Arginase II Restricts Host Defense to Helicobacter pylori by Attenuating Inducible Nitric Oxide Synthase Translation in Macrophages


J Immunol 2010; 184:2572-2582; Prepublished online 22 January 2010;
doi: 10.4049/jimmunol.0902436
http://www.jimmunol.org/content/184/5/2572

References
This article cites 60 articles, 37 of which you can access for free at:
http://www.jimmunol.org/content/184/5/2572.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2010 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Arginase II Restricts Host Defense to Helicobacter pylori by Attenuating Inducible Nitric Oxide Synthase Translation in Macrophages

Nurudddeen D. Lewis,*† Mohammad Asim,*‡ Daniel P. Barry,*‡ Kshipra Singh,*‡ Thibaut de Sablet,*‡ Jean-Luc Boucher,‡ Alain P. Gobert,*‡ Rupesh Chaturvedi,*‡ and Keith T. Wilson*‡

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 inducible NO synthase (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 arginase II (Arg2) mRNA and protein, which localized to mitochondria, whereas arginase I was not induced. Increased iNOS protein and NO levels were also demonstrated by small interfering RNA 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. The Journal of Immunology, 2010, 184: 2572–2582.

H helicobacter pylori is a microaerophilic, Gram-negative bacterium that selectively colonizes the human stomach. It is one of the most successful human pathogens because it infects half of the world’s population (1). All infected individuals exhibit chronic active gastritis, and a substantial proportion develops peptic ulcer disease or gastric adenocarcinoma. Importantly, gastric cancer is the second leading cause of cancer deaths worldwide (2). The infection is usually acquired in childhood and persists for the life of the host despite eliciting a vigorous innate and adaptive immune response (1). 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 noninvasive 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 (3–5). 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 inducible NO synthase (iNOS) (6). Our laboratory and others have demonstrated that H. pylori induces the expression and activity of iNOS in macrophages both in vivo (7, 8) and in vitro (9–14). Further, we have reported that macrophages cocultured with H. pylori have the ability to kill the bacterium by an NO-dependent mechanism (11, 13, 14). 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 (15) and down-regulatory effects of regulatory T cells (16) have been directly implicated in the persistence of the organism. Thus, although 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 (17, 18).
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 (13). 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 (19, 20) and above the $K_m$ of the iNOS enzyme for L-arginine, which is in the range of 10 μM (21, 22). 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 (13).

Arginase enzymes are the endogenous antagonists to iNOS because they compete for the same substrate by metabolizing L-arginine into urea and L-ornithine (23, 24). The latter is metabolized by ornithine decarboxylase (ODC) to produce the polyamines putrescine, spermidine, and spermine (25). There are two isoforms of arginase: arginase I (Arg1) is ubiquitous, but is especially abundant in liver, and arginase II (Arg2) is abundant in kidney and possesses a mitochondrial localization sequence (26–28). We have reported that...

**FIGURE 1.** Arginase inhibition enhances H. pylori-stimulated macrophage NO production and iNOS protein expression by enhancing iNOS translation. A total of $1 \times 10^6$ RAW 264.7 macrophages grown in 1 ml 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 NO 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 13 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 versus 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 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 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. $p < 0.05$; $**p < 0.01$ 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 concentration of L-arginine. F, iNOS protein levels at various time points. Data were normalized to $β$-actin. Data are the mean ± SEM of three experiments. $p < 0.05$; $**p < 0.01$ 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 [35S]methionine. After 4 h, iNOS protein was immunoprecipitated and resolved by SDS-PAGE and phosphorimaged. I, [35S]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 with controls without BEC. $**p < 0.01$ compared with HP-stimulated cells treated with BEC.
Arg2, but not Arg1, is upregulated in *H. pylori*-stimulated macrophages (10). Induction of arginine activity by pathogens has been reported to modulate macrophage NO production in the case of both Arg1 (29–32) and Arg2 (33, 34), 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 and that this occurs by limiting iNOS protein expression due to an required for iNOS synthesis. We now report that was followed by addition of desired concentrations of L-arginine (13).

Serum-free DMEM supplemented with 0.3% BSA as described (13). This thereby restricting NO production and bacterial killing.

**Materials and Methods**

**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). 5\-2\(\text{-bromo-nitro-}\)l-lysine (BEC), a slow-binding competitive inhibitor of arginase (35, 36), was synthesized by J.-L. Boucher (37). cPTIO [2\-(4-carboxyphenyl)-4\-4.5.5\-tetramethylimidazol-1\-oxyl-3-oxide] was from Cayman Chemical (Ann Arbor, MI). Small interfering RNA (siRNA) to ODC and Arg2 were from Ambion (Austin, TX). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

**Bacteria, cells, and culture conditions**

*H. pylori* SS1 was grown and used as described (12, 38), and multiplicity of infection (MOI) was defined as the ratio of bacteria to eukaryotic cells. *Citrobacter rodentium* was grown and used as described previously (39, 40). Macrophages were activated with either live bacteria or lysates prepared with a French press (13), and MOIs were determined in lysates as described (12). For bactericidal studies, live *H. pylori* was separated from macrophages by filter supports (13). When live bacteria were added, medium without antibiotics was used (11, 13, 14). 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 separated from macrophages (10). Induction of arginase activity by pathogens has been described (14). For Arg2 knockdown, we used siRNA duplex-targeting nucleotides 1168–1186 as follows: sense, 5\-'GGCAUUCGAGGGCAUGA\-3\'; antisense, 5\-'AU\-CU\-GU\-GU\-CUU\-GAG\-X3'. Scrambled siRNA and conditions for transfection and activation were as described (14, 38).

**Mice**

All animal experiments were approved by the Vanderbilt University Animal Care and Use Committee (Nashville, TN). C57BL/6 wild-type (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, Houston, TX) and were bred at the Nashville Veterans Affairs Medical Center. Male mice were used at 8 wk of age. Peritoneal macrophages were isolated as described (14) and used in l-arginine-free, serum-free DMEM. In addition, mice were gavaged with 5 \(\times\) 10\(^8\) *H. pylori* SS1 in 100 \(\mu\)l Brucella broth or broth alone. In some studies, mice were treated with 0.1% (w/v) BEC in the drinking water (40), starting 1 d preinfection and continuing for a total of 3 d. Mice were sacrificed 2 d postinoculation, and stomachs were excised and used for isolation of gastric macrophages, which was performed as described (15).

**Measurement of NO**

The concentration of the oxidized metabolite of NO, nitrite (NO\(_2^-\)), was assessed by the Griess reaction (9–11, 13, 14).

**Real-time PCR**

Primers for iNOS, Arg1, and \(\beta\)-actin were used as described (10, 13). For Arg2, we used primers that generated a 234-bp product as follows: sense, 5\-'GGATCCAGAAGGTGATGGAA\-3'; antisense, 5\-'AAGCTGACAGC-
**Immunofluorescence staining for Arg2 and mitochondria**

RAW 264.7 cells (1 × 10⁴) were plated in four-well chamber glass slides. Posttreatments, 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 Ab to Arg2 (1:200 dilution; Santa Cruz Biotechnology), followed by incubation with FITC-labeled donkey anti-goat secondary Ab (1:1000 dilution) for 45 min at room temperature. Cells were counterstained with DAPI for nuclear staining. Slides were dried, mounted, and visualized as described (39).

**Analysis of NO production and iNOS protein expression in gastric macrophages**

Isolated gastric macrophages were plated in 96-well plates. NO₂⁻ concentration was assayed by the Griess reaction and iNOS protein assessed by flow cytometry as described (13).

**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 t test was used. Prism 5.0 (GraphPad, 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 (11), but this enzyme is inactive in lysates (9, 10). Because we have previously reported that NO production increases in an L-arginine–dependent manner (13), we studied the effect of arginase inhibition over broad concentrations of L-arginine from 0–1.6 mM (Fig. 1A). In the presence of BEC, a specific inhibitor of arginase that does not directly affect iNOS expression or activity (35, 36), we detected a significant increase in NO production, measured as the concentration of the stable metabolite, NO₂⁻, at all concentrations of L-arginine between 0.1 and 1.6 mM, but not in the absence of L-arginine (Fig. 1A). It should be noted that concentrations of BEC from 30–150 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. 1B). 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. 1C). Quantification of iNOS protein levels by densitometry revealed increases in iNOS protein levels in the presence of BEC (Fig. 1D). Furthermore, we examined the effect of arginase inhibition at time points from 6–24 h poststimulation and found that NO production (Fig. 1E) and iNOS protein levels (Fig. 1F, 1G) were both significantly increased at 18 h and 24 h in the presence of BEC. Additionally, at 12 h poststimulation, there was an increase

---

**FIGURE 3.** Arg2 is upregulated by *H. pylori* stimulation in macrophages and localizes to the mitochondria. Arg1 (A) and Arg2 (B) mRNA expression was assessed by real-time PCR. Data were standardized to β-actin and presented as fold increase versus 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 poststimulation with 20 μg protein loaded per lane. Blots from samples treated with and without BEC were incubated together with the same Ab and exposed on the same film. D. Arginase activity in mitochondrial and cytosolic fractions from *H. pylori*-stimulated macrophages, assessed at 18 h postactivation. 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 Ab to mouse Arg2 detected with FITC (green); mitochondria were labeled with MitoTracker (red) and nuclei were stained with DAPI.
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 (13), 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 Ab to iNOS and [35S]methionine incorporation measured, there was increased iNOS translation in the presence of BEC when assessed by both SDS-PAGE (Fig. 1†) and quantification of radiolabeled counts (Fig. 1‡).

Because we have reported that knocking down of ODC increased NO production in *H. pylori*-stimulated macrophages by blocking spermine synthesis (14), we compared this effect to that of BEC (Fig. 2). Knockdown of ODC or inhibition of arginase resulted in an identical increase in NO production (Fig. 2A) with *H. pylori* stimulation. There was an additive effect with both treatments, such that there was a significant potentiation of NO production (Fig. 2A). 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. 2B).

**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* (10, 12, 13, 42), and Arg1 and Arg2 mRNA levels were quantified by real-time PCR (Fig. 3A). Arg2 mRNA levels were increased with *H. pylori* stimulation 4-fold in the presence of l-arginine (from 0.1–1.6 mM), and BEC had no effect on this induction (Fig. 3A). 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. 1B), which is likely due to cellular stress from l-arginine depletion, as we have described (13). We confirmed that Arg1 mRNA levels are not upregulated with *H. pylori* stimulation and that BEC did not affect Arg1 levels (Fig. 3B).

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. 3C). 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 (13).

Arg2 has a leader sequence that can target it to mitochondria (43). 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. 3D). Consistent with this, *H. pylori* induced a marked increase in Arg2 staining by immunofluorescence detection that colocalized with mitochondria (Fig. 3E).

**Effect of arginase inhibition on iNOS protein expression is not observed with another enteric pathogen, *C. 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*. To address this question, we used the mouse colitis-inducing pathogen *C. rodentium*, which we have shown induces both iNOS and Arg1 in vivo (40). As with *H. pylori*, *C. rodentium* lysate stimulated NO production in RAW 264.7 cells that was enhanced by BEC (Fig. 4A). 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. 4B).

Although *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 differ 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 (35, 36). 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. 5A). In cells transfected with Arg2 siRNA, we observed significant increases in *H. pylori*-stimulated NO production (Fig. 5B). Although knockdown of Arg2 had no effect on *H. pylori*-stimulated iNOS mRNA levels (Fig. 5C), it resulted in a consistent potentiation of iNOS protein levels by Western blot analysis (Fig. 5D), which was confirmed by densitometry (Fig. 5E).

---

**FIGURE 4.** 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 mU). NO3− 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 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 Ab and exposed on the same autoradiograph film.
Arg2-deficient peritoneal macrophages produce more NO and have higher iNOS protein levels when stimulated with H. pylori ex vivo

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. 6A), whereas Arg1 levels were not increased (Fig. 6B). iNOS mRNA levels were also increased in the peritoneal macrophages from both WT and Arg2−/− mice (Fig. 6C). After 24 h of coculture with H. pylori, Arg2-deficient peritoneal macrophages produced significantly more NO than WT macrophages (Fig. 6D).

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 Arg2 stimulation in WT cells that was further upregulated in Arg2-deficient peritoneal macrophages (Fig. 6D, 6E).

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 (13, 14). In the current study, we have shown that Arg2 restricts NO production and iNOS translation in 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. 7A). In parallel, there was enhanced killing of the H. pylori above the filter supports (Fig. 7B). To determine if the changes in killing with BEC were NO-dependent, we used cPTIO, an NO scavenger that rapidly converts NO to NO2 that is then converted to NO2− (44). As expected, cPTIO increased the NO2− 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 50–350 μM and used 100 μM for these studies because the effect on NO2− 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. 7B). 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 (10), we now determined if arginase inhibits iNOS in vivo. The peak time point of macrophage infiltration is 48 h postinoculation (45). Therefore, we determined the effect of BEC (0.1% w/v) administration in the drinking water (40) on...

FIGURE 5. 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 NO2 production by H. pylori-stimulated macrophages measured after 24 h. *p < 0.05; **p < 0.01; and ***p < 0.001 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 versus 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.
levels of iNOS protein and NO in gastric macrophages isolated from mice 48 h postinoculation 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. 8A). 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. 8B, 8C). 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

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. 8), BEC administration increased NO production (Fig. 9A) and iNOS protein levels (Fig. 9B, 9C) in WT gastric macrophages isolated from infected mice. Arg2−/− gastric macrophages produced similar levels of NO (Fig. 9A) and iNOS protein (Fig. 9B, 9C) as cells from WT mice treated with BEC. Importantly, BEC administration to Arg2−/− mice did not further increase NO production (Fig. 9A) or iNOS protein levels (Fig. 9B, 9C) 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.

Discussion

NO production by classically activated type I macrophages is required to control a variety of parasitic and bacterial pathogens (46, 47). 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* (31) and *Toxoplasma gondii* (30) and the intracellular bacteria *Mycobacterium tuberculosis* (30); and to induction of Arg2 by the extracellular parasite *Trypanosoma brucei brucei* (34) and the intracellular bacteria *Chlamydia psittaci* and *Chlamydia pneumoniae* (33).

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 poststimulation, 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, be-
cauuse when we employed siRNA knockdown of Arg2 or used
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
stimulation, and treat-
H. pylori
survival in its acidic niche (48). Although
H. pylori possesses urease, which metabolizes urea into
amines.
H. pylori
possesses its own arginase, which acts to generate an intracellular
Arg2 metabolizes L-arginine to produce L-ornithine plus urea.
L-ornithine can then be metabolized by ODC to produce poly-
nitric acids. TheJournal of Immunology 2579
FIGURE 7. 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 × 10⁶ RAW 264.7 cells in arginine- and serum-free DMEM
with 0.4 mM 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 NO₂⁻ 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. Effect of arginase inhibition
and NO scavenger on bacterial killing (%). Data are the mean ± SEM
of three separate experiments.

FIGURE 8. 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 × 10⁸ H. pylori bacteria. Treatment with BEC was
continued for an additional 48 h and mice were sacrificed, followed by
isolation of gastric macrophages.
A. 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 comparing HP + BEC to HP
alone.
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 comparing HP + BEC to HP
alone. 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.

arginate metabolism 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.
not shift the iNOS level in the cells from the Arg2 protein levels in gastric macrophages; note that the BEC treatment does not increase NO production in Arg2−/− 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 × 10⁸ 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 NO₂⁻ 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.

FIGURE 9. 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 × 10⁸ 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 NO₂⁻ 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.

It has been reported that although iNOS is a cytoplasmic enzyme, Arg2 contains a mitochondrial localization domain (51, 52). 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 ω-arginine. These findings suggest that future studies of ω-arginine transport by mitochondria could prove to be a fruitful area of investigation. Consistent with our previous work (13), 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 ω-arginine tested, including at a marked excess of ω-arginine. Because the circulating level of ω-arginine in mammalian serum is ∼0.1 mm (19, 20), which is well above the Kₘ of iNOS (21, 22), 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 although uptake of extracellular ω-arginine is required for macrophage NO production (13, 53), the competitive effect of arginase is of critical importance in regulating ω-arginine substrate 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 postinoculation with H. pylori. It should be noted that we have determined that at 4 mo postinoculation 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 (N.D. Lewis, et al., manuscript in preparation).

There is substantial literature focused on the induction of Arg1 in macrophages, resulting in a type II phenotype (54, 55), which is characterized by the response to Th2 cytokines and parasites and a lack of iNOS mRNA expression (56). Although we previously showed that H. pylori induces Arg2 rather than Arg1 (10), we now demonstrate that although Arg2 and iNOS are coexpressed at the mRNA level, Arg2 acts to restrict iNOS translation and hence NO generation. ω-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, whereas the phosphorylated form inhibits it (57, 58). In the case of iNOS, it has been suggested that inhibition of the eIF2α phosphorylation could enhance iNOS translation; however, we recently showed that inhibition of eIF2α phosphorylation did not increase NO production (59). We therefore speculate that inhibition of eIF2α might contribute to the decreased NO production observed in Arg2−/− macrophages; however, additional studies will be required to test this hypothesis.
that dephosphorylation 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 (13). 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 (60). 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 macrophages, such as \(Trypanosoma brucei brucei\) (34) and \(Chlamydia psittaci\) (33). 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 (10). This raises the possibility that there could be differences in Arg2 levels or gene polymorphisms in human subject groups at varying risk for gastric 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.

**Acknowledgments**

We thank Jordan Wolff for technical assistance with arginine activity assays.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


