotic piglets, and ferrets (14, 15). Most of the work discussed herein will focus on experimental data derived from humans, mice, and Mongolian gerbils. The experimental model will be noted in the discussions that follow. Below is a summary of recent work on the immune response to *H. pylori*.

Adaptive immunity. *H. pylori* infection of mice induces the infiltration of lymphocytes into the stomach of mice 10 d post-inoculation (16). Both T and B cells are present in the infected gastric mucosa, with most of the T cells being $CD4^+$ (17, 18). *H. pylori* infection induces the expression of several pro-inflammatory cytokines, such as

IFN-γ, IL-12, IL-17, TNF, and IL-6 (19, 20). However, there is little expression of IL-4 (21). Furthermore, splenocytes isolated from infected mice also produce more IFN-γ and IL-2 than IL-4 and IL-5, suggesting a Th1 response (22). Such a response is similarly found in humans (23). Th1 immunity typically develops in response to bacteria that reside intracellularly. Because *H. pylori* is an extracellular bacterium, a Th1 response may be unnecessary. However, several studies suggest that the defect in the immune response may be that the Th1 response is not vigorous enough (6).

The infiltration of T cells into the gastric mucosa of infected mice is necessary for the onset of gastric inflammation (24). In severe combined immunodeficient mice (SCID, mice lacking mature T and B cells), there is minimal inflammation in the stomach (25). Furthermore, adoptive transfer of splenocytes from infected immunocompetent mice into SCID mice induces more severe gastric inflammation (26). As mentioned above, splenocytes stimulated with *H. pylori* produce high-levels of IFN-γ. Consistent with this data, IFN-$\gamma^{-/-}$ mice have decreased gastritis, providing evidence that Th1 responses are associated with increased gastritis (27).

In addition to IFN-γ-dominant Th1 responses, there is evidence that Th17 responses are also important (28, 29). IL-17 levels are increased in infected mice and humans and have been linked to chemokine-mediated neutrophil infiltration (16, 30), a hallmark of *H. pylori* infection. Additionally, prophylactic immunization of mice induces high-level expression of IL-17 that is associated with enhanced gastritis and decreased bacterial colonization (31). These data provide evidence suggesting that a tempered Th17 response may be responsible for persistent bacterial colonization.

The inflammatory response to infection with *H. pylori* is also affected by the infiltration of regulatory T cells (Tregs) expressing CD4, CD25, and Foxp3 (32). Tregs can suppress T cell proliferation and cytokine production (33). Furthermore, Tregs produce anti-inflammatory cytokines, such as IL-10, which counteract the pro-inflammatory cytokines produced by Th1-type T cells (33). Additionally, depletion of Tregs can reverse these effects in circulating memory T cells (34). Further evidence has accumulated which suggests that Treg induction may be detrimental to host immunity against *H. pylori*. In mice lacking T cells, adoptive transfer of lymphocytes depleted of Tregs resulted in a significant reduction in *H. pylori* colonization and increased gastric inflammation as compared to mice receiving non-depleted lymphocytes (34). Furthermore, there was enhanced production of IFN-γ. Additionally, depletion of Tregs in wild-type (WT) mice also enhanced gastric inflammation, pro-inflammatory cytokine production, and decreased bacterial colonization (32). Humans also have enhanced Treg infiltration of the stomach during *H. pylori* infection which may contribute to bacterial persistence (35). However, Tregs may play an important role in tempering the immune response to decrease overall tissue damage caused by the inflammatory response (36).

Infection by extracellular bacteria typically induces the production of antibodies. These antibodies may act in several ways in order to lessen the burden of the infection, which may include binding to bacteria and inducing complement activation that can result in formation of holes in the bacterial membrane, inactivation of bacterial toxins, and induce the phagocytosis of the bacterium (37). *H. pylori* elicits an antibody response and immunoglobulin deposits have been shown on *H. pylori* on the gastric surface (38,

39). In patients, *H. pylori* infection induces autoreactive antibody responses due to the molecular mimicry between *H. pylori* proteins and H+K+-ATPase (40). Thus, *H. pylori* infection may contribute to autoimmune gastritis by promoting an immune response against self antigens. Immunization with *H. pylori* antigens can elicit protective immunity that is not dependent upon B cells (41). In mice with active immunization with *H. pylori* antigens, MHC class II-deficient mice failed to develop protection (42), whereas B cell-deficient mice (μMT) were fully protected after immunization (41). Other studies have demonstrated that antibody responses are not only dispensable during *H. pylori* infection, but they are detrimental to the elimination of the bacteria. In B cell-deficient and WT mice infected with *H. pylori* for 8 and 16 weeks, the B cell-deficient mice demonstrated more than a two log order decrease in bacterial colonization as compared to the WT mice (41); however, the bacteria were not completely eradicated. Furthermore, IgA antibody responses have also been implicated to have a negative effect in *H. pylori* infection (43). One area in humoral immunity that has received attention is the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma which arises from B cells. Our laboratory has demonstrated that B cells are protected from spontaneous apoptosis and undergo proliferation in response to stimulation with a low inoculum of *H. pylori* (44).

Innate immunity. *H. pylori* causes an inflammatory reaction with both polymorphonuclear and mononuclear cells (45). These cells produce pro-inflammatory cytokines and chemokines including IL-1β, IL-6, TNF-α and IL-8 (19, 20). Products of *H. pylori* can also activate immune cells *in vitro*, and have been detected in the lamina

propria (46). *H. pylori* has been considered noninvasive, but studies have demonstrated *H. pylori* invasion of gastric epithelial cells *in vitro* (47) and *in vivo* (48), and into microvessels of the lamina propria (49). Importantly, *H. pylori* has recently been demonstrated to be in direct contact with immune cells of the lamina propria in the majority of both gastritis and gastric cancer cases using transmission electron microscopy and immunogold detection (50). Peripheral blood mononuclear cells stimulated with *H. pylori* show larger increases in IL-12 than IL-10 (51). IL-12 drives Th1 lymphokine (IFN-γ) responses to *H. pylori*, and bacterial products inhibit mitogen-activated lymphocyte proliferation (52). Gastric epithelial cells act as antigen presenting cells and activate $CD4^+$ T cells (53). Upon activation, dendritic cells (DCs) can stimulate either a Th1 or Th2/regulatory T cell response (54). DCs activated by *H. pylori* undergo maturation (55, 56), allowing them to elicit a Th1 response in T cells (57), but the IL-12 response may be attenuated compared to that observed with other pathogens (58).

Recognition of H. pylori. Additionally, the innate immune system plays a vital role in the recognition of *H. pylori* by stimulation and activation of certain pattern-recognition receptors. These are evolutionarily conserved receptors that are responsible for identifying pathogen-associated molecular patterns, which are associated with microbial pathogens (59). In *H. pylori* infection, there is accumulating evidence suggesting the importance of toll-like receptors (TLRs) in the recognition of the bacterium (60, 61).

There are several TLRs that have been discovered which recognize various microbial molecular patterns (62). Much of the work to determine the role of TLRs

during *H. pylori* infection has focused upon TLR4, TLR2, and TLR5. The microbial patterns that stimulate these receptors are bacterial lipopolysaccharide (LPS), fungal mannans, and host heat shock proteins for TLR4, bacterial peptidoglycan, lipoprotein, and lipoteichoic acid for TLR2, and bacterial flagellin for TLR5 (63). Stimulation of epithelial cells results in upregulation of TLR4, leading to the activation of NF-κB and IL-8 production (64). However, others have suggested that IL-8 expression in epithelial cells activated with *H. pylori* is independent of TLR4 because blocking TLR4 stimulation using a monoclonal antibody does not prevent IL-8 expression (65). Additionally, one explanation as to why *H. pylori* LPS may not be important for *H. pylori* recognition and TLR4 stimulation is because it is an ineffective activator of the immune response compared to LPS from other Gram-negative bacteria, due to modifications in the LPS lipid A core (66).

Epithelial cells in the stomach and intestine also express TLR2 (67). *H. pylori* stimulation of HEK293 cells transfected with TLR2 allowed these cells to respond to the bacterium and produce IL-8 (67). However, transfection with TLR4 did not induce IL-8 production. One of *H. pylori's* virulence factors, neutrophil-activating protein, is a known TLR2 agonist that stimulates production of oxygen radicals from neutrophils (68, 69). Additionally, TLR2 polymorphisms have been associated with gastric cancer (70).

TLR5 responds to stimulation with flagellin and may play an important role in recognition of *H. pylori* because the bacterium is flagellated. TLR5 is present on gastric epithelial cells and transfection of HEK293 cells with TLR5 leads to IL-8 production upon stimulation with *H. pylori* (67). However, others suggest that TLR5 may not be

important in recognition of *H. pylori* because some data suggest that *H. pylori* flagella have low immune-stimulating activity (71). Some have provided evidence suggesting that *H. pylori* flagellin contains specific amino acid substitutions within the TLR5 recognition site that prevents its recognition (72).

Protective immunity against *H. pylori*

Several animal models have now provided insight into the failure of the immune response to eliminate the infection with *H. pylori* by demonstrating various mechanisms by which bacterial colonization can be decreased or eradicated and eliciting protective immunity. Vaccination studies have provided a model to study protective immunity against *H. pylori* because most vaccination protocols against *H. pylori* are effective in either preventing infection with *H. pylori* or eliminating an established infection (73). Initially studies suggested that protective immunity was mediated by antibodies specific against *H. pylori*. However, immunization of mice unable to produce antibodies still provided protective immunity (41).

Further studies sought to determine which cell types were important for protective immunity during immunization. Mice that are lacking $CD8^+$ T cells still exhibit protective immunity following immunization; however, mice lacking $CD4^+$ T cells do not (42). Furthermore, $CD4^+$ T cells isolated from an immunized mouse can provide protective immunity when adoptively-transferred into mice lacking mature T and B cells (74). Protective immunity does not occur in mice lacking IL-12 or IFN-γ (17, 27), but does

occur in mice lacking IL-4 (75), further suggesting that an enhanced Th1 response is needed in order to eliminate the infection.

Additionally, mast cells have been reported to play an essential role in protective immunity against *H. pylori*. Using mast cell-deficient mice ($Kit^{W/W\text{-}v}$), one group has demonstrated that mast cells are needed to provide protective immunity in a vaccination model (76). However, this study has been criticized because the mice used in this study have been shown to develop mast cell populations during cutaneous and intestinal chronic inflammation. Furthermore, the authors used a mouse model of *Helicobacter felis*, instead of *H. pylori*. Another group repeated similar experiments using a different mast cell-deficient mouse model ($Kitl^{Sl/Sl\text{-}d}$). They found that protective immunity still occurred in these mice (77). However, the authors demonstrated that mast cells do contribute to vaccine-induced immunity, possibly by contributing to neutrophil recruitment and inflammation.

Neutrophils have been shown to be important for vaccine-induced immunity. Antibody-mediated depletion of neutrophils has been shown to prevent protective immunity against *H. pylori* (78). IL-$10^{-/-}$ mice, which can spontaneously eliminate *H. pylori* infection, have increased levels of neutrophil infiltration upon infection (79). Enhanced neutrophil responses in IL-$10^{-/-}$ mice appears to be related to increased IL-17 production by these mice (31). Furthermore, antibody-mediated neutralization of IL-17 in vaccinated mice that were subsequently challenged with *H. felis* resulted in reduced infiltration of neutrophils with no reduction in bacterial colonization (80). Additionally, depletion of neutrophils in IL-$10^{-/-}$ mice delays clearance of an *H. pylori* infection (78).

H. pylori immune evasion strategies

Of the many bacterial species that have been discovered thus far, very few are capable of colonizing human beings. Of those that colonize humans, only a handful will actually cause disease. This is due, in part, to the expertise of the human immune system. Nevertheless, several bacteria have devised brilliant strategies to bypass critical components of our immune system. Included in this group is *H. pylori,* which may be considered the most successful human pathogen due to its ability to colonize its human host for the duration of the host's life and its remarkable ability to colonize approximately 50% of the total world's population (81). The success of *H. pylori* is possibly due, in part, to the fact that it has coexisted with humans for a very long time (82). Recent genetic studies have indicated that *H. pylori* spread during human migrations from east Africa around 58,000 years ago (83). Consequently, it has been proposed that infection with *H. pylori* may provide a selective evolutionary advantage due to the long-term relationship between humans and *H. pylori* (81, 84). Additionally, only a small percentage of infected individuals develop peptic ulcer disease or gastric adenocarcinoma, and this usually takes several decades of infection (1). Due to this long-term cohabitation between humans and *H. pylori*, these bacteria have devised several strategies to avoid being eradicated by the immune response and provides an excellent model of chronic colonization and inflammation.

A major blockade for bacterial colonization of the stomach is the acidic environment. Most bacterial species are killed upon encounter with the low pH levels found in the human stomach. However, *H. pylori* overcomes this obstacle by the

production of ammonia, which acts as a buffer to the surrounding acidity (85) (86). The production of ammonia is performed by the *H. pylori* enzyme urease, which converts urea into carbon dioxide and ammonia. Consequently, mutant strains of *H. pylori* that lacks urease, or the urea transporter, are unable to colonize mice (87, 88). Additionally, the bacteria are able to burrow themselves deep into the mucus layer of the stomach, directly above the epithelial cells where the pH is closer to neutral (89). The ability to establish this location is facilitated by *H. pylori*'s flagella. Similar to urease, flagella are also required for bacterial colonization (90).

One of *H. pylori*'s virulence factors, neutrophil-activating protein, induces the infiltration and activation of neutrophils and monocytes (69). Upon activation, neutrophils produce reactive oxygen species which cause a wide range of damage to proteins, DNA, and lipids and are effective at killing several types of bacteria (91). *H. pylori* is capable of avoiding killing by reactive oxygen species by expressing enzymes that can scavenge reactive oxygen species such as catalase, superoxide dismutase, and an NADPH-dependent quinone reductase. These three enzymes scavenge hydrogen peroxide, superoxide, and NADPH respectively. Depletion of NADPH is important for bacterial survival because it is needed by neutrophils in order to produce superoxide. It has been suggested that the purpose of neutrophil-activating protein is to induce inflammation in order to obtain nutrients from the inflamed tissue (92).

H. pylori also induces the activation of macrophages to produce the enzyme inducible nitric oxide (NO) synthase (iNOS), which metabolizes L-arginine into NO that can kill *H. pylori* (93). The importance of iNOS in the innate immune response is

demonstrated by the increased susceptibility to various bacterial infections in mice deficient of iNOS (94). iNOS produces NO in copious amounts that, similar to reactive oxygen species, is antimicrobial and can cause damage to proteins and DNA (95). To avoid killing by NO, *H. pylori* expresses the enzyme arginase which effectively steals the substrate L-arginine away from macrophage iNOS (96). Consequently, a mutant strain of *H. pylori* that is deficient for arginase is effectively killed by macrophages in a coculture system, whereas WT *H. pylori* is not (96). Additionally, NO can react with superoxide to form peroxynitrite, a very volatile, cytotoxic, and mutagenic reactive nitrogen species that can kill bacteria (95). *H. pylori* possesses a peroxiredoxin, a homolog of *Salmonella typhimurium's* alkylhydroperoxide reductase subunit C, that can effectively detoxify peroxynitrite and evade being killed by this reactive nitrogen species (97).

Intriguingly, *H. pylori* arginase has also been shown to be detrimental to T cell responses. It has been demonstrated that L-arginine is needed for the re-expression of the CD3ζ chain of the T cell receptor (98). Consequently, *H. pylori* arginase steals away the L-arginine from T cells, thereby inhibiting the re-expression of the CD3ζ chain and inhibiting T cell proliferation (99).

Furthermore, it has been demonstrated that *H. pylori* upregulates in macrophages the enzyme ornithine decarboxylase (ODC) that leads to the production of polyamines (100). The polyamine spermine has been shown to inhibit macrophage NO production by blocking L-arginine uptake (93). The L-arginine transporter cationic amino acid transporter 2 (CAT2) is necessary for NO production by iNOS in macrophages (101). By blocking L-arginine uptake in macrophages, spermine synthesis by ODC contributes to

bacterial survival (102). Importantly, chemical inhibition of ODC during *H. pylori* infection led to enhanced NO production by macrophages and a significant decrease in bacterial colonization after 4 months (102).

In addition to scavenging reactive nitrogen and oxygen species, *H. pylori* has also adopted mechanisms to avoid killing by phagocytosis. It has been elegantly demonstrated that cholesterol promotes the phagocytosis of *H. pylori* (103). Interestingly, *H. pylori* expresses an enzyme, cholesterol-α-glucosyltransferase, that glucosylates cholesterol and inhibits phagocytosis by macrophages (103). The mechanism by which glucosylated cholesterol inhibits phagocytosis is unclear. Additionally, it has been demonstrated that a cholesterol-rich diet during bacterial challenge leads to a T cell-dependent reduction of the *H. pylori* burden in the stomach (103). Consequently, glucosylation of cholesterol inhibits T cell responses in an antigen-specific manner (103). However, if cholesterol is present and *H. pylori* is phagocytosed, the bacterium possesses additional strategies to avoid being killed by phagocytosis. Upon being phagocytosed, *H. pylori* delays the maturation of phagosomes and causes the formation of large phagosomal structures called megasomes (104). By delaying phagosome maturation and preventing its death, the bacterium could potentially induce apoptosis in these cells and possibly escape out of the cells. It has been reported that inhibition of phagosome maturation only occurs with virulent, type I strains of *H. pylori* (strains that express both CagA and VacA) (104, 105); however, this has been disputed (106).

Another *H. pylori* virulence factor, VacA, has been shown to have multiple roles in the evasion of host immune responses. VacA has been reported to interfere with the

presentation of antigens by B cells (107). Antigen presentation by B cells to T cells was defective when B cells were exposed to VacA (107). Additionally, VacA is detrimental to proper T cell function. It has been reported that VacA interferes with the activity of nuclear factor of activated T cells (NFAT), which results in inhibition of IL-2 expression, decreased T cell proliferation, and cell cycle arrest (108).

Role of iNOS

Since the discovery of NO as a molecule with an extensive array of biological functions and the identification of iNOS that is responsible for high-output NO production, there has been intense interest in iNOS as a regulator of mucosal inflammation and injury. Studies in the intestine have shown inconsistent results (109). In the case of iNOS in *H. pylori* infection, our laboratory reported that iNOS is induced by *H. pylori in vitro* in macrophages (Fig. 1) (110) and *in vivo* in human gastritis tissues (111), and that soluble factors released by *H. pylori* can induce macrophage iNOS in the absence of bacterial contact with the cells (112). The generation of NO in response to *H. pylori* infection has an important role in host defense. Our laboratory reported that macrophages kill *H. pylori* by an NO-dependent mechanism (93, 96) and that the bacterium possesses an arginase enzyme that facilitates survival by competing with iNOS for the same substrate, L-arginine (96). Our laboratory has also shown that activation of ODC by *H. pylori* results in loss of innate immunity via spermine-mediated inhibition of iNOS at the level of protein translation (93). The mechanism by which this occurs is by the inhibition of L-arginine uptake by CAT2 (102), as has been discussed previously. We

recently reported that the generation of NO by macrophages in response to *H. pylori* is entirely dependent on the availability of L-arginine and that this results in increased expression of iNOS protein, without altering the induction of mRNA expression (113). Specifically, we found that the addition of increasing levels of extracellular L-arginine results in a proportionate increase in NO production even at concentrations well above the circulating levels of L-arginine in humans and mice of 0.1 mM (114, 115), and above the K_m of the iNOS enzyme for L-arginine, which is in the range of 10 μM (116, 117). 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 (113).

Importance of arginase

Many have concluded that it is difficult to predict the effects of NO in the milieu of mucosal inflammation. This may be due in part to arginase. Arginase converts L-arginine to L-ornithine (Fig. 1). Ornithine is acted upon by ODC to form polyamines. Thus, arginase is the endogenous competitive inhibitor for iNOS. Two forms of arginase have been purified and cloned. Arginase I (Arg1) is cytosolic in location, and abundant in liver; it has been referred to as "hepatic" arginase (118). Arginase II (Arg2) is mitochondrially-associated, present in most tissues, and referred to as the "extrahepatic" form (119). The K_m values for L-arginine of arginase I and II are ~10 mM and are much higher than the K_m value for iNOS, which is ~5 μM. However, the V_{max} of arginase I and II are 10^3-10^4 times higher than that of iNOS in activated macrophages. Therefore, the

V_{max}/K_m values are similar and these enzymes are expected to compete for L-Arg in the cells (120). Our laboratory has shown that Arg1 is the predominant form upregulated in colitis models, where it has a beneficial role (121), while Arg2 is upregulated in *H. pylori* gastritis (122), and induction of Arg2 in *H. pylori*-stimulated macrophages results in apoptosis (122). Induction of arginase activity by pathogens has been reported to modulate macrophage NO production in the case of both Arg1 (123-126), and Arg2 (127, 128), which can restrict effective immunity. These effects have been attributed to substrate competition, but alterations in iNOS expression have not been studied.

The downstream effectors of arginase activity: polyamines

The mammalian polyamines are putrescine, spermidine, and spermine (129). ODC forms putrescine from L-ornithine. Spermidine is formed from putrescine by spermidine synthase and spermine is formed by spermine synthase (Fig. 1). Polyamines have been implicated in carcinogenesis (130). Spermine can inhibit LPS-stimulated pro-inflammatory cytokine production in monocytes, macrophages, and lymphocytes (131, 132). Injection of spermine inhibits acute footpad inflammation (131). While polyamines may be anti-inflammatory in acute inflammation, in chronic inflammation, as in *H. pylori* infection, suppression of immune responses could contribute to persistence and progression of the disease. Polyamine levels have been shown to be increased in the gastric mucosa in human *H. pylori* infection (133).

ODC and polyamine oxidation

Although ODC is the rate-limiting step in polyamine synthesis, availability of L-ornithine substrate can become a factor, as with our observation that inhibition of either arginase or ODC blocked macrophage apoptosis (Fig. 1) (122). Regulation of polyamine homeostasis occurs through metabolism and efflux of intracellular polyamines. In one pathway, spermine or spermidine are acetylated by spermidine/spermine N^1-acetyltransferase, and this allows efflux of polyamines from cells, which occurs in the acetylated form (134). Our collaborator, Robert Casero, and others have identified an enzyme, polyamine oxidase 1, later renamed spermine oxidase (SMO), which is inducible and selectively uses spermine as substrate. Catabolism of polyamines by SMO produces H_2O_2 , which can result in induction of apoptosis (135). Oxidation of polyamines has been shown to induce mitochondrial uncoupling and release of cytochrome *c* (136), which is known to activate caspases, and thus apoptosis. Our laboratory has implicated this pathway in *H. pylori*-induced apoptosis in macrophages and epithelial cells (137).

Apoptosis in *H. pylori* gastritis

Programmed cell death (apoptosis) has been implicated in the pathogenesis of *H. pylori*-induced inflammation. Induction of apoptosis in gastric epithelial cells has been well-demonstrated *in vitro* (138) and *in vivo* (139). *H. pylori* causes lymphocyte apoptosis *in vitro*, which has been postulated to contribute to the ability of *H. pylori* to evade the immune response (140). Studies from our laboratory have demonstrated that *H. pylori* also induces macrophage apoptosis and that this occurs by the activation of Arg2,

ODC, and SMO (Fig. 1) (93, 122, 137). We contend that this apoptosis contributes to the persistence of *H. pylori* by attenuating innate immunity. It has been demonstrated that macrophages are important in the immune response to *H. pylori* and their removal results in increased bacterial colonization (141). Furthermore, macrophages have been shown to release the pro-inflammatory cytokine IL-1ß during apoptosis in other models (142, 143). This may contribute to the severity of gastritis in *H. pylori* infection.

Significance, summary, and dissertation goals

The demonstration that *H. pylori* is the primary cause of chronic gastritis and peptic ulcer disease in humans (10) was one of the major medical discoveries of the 20th century and merited the Nobel Prize in Medicine in 2005. The identification of *H. pylori* as the etiological agent for gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma further emphasizes the clinical relevance of this pathogen. Although these epidemiological associations are established, strategies for disease risk assessment are inadequate. *H. pylori* infection is characterized by the presence of chronic active gastritis, i.e. both mononuclear cell (lymphocytic) and polymorphonuclear cell (neutrophilic) infiltration, and persistence of the bacterium despite many years of infection. Thus, the immune response is ineffective. The focus in our laboratory has been to provide insight into this failure to eradicate the organism and the causes of the inflammation. Our data support the concept that there are fundamental defects in the host response that can be attributed to alterations of L-Arg metabolism (Fig. 1). We propose that diversion of L-Arg to the arginase pathway is detrimental in the immune response

against *H. pylori*. Using *in vitro* and *in vivo* models we seek to develop clues into the regulation of cellular function in the setting of *H. pylori* infection that may prove fundamentally important in establishing how the bacterium evades the immune response. Knowledge gained will have relevance to other gastrointestinal inflammatory conditions, infections, and neoplasias.

In Chapter II, we examined the importance of *H. pylori*-induced expression of macrophage Arg2 *in vitro* and determined its effects on iNOS activity. We demonstrated for the first time that upregulation of macrophage Arg2 by an extracellular bacterium results in diminished NO-dependent killing. Moreover, our studies demonstrating that inhibition of macrophage arginase results in enhanced iNOS-derived NO production in response to *H. pylori in vivo* indicate that such a strategy could enhance the ability of the immune system to control or eliminate *H. pylori* infection.

In Chapter III, we examined the importance of *H. pylori*-induced expression of Arg2 *in vivo* in a chronic infection model. We demonstrated that *H. pylori* upregulates Arg2 in the gastric mucosa that localizes to $F4/80^{+}$ macrophages, thereby restricting iNOS protein levels and NO production, and enhancing macrophage apoptosis. These data suggest that Arg2 induction contributes to the defective immune response in WT mice.

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. Because we have reported that Arg2 is upregulated in *H. pylori* gastritis tissues from human subjects (122), insights into the importance of

Arg2 could be gained from molecular epidemiology studies of Arg2 levels in human subject groups from populations with high prevalence rates of *H. pylori* infection.

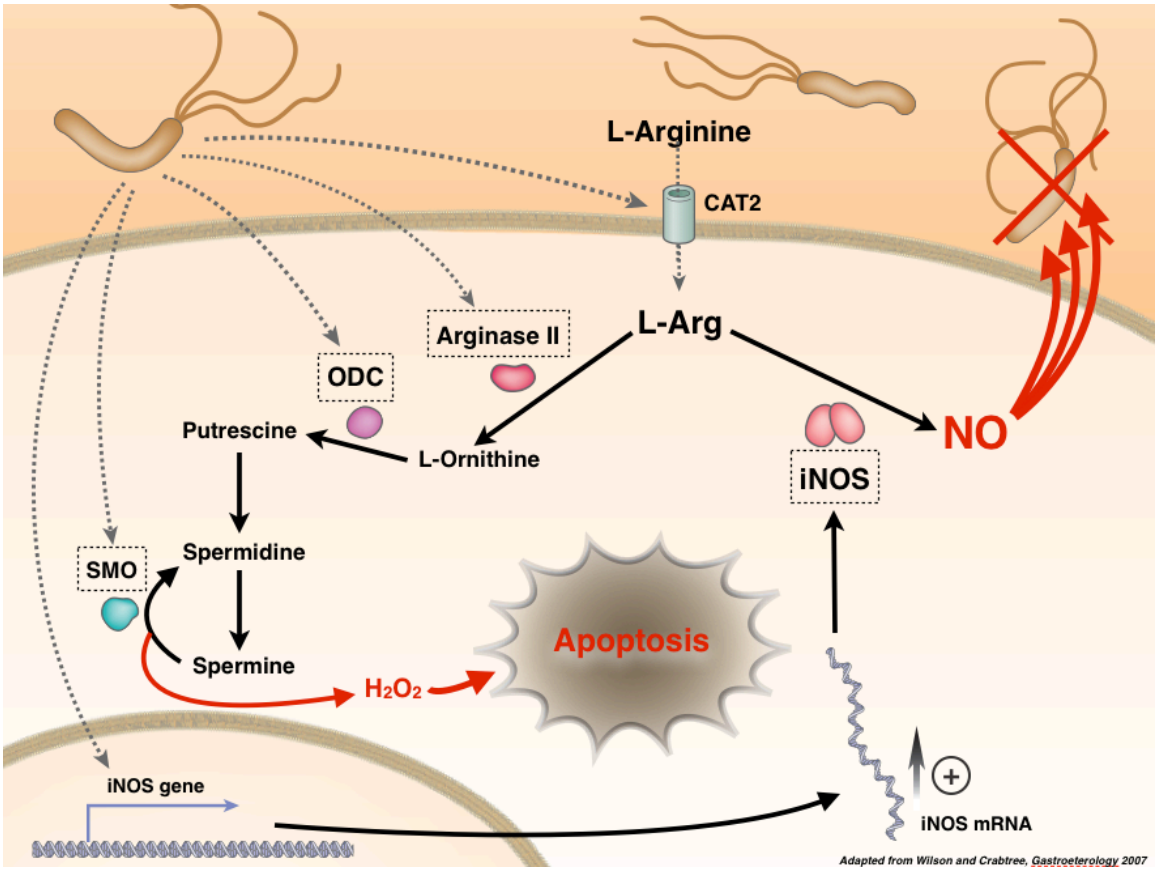

FIGURE 1. L-arginine metabolism in macrophages stimulated with *H. pylori*. This figure demonstrates the enzymes that are upregulated in macrophages stimulated with *H. pylori*. The figure was adapted from Wilson and Crabtree, Gastroenterology 2007 (6).

CHAPTER II

ARGINASE II RESTRICTS HOST DEFENSE TO *HELICOBACTER PYLORI* BY ATTENUATING INDUCIBLE NITRIC OXIDE SYNTHASE TRANSLATION IN MACROPHAGES

Summary

Helicobacter pylori infection of the stomach causes peptic ulcer disease and gastric cancer. Despite eliciting a vigorous immune response, the bacterium persists for the life of the host. An important antimicrobial mechanism is the production of NO derived from iNOS. We have reported that macrophages can kill *H. pylori in vitro* by an NO-dependent mechanism, but supraphysiologic levels of the iNOS substrate L-arginine are required. Because *H. pylori* induces arginase activity in macrophages, we determined if this restricts NO generation by reducing L-arginine availability. Inhibition of arginase with S-(2-boronoethyl)-L-cysteine (BEC) significantly enhanced NO generation in *H. pylori*-stimulated RAW 264.7 macrophages by enhancing iNOS protein translation, but not iNOS mRNA levels. This effect resulted in increased killing of *H. pylori* that was attenuated with an NO scavenger. In contrast, inhibition of arginase in macrophages activated by the colitis-inducing bacterium *Citrobacter rodentium* increased NO without affecting iNOS levels. *H. pylori* upregulated levels of Arg2 mRNA and protein, which localized to mitochondria, while Arg1 was not induced. Increased iNOS protein and NO levels were also demonstrated by siRNA knockdown of Arg2 and in peritoneal macrophages from C57BL/6 $Arg2^{-/-}$ mice. In *H. pylori*-infected mice, treatment with BEC or deletion of Arg2 increased iNOS protein levels and NO generation in gastric

macrophages, but treatment of Arg2$^{-/-}$ mice with BEC had no additional effect. These studies implicate Arg2 in the immune evasion of *H. pylori* by causing intracellular depletion of L-arginine, and thus reduction of NO-dependent bactericidal activity.

Introduction

Helicobacter pylori is a microaerophilic, gram-negative bacterium that selectively colonizes the human stomach. It is one of the most successful human pathogens, since it infects half of the world's population (6). All infected individuals exhibit chronic active gastritis and a substantial proportion develop peptic ulcer disease or gastric adenocarcinoma. Importantly, gastric cancer is the second leading cause of cancer deaths worldwide (3). The infection is usually acquired in childhood and persists for the life of the host despite eliciting a vigorous innate and adaptive immune response (6). This raises the question as to how *H. pylori* is consistently able to evade this cellular and humoral immune response. *H. pylori* has generally been considered to be a non-invasive pathogen because it resides in the mucus layer of the stomach in contact with the epithelium. However, multiple reports have demonstrated that *H. pylori* can invade the mucosa, with elegant studies depicting the organism in direct contact with lamina propria immune cells (49, 50, 148). These findings strongly suggest that the failure of the immune response could be directly related to the inability of effector cells to kill the organism 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 iNOS (149). Our laboratory and others have

demonstrated that *H. pylori* induces the expression and activity of iNOS in macrophages both *in vivo* (111, 150) and *in vitro* (93, 96, 110, 112, 113, 122). Further, we have reported that macrophages cocultured with *H. pylori* have the ability to kill the bacterium by an NO-dependent mechanism (93, 96, 113). 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. Recently, it has been emphasized that other components of the innate immune response that orchestrate the cellular immunity to *H. pylori* are attenuated; for example, inadequate dendritic cell activation (151) and downregulatory effects of regulatory T cells (32) have been directly implicated in the persistence of the organism. Thus, while the Th1 response to *H. pylori* has been well documented, adoptive transfer experiments have suggested that the cellular immune response is not vigorous enough to lead to clearance of the infection (24, 27).

This reasoning has led our laboratory to consider the possibility that the iNOS-mediated host defense to *H. pylori* is suboptimal. We recently reported that the generation of NO by macrophages in response to *H. pylori* is entirely dependent on the availability of L-arginine and that this results in increased expression of iNOS protein, without altering the induction of mRNA expression (113). Specifically, we found that the 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 (114, 115), and above the K_m of the iNOS enzyme for L-arginine, which is in the range of 10 μM (116, 117). 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 (113).

Arginase enzymes are the endogenous antagonists to iNOS, since they compete for the same substrate by metabolizing L-arginine into urea and L-ornithine (152, 153). The latter is metabolized by ornithine decarboxylase (ODC) to produce the polyamines putrescine, spermidine, and spermine (154). There are two isoforms of arginase: Arg1 is ubiquitous, but is especially abundant in liver, and Arg2 is abundant in kidney and possesses a mitochondrial localization sequence (155-157). We have reported that Arg2, but not Arg1, is upregulated in *H. pylori*-stimulated macrophages (122). Induction of arginase activity by pathogens has been reported to modulate macrophage NO production in the case of both Arg1 (123, 125, 126, 158), and Arg2 (127, 128), which can restrict effective immunity. These effects have been attributed to substrate competition, but alterations in iNOS expression have not been studied.

We hypothesized that Arg2 expression can restrict NO-mediated defense against *H. pylori* by limiting L-arginine availability, and that this may explain the large amounts of L-arginine that are required for iNOS synthesis. We now report that *H. pylori*-stimulated NO production is attenuated by Arg2 activity *in vitro* and *in vivo*, and that this occurs by limiting iNOS protein expression due to an effect on iNOS translation. Inhibition of arginase enhanced killing of *H. pylori* by macrophages. Moreover, we show that in *H. pylori*-infected mice treated with an arginase inhibitor there was markedly increased iNOS protein expression and NO production in gastric macrophages. To our knowledge, this is the first report that an extracellular bacterium can manipulate host L-

arginine metabolism and iNOS protein synthesis by an Arg2-dependent mechanism, thereby restricting NO production and bacterial killing.

Experimental procedures

Reagents: All reagents used for cell culture and RNA extraction were from Invitrogen (Carlsbad, CA). Real-time PCR reagents were from Bio-Rad (Hercules, CA). *S*-(2-boronoethyl)-L-cysteine (BEC), a slow-binding competitive inhibitor of arginase (159, 160), was synthesized by J.-L.B (161). 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) was from Cayman Chemical (Ann Arbor, MI). siRNA to ODC and Arg2 were from Ambion (Austin, TX). All other chemicals were from Sigma (St. Louis, MO).

Bacteria, cells, and culture conditions: H. pylori SS1 was grown and used as described (110, 137) and multiplicity of infection (MOI) was defined as the ratio of bacteria to eukaryotic cells. *C. rodentium* was grown and used as described previously (121, 162). Macrophages were activated with either live bacteria or lysates prepared with a French press (113), and MOIs were determined in lysates as described (110). For bactericidal studies, live *H. pylori* was separated from macrophages by filter supports (113). When live bacteria were added, medium without antibiotics was used (93, 96, 113). Bacterial killing was determined by serial dilution and culture after 24 h of coculture, and colony growth was compared between samples cocultured with or without macrophages. The murine macrophage cell line RAW 264.7 was maintained in complete

DMEM (113). For experiments, medium was changed to L-arginine-free, serum-free DMEM supplemented with 0.3% bovine serum albumin as described (113). This was followed by addition of desired concentrations of L-arginine (113).

Mice: All animal experiments were approved by the Vanderbilt University Animal Care and Use Committee. C57BL/6 WT mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 Arg2$^{-/-}$ mice were a gift from Brendan Lee (Baylor College of Medicine) and were bred at the Nashville Veterans Affairs Medical Center. Male mice were used at 8 weeks of age. Peritoneal macrophages were isolated as described (93) and used in L-arginine-free, serum-free DMEM. In addition, mice were gavaged with 5×10^8 *H. pylori* SS1 in 100 μl of Brucella broth, or broth alone. In some studies, mice were treated with 0.1% (w/v) BEC in the drinking water (121), starting one day pre-infection, and continuing for a total of 3 days. Mice were sacrificed 2 days post-inoculation, and stomachs were excised and used for isolation of gastric macrophages, which was performed as described (113).

Measurement of NO: The concentration of the oxidized metabolite of NO, nitrite (NO_2^-), was assessed by the Griess reaction (93, 96, 112, 113, 122).

Real-time PCR: Primers for iNOS, Arg1, and β-actin were used as described (113, 122). For Arg2, we used primers that generated a 234-bp product as follows: sense, 5'-GGATCCAGAAGGTGATGGAA-3' and antisense, 5'-

AFAGCTGACAGCAACCCTGT-3'. mRNA was extracted and cDNA was synthesized as described (113), and 2 μl were used for real-time PCR for iNOS, Arg1, Arg2, and β-actin with the iQ SYBR green Supermix (Bio-Rad). The thermal cycling conditions and the method used to calculate relative expression were as described (113).

Immunoblot analysis: RAW 264.7 cells were treated with *H. pylori* lysates for various time points as indicated in each of the figures. Macrophages were lysed and Western blotting for iNOS and β-actin was performed as described (113). For Arg2 detection, we used a goat polyclonal antibody (L-20) generated against murine Arg2 from Santa Cruz Biotechnology (Santa Cruz, CA) at a dilution of 1:500. For Arg1 detection, a mouse polyclonal antibody generated against human Arg1 (BD Transduction Laboratories, San Jose, CA) was used at a dilution of 1:2000.

Detection of iNOS protein by flow cytometry: After stimulation, cells were washed, fixed, permeabilized, and stained with anti-iNOS antibody, and flow cytometry was performed as described (113).

Translation analysis for iNOS: After stimulation of RAW 264.7 macrophages with or without *H. pylori* lysates in the presence and absence of BEC, proteins were metabolically labeled with ^{35}S-methionine and analyzed for iNOS translation (93, 113).

Transient transfection of siRNA in macrophages: ODC siRNA transfection was

performed as described (93). For Arg2 knockdown, we used siRNA duplex targeting nucleotides 1168–1186 as follows: sense, 5'-GGCAUUCGAAGGGACAGAUtt-3'; antisense, 5'-AUCUGUCCCUUCGAAUGCCtt-3'. Scrambled siRNA and conditions for transfection and activation were as described (93, 137).

Arginase activity in cytosolic and mitochondrial fractions: RAW 264.7 cells were lysed and fractionated using the Cytosolic/Mitochondrial Fractionation Kit from Calbiochem (La Jolla, CA), according to the manufacturer's instructions. Proteins from both fractions were incubated with 0.1 μM L-guanido-^{14}C-Arg (NEN, Boston, MA) for 24 h at 37°C, and arginase activity was assayed as described (163).

Immunofluorescence staining for Arg2 and mitochondria: RAW 264.7 cells (1 x 10^4) were plated in 4-well chamber glass slides. After treatments, MitoTracker Red (Invitrogen) was added to the cell cultures at a concentration of 100 nM for 30 min. Cells were washed with PBS and fixed in 3.7% formaldehyde. Cells were then permeabilized in 100% chilled methanol, washed, and blocked with Background Sniper (Biocare Medical, Concord, CA) for 1 h at room temperature. Cells were incubated at room temperature for 1 h with primary antibody to Arg2 (1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz CA), followed by incubation with FITC-labeled donkey anti-goat secondary antibody (1:1000 dilution) for 45 min at room temperature. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining. Slides were dried, mounted, and visualized as described (162).

Analysis of NO production and iNOS protein expression in gastric macrophages: Isolated gastric macrophages were plated in 96-well plates. NO_2^- concentration was assayed by the Griess reaction and iNOS protein assessed by flow cytometry as described (113).

Statistical Analysis: Quantitative data are expressed as means ± SEM. For comparisons between multiple groups ANOVA followed by the Student-Newman-Keuls multiple comparisons test was used, and for single comparisons between two groups the Student's *t* test was used. Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) was used for all analyses.

Results

Inhibition of arginase enhances H. pylori-induced NO production by macrophages via enhancement of iNOS translation

We sought to determine if arginase expression and activity in macrophages impairs the host response to *H. pylori* by reducing iNOS-derived NO production. Lysates of *H. pylori* were used for these studies, because we have reported that *H. pylori* itself possesses an arginase enzyme that can compete with host cells for L-arginine utilization (96), but this enzyme is inactive in lysates (112, 122). Because we have previously reported that NO production increases in an L-arginine-dependent manner (113), we studied the effect of arginase inhibition over broad concentrations of L-arginine from 0 - 1.6 mM (Fig. 2*A*). In the presence of BEC, a specific inhibitor of arginase that does not

directly affect iNOS expression or activity (160, 164), we detected a significant increase in NO production, measured as the concentration of the stable metabolite, NO_2^-, at all concentrations of L-arginine between 0.1 and 1.6 mM, but not in the absence of L-arginine (Fig. 2*A*). It should be noted that concentrations of BEC from 30 to 150 μM were tested in this system, and the peak effect on NO production occurred at 90 μM with no detectable cytotoxicity (data not shown); thus the 90 μM dose was used for the remainder of the experiments.

Arginase inhibition had no effect on *H. pylori*-stimulated iNOS mRNA expression, and increasing L-arginine concentration did not increase iNOS mRNA expression (Fig. 2*B*). In contrast, iNOS protein expression increased with the addition of L-arginine, and these levels were markedly increased with inhibition of arginase at each level of L-arginine tested (Fig. 2*C*). Quantification of iNOS protein levels by densitometry revealed increases in iNOS protein levels in the presence of BEC (Fig. 2*D*). Furthermore, we examined the effect of arginase inhibition at time points from 6 to 24 h post-stimulation, and found that NO production (Fig. 2*E*) and iNOS protein levels (Figs. 2*F* and *G*) were both significantly increased at 18 h and 24 h in the presence of BEC. Additionally, at 12 h post-stimulation there was an increase in iNOS protein in the BEC-treated cells that was not present in the cells treated with *H. pylori* alone. Taken together, these data show that there is a similar degree of increase in steady-state iNOS protein levels at 12 - 24 h with BEC treatment.

Because we have reported that increased extracellular L-arginine availability augments iNOS translation (113), we sought to determine if reduced competition for

intracellular L-arginine by experimental inhibition of arginase also enhances iNOS translation. When lysates from *H. pylori*-stimulated cells were immunoprecipitated with antibody to iNOS, and ^{35}S-methionine incorporation measured, there was increased iNOS translation in the presence of BEC when assessed by both SDS-PAGE (Fig. 2*H*) and quantification of radiolabeled counts (Fig. 2*I*).

Because we have reported that knockdown of ODC increased NO production in *H. pylori*-stimulated macrophages by blocking spermine synthesis (93), we compared this effect to that of BEC (Fig. 3). Knockdown of ODC or inhibition of arginase resulted in an identical increase in NO production (Fig. 3*A*) with *H. pylori* stimulation. There was an additive effect with both treatments, such that there was a significant potentiation of NO production (Fig. 3*A*). When assessed by Western blot analysis, each treatment caused an increase in *H. pylori*-induced iNOS protein expression, but when added together there was not a further increase in iNOS levels (Fig. 3*B*).

H. pylori induces macrophage Arg2 that is localized to the mitochondria

Because we found that inhibition of arginase had a significant effect on iNOS and NO production, we explored the effect of *H. pylori* on the induction of arginase isoforms in more depth. RAW 264.7 macrophages were stimulated with *H. pylori* for 6 h, the time point at which we have demonstrated peak mRNA expression responses to *H. pylori* (100, 110, 113, 122), and Arg1 and Arg2 mRNA levels were quantified by real-time PCR (Fig. 4*A*). Arg2 mRNA levels were increased with *H. pylori* stimulation by 4–fold in the presence of L-arginine (from 0.1 – 1.6 mM) and BEC had no effect on this induction

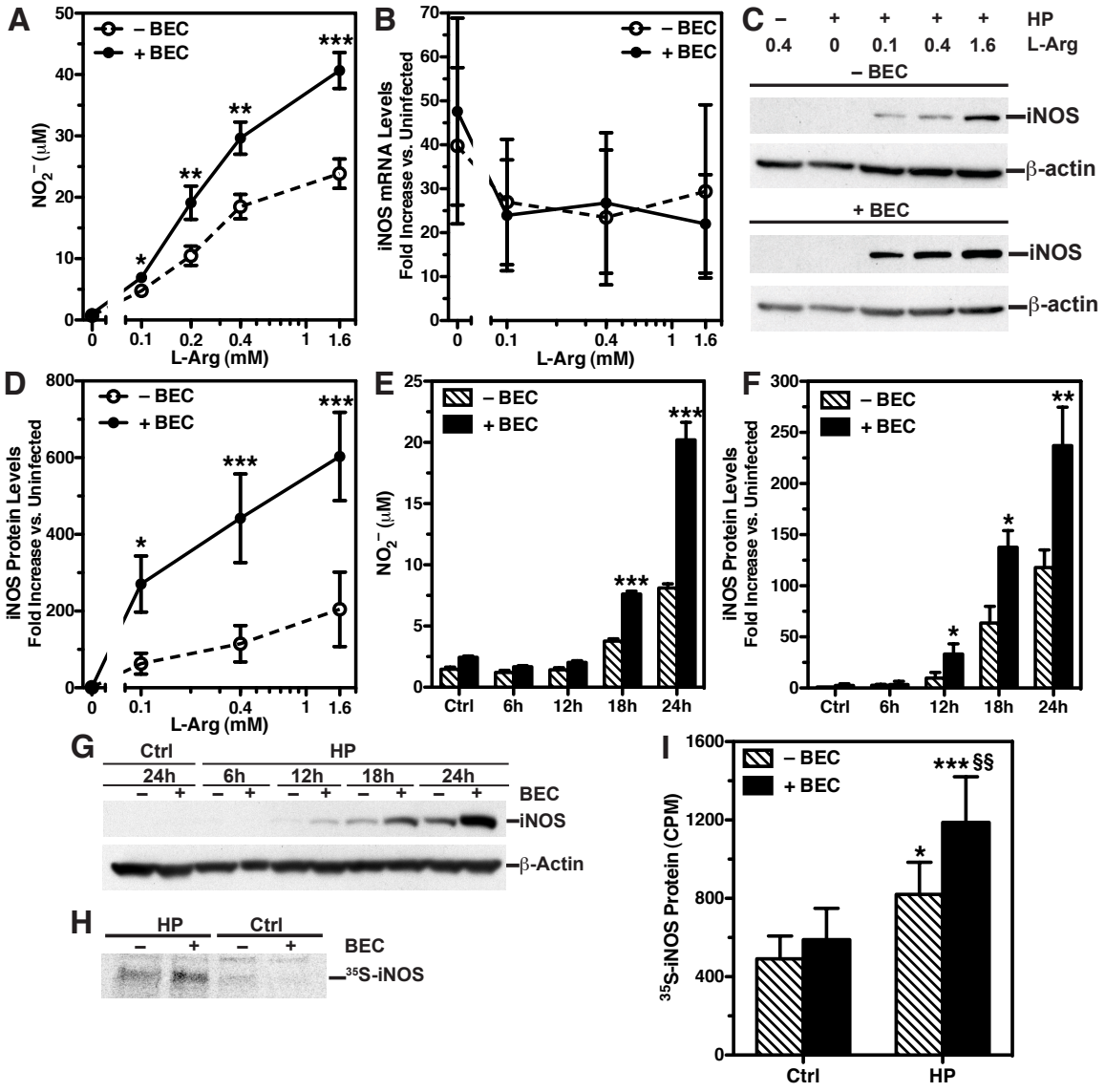

FIGURE 2. Arginase inhibition enhances *H. pylori*-stimulated macrophage NO production and iNOS protein expression by enhancing iNOS translation. 1×10^6 RAW 264.7 macrophages grown in 1 ml of medium were stimulated with *H. pylori* (HP) lysates at an MOI of 100. Experiments were conducted in arginine- and serum-free DMEM to which known concentrations of L-arginine were added. *A*, Cells were stimulated with *H. pylori* lysates for 24 h in the presence or absence of the arginase inhibitor BEC (90 μM). NO was measured as NO_2^- in the supernatant. Data are the mean ± SEM of thirteen separate experiments. *, $p < 0.05$, **, $p < 0.01$, *** $p < 0.001$ comparing – BEC (medium alone) to + BEC at the same concentration of L-arginine. *B*, iNOS mRNA levels were assessed after 6 h by real-time PCR. Data were standardized to β-actin and presented as fold increase vs. uninfected control at the 0.4 mM concentration of L-arginine. Data are

the mean ± SEM for three separate experiments. *C*, Western blotting was performed after 24 h for iNOS and β-actin, with 30 μg of protein loaded per lane. A representative Western blot is shown; similar results were observed in five experiments. Samples from cells treated with or without BEC were run on separate gels and the blots were then incubated together in the same container with the same antibody and exposed together on the same autoradiograph film. *D*, Densitometry from Western blotting for iNOS. Data were normalized to β-actin. Data are the mean ± SEM for five separate Western blots. *, $p < 0.05$, ***, $p < 0.001$ comparing – BEC to + BEC at the same concentration of L-arginine. *E*, NO production was measured at various time points from RAW 264.7 cells stimulated with *H. pylori* at an MOI of 100. Data are the mean ± SEM of three experiments. ***, $p < 0.001$ comparing – BEC to + BEC at the same time point. *F*, Densitometry from Western blotting for iNOS protein levels at various time points. Data were normalized to β-Actin. Data are the mean ± SEM of three experiments. *, $p < 0.05$, ***, $p < 0.001$ comparing – BEC to + BEC at the same time point. *G*, Representative Western blot for iNOS at various time points in the presence or absence of BEC. *H*, iNOS translation was measured by stimulating cells with *H. pylori* for 18 h and adding ^{35}S-methionine. After 4 h, iNOS protein was immunoprecipitated and resolved by SDS-PAGE and phosphorimaged. *I*, ^{35}S-methionine incorporation in immunoprecipitates was determined by scintillation counting. Data are the mean ± SEM of four separate experiments. *, $p < 0.05$, ***, $p < 0.001$ compared to controls without BEC. §§, $p < 0.01$ compared to HP-stimulated cells treated with BEC.

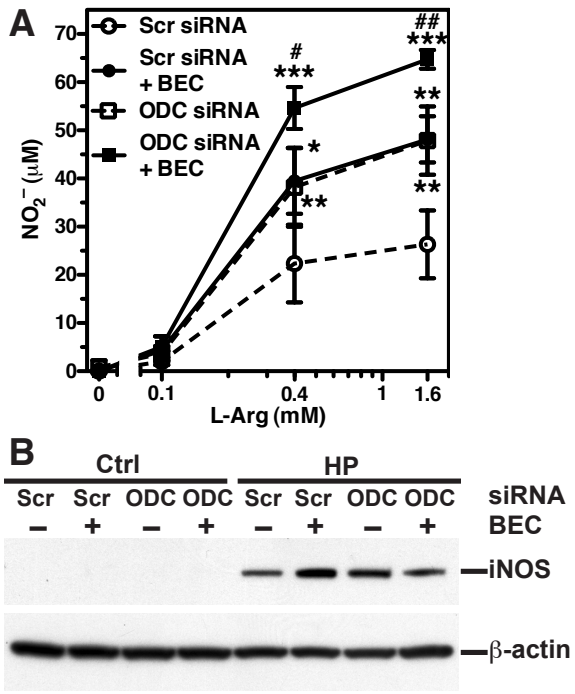

FIGURE 3. ODC knockdown and arginase inhibition have an additive effect on *H. pylori*-stimulated NO production in macrophages. RAW 264.7 cells were treated with ODC siRNA or scrambled control (Scr) siRNA and stimulated with *H. pylori* lysates in the presence or absence of BEC (90 μM). *A*, Effect of ODC siRNA and BEC on NO_2^- levels, at various concentrations of L-arginine. Data are the mean ± SEM of three separate experiments. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ compared to Scr siRNA at the same concentration of L-arginine. #, $p < 0.05$, ##, $p < 0.01$, comparing ODC siRNA + BEC to Scr siRNA + BEC at the same concentration of L-arginine. *B*, Western blot analysis for iNOS and β-actin. Experiments were conducted at 0.4 mM L-arginine. Results are representative of three separate experiments.

(Fig. 4*A*). It should be noted that there were higher levels of Arg2 mRNA in the absence of L-arginine, similar to the effect for iNOS (Fig. 2*B*), which is likely due to cellular stress from L-arginine depletion as we have described (113). We confirmed that Arg1 mRNA levels are not upregulated with *H. pylori* stimulation, and that BEC did not affect Arg1 levels (Fig. 4*B*).

When Arg2 protein levels were assessed, there was a significant increase with *H. pylori* stimulation in the presence of L-arginine. This did not change with increasing L-arginine levels above 0.1 mM, and BEC had no effect on stimulated Arg2 levels (Fig. 4*C*). When cells were depleted of L-arginine, there was no detectable Arg2 protein expression with *H. pylori* stimulation despite increased Arg2 mRNA levels. This is consistent with our previous findings that a physiologic level of L-arginine (0.1 mM) is required for global protein translation to occur in macrophages (113).

Arg2 has a leader sequence that can target it to mitochondria (165). We therefore determined the subcellular localization of Arg2 in macrophages under conditions of *H. pylori* stimulation. When arginase activity was compared in cytosolic and mitochondrial fractions, there was a significant increase only in the mitochondrial fraction after *H. pylori* stimulation (Fig. 4*D*). Consistent with this, *H. pylori* induced a marked increase in Arg2 staining by immunofluorescence detection that colocalized with mitochondria (Fig. 4*E*).

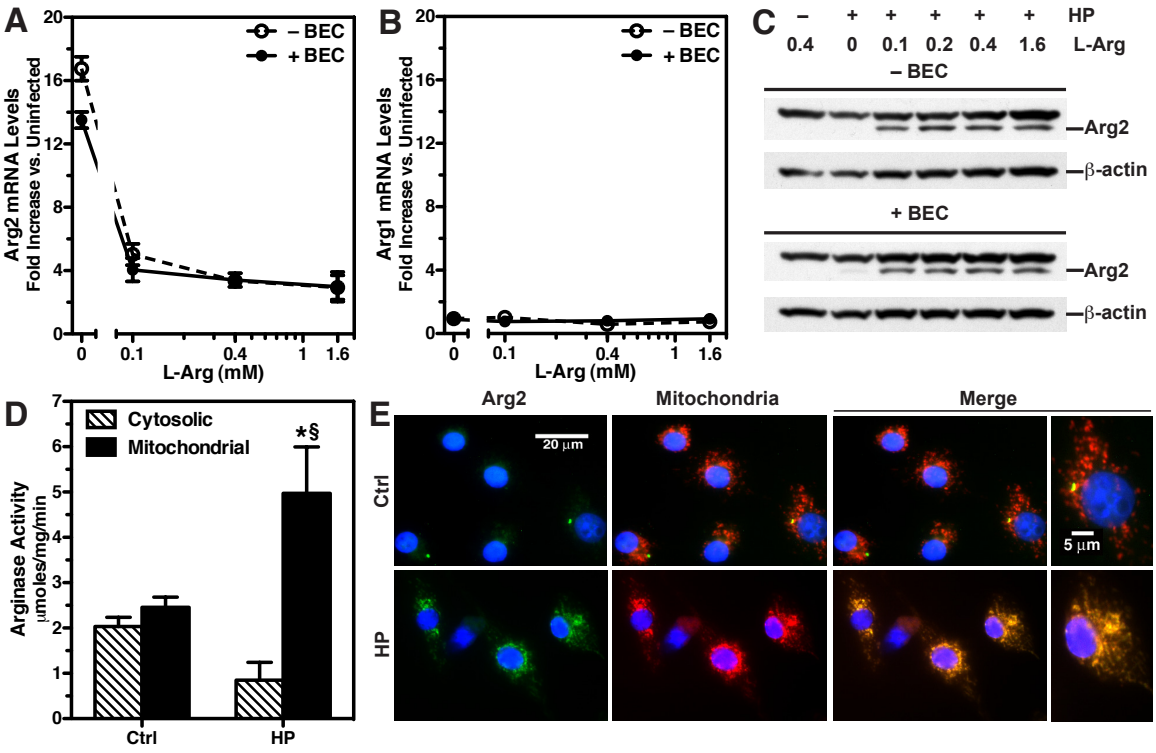

FIGURE 4. Arg2 is upregulated by *H. pylori* stimulation in macrophages and localizes to the mitochondria. *A*, Arg1 and *B*, Arg2 mRNA expression was assessed by real-time PCR. Data were standardized to β-actin and presented as fold increase vs. uninfected control cells at the 0.4 mM concentration of L-arginine. Data are the mean ± SEM for three separate experiments. *C*, Representative Western blot is shown for Arg2 and β-actin in the presence or absence of BEC (90 μM) with cells harvested at 24 h after stimulation, with 20 μg of protein loaded per lane. Blots from samples treated with and without BEC were incubated together with the same antibody and exposed on the same film. *D*, Arginase activity in mitochondrial and cytosolic fractions from *H. pylori*-stimulated macrophages, assessed at 18 h after activation. Data are the mean ± SEM of two separate experiments. *, $p < 0.05$ comparing the mitochondrial fraction from *H. pylori*-stimulated cells to the cytosolic fraction from unstimulated control cells; §, $p < 0.05$ comparing the mitochondrial fraction to the cytosolic fraction of *H. pylori*-stimulated cells. *E*, Immunofluorescence photomicrographs of macrophages stained with antibody to mouse Arg2 detected with FITC (green); mitochondria were labeled with MitoTracker (red) and nuclei were stained with DAPI.

Effect of arginase inhibition on iNOS protein expression is not observed with another enteric pathogen, Citrobacter rodentium

Because we were able to demonstrate that Arg2 restricts NO production by an effect on iNOS protein levels in *H. pylori*-stimulated macrophages, we sought to determine if such an effect was specific to *H. pylori*. In order to address this question, we used the mouse colitis-inducing pathogen *Citrobacter rodentium*, which we have shown induces both iNOS and Arg1 *in vivo* (121). As with *H. pylori*, *C. rodentium* lysate stimulated NO production in RAW 264.7 cells that was enhanced by BEC (Fig. 5*A*). In contrast to its effect on *H. pylori*-stimulated cells, addition of BEC did not result in increased iNOS protein levels when cells were activated with *C. rodentium* (Fig. 5*B*). While *H. pylori* induced only Arg2 protein expression, *C. rodentium* stimulation resulted in a modest increase in Arg2, and a more significant increase in Arg1 levels. Taken together, these data suggest that the mechanisms of inhibition of NO production in macrophages by arginase enzymes differs for these pathogens.

Knockdown of Arg2 increases NO production and iNOS protein levels

Because *H. pylori* selectively induces Arg2, we sought to determine if knockdown of Arg2 would have the same effect as BEC, which inhibits Arg1 as well as Arg2 (159, 160). Using transfection of siRNA, we were able to achieve a 65 ± 8.2% knockdown of Arg2 in *H. pylori*-stimulated RAW 264.7 macrophages (Fig. 6*A*). In cells transfected with Arg2 siRNA, we observed significant increases in *H. pylori*-stimulated NO production (Fig. 6*B*). While knockdown of Arg2 had no effect on *H. pylori*-stimulated iNOS mRNA

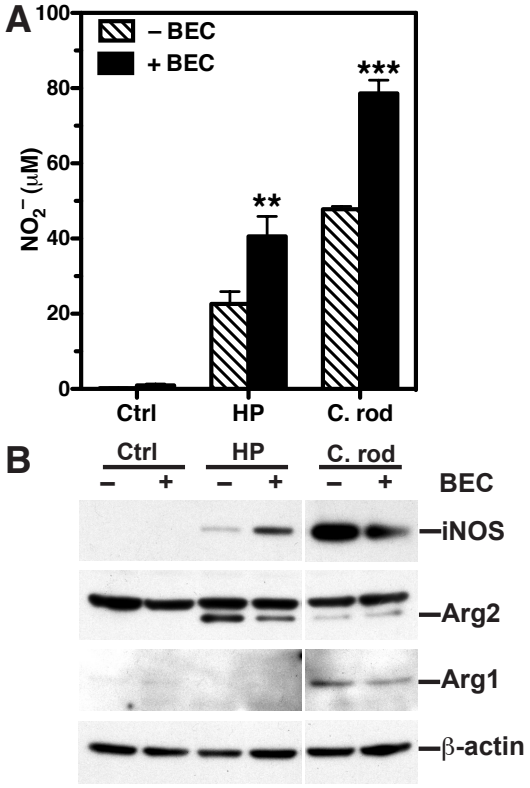

FIGURE 5. Arginase inhibition increases NO production, but not iNOS protein levels in *C. rodentium*-stimulated macrophages. RAW 264.7 macrophages were cultured in arginine- and serum-free DMEM with 0.4 mM of L-arginine. *A*, Cells were stimulated with *H. pylori* or *C. rodentium* (C. rod) lysates for 24 h in the presence or absence of the Arginase Inhibitor BEC (90 μM). NO_2^- was measured in the supernatant. Data are the mean ± SEM of four separate experiments. **, $p < 0.01$, ***, $p < 0.001$ comparing – BEC (medium alone) to + BEC in cells treated with the same bacterium. *B*, Western blotting was performed after 24 h for iNOS, Arg2, Arg1, and β-actin, with 20 μg of protein loaded per lane. Representative Western blots are shown; similar results were observed in two additional experiments. The same membrane was used to assay for all protein levels shown. Blots from samples stimulated with *H. pylori* or *C. rodentium* were incubated together with the same antibody and exposed on the same autoradiograph film.

levels (Fig. 6*C*), it resulted in a consistent potentiation of iNOS protein levels by Western blot analysis (Fig. 6*D*), which was confirmed by densitometry (Fig. 6*E*).

Arg2-deficient peritoneal macrophages produce more NO and have higher iNOS protein levels when stimulated with H. pylori ex vivo

In order to confirm our findings that Arg2 restricts macrophage NO production, we also used primary peritoneal macrophages isolated from WT and Arg2$^{-/-}$ mice. After 6 h of culture with *H. pylori* lysates, mRNA levels of Arg2 were significantly increased in cells from WT mice (Fig. 7*A*), whereas Arg1 levels were not increased (Fig. 7*B*). iNOS mRNA levels were also increased in the peritoneal macrophages from both WT and Arg2$^{-/-}$ mice (Fig. 7*C*). After 24 h of coculture with *H. pylori*, Arg2-deficient peritoneal macrophages produced significantly more NO than WT macrophages (Fig. 7*D*). Because of the limited number of cells, we used flow cytometry for detection of iNOS protein, which we have previously demonstrated to be highly sensitive and to correlate well with iNOS protein levels assessed by Western blotting in macrophages (13). There was a concomitant increase in iNOS protein levels with *H. pylori* stimulation in WT cells that was further upregulated in Arg2-deficient peritoneal macrophages (Fig. 7*D* and 7*E*).

Inhibition of arginase in H. pylori-stimulated macrophages enhances NO production and bacterial killing

We have reported that iNOS-dependent NO production by macrophages can kill *H. pylori* and that increased L-arginine availability enhances this effect (93, 113). In the current study, we have shown that Arg2 restricts NO production and iNOS translation in

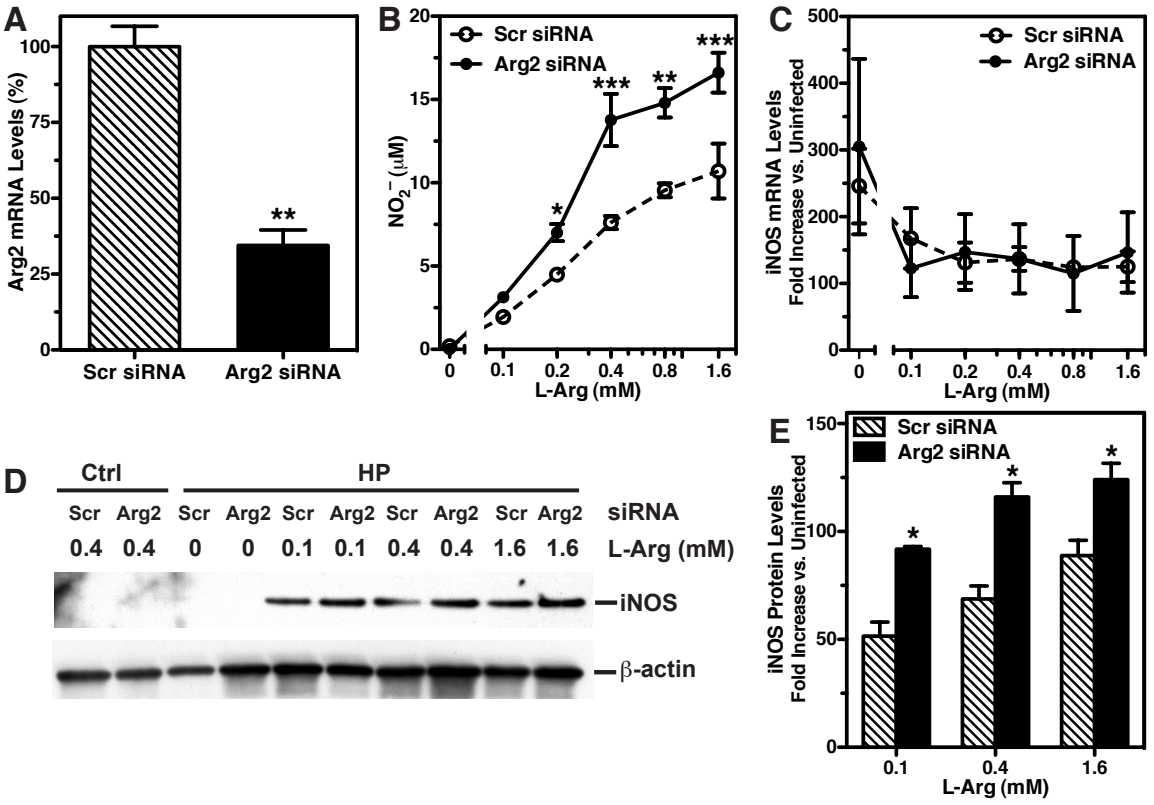

FIGURE 6. Knockdown of Arg2 in *H. pylori*-stimulated macrophages increases NO production and iNOS protein expression. RAW 264.7 macrophages were transfected with Arg2 siRNA or scrambled control siRNA (Scr) and stimulated with *H. pylori* lysates at an MOI of 100. Experiments were performed at the concentrations of L-arginine shown. *A*, Knockdown of Arg2 in *H. pylori*-stimulated macrophages cultured in 0.4 mM of L-arginine, assessed by real-time PCR of mRNA expression after 6 h of stimulation. *B,* Effect of knockdown of Arg2 on NO_2^- production by *H. pylori*-stimulated macrophages measured after 24 h. *, $p < 0.05$, **, $p < 0.01$ comparing Arg2 siRNA to the scrambled control at the same concentration of L-arginine. Data are the mean ± SEM of two separate experiments performed in duplicate. *C,* iNOS mRNA levels were assessed by real-time PCR after 6 h of stimulation. Data were standardized to β-actin and presented as fold increase vs. uninfected control at 0.4 mM L-arginine. *D,* iNOS protein levels were assessed by Western blotting after 24 h of stimulation. A representative blot is shown. Similar results were observed in two experiments. *E,* iNOS Western blots were analyzed by densitometry and were normalized to β-actin. Data are the mean ± SEM of two separate blots. *, $p < 0.05$ comparing Arg2 siRNA to the scrambled control.

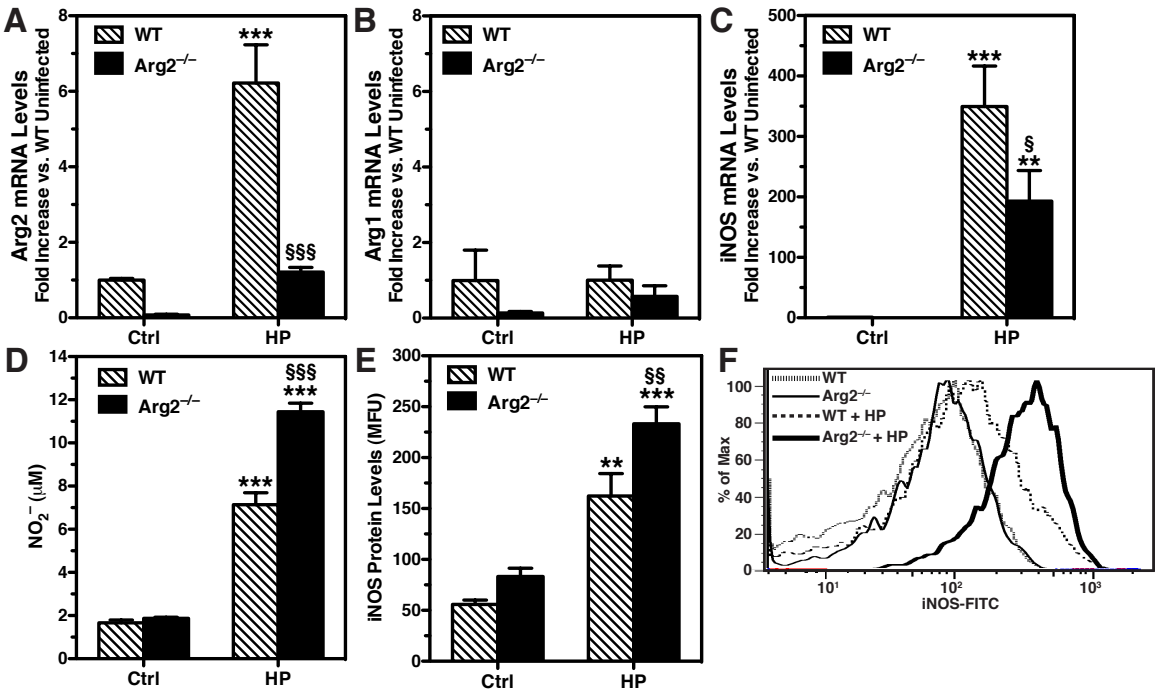

FIGURE 7. Arg2-deficient peritoneal macrophages produce more NO and express higher levels of iNOS protein when stimulated with *H. pylori ex vivo*. Macrophages were isolated from C57BL/6 WT and Arg2$^{-/-}$ mice, counted, and plated. Cells were stimulated with *H. pylori* lysates at an MOI of 100 for 6 h for examination of mRNA levels and 24 h for protein levels. *A*, Arg2, *B*, Arg1, and *C*, iNOS mRNA expression by real-time PCR. All real-time PCR data were standardized to β-actin and presented as fold increase vs. uninfected control at the 0.4 mM concentration of L-arginine. Real-time PCR data are the mean ± SEM for four separate experiments. ***, $p < 0.001$ versus WT Ctrl; §, $p < 0.05$ and §§§, $p < 0.001$ versus WT + HP (panels *A* and *C*). *D,* NO_2^- production. Data are the mean ± SEM from two separate experiments each using three mice per group. ***, $p < 0.001$ compared to WT control; §§§, $p < 0.001$ comparing Arg2$^{-/-}$to WT. *E,* iNOS protein levels were assessed by flow cytometry. Data are the mean ± SEM of four separate experiments. **, $p < 0.01$ and ***, $p < 0.001$ compared to WT control; §§, $p < 0.01$ comparing Arg2$^{-/-}$ to WT. *F,* Representative histogram of iNOS protein levels in peritoneal macrophages; note the greater shift to the right for the Arg2$^{-/-}$ + HP when compared to WT + HP.

macrophages. Therefore, we sought to determine if inhibiting arginase would enhance bacterial killing. For these experiments, we used a transwell system in which *H. pylori* were placed above a filter support to separate the bacteria from the macrophages to prevent bacterial killing by phagocytosis. In this model, arginase inhibition also allowed macrophages to produce significantly more NO in response to *H. pylori* (Fig. 8*A*). In parallel, there was enhanced killing of the *H. pylori* above the filter supports (Fig. 8*B*). In order to determine if the changes in killing with BEC were NO-dependent, we utilized cPTIO, an NO scavenger that rapidly converts NO to NO_2 that is then converted to NO_2^- (166). As expected, cPTIO increased the NO_2^- levels in the macrophage supernatants from cells exposed to either *H. pylori* alone or *H. pylori* plus BEC. It should be noted that we tested cPTIO in a dose range of 50 to 350 μM, and utilized 100 μM for these studies because the effect on NO_2^- generation plateaued at this concentration. Importantly, bacterial killing was significantly reduced in both the *H. pylori* alone or *H. pylori* plus BEC groups by the cPTIO (Fig. 8*B*). These data suggest that the restriction of NO production by arginase in response to extracellular *H. pylori* contributes to the survival of this pathogen.

In Vivo inhibition of arginase increases NO production and iNOS protein levels in gastric macrophages from H. pylori-infected mice

Because we have reported that Arg2 is increased in *H. pylori*-infected gastric tissues (122), we now determined if arginase inhibits iNOS *in vivo*. The peak time point of macrophage infiltration is 48 h post-inoculation (16). Therefore, we determined the effect of BEC (0.1% w/v) administration in the drinking water (121) on levels of iNOS protein

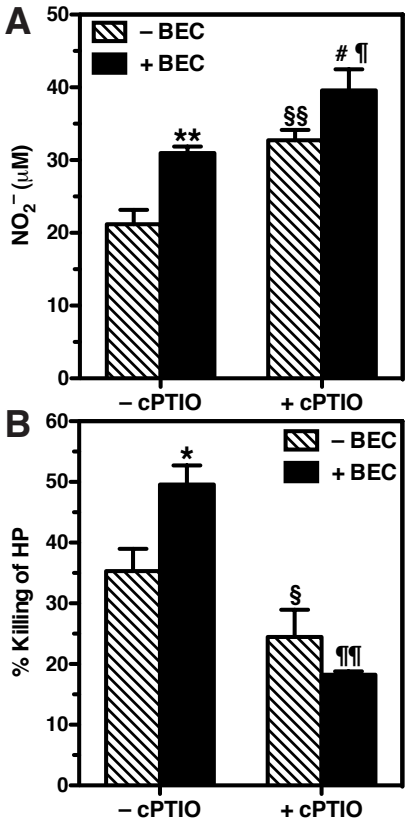

FIGURE 8. Arginase inhibition increases macrophage NO production and killing of *H. pylori* that is attenuated with an NO scavenger. Live *H. pylori* placed above transwell filter supports were incubated in 24-well plates with 1×10^6 RAW 264.7 cells in arginine- and serum-free DMEM with 0.4 mM of L-arginine for 24 h at an MOI of 100. BEC was used at a concentration of 90 μM, and the NO scavenger cPTIO was used at a concentration of 100 μM. Bacterial killing was determined by serial dilution and culture after 24 h of coculture. *A,* Effect of arginase inhibition and NO scavenger on NO_2^- levels. **, $p < 0.01$ for + BEC versus – BEC in the – cPTIO group; #, $p < 0.05$ for + BEC versus – BEC in the + cPTIO group; §§, $p < 0.01$ for + cPTIO versus – cPTIO in the – BEC group; ¶, $p < 0.05$ for + cPTIO versus – cPTIO in the + BEC group. *B,* Bacterial killing (%) was determined by comparing colony growth from samples cocultured with or without macrophages in the presence or absence of BEC and/or cPTIO. *, $p < 0.05$ for + BEC versus – BEC in the – cPTIO group; §, $p < 0.05$ for + cPTIO versus – cPTIO in the – BEC group; ¶¶, $p < 0.01$ for + cPTIO versus – cPTIO in the + BEC group. Data are the mean ± SEM of three separate experiments.

and NO in gastric macrophages isolated from mice 48 h post-inoculation with *H. pylori*. In mice receiving drinking water alone, there was only a small increase in NO production, but macrophages from *H. pylori*-infected mice that were treated with BEC exhibited a 2.6 ± 0.5-fold increase in NO production (Fig. 9*A*). Furthermore, there was a significant increase in iNOS protein levels from gastric macrophages isolated from *H. pylori*-infected mice that were treated with BEC (Fig. 9*B* and 9*C*). In contrast, iNOS mRNA levels were not increased by BEC treatment of mice (data not shown). These data show that *in vivo* inhibition of arginase effectively restores NO production by gastric macrophages in response to *H. pylori* by enhancing iNOS protein synthesis.

Arginase-mediated effects on NO production and iNOS protein levels in gastric macrophages is specifically derived from Arg2 in H. pylori-infected mice

In order to determine if Arg1 could also play a role in our *in vivo* model, we infected both WT and Arg2$^{-/-}$ mice with *H. pylori* and determined the effect of BEC administration on levels of iNOS protein and NO in isolated gastric macrophages. Similar to our previous data (Fig. 9), BEC administration increased NO production (Fig. 10*A*) and iNOS protein levels (Fig. 10*B* and *C*) in WT gastric macrophages isolated from infected mice. Arg2-deficient gastric macrophages produced similar levels of NO (Fig. 10*A*) and iNOS protein (Fig. 10*B* and *C*) as cells from WT mice treated with BEC. Importantly, BEC administration to Arg2$^{-/-}$ mice did not further increase NO production (Fig. 10*A*) or iNOS protein levels (Fig. 10*B* and *C*) in isolated gastric macrophages. These data indicate that Arg1 does not affect gastric macrophage NO production and iNOS protein levels during *H. pylori* infection *in vivo*.

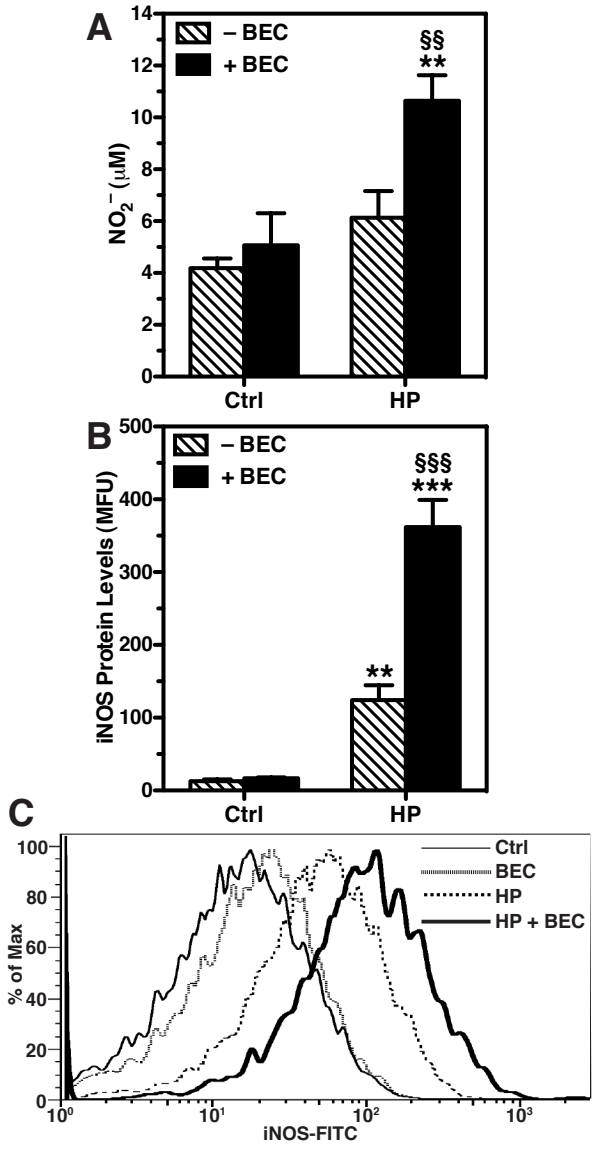

FIGURE 9. *In Vivo* inhibition of arginase increases NO production and iNOS protein levels in gastric macrophages. C57BL/6 mice were given a 0.1% BEC solution continuously in their drinking water for 24 h before being gavaged with 5×10^8 *H. pylori* bacteria. Treatment with BEC was continued for an additional 48 h and mice were sacrificed, followed by isolation of gastric macrophages. *A,* Cells were plated for 72 h and NO_2^- production was measured in the supernatant. Data are the mean ± SEM of two separate experiments, each using three mice per group. **, $p < 0.01$ comparing HP + BEC with Ctrl – BEC. §§, $p < 0.01$ comparing HP + BEC to HP – BEC. *B,* iNOS protein levels were assessed by flow cytometry immediately upon isolation of gastric macrophages. Data are the mean ± SEM of three mice per group. **, $p < 0.01$, ***, $p < 0.001$ compared to WT – BEC. §§§, $p < 0.01$ comparing HP + BEC to HP – BEC. *C,*

Representative histogram of iNOS protein levels in gastric macrophages; note the increased shift to the right, indicating higher iNOS levels, in cells from *H. pylori*-infected mice treated with BEC versus water alone.

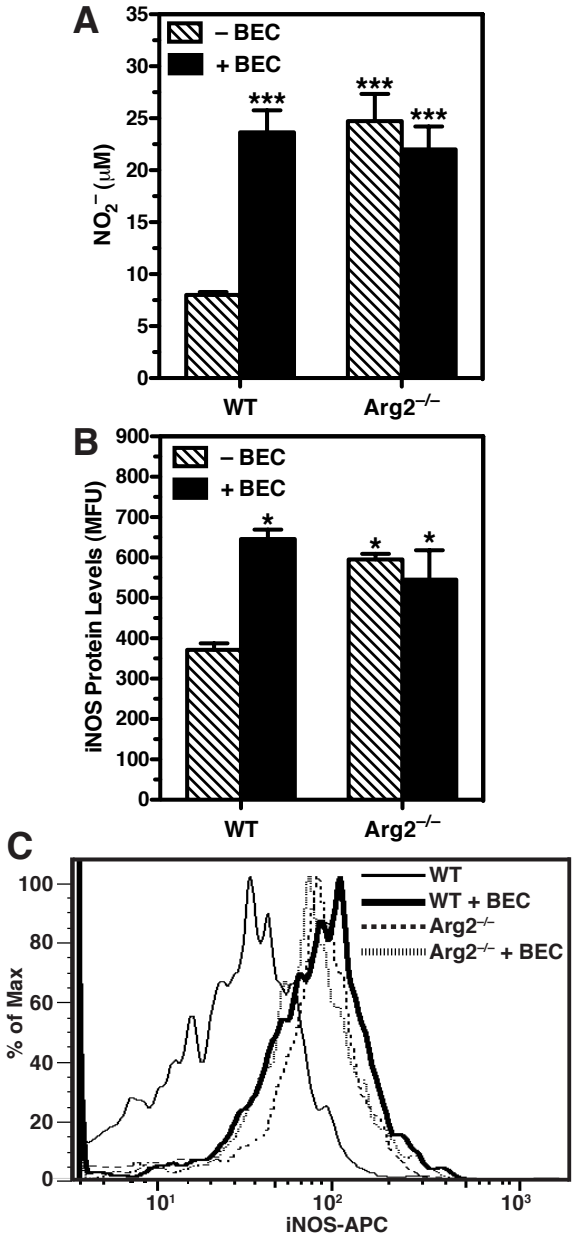

FIGURE 10. *In Vivo* inhibition of arginase does not increase NO production or iNOS protein levels in Arg2$^{-/-}$ gastric macrophages from *H. pylori*-infected mice. C57BL/6 WT and Arg2$^{-/-}$ mice were given a 0.1% BEC solution continuously in their drinking water for 24 h before being gavaged with 5×10^8 *H. pylori* bacteria. Treatment with BEC was continued for an additional 48 h and mice were sacrificed, followed by isolation of gastric macrophages. *A,* Cells were plated for 72 h and NO_2^- production was measured in the supernatant. Data are the mean ± SEM of three mice per group. ***, $p < 0.001$ versus

WT – BEC. *B,* iNOS protein levels were assessed by flow cytometry immediately upon isolation of gastric macrophages. Data are the mean ± SEM of three mice per group. *, $p < 0.05$ versus WT – BEC. *C*, Representative histogram of iNOS protein levels in gastric macrophages; note that the BEC treatment does not shift the iNOS level in the cells from the $Arg2^{-/-}$ mice.

Discussion

Nitric oxide production by classically-activated Type I macrophages (M1) is required to control a variety of parasitic and bacterial pathogens (167, 168). Microbes can avoid NO-dependent killing by suppressing production of NO by the host, through the induction of macrophage arginase. For example, downregulation of NO production by macrophages has been attributed to induction of Arg1 by the intracellular parasites *Leishmania major* (125) and *Toxoplasma gondii* (124), and the intracellular bacterium *Mycobacterium tuberculosis* (124); and to induction of Arg2 by the extracellular parasite *Trypanosoma brucei brucei* (169) and the intracellular bacteria *Chlamydia psittaci* and *C. pneumoniae* (127).

In the current report, we have demonstrated for the first time that upregulation of macrophage Arg2 by an extracellular bacterium results in diminished NO-dependent killing. We have demonstrated that an inhibitor of macrophage arginase (BEC) enhanced NO production and killing of *H. pylori*, and that these effects are due to enhanced translation of iNOS protein, and not to an effect on iNOS mRNA expression levels. The effect of BEC occurred in a similar manner at time points tested from 12 to 24 h post-stimulation, indicating Arg2 has early and consistent suppressive effects on the iNOS component of the innate immune response. These findings cannot be attributed to any toxic effects of BEC, because macrophage cell viability testing demonstrated that BEC had no deleterious effects on these cells (data not shown). Additionally BEC had no toxic effects on *H. pylori* when tested alone in our killing assay model. We have shown that the enhancement of NO production in *H. pylori*-stimulated cells is specifically attributable to

Arg2, because when we employed siRNA knockdown of Arg2 or utilized macrophages from Arg2$^{-/-}$ mice we again demonstrated a marked increase in iNOS protein and NO production. We have excluded a role for Arg1, because Arg1 was not induced in RAW 264.7 cells or peritoneal macrophages with *H. pylori* stimulation, and treatment of *H. pylori*-infected Arg2$^{-/-}$ mice with a non-selective inhibitor of arginase had no additional effect on iNOS or NO levels in gastric macrophages. Additionally, we have reported that in contrast to Arg2, Arg1 mRNA levels were not upregulated in mouse or human *H. pylori* gastritis tissues (122). Furthermore, we have found that there is no compensatory upregulation of Arg1 in chronic gastritis tissues from *H. pylori* infected Arg2$^{-/-}$ mice , as we demonstrate in Chapter III of this dissertation (144). An implication of our findings is that induction of Arg2 in the host leads to a defective innate immune response manifested as diminished iNOS protein translation and NO production by macrophages. Moreover, our studies demonstrating that inhibition of macrophage arginase results in enhanced iNOS-derived NO production in response to *H. pylori in vivo* indicate that such a strategy could enhance the ability of the immune system to control or eliminate *H. pylori* infection.

Arg2 metabolizes L-arginine to produce L-ornithine plus urea. L-ornithine can then be metabolized by ODC to produce polyamines. *H. pylori* possesses urease, which metabolizes urea into ammonia that neutralizes hydrochloric acid and facilitates *H. pylori* survival in its acidic niche (170). While *H. pylori* possesses its own arginase, which acts to generate an intracellular supply of urea (96), it is possible that urea from host arginase could be utilized by the bacterium. The importance of this pathway is substantiated by the

evidence that *H. pylori* lacking UreI, the protein responsible for uptake of urea into the bacterium, is unable to colonize mice or gerbils (171). Furthermore, host polyamine production has been shown to enhance the growth and survival of *Leishmania* species within macrophages (172). It is unknown whether *H. pylori* utilizes polyamines for growth and survival; this is an area of active investigation in our laboratory.

Arg2 expression could restrict macrophage NO production by competing with iNOS for the substrate L-arginine, or by contributing to the production of the polyamine spermine. We considered the latter possibility because we have reported that spermine inhibits *H. pylori*-stimulated iNOS translation and NO production in macrophages (93). When ODC siRNA was used, which abolishes the increase in spermine synthesis due to *H. pylori* stimulation of macrophages (93), there was an increase in NO production, but there was an additional increase in NO generation when this was combined with arginase inhibition. If Arg2 were restricting NO production only by enhancing polyamine synthesis, then this additive effect of the two inhibitors should not have occurred. Thus, we conclude that there is a combined deleterious effect on innate immunity by both Arg2 and ODC each acting to inhibit NO production. Intriguingly, we found that there was not an additive increase in iNOS protein levels when BEC and ODC siRNA were combined, despite the potentiation of NO levels. We speculate that while inhibition of ODC and arginase each enhances iNOS protein synthesis, combined inhibition of both enzymes increases L-arginine substrate availability for iNOS sufficiently to increase its functional activity and cause even more NO production.

It has been reported that while iNOS is a cytoplasmic enzyme, Arg2 contains a mitochondrial localization domain (173, 174). Our studies demonstrate that Arg2 is localized in the mitochondria upon activation by *H. pylori*. Yet despite this physical separation of Arg2 and iNOS, there is competition for the common substrate, intracellular L-arginine. These findings suggest that future studies of L-arginine transport by mitochondria could prove to be a fruitful area of investigation. Consistent with our previous work (113), *H. pylori*-induced macrophage NO production occurred in a concentration-dependent manner. Somewhat surprisingly, in the presence of the Arginase Inhibitor BEC, or with knockdown of Arg2, the increase in NO production occurred at all concentrations of L-arginine tested, including at a marked excess of L-arginine. Because the circulating level of L-arginine in mammalian serum is approximately 0.1 mM (114, 115), which is well above the K_m of iNOS (116, 117), one might expect that at the concentrations of 0.4 mM or 1.6 mM, the effect of inhibition of arginase on iNOS-derived NO production would be lost. Instead, our data suggest that while uptake of extracellular L-arginine is required for macrophage NO production (113, 175), the competitive effect of arginase is of critical importance in regulating L-arginine availability for iNOS, and this consequently affects both iNOS translation and NO production in response to *H. pylori* infection.

We demonstrated that inhibition of arginase *in vivo* enhanced NO production and iNOS protein levels in macrophages during acute infection with *H. pylori*. Due to limited availability of the Arginase Inhibitor BEC, we were unable to perform experiments at longer time points post-inoculation with *H. pylori*. It should be noted that we have

determined that at 4 months post-inoculation with *H. pylori*, Arg2$^{-/-}$ mice exhibit a correlation between increased gastritis and decreased bacterial colonization that is not observed with WT mice, that iNOS expression is increased in the gastric macrophages of *H. pylori*-infected Arg2$^{-/-}$ mice, and that Arg2$^{-/-}$ mice exhibit alterations in T cell responses as demonstrated in Chapter III of this dissertation (144).

There is substantial literature focused on the induction of Arg1 in macrophages, resulting in a Type II (M2) phenotype (176, 177), which is characterized by the response to Th2 cytokines and parasites, and a lack of iNOS mRNA expression (178). While we previously showed that *H. pylori* induces Arg2 rather than Arg1 (122), we now demonstrate that while Arg2 and iNOS are co-expressed at the mRNA level, Arg2 acts to restrict iNOS translation and hence NO generation. L-arginine is one of the amino acids that has been shown to modulate the phosphorylation status of eukaryotic translation initiation factor 2α (eIF2α); specifically, the dephosphorylated form enhances translation, while the phosphorylated form inhibits it (179, 180). In the case of iNOS, it has been suggested that desphosphorylation of eIF2α has a central role in the facilitation of iNOS translation in astrocytes (59). However, we have reported that in *H. pylori*-stimulated macrophages, dephosphorylation of eIF2α occurs with addition of extracellular L-arginine to L-arginine-starved cells, but there is a further increase in iNOS translation with addition of excess L-arginine beyond 0.1 mM, despite no further dephosphorylation of eIF2α (113). These data suggested that additional translational control mechanisms are involved. Proteomic studies are in progress in our laboratory to seek additional candidate proteins that may be involved in the regulation of iNOS translation by L-arginine in *H.*

pylori-stimulated macrophages. Based on our data in the current report, additional studies related to the effects of altered intracellular availability of L-arginine on protein translation in general and on iNOS translation, specifically, are under development in our laboratory.

Similar to our results, it has been reported that induction of Arg1 by Th2 cytokines can result in impaired iNOS translation in macrophages (181). In contrast, we found that when the colitis-inducing extracellular pathogen *C. rodentium* induced Arg1, inhibition of arginase resulted in increased NO production without enhancing iNOS protein expression. These results suggest that our findings with *H. pylori* may be specific in terms of the intracellular competition for L-arginine resulting in an effect on iNOS translation. Other pathogens have been shown to induce Arg2 in macrophages, such as *Trypanosoma brucei brucei* (169) and *Chlamydia psittaci* (127). However, the mechanisms underlying effects on NO production have not been elucidated in these studies.

Our data indicate that induction of Arg2 by *H. pylori* could be a potential mechanism by which the pathogen escapes the host innate immune response. We previously reported that Arg2, rather than Arg1, is upregulated in *H. pylori* gastritis tissues from human subjects and experimentally infected mice (122). This raises the possibility that there could be differences in Arg2 levels or gene polymorphisms in human subject groups at varying risk for gastric cancer, the long-term consequence of *H. pylori* infection. Studies related to this issue may be a promising area for future investigation.

CHAPTER III

IMMUNE EVASION BY *HELICOBACTER PYLORI* IS MEDIATED BY INDUCTION OF MACROPHAGE ARGINASE II

Summary

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 Arg2 inhibits iNOS translation, causes apoptosis, and restricts bacterial killing. We now determined if Arg2 impairs host defense *in vivo*, using a chronic *H. pylori* infection model. In C57BL/6 mice, expression of Arg2, but not Arg1, was abundant and localized to gastric macrophages. $Arg2^{-/-}$ mice had increased histologic gastritis and decreased bacterial colonization compared to WT mice. Increased gastritis scores correlated with decreased colonization in individual $Arg2^{-/-}$ mice, but not 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 pro-inflammatory cytokine responses.

Introduction

Helicobacter 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 (182). *H. pylori* infects approximately 50% of the world's population, and, more importantly, the associated gastric cancer is the second leading cause of cancer-related death worldwide (82). The infection is usually acquired in childhood and persists for the life of the host despite eliciting a seemingly vigorous immune response (36). 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 less than 50% in some regions of the world (7).

While *H. pylori* is typically considered to be noninvasive because most of the bacteria reside in the mucus 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 (49, 50, 148). 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 (6). While 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 Tregs and B cells (36). 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 (31, 32, 183, 184). 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.

In chapter II of this dissertation and in previous publications, we demonstrated that *H. pylori* induces the expression and activity of iNOS in macrophages both *in vivo* and *in vitro* (102, 110, 111, 144). Further, we have reported that macrophages cocultured with *H. pylori* can kill the bacterium by an NO-dependent mechanism (113, 144). 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 (102, 111). This reasoning has led our laboratory to consider the possibility that iNOS-mediated host defense to *H. pylori* is suboptimal. Importantly, infection of iNOS$^{-/-}$ mice with *H. pylori* results in similar levels of bacterial colonization as wild-type (WT) mice (75), further suggesting a defect in iNOS-dependent host defense.

In chapter II of this dissertation, we have reported that Arg2, but not Arg1, is upregulated in *H. pylori*-stimulated macrophages (144). We reported that induction of

macrophage Arg2 by *H. pylori* inhibits iNOS translation, NO production, and bacterial killing *in vitro* (144). 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 (144). 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 (93). Moreover, back-conversion of spermine into spermidine by the enzyme spermine oxidase, which is also induced by *H. pylori*, releases hydrogen peroxide that causes apoptosis (137, 185). Consequently, we have found that inhibition of arginase blocks *H. pylori*-induced macrophage apoptosis in our *in vitro* studies (122).

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 as compared to 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 pro-inflammatory 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.

Experimental procedures

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 (110, 137). Prior to infection, bacteria were grown in a Brucella broth culture for 16 – 20 h. Concentrations of bacteria were determined by optical density at 600 nm (OD of 1 = 10^9 CFU/ml) (110).

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 wks of age as described (144). Mice were gavaged with 5×10^8 *H. pylori* SS1 in 100 μl of Brucella broth, or 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 (102, 113). 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 (102). 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 (102, 186).

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 (44, 187).

Real-time PCR: RNA was extracted using the RNeasy Mini Kit from Qiagen (Valencia, CA). PCR methods were performed as described (113, 121, 122, 144). Primer sequences used were as follows: IL-10, 5′-CCAAGCCTTATCGGAAATGA-3′ (forward) and 5′-TCACTCTTCACCTGCTCCAC-3′ (reverse); IL-12p40, 5′-GATTCAGACTCCAGGGGACA-3′ (forward) and 5′-CATCTTCTTCAGGCGTGTCA-3′ (reverse); IL-17a, 5′-GCTCCAGAAGGCCCTCAGA-3′ (forward) and 5′-CTTTCCCTCCGCATTGACA-3′ (reverse); Foxp3, 5′-AGAGCCCTCACAACCAGCTA-3′ (forward) and 5′-CCAGATGTTGTGGGTGAGTG-3′ (reverse); IL-6, 5′-AGTTGCCTTCTTGGGACTGA-3′ (forward) and 5′-TCCACGATTTCCCAGAGAAC-3′ (reverse); and IL-23p19, 5′-CATGGGGCTATCAGGGAGTA-3′ (forward) and 5′-AATAATGTGCCCCGTATCCA-3′ (reverse). Primer sequences for IFN-γ (121), Arg1 (122), Arg2 (144), β-actin (113), and iNOS (113) 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 (102). iNOS was detected with a rabbit polyclonal antibody (1:!00 dilution, BD Biosciences, San Diego, CA). Arg2 was detected with a goat polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoperoxidase staining was performed as described (102). Nitrotyrosine was detected with a mouse monoclonal anti-vertebrate nitrotyrosine antibody (1:100 dilution; Millipore, Billerica, MA). Cleaved caspase-3 was detected with a rabbit polyclonal antibody (1:200 dilution; Cell Signaling Technology, Danvers, MA). Quantification of cleaved caspase-3 staining was performed by counting all positively-stained cells amongst 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 600× magnification under oil immersion.

Gastric macrophages: Immune cells were isolated from the glandular stomach by enzymatic digestion as described (102, 113, 187). 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 of dispase, 0.25 mg/ml of 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, Inc., San Diego, CA). Where two groups were compared, Student's *t* test was used. Data with more than two groups were analyzed by ANOVA and the Student-Newman Keuls post-hoc multiple comparisons test. $p < 0.05$ was considered statistically significant. The relationship between gastritis and colonization was determined using the 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 (102). Arg2 mRNA expression was increased by >9–fold in *H. pylori*-infected WT mice when assessed by real-time PCR, while Arg1 was not induced (Fig. 11*A* and *B*). Furthermore, Arg1 was not upregulated in Arg2$^{-/-}$ mice (Fig. 11*B*). Additionally, iNOS mRNA expression increased in infected WT mice, and this was further enhanced in Arg2$^{-/-}$ mice (Fig. 11*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. 11*D*). This staining demonstrated increased Arg2 levels in *H. pylori*-infected tissues from

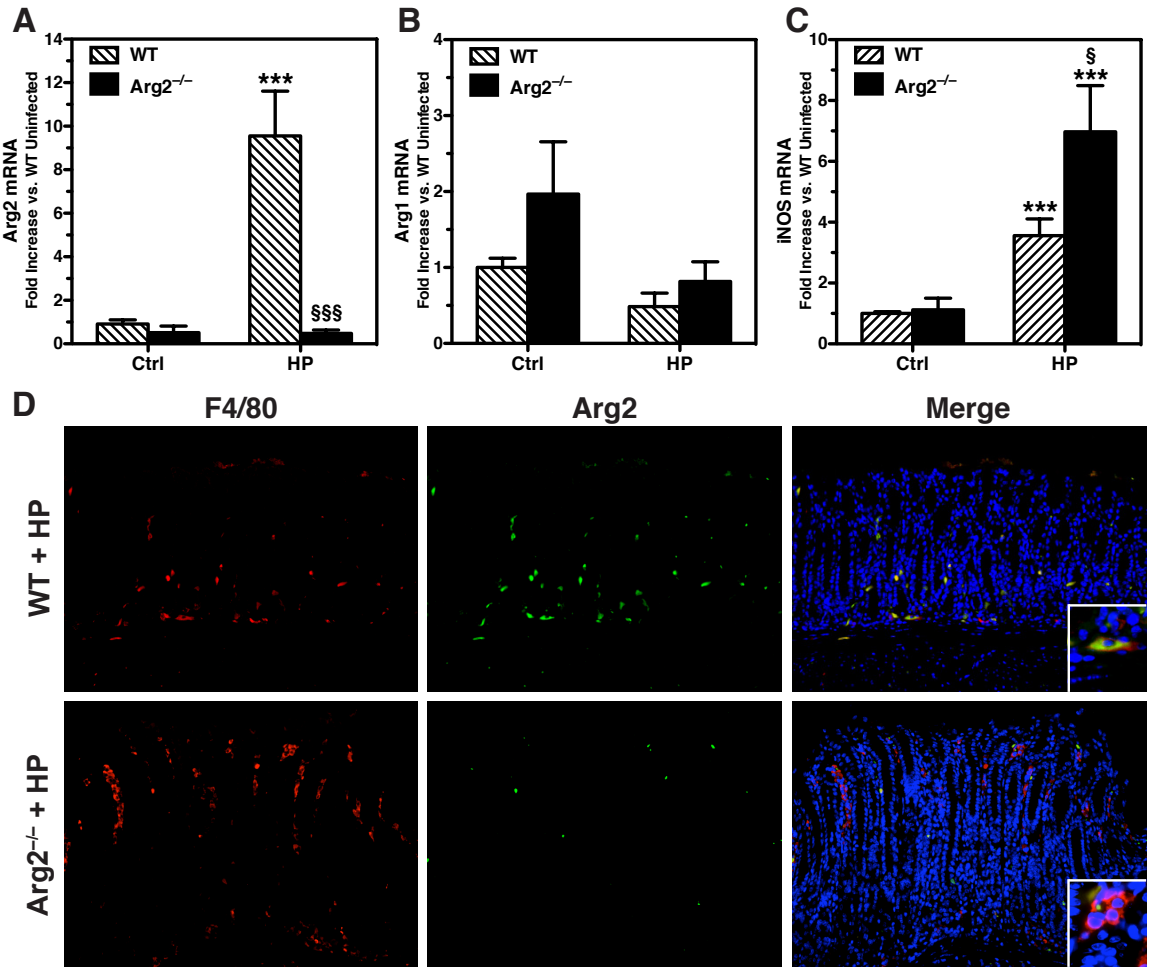

FIGURE 11. *H. pylori* infection induces iNOS and Arg2, but not Arg1, in gastric tissues. C57BL/6 WT and Arg2$^{-/-}$ mice were infected with *H. pylori* strain SS1. At 4 mo postinfection, mice were sacrificed, gastric tissues were removed, and RNA was extracted. iNOS (*A*), Arg2 (*B*), and Arg1 (*C*) 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. $^{§}p < 0.05$; $^{§§§}p < 0.001$ compared with WT infected mice. *D*, Photomicrographs, displayed at 200× and 600×, 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 3-4 mice per group.

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. 12*A*). In addition to enhanced gastritis levels, we found a significant decrease in *H. pylori* colonization levels in Arg2$^{-/-}$ mice (Fig. 12*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 hematoxylin and eosin-stained gastric sections are shown for uninfected and infected WT and Arg2$^{-/-}$ mice (Fig. 12*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. 12*E*).

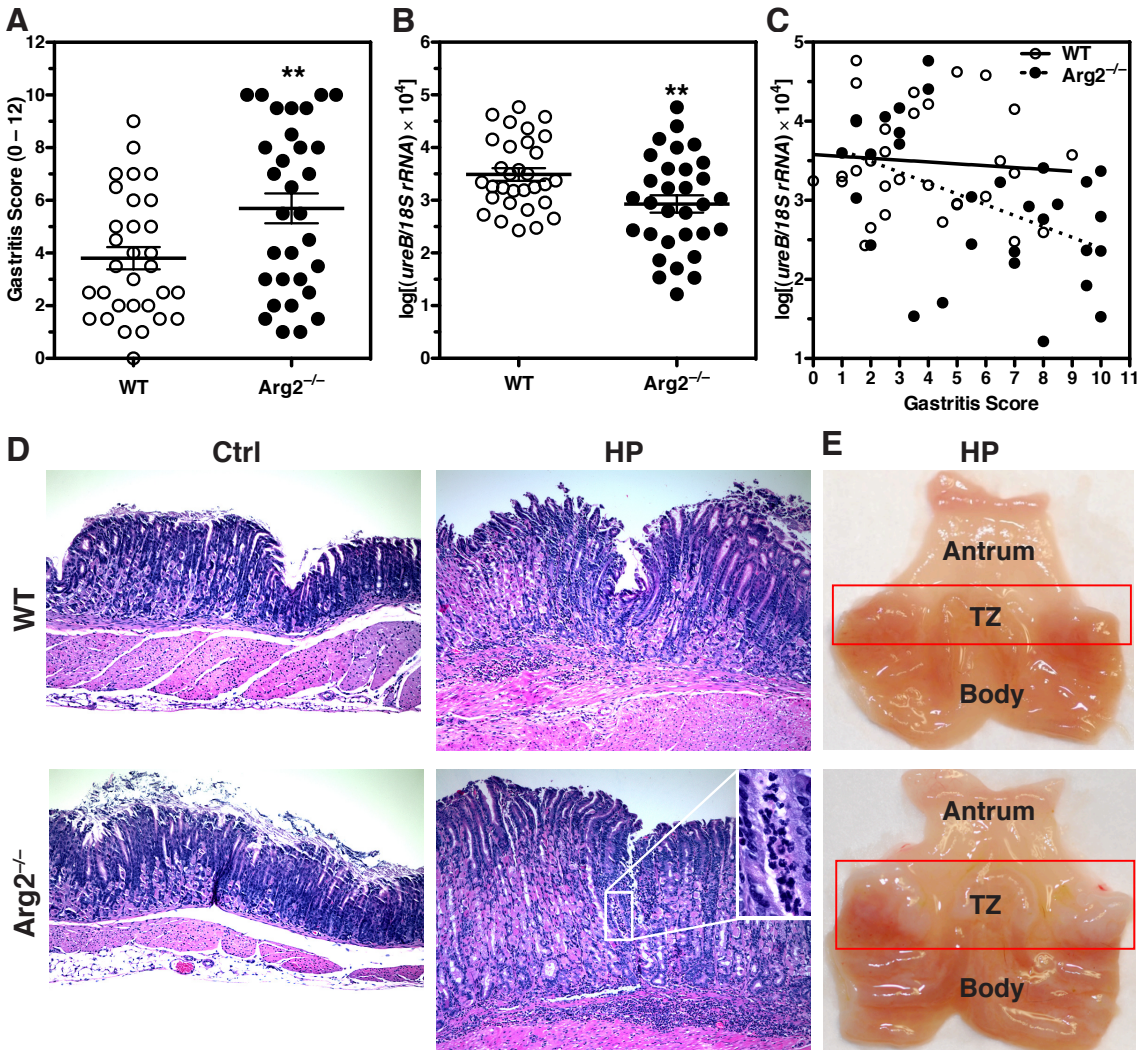

FIGURE 12. Increased gastritis and decreased bacterial colonization in Arg2$^{-/-}$ mice. C57BL/6 WT and Arg2$^{-/-}$ mice were infected with *H. pylori* for 4 mo, sacrificed, and their stomachs were removed. *A*, Hematoxylin and eosin-stained slides were prepared from a strip of the glandular stomach containing both the antrum and body. Acute and chronic inflammation were each scored 0–3 in the antrum and body, and the scores added for a 0–12 scale. $^{**}p < 0.01$. *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 hematoxylin and eosin-stained sections are shown for both uninfected and infected WT and Arg2$^{-/-}$ mice. Photomicrographs are depicted at 200× magnification and 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.

Chronic infection of H. pylori induces pro-inflammatory 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. 13*A*, *B*, and *C*, respectively). IL-10 expression, a hallmark of the counter-regulatory response to Th1 and Th17 responses in *H. pylori* infection (79, 184), was increased in infected WT, but not Arg2$^{-/-}$ mice (Fig. 13*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 (Fig. 14).

Arg2$^{-/-}$ macrophages are more abundant, express more iNOS, and have increased nitrotyrosine staining as compared to 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* (144). Furthermore, we have shown that Arg2$^{-/-}$ gastric macrophages isolated 48 h postinoculation with *H. pylori* express more iNOS protein and produce more NO as compared to WT macrophages (144). Therefore, to determine if such an effect occurred during chronic infection with *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. 15), along with the appropriate isotype controls (Fig. 16). Consistent with our recent report (102), there was increased macrophage

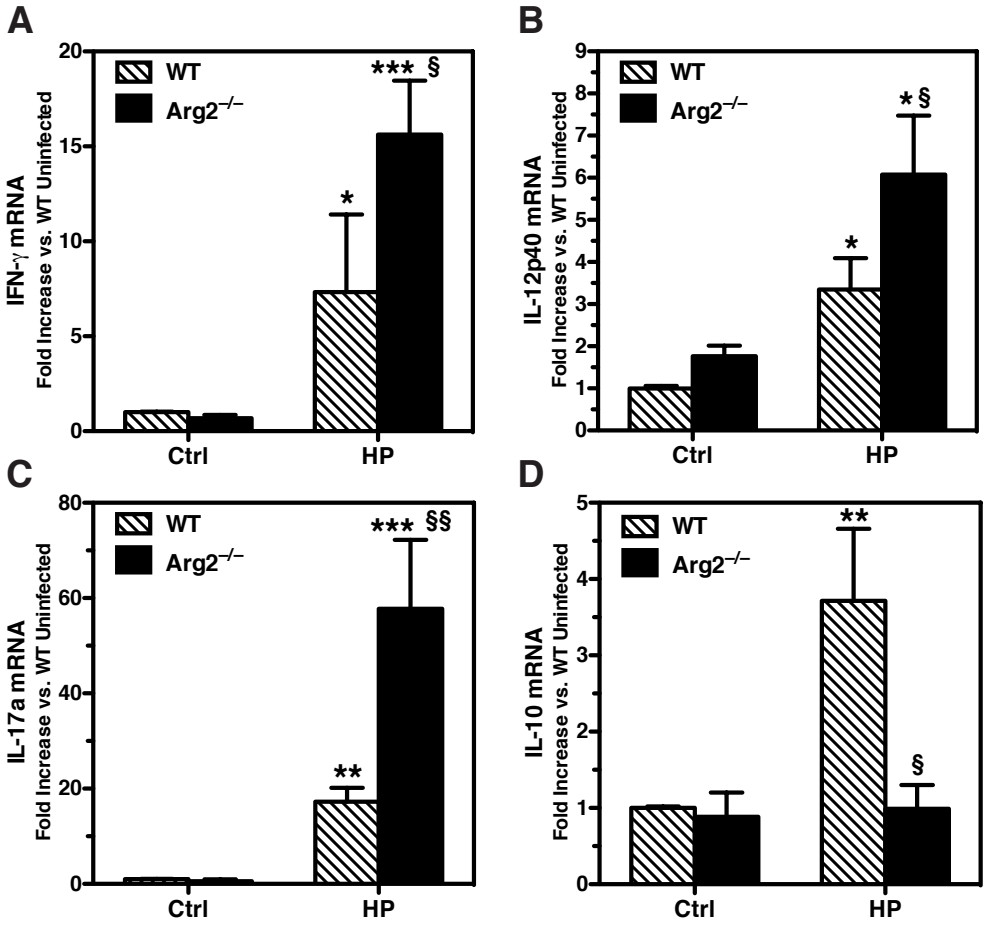

FIGURE 13. Chronic infection with *H. pylori* induces proinflammatory cytokine production that is further enhanced in Arg2$^{-/-}$ mice. mRNA was extracted from the gastric antrum of uninfected and infected WT and Arg2$^{-/-}$ mice at 4 mo postinoculation, converted to cDNA, and real-time PCR was performed 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. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to uninfected WT mice. $^{\S}p < 0.05$; $^{\S\S}p < 0.01$ compared to infected WT mice. For uninfected mice, n = 3-6 per group, and for infected mice, n = 5-10 per group.

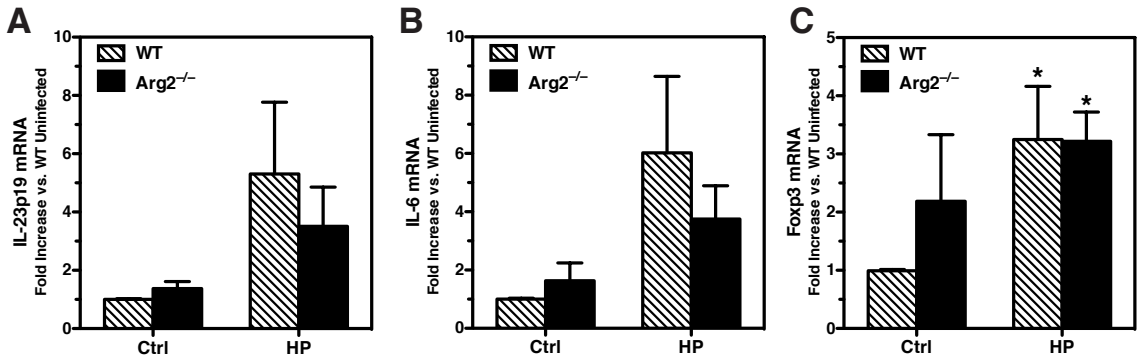

FIGURE 14. Chronic infection with *H. pylori* induces IL-23p19, IL-6, and Foxp3 expression. mRNA was extracted from the gastric body of uninfected and infected WT and Arg2$^{-/-}$ mice at 4 mo postinoculation, converted to cDNA, and real-time PCR was performed for IL-23p19 (*A*), IL-6 (*B*), and FoxP3 (*D*). Data were standardized to β-actin and presented as fold increase versus uninfected WT mice. *$p < 0.05$ compared to uninfected WT mice. For uninfected mice, n = 3-4 per group, and for infected mice, n = 5-7 per group.

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. 15). 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. 15). This staining was present in the lamina propria, with trails of iNOS^{+} macrophages migrating towards the lumen, as well as in the submucosal region.

To confirm our observation that there are more iNOS^{+} macrophages in infected Arg2$^{-/-}$ mice as compared to 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. 17*A*) and iNOS^{+} macrophages (Fig. 17*B*) in infected Arg2$^{-/-}$ mice compared to 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. 17*C*). Importantly, the Arg2$^{-/-}$ gastric macrophages expressed more iNOS protein than the cells from the WT mice (Fig. 17*D* and *E*).

Because we found that gastric macrophages isolated from *H. pylori*-infected Arg2$^{-/-}$ mice express higher iNOS protein levels than 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

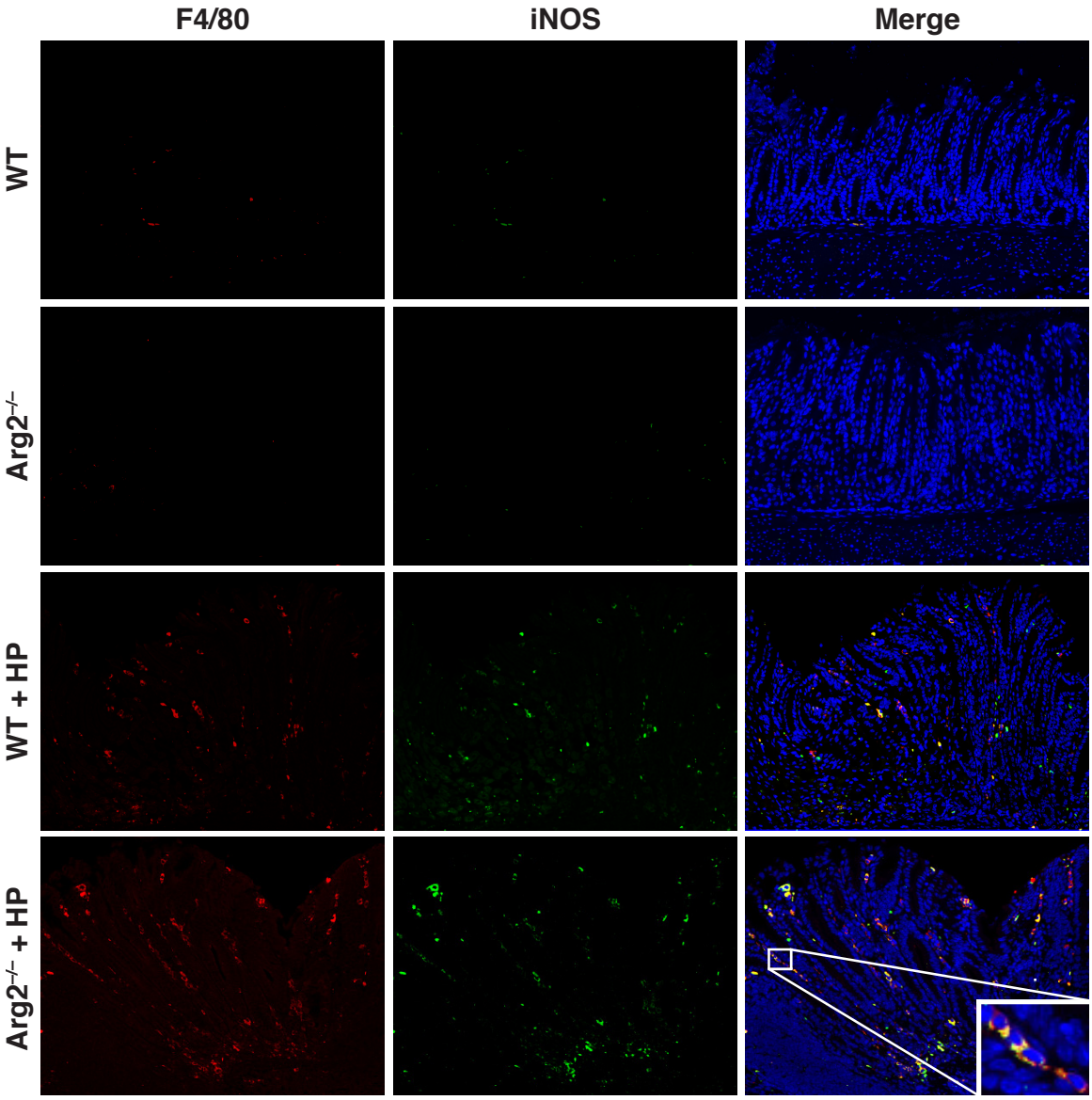

FIGURE 15. Arg2$^{-/-}$ mice have increased iNOS^{+} macrophages during chronic infection with *H. pylori*. Representative immunofluorescence staining from uninfected and infected WT and Arg2$^{-/-}$ mice are shown. The macrophage marker F4/80 was detected with tetramethyl rhodamine isothiocyanate (red), iNOS was detected with FITC (green), and nuclei were stained with DAPI (blue); co-localization is shown in merged images by the yellow color. Photomicrographs are shown at 200× magnification and 600× for the inset.

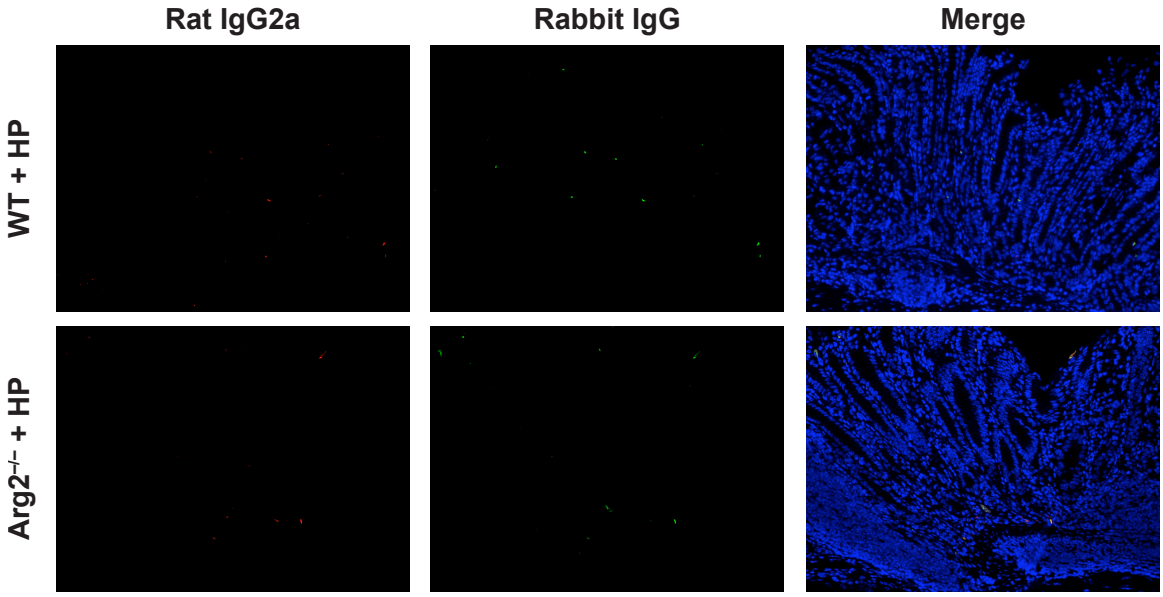

FIGURE 16. Isotype control staining for F4/80 and iNOS during chronic infection with *H. pylori*. Representative immunofluorescent staining from WT and Arg2$^{-/-}$ mice is shown. Rat IgG2a was used as an isotype control antibody for F4/80 and was detected with tetramethyl rhodamine isothiocyanate (red). Rabbit IgG was used as an isotype control antibody for iNOS and was detected with FITC (green). Nuclei were stained with ′DAPI (blue). Photomicrographs are shown at 200× magnification.

NO with superoxide (O_2^-), and this has been used as a marker of the production of reactive nitrogen species (158, 188-190). We stained tissues from chronically-infected WT and Arg2$^{-/-}$ mice and found that inflammatory cells in Arg2$^{-/-}$ mice had increased nitrotyrosine staining as compared to WT mice (Fig. 17*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. 11*D* and Fig. 15.

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 (122), 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 maximal macrophage infiltration into the stomach, and maximal apoptosis at this timepoint postinoculation with *H. pylori* (187). Additionally, we have demonstrated that Arg2 restricts NO production in gastric macrophages at this timepoint (113, 144). Consistent with our *in vitro* data, we found that *H. pylori* infection induced macrophage apoptosis, and that this was abolished in Arg2$^{-/-}$ macrophages (Fig. 18*A*). Representative flow cytometric dot plots demonstrating annexin V and 7-AAD staining in infected WT and Arg2$^{-/-}$ macrophages are shown in Fig. 18*B*.

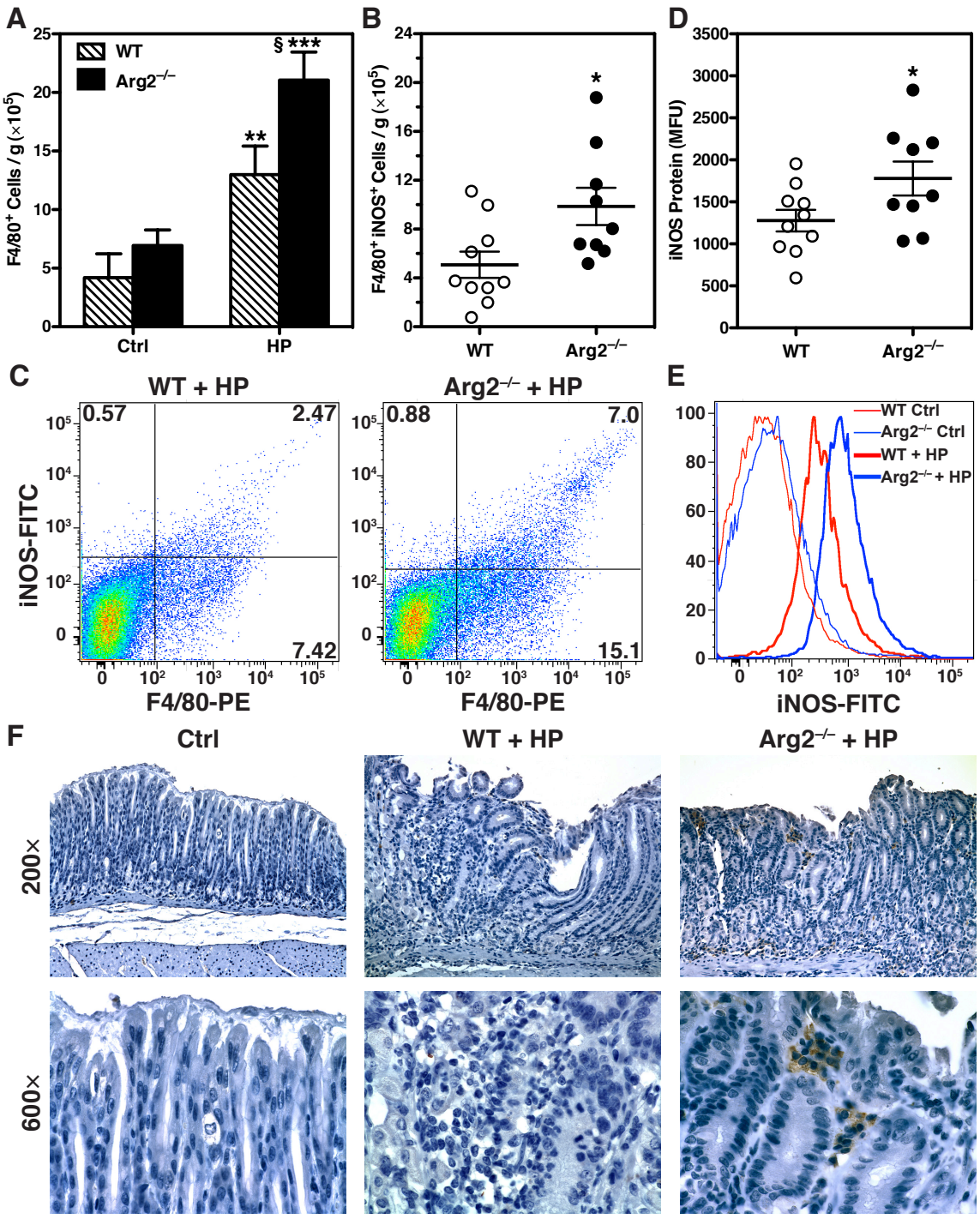

FIGURE 17. Arg2$^{-/-}$ macrophages are more abundant, express more iNOS, and have increased nitrotyrosine staining as compared to WT macrophages during *H. pylori* infection. Gastric cells were isolated from infected WT and Arg2$^{-/-}$ mice and analyzed by flow cytometry for the expression of the macrophage marker F4/80 and iNOS. The total number of F4/80^{+} cells (*A*, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs uninfected WT macrophages;

§$p < 0.05$ vs infected WT macrophages, $n = 3$ for uninfected groups, and $n = 9$-10 for infected groups) and $F4/80^+$ $iNOS^+$ cells (*B*, *$p < 0.05$) 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 200× and 600× magnification. Data are representative of 2 mice in the control group and 4-6 in the infected groups.

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 (191). In infected WT mice there was abundant staining in the mononuclear inflammatory cells with marked staining of cells with apoptotic bodies (Fig. 18*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. 17*A*. Additionally, quantification of this staining revealed a decrease in cleaved caspase-3 staining among the inflammatory cells in the Arg2$^{-/-}$ mice compared to WT mice (Fig. 18*D*).

Discussion

H. pylori infection induces a vigorous immune response, and in the murine model there is a rapid influx of macrophages (16, 187) and neutrophils (16) 48 h postinfection, followed by infiltration of lymphocytes 10 d postinfection (16). 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 (79, 183). 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

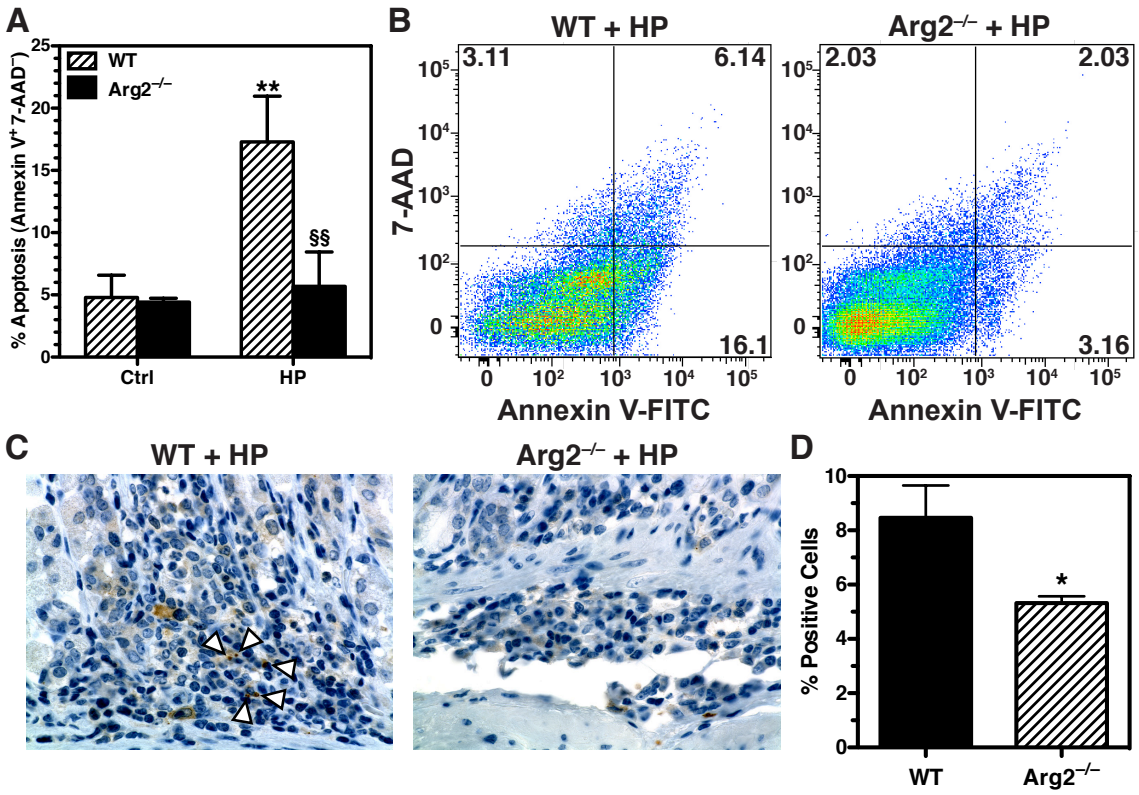

FIGURE 18. Arg2$^{-/-}$ macrophages undergo less apoptosis than WT macrophages during *H. pylori* infection. *A*, Gastric macrophages from WT and Arg2$^{-/-}$ mice were isolated 48 h postinoculation with *H. pylori* SS1 and stained for flow cytometry with annexin V-FITC and 7-AAD. The percentage of annexin-V^{+} 7-AAD^{-} cells are shown. $^{**}p < 0.01$ compared to uninfected WT mice. $^{§§}p < 0.01$ compared to infected WT mice, $n = 4$ mice per group. *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 of slides from chronically-infected WT and Arg2$^{-/-}$ mice stained for cleaved caspase-3. The arrows are used to highlight apoptotic bodies. Data are representative of 7-8 mice per group. *D*, Quantification of cleaved caspase-3 staining. The percentage of positively-stained inflammatory cells is shown.

macrophage apoptosis. Consequently, this restricts host defense against *H. pylori*. This is the first report to demonstrate that macrophage Arg2 expression has a deleterious impact on the effectiveness of host immunity by impairing the inflammatory response *in vivo*.

Herein, we have demonstrated that induction of Arg2 during chronic *H. pylori* infection restricts macrophage iNOS protein levels, limits the pro-inflammatory 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 (144). 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* (122, 144). Furthermore, we have shown that Arg2 gene expression is upregulated in human gastric tissues infected with *H. pylori* (122). 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 (177, 192). 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* (125) and *Toxoplasma gondii* (158) and the bacterium *Mycobacterium tuberculosis* (158); and to induction of Arg2 by the parasite *Trypanosoma brucei brucei* (128) and the bacteria *Chlamydia psittaci* and *Chlamydia pneumoniae* (127). 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 (31, 79, 183). In fact, persistence of the bacterial infection is primarily thought to be due to an immune response that is not vigorous enough (6). 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 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 completely eliminate the infection, which correlates with what others have demonstrated with lymphocyte adoptive transfer experiments (24, 183).

We previously demonstrated that Arg2 localizes to mitochondria while iNOS resides in the cytoplasm (144). 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 (144). 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 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 (114). 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 (193). Furthermore, it has been shown that Arg1-expressing macrophages can inhibit both the re-expression of T cell receptors and T cell proliferation *in vitro* (98, 194) 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 (176) of macrophages in *H. pylori* infection. However, the competing alternative (M2) activation nomenclature has been used to refer to cells with induction of Arg1 rather than Arg2 (176). 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 (195). Another factor in the utilization of L-arginine in host defense is that its uptake into macrophages is required to allow generation of NO (113, 196). 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 (102). 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 (122). This apoptosis was associated with decreased abundance of macrophages in the gastric mucosa in WT mice when compared to $Arg2^{-/-}$ mice. Another contributing factor to the increase in macrophages in the $Arg2^{-/-}$ mice may be increased stimulation of mononuclear cell infiltration, since enhanced Th1 and Th17 responses, as we have detected, have been correlated with increased inflammatory cells in the gastric mucosa (31, 79, 183). Taken together, our data suggest that the limited numbers of macrophages associated with Arg2 induction results in diminished gastric inflammation and pro-inflammatory 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, since depletion of macrophages from mice with clodronate-loaded liposomes resulted in reduced levels of histologic gastritis (197). It should be noted that the reduction in macrophages had no effect on *H. pylori* colonization levels in that study (197), 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, since 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 (198). 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* (145-147). Studies related to this issue may be a promising area for future investigation.

CHAPTER IV

CONCLUSION

Summary

H. pylori is an important human pathogen and research to determine the failure of the immune response during this infection is clinically important. This has been the focus of our laboratory and my dissertation in particular. In this dissertation, I have presented research demonstrating our discovery of a mechanism by which *H. pylori* escapes the host immune response. By upregulating Arg2, *H. pylori* effectively reduces NO production and induces apoptosis in macrophages. We have previously demonstrated that Arg2 is upregulated in humans infected with *H. pylori*. Consequently, an important next step for this project is to determine if the mechanisms we have demonstrated herein are occurring in humans. This could potentially be a promising area for future investigation.

In Chapter II, we examined the importance of *H. pylori*-induced expression of macrophage Arg2 *in vitro* and determined its effects on iNOS activity. We demonstrated for the first time that upregulation of macrophage Arg2 by an extracellular bacterium results in diminished NO-dependent killing. We have demonstrated that an inhibitor of macrophage arginase (BEC) enhanced NO production and killing of *H. pylori*, and that these effects are due to enhanced translation of iNOS protein, and not to an effect on iNOS mRNA expression levels. The effect of BEC occurred in a similar manner at time points tested from 12 to 24 h post-stimulation, indicating Arg2 has early and consistent

suppressive effects on the iNOS component of the innate immune response. We have shown that the enhancement of NO production in *H. pylori*-stimulated cells is specifically attributable to Arg2, because when we employed siRNA knockdown of Arg2 or utilized macrophages from Arg2$^{-/-}$ mice we again demonstrated a marked increase in iNOS protein and NO production. An implication of our findings is that induction of Arg2 in the host leads to a defective innate immune response manifested as diminished iNOS protein translation and NO production by macrophages. Moreover, our studies demonstrating that inhibition of macrophage arginase results in enhanced iNOS-derived NO production in response to *H. pylori in vivo* indicate that such a strategy could enhance the ability of the immune system to control or eliminate *H. pylori* infection.

In Chapter III, we examined the importance of *H. pylori*-induced expression of Arg2 *in vivo*. We demonstrated that *H. pylori* upregulates Arg2 in the gastric mucosa that localizes to F4/80^{+} macrophages, 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 demonstrate that macrophage Arg2 expression has a deleterious impact on the effectiveness of host immunity by impairing the inflammatory response *in vivo*. These data confirm our recent studies presented in chapter II of this dissertation showing that inhibition of macrophage arginase *in vitro* enhances iNOS translation and NO production and, consequently, causes more bacterial killing (144). 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. 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 to the defective immune response in WT mice.

In summary, we have demonstrated that induction of Arg2 by *H. pylori* is detrimental to the macrophage response against the infection. We have demonstrated that induction of Arg2 by *H. pylori* causes decreased iNOS translation, decreased NO production, decreased bacterial killing, and enhanced macrophage apoptosis. The effects of Arg2 induction in macrophages is demonstrated in Fig. 19. Therefore, induction of macrophage Arg2 restricts host defense against *H. pylori* and contributes to the immune evasion of *H. pylori*.

Future directions

Determine the H. pylori factor responsible for upregulation of Arg2

We have demonstrated that *H. pylori* induces Arg2 expression in macrophages and this contributes to the defective immune response against *H. pylori*. One important aspect surrounding this project that has yet to be identified is the *H. pylori* factor (e.g. protein(s) or other components such as lipids) responsible for upregulating Arg2. Our data indicates that this product may be secreted, as experiments conducted in which *H. pylori* was

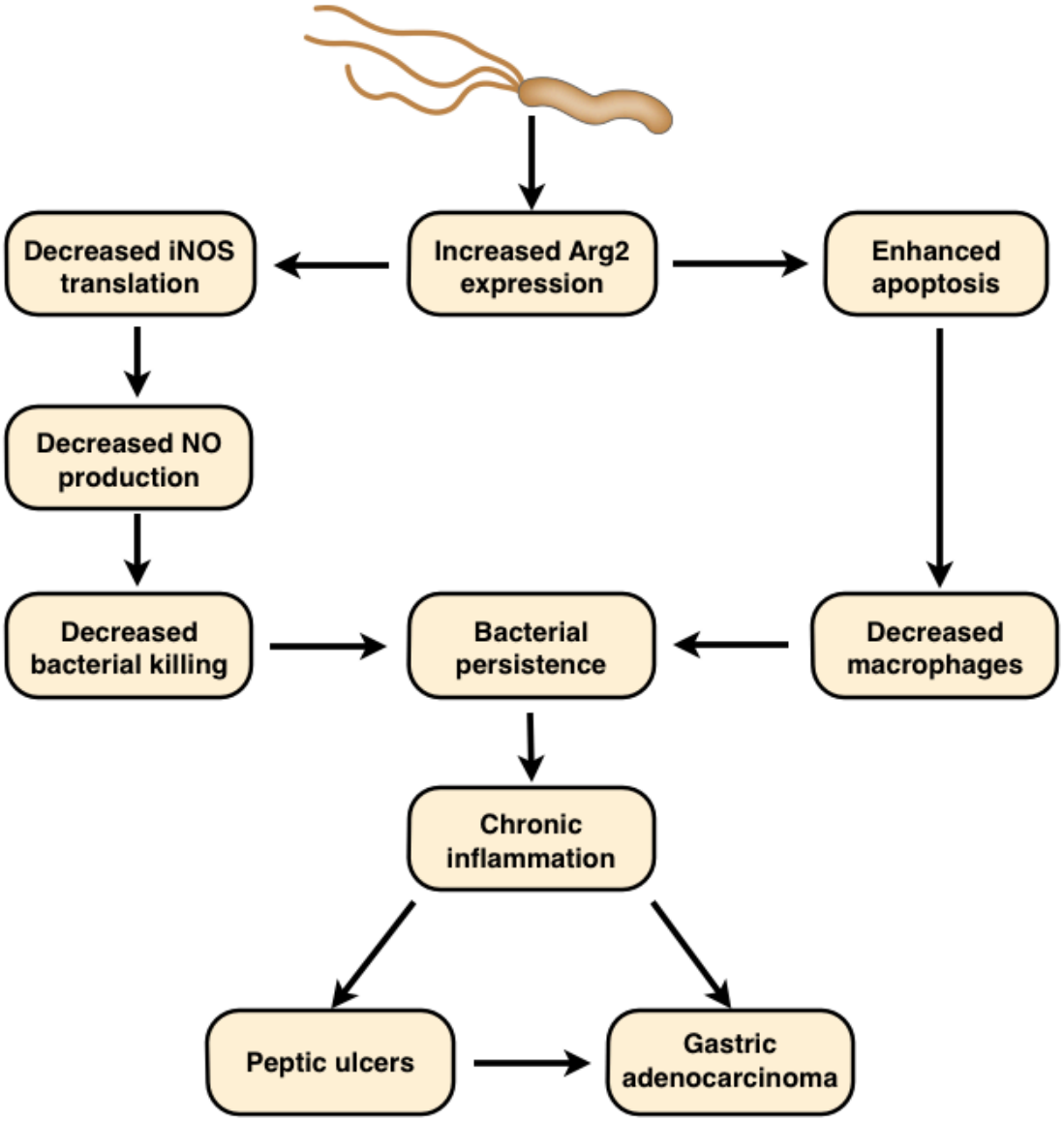

FIGURE 19. Working model demonstrating the consequences of Arg2 induction. This figure summarizes the main findings of this dissertation and shows how the results of Arg2 induction contribute to human disease induced by *H. pylori.*

suspended over a transwell and macrophages were cultured underneath still allow for induction of Arg2 after 6 hr. Consequently, inhibition of arginase using this transwell system leads to increased NO production and bacterial killing. In order to determine if there is a protein responsible for upregulation of Arg2, we began testing different isogenic mutant strains of *H. pylori* 7.13. Our goal was to discover a mutant strain of *H. pylori* that would not induce Arg2, thereby providing a clue as to which protein may be responsible for the induction of Arg2. The mutants we tested were *cagA*, *cagM*, *cagE*, *vacA*, *oipA*, *slt*, and *ureA*. Each of these mutant strains are viable, although some exhibit slower growth on blood agar plates. We used these mutants to activate the macrophage RAW264.7 cell line, with or without a transwell and measured Arg2 mRNA expression by real-time PCR. At the time of writing this dissertation, these preliminary experiments have not revealed any mutants that do not induce Arg2. Another method of identifying an *H. pylori* protein responsible for induction of Arg2 is to grow a broth culture of *H. pylori* and fractionate the supernatant. One could then stimulate macrophages using each one of these fractions and determine which fraction induces Arg2 expression. After identifying the fraction capable of inducing Arg2, mass spectrometry could be used to identify the various proteins within that fraction. This could be followed by further experiments to test each protein individually to determine if it possesses the activity necessary to induce Arg2.

Determine the signaling pathway leading to induction of Arg2

Another additional aspect of this project that has yet to be resolved is to identify which intracellular signaling pathway is responsible for induction of Arg2. We planned to address this question by activating RAW 264.7 cells with *H. pylori* in the presence of various signaling pathway inhibitors. Our goal was to find an inhibitor that would block the induction of Arg2 by *H. pylori*. This would provide evidence that induction of Arg2 is dependent upon the identified pathway. We planned to inhibit several pathways by using various inhibitors including: NFκB inhibitor (Bay 11-7082), MEK inhibitor (PD98059), ERK 1/2 inhibitor [ERKi (3-(2-aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione, HCl], JNK inhibitor (SP600125), p38 inhibitor (SB203580), PI3K inhibitor (LY294002), Src inhibitor (PP1), and the HIF-1 inhibitor [HIFi (3-(2-(4-adamantan-1-yl-phenoxy)-acetylamino)-4-hydroxybenzoic acid methyl ester]. These inhibitors cover a broad range of signaling pathways, one of which should play a role in *H. pylori*-induced Arg2 upregulation. This is closely related to the future direction above to identify the *H. pylori* factor responsible for induction of Arg2, and it could possibly help to narrow down the list of targets that may possess this ability to induce Arg2. By identifying the signaling pathway that is responsible for induction of Arg2, one could then determine which receptor senses *H. pylori* and leads to Arg2 induction. It is possible that TLRs may play a role in induction of Arg2. Several TLRs have now been identified along with their respective ligands. Importantly, however, many TLRs can bind multiple targets. The importance of TLRs for induction of macrophage Arg2 can be determined using macrophages isolated from myeloid differentiation primary response gene (88)

(MyD88)-deficient mice. MyD88 is a universal adaptor protein that is used by all TLRs, except TLR3, to activate the transcription factor NFκB. If a certain TLR was found to be involved, this would potentially narrow down the list of targets to those TLR ligands. However, there is a chance that the protein has not been previously identified to bind that specific TLR.

Determine the mechanism by which L-arginine affects iNOS translation

An interesting finding in our studies concerning the role of Arg2 in *H. pylori* infection was that inhibition of arginase, selective knockdown of Arg2, and genetic knockout of Arg2 all resulted in enhanced iNOS protein levels without effecting iNOS mRNA levels. Using RAW 264.7 cells, we demonstrated that this increase in iNOS protein expression was due to enhanced iNOS translation. We have previously demonstrated that exogenous addition of the iNOS and arginase substrate, L-arginine, to the medium of *H. pylori*-stimulated macrophages also caused an increase in iNOS translation. Consequently, we believe that the increase in iNOS translation that we see upon inhibition of Arg2 is due to increased levels of intracellular L-arginine. By blocking arginase activity, there should be more L-arginine inside the cell for iNOS. In our first paper describing that addition of L-arginine to *H. pylori*-stimulated macrophages enhances iNOS translation, we demonstrated that the enhancement of iNOS protein levels was due to enhanced translation and not due to increased stability of iNOS protein. Furthermore, we demonstrated that the increases we saw in iNOS protein levels upon addition of L-arginine were specific to iNOS and did not enhance global translation.

Moreover, only L-arginine was found to cause this effect, not the addition of other amino acids. We attempted to determine the mechanism by which addition of L-arginine enhances iNOS translation by examining eIF2α. It has been shown that different levels of amino acids, including L-arginine, can alter the phosphorylation status of eIF2α. The dephosphorylated form enhances translation, whereas the phosphorylated form is considered to be a global inhibitor of protein translation. Dephosphorylation of eIF2α has been associated with facilitation of iNOS translation in brain astrocytes (31), but this phenomenon had not been previously studied in immune cells or in cells exposed to an infectious stimulus. We therefore assessed levels of the eIF2α phosphoprotein and found that the levels were high in unstimulated cells even in the presence of high levels of L-arginine, indicating that L-arginine alone is unlikely to be sufficient to activate protein translation in macrophages. In cells activated with *H. pylori*, phospho-eIF2α levels rapidly decreased as L-arginine levels were increased from 0 to 0.1 mM, but there was not a significant change at higher concentrations of L-arginine. These data were consistent with the increase in global protein translation at 0.1 mM L-arginine that did not increase further at higher concentrations of L-arginine that we observed. However, these results were not consistent with the continued increase in iNOS protein expression and iNOS protein translation with concentrations of L-arginine above 0.1 mM, indicating that eIF2α is not responsible for enhanced iNOS translation. Proteomic experiments are currently being performed in order to determine potential proteins that may regulate iNOS translation.

Final remarks

It is truly amazing that *H. pylori* has infected 50% of the total human population for the past 50,000 years, caused gastritis in 100% of those infected, caused peptic ulcers in approximately 10%, and initiated the pathogenic environment leading to gastric adenocarcinoma or MALT lymphoma in approximately 1%. Given these numbers we can estimate that there are approximately 3.45 billion people infected with *H. pylori* in the world. That is a lot of stomach aches. Additionally, we can estimate that 345 million people have *H. pylori*-induced peptic ulcers and 34.5 million people have gastric cancer. The detrimental effects of this pathogen are astronomical, and only recently has it become known that these diseases are caused by *H. pylori*. We know that the infection can potentially be cured with antibiotics and that diseases can be prevented, but is it feasible to treat half of the world with antibiotics? Methicillin-resistant *Staphylococcus aureus* (MRSA) is causing high rates of morbidity and mortality because of its resistance to certain antibiotics. Prescribing half of the world antibiotics would possibly cause an increase in antibiotic resistance of pathogenic bacteria, including *H. pylori*, as has been previously reported (7). Although some may argue that infection with *H. pylori* may provide a selective advantage due to the long period of co-evolution with humans, the massive detriment to human health caused by this bacterium cannot be overlooked. The discovery of this bacterium, along with its role in causing disease, has provided scientists with a starting point to learn effective mechanisms to alleviate this disease burden. My dissertation project is relevant to this because I have sought to determine the mechanisms

responsible for the persistent colonization of *H. pylori* and how altering the immune response may contribute to clearance of the infection.

The immune system is ineffective at resolving an *H. pylori* infection. The reason for this ineffectiveness of the immune response is one of the most important topics of this field. Moreover, the results found by studying the immune response against *H. pylori* can be applied to other chronic infections and the immune system in general. Using *H. pylori* as a model, scientists have learned a great deal on how a chronic immune response is detrimental for humans and this may contribute to gastric cancer. It is difficult for me to conceive that the immune system cannot eliminate this infection. The immune system has been responsible for protecting the human race against pathogens for many years. It is possible that the immune system does eliminate the infection in some individuals, although this is difficult to determine. It may be that in some parts of the world, due to the high prevalence of the bacterium, some individuals may eliminate the infection only to be reinfected later on in life. Because *H. pylori* usually causes mild clinical symptoms, and most infected persons live a full life, there may be less selective pressure upon the immune system to eliminate the infection.

My project has sought to determine possible mechanisms by which the immune system is unable to eliminate the infection. We have discovered a potential mechanism by which *H. pylori* is able to evade the immune response and persist for the life of the host. I have demonstrated in the past two chapters that *H. pylori* induces Arg2 in macrophages. Induction of Arg2 inhibits the macrophage response by blocking iNOS translation. Furthermore, L-arginine metabolism by Arg2 contributes to the production of spermine,

which is necessary for *H. pylori*-induced apoptosis. By upregulating Arg2, *H. pylori* is able to decrease the production of reactive oxygen and nitrogen species that can kill it. Furthermore, it causes the death of macrophages which are capable of killing *H. pylori* utilizing several different mechanisms. The most interesting result that I found was that in WT mice, increased gastric inflammation had no effect on bacterial colonization, but in mice deficient in Arg2, increased gastric inflammation correlated with decreased bacterial colonization. This data suggests that Arg2 blocks effective immunity. We have demonstrated that Arg2 is also upregulated in the stomach of humans infected with *H. pylori*. It will be interesting to determine if Arg2 restricts iNOS-dependent host defense and causes macrophage apoptosis in humans.

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