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Arginase 2 deletion leads to enhanced M1 macrophage activation and upregulated polyamine metabolism in response to *Helicobacter pylori* infection

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Abstract

We reported that arginase 2 (ARG2) deletion results in increased gastritis and decreased bacterial burden during *Helicobacter pylori* infection in mice. Our studies implicated a potential role for inducible nitric oxide (NO) synthase (NOS2), as *Arg2*$^{-/-}$ mice exhibited increased NOS2 levels in gastric macrophages, and NO can kill *H. pylori*. We now bred *Arg2*$^{-/-}$ to *Nos2*$^{-/-}$ mice, and infected them with *H. pylori*. Compared to wild-type mice, both *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice exhibited increased gastritis and decreased colonization, the latter indicating that the effect of ARG2 deletion on bacterial burden was not mediated by NO. While *Arg2*$^{-/-}$ mice demonstrated enhanced M1 macrophage activation, *Nos2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice did not demonstrate these changes, but exhibited increased CXCL1 and CXCL2 responses. There was an increased expression of the Th1/ Th17 cytokines, interferon gamma and interleukin 17, in gastric tissues and splenic T-cells from *Arg2*$^{-/-}$, but not *Nos2*$^{-/-}$ or *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice. Gastric tissues from infected *Arg2*$^{-/-}$ mice demonstrated increased expression of arginase 1, ornithine decarboxylase,

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adenosylmethionine decarboxylase 1, spermidine/spermine N^1-acetyltransferase 1, and spermine oxidase, along with increased spermine levels. These data indicate that ARG2 deletion results in compensatory upregulation of gastric polyamine synthesis and catabolism during *H. pylori* infection, which may contribute to increased gastric inflammation and associated decreased bacterial load. Overall, the finding of this study is that ARG2 contributes to the immune evasion of *H. pylori* by restricting M1 macrophage activation and polyamine metabolism.

Keywords

Helicobacter pylori; Immune evasion; Polyamines; Macrophage activation

Introduction

Helicobacter pylori is a Gram-negative, microaerophilic bacterium that selectively colonizes the gastric mucosa of humans (Cover and Blaser 2009; Hardbower et al. 2014; Sibony and Jones 2012). It is estimated that more than 50 % of the human population is infected with *H. pylori*, leading to chronic gastritis and peptic ulcer disease (Cover and Blaser 2009; Sibony and Jones 2012). Importantly, *H. pylori* is the greatest risk factor for the development of gastric adenocarcinoma, the third leading cause of cancer deaths worldwide (Bonequi et al. 2013; Hardbower et al. 2013, 2014; Nomura et al. 1991; Parsonnet et al. 1991; Peek and Blaser 2002; Ferlay et al. 2015). The global prevalence of infection and the high degree of gastric cancer mortality clearly indicate that *H. pylori* is a significant public health issue.

H. pylori infection induces both innate and adaptive immune responses, but these responses are inadequate to clear the infection and result in pro-carcinogenic, chronic inflammation (Hardbower et al. 2013, 2014; Peek et al. 2010; Robinson et al. 2007; Wilson and Crabtree 2007). Macrophages represent a dynamic subset of innate immune cells and serve to coordinate the immune response to *H. pylori* (Murray and Wynn 2011; Peek et al. 2010; Robinson et al. 2007; Wilson and Crabtree 2007). Macrophage activation is a critical component in the response to bacterial pathogens (Benoit et al. 2008). There are three classes of activated macrophages—M1, M2, and Mreg (Martinez and Gordon 2014; Mosser 2003; Mosser and Edwards 2008). M1 macrophages are highly pro-inflammatory: expressing high levels of inducible nitric oxide synthase (NOS2), interleukin (IL)-1β, and tumor necrosis factor (TNF)-α; they are aimed at the clearance of pathogens (Benoit et al. 2008; Martinez and Gordon 2014; Mosser 2003; Mosser and Edwards 2008; Murray and Wynn 2011; Strauss-Ayali et al. 2007). Alternatively activated M2 macrophages are specialized for wound healing and responses to parasites, with enhanced expression of arginase 1 (ARG1), chitinase 1 (CHIA1) and resistin-like molecule alpha (RETNLA) (Anderson and Mosser 2002; Martinez and Gordon 2014; Mosser and Edwards 2008). M2 macrophages are also referred to as tumor-associated macrophages, given their pro-angiogenic and pro-tumorigenic properties (Ostuni et al. 2015; Pander et al. 2011). Regulatory macrophages, Mreg, are anti-inflammatory and secrete high levels of IL-10 and transforming growth factor (TGF)-β (Fleming and Mosser 2011; Mosser and Edwards 2008; Murray and Wynn 2011). *H. pylori* infection most commonly results in M1 and Mreg macrophage activation (Gobert et al. 2014).

H. pylori has developed effective mechanisms to thwart the macrophage response, which appears to be centralizing around the roles of NOS2 and arginase 2 (ARG2) in macrophage function. Infection with a bacterial pathogen, including *H. pylori*, leads to induction of NOS2 and production of nitric oxide (NO), a potent anti-microbial molecule, from L-arginine (L-Arg) (Shapiro and Hotchkiss 1996; Wilson et al. 1996; Gobert et al. 2002b). *H. pylori* diminishes the efficacy of the NO response via production of the bacterial arginase, RocF, leading to depletion of L-Arg that is available to the host (Gobert et al. 2001, 2011; Chaturvedi et al. 2007). Additionally, *H. pylori* infection leads to induction of ARG2, which also utilizes L-Arg as a substrate (Lewis et al. 2010, 2011). Induction of ARG2 further reduces L-Arg availability to NOS2, and also leads to macrophage apoptosis (Gobert et al. 2002a; Gogoi et al. 2015; Lewis et al. 2010, 2011). By modulating L-Arg levels, the efficacy of both NOS2 and ARG2, and macrophage viability, *H. pylori* effectively hinders the macrophage response, allowing for the establishment of a pro-tolerogenic environment in which to survive over the lifetime of the host (Chaturvedi et al. 2010, 2012; Wilson and Crabtree 2007). The purpose of the current study was to determine the role of NO or other mechanisms in the altered immunity to *H. pylori* attributable to ARG2.

Results

The role of ARG2 in *H. pylori* immunopathogenesis is not NOS2-dependent

We have previously reported that *Arg2*$^{-/-}$ mice had significantly increased *H. pylori*-induced gastritis, with a concomitant decrease in bacterial burden (Lewis et al. 2011). We determined that ARG2 attenuated NOS2 translation, and thus, we hypothesized that the augmented gastritis in the Arg2$^{-/-}$ mice was due to enhanced NOS2 translation and NO production (Lewis et al. 2010, 2011). To determine if the ARG2 phenotype was dependent on NOS2, we crossed *Nos2*$^{-/-}$ mice and *Arg2*$^{-/-}$ mice, creating *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice. ARG2 and NOS2 are both highly inducible in macrophages during *H. pylori* infection (Gobert et al. 2002a; Shapiro and Hotchkiss 1996; Wilson et al. 1996). Thus, we took advantage of this fact and utilized macrophages to confirm *Arg2* and *Nos2* knockout in bone marrow-derived macrophages (BMmacs) infected ex vivo with *H. pylori* (Supp. Fig. 1a, b), as differences in ARG2 and NOS2 levels would be most apparent in macrophages. Wild type (WT), *Arg2*$^{-/-}$, *Nos2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice were infected with *H. pylori* SS1 for 4 months, after which the mice were killed and their stomachs isolated for analysis. Consistent with our previous studies (Lewis et al. 2011), *Arg2*$^{-/-}$ mice had significantly increased gastritis versus WT mice (Fig. 1a). *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice also had significantly increased gastritis when compared to WT mice (Fig. 1a). *Nos2*$^{-/-}$ mice had similar gastritis as WT mice (Fig. 1a). The representative images of the gastritis further demonstrate the increased inflammation in *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice, as compared to WT and *Nos2*$^{-/-}$ mice (Fig. 1b). Consistent with the increase in gastritis, *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice both showed significantly decreased bacterial burden when compared to WT mice (Fig. 1c). These data, especially the finding that *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice did not lose the reduced colonization of *Arg2*$^{-/-}$ mice, indicate that the role of ARG2 in *H. pylori* pathogenesis is not dependent on NOS2, but rather some other aspect of the immune response to the pathogen. Additionally, *Nos2*$^{-/-}$ mice exhibited a modest decrease in colonization compared to WT

mice (Fig. 1c), further suggesting that NOS2-derived NO is not the primary effector that controls colonization in this infection model.

ARG2 reduces pro-inflammatory cytokine expression and immune cell-derived chemokine production in response to *H. pylori*

To begin to address how ARG2 alters the immune response to *H. pylori*, we performed Luminex analysis on gastric tissues from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$ and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice after chronic infection to assess differences in cytokine and chemokine levels in tissues. We found that *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice had significantly increased protein levels of C–C chemokine ligand (CCL) 3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES), as compared to WT and *Nos2*$^{-/-}$ mice (Fig. 2a). CCL3, CCL4, and CCL5 are typically produced by immune cells, such as macrophages, in response to pathogens (Algood et al. 2004). These data indicate that ARG2 regulates the innate immune response to *H. pylori*. Specifically, ARG2 deletion may affect M1 macrophage activation, as M1 macrophages are pro-inflammatory drivers of the innate immune response to many pathogens (Benoit et al. 2008; Martinez and Gordon 2014; Mosser and Edwards 2008; Murray and Wynn 2011). Thus, we assessed mRNA expression levels of the M1 macrophage markers, *Nos2*, *Il-1β*, and *Tnf-α* (Mosser and Edwards 2008) in gastric tissues from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice. *Nos2*$^{-/-}$ and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice did not express *Nos2*, further confirming knockout of the gene (Fig. 2b). *Arg2*$^{-/-}$ mice had significantly enhanced mRNA levels of the M1 markers, compared to the other three genotypes (Fig. 2b). Markers of M2 and Mreg macrophage activation were also assessed in these gastric tissues. M2 markers, *Chia1* (also known as *Ym1)* and *Retnla* (also known as *Fizz1/Relm-α*) (Anderson and Mosser 2002; Mosser and Edwards 2008), were not induced by *H. pylori* infection (data not shown). Moreover, Mreg markers, *Il-10* and *Tgf-β* (Anderson and Mosser 2002; Mosser and Edwards 2008; Murray and Wynn 2011), were not significantly altered between the four genotypes (data not shown), indicating that the effect of ARG2 knockout is specifically on M1 activation.

Interestingly, as shown in Fig. 3, NOS2 knockout, in both *Nos2*$^{-/-}$ and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice, resulted in significantly increased levels of the chemokines, C–X–C chemokine ligand (CXCL) 1 (KC) and CXCL2 (MIP2), which have been shown to be primarily produced by epithelial cells (Schleimer et al. 2007). These data indicate that the effects of the *Nos2* deletion may be more significant in epithelial cell function than in immune cell function.

ARG2 diminishes Th1/Th17 T-cell differentiation in response to chronic *H. pylori* infection

A second essential component to *H. pylori*-driven gastritis is the adaptive T-cell response (Robinson et al. 2007). To evaluate the T helper (Th) cell response to *H. pylori* infection, gastric tissues from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice were assessed for gene expression of Th1-, Th17-, Th2-, and Treg-specific markers. The mRNA levels of Th1 marker, *Ifn-γ*, and Th17 marker, *Il-17*, were significantly increased in *Arg2*$^{-/-}$ mice, when compared to WT mice (Fig. 4a). This correlates with the significantly increased histologic gastritis observed in these mice (Fig. 1a). However, WT, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice demonstrated similar levels of *Ifn-γ* and *Il-17a* expression in gastric tissues, and each had significantly lower expression than in *Arg2*$^{-/-}$ mice (Fig. 4a). These data suggest that the

increase in the Th1/Th17 response to *H. pylori* in *Arg2*$^{-/-}$ mice is dependent on NOS2, as *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice demonstrated the same Th1/Th17 phenotype as *Nos2*$^{-/-}$ mice. mRNA expression of Th2 marker, *Il-4*, and expression of Treg marker, *Foxp3*, were also measured in gastric tissues from these mice and no significant differences were found between genotypes (data not shown).

To confirm the enhanced Th1/Th17 findings in *Arg2*$^{-/-}$ mice, splenocytes were isolated from *H. pylori*-infected WT and *Arg2*$^{-/-}$ mice, and cultured in the presence of CD3 and CD28 to promote T-cell proliferation. The splenic T-cells were then treated with *H. pylori* SS1 French-pressed lysate (FPL) to allow *H. pylori*-specific T-cells to proliferate, and levels of secreted IFN-γ and IL-17 were assessed. Consistent with the mRNA findings in gastric tissue, *Arg2*$^{-/-}$ mice had significantly increased IFN-γ and IL-17 secretion from splenic T-cells (Fig. 4b). Additionally, the Th1 population within the *H. pylori*-stimulated splenic T-cells was assessed by flow cytometry for CD4, a T helper cell marker, and intracellular IFN-γ. Again, *Arg2*$^{-/-}$ mice had a significantly increased CD4^{+}/IFN-γ^{+} Th1 population, as compared to *H. pylori*-stimulated WT splenic T-cells (Fig. 4c).

ARG2 mediates M1 macrophage activation and function, but not M2 or Mreg activation, in response to *H. pylori*

As previously reported, ARG2 and NOS2 are highly inducible in macrophages during *H. pylori* infection (Gobert et al. 2002a, b; Lewis et al. 2010). Bone marrow-derived macrophages (BMmacs) were isolated from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice to specifically evaluate the affect of ARG2 and NOS2 knockout on macrophage activation and function. BMmacs from each of the four genotypes in this study were co-cultured with *H. pylori* for 24 h, followed by assessment of M1, M2, and Mreg activation markers by real-time polymerase chain reaction (RT-PCR). Gene expression levels of *Nos2*, *Il-1β*, and *Tnf-α* were utilized as representative M1 markers, *Chia1* and *Retnla* as M2 markers, and *Il-10*, *Tgf-β*, and *Tnfsf14* as Mreg markers. As expected, *Nos2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ BMmacs did not expresses *Nos2* (Fig. 5a). *Arg2*$^{-/-}$ BMmacs demonstrated enhanced M1 activation, as evidenced by significantly increased *Nos2*, *Il-1β*, and *Tnf-α*, as compared to WT and *Nos2*$^{-/-}$ BMmacs (Fig. 5a). Interestingly, *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ BMmacs demonstrated increased *Il-1β* expression to a similar level as *Arg2*$^{-/-}$ BMmacs, while *Tnf-α* expression remained similar to WT and *Nos2*$^{-/-}$ BMmacs, rather than increasing as in *Arg2*$^{-/-}$ BMmacs (Fig. 5a). The effects of ARG2 and NOS2 on macrophage activation were also studied in BMmacs from each genotype, utilizing IFN-γ/LPS activation as a classical M1 stimulus. Under these conditions, both *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ BMmacs had enhanced M1 activation, when compared with WT and *Nos2*$^{-/-}$ BMmacs (Supp. Fig. 2a). *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ BMmacs had the highest expression of *Il-1β*, even when compared to *Arg2*$^{-/-}$ BMmacs (Supp. Fig. 2a).

M2 and Mreg markers were also assessed in BMmacs from all four genotypes. Consistent with the gastric tissue mRNA data, *H. pylori* infection did not induce expression of *Arg1*, *Chia1,* or *Retnla* in BMmacs, from any of the genotypes (data not shown). As *H. pylori* did not induce an M2 response, BMmacs from each of the four genotypes were stimulated with IL-4, the canonical M2 stimulus. Stimulation with IL-4 induced an M2 response in these

mice, but there were no significant differences between the genotypes (Supp. Fig. 2b). Moreover, *H. pylori* infection had a very modest effect on Mreg activation between the four genotypes. *Arg2*$^{-/-}$ BMmacs had significantly increased *Il-10* expression, when compared to each of the other genotypes (data not shown). Additionally, there was no difference in *Tgf-β* or *Tnfsf14* expression between each of the genotypes (data not shown).

To confirm the effect of ARG2 on M1 macrophage activation in vivo, splenic macrophages were isolated from chronically infected WT and *Arg2*$^{-/-}$ mice, and stimulated with *H. pylori* SS1 FPL. As expected, splenic macrophages from *Arg2*$^{-/-}$ mice had significantly increased levels of secreted IL-1β and TNF-α (Fig. 5b). Moreover, *Arg2*$^{-/-}$ BMmacs produced significantly increased levels of NO, in response to *H. pylori* infection ex vivo (Fig. 5c). Taken together, these data demonstrate that ARG2 can modulate M1 macrophage responses to *H. pylori* and other stimuli, but ARG2 does not have a role in regulating M2 and Mreg activation.

ARG2 deletion results in a compensatory upregulation in polyamine metabolism in response to *H. pylori*

ARG1 and ARG2 are redundant enzymes responsible for the conversion of L-Arg to l-ornithine, which is the substrate for ornithine decarboxylase (ODC) (Chaturvedi et al. 2010, 2012). ARG1 is essential for life, and can be induced by certain stimuli; ARG2 is also inducible under conditions of stress, such as bacterial infections (Chaturvedi et al. 2012; Gobert et al. 2002a; Gogoi et al. 2015; Lewis et al. 2010, 2011). *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice are lacking this inducible form of the enzyme, potentially altering both the response to pathogens and polyamine metabolism. We hypothesized that the loss of *Arg2* could lead to an upregulation of *Arg1* expression, and an increase in gene expression related to polyamine metabolism. We assessed a panel of genes involved in polyamine metabolism, including *Arg1,* which generates substrates for polyamine metabolism, polyamine synthetic enzymes, *Odc* and adenosylme-thionine decarboxylase 1 (*Amd1)*, and polyamine catabolic enzymes, spermidine/spermine N^1-acetyltransferase 1 (*Sat1)* and spermine oxidase (*Smox)*. Expression of each of these genes was examined in chronically infected gastric tissues from WT, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice by RT-PCR. *Arg2*$^{-/-}$ mice had significantly increased *Arg1* and *Sat1* levels compared to each of the other genotypes (Fig. 6a). Both *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice had significantly enhanced *Odc*, *Amd1*, and *Smox* expression, as compared to WT and *Nos2*$^{-/-}$ mice (Fig. 6a). Thus, ARG2 knockout enhanced the expression of polyamine metabolism genes, while NOS2 deletion had no effect on expression of these genes.

Additionally, *Arg1* and *Odc* gene expression was increased in BMmacs from both *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice (Fig. 6b). However, *Amd1*, *Sat1*, and *Smox* were not significantly induced by *H. pylori* infection in BMmacs, nor were differences between the genotypes detected (data not shown). These data suggest that ARG2 in macrophages has a role in polyamine synthesis, but not in polyamine catabolism.

Furthermore, the levels of putrescine, spermidine, and spermine were measured in gastric tissues by high-performance liquid chromatography. *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice demonstrated significantly increased spermine levels (Fig. 7a), and higher spermidine and

total polyamine levels as compared to WT and *Nos2*$^{-/-}$ mice, although the latter differences did not reach significance (Fig. 7a, b). Further, the significant decrease in putrescine (Fig. 7a) in the *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice suggests an increased conversion to spermidine and spermine. These data indicate that the loss of ARG2 drives enhanced polyamine metabolism in *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice. Taken together, these data indicate that the loss of ARG2 enhances polyamine metabolism.

Discussion

In this study, we show that ARG2 deletion leads to increased gastritis following chronic *H. pylori* infection. This is similar to previously published data from our laboratory (Lewis et al. 2011), which implied that effects of ARG2 deletion were mediated by enhanced NOS2 expression and NO production (Lewis et al. 2010, 2011). However, the present study demonstrated that the role of ARG2 in *H. pylori* infection is more complex. ARG2 knockout leads to enhanced overall M1 macrophage activation, and upregulated polyamine metabolism. There appeared to be a partial role for NOS2 in regulation of M1 activation, as some changes observed in *Arg2*$^{-/-}$ mice were observed in *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice, and others were not.

Our previous studies led us to hypothesize that deletion of both ARG2 and NOS2 in *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice would restore WT colonization levels, such that reduced colonization in *Arg2*$^{-/-}$ mice would be lost when the *Nos2* gene was also deleted. However, *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice demonstrated similar colonization and gastritis levels as *Arg2*$^{-/-}$ mice, indicating that the role of ARG2 is NOS2-independent. While *Nos2*$^{-/-}$ mice demonstrated a similar level of gastritis as WT mice, colonization was decreased, and statistically similar to the *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice. This may be explained by an increased production of epithelial-derived chemokines, which activate inflammatory cells, in *Nos2*$^{-/-}$ and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice, indicating that NOS2 has an important role in modulating responses in epithelial cells. This is unexpected given that the major source of NOS2 during infection has been reported to be macrophages and neutrophils (Goto et al. 1999; Mannick et al. 1996; Sakaguchi et al. 1999; Lewis et al. 2011). Future studies are required to determine the role of NOS2 in epithelial cells during *H. pylori* infection.

We focused on the role that ARG2 plays in innate and adaptive immune response to *H. pylori* infection. Consistent with the increase in gastritis, ARG2 knockout led to increased M1 macrophage activation in tissues and macrophages. *Il-1β* expression was increased in *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ BMmacs with either *H. pylori* infection or the classical M1 stimuli, IFN-γ and LPS. These findings were limited to mice carrying the ARG2 deletion and not found in WT or *Nos2*$^{-/-}$ mice, indicating that these findings are NOS2/NO-independent. The overall increase in *Il-1β* gene expression, despite the stimulus, could indicate that ARG2 plays a role in inflammasome function within macrophages, as pro-IL-1β cleavage into mature IL-1β is the primary pro-inflammatory function of the inflammasome (Latz et al. 2013). This may contribute to the enhanced inflammation observed in *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice after chronic *H. pylori* infection.

ARG2 does not appear to play a global role in macrophage activation. While loss of ARG2 enhanced M1 macrophage activation, M2 macrophage activation was not affected by ARG2. Our laboratory reported that *H. pylori* does not induce a significant M2 response (Gobert et al. 2014). However, we now show that IL-4 stimulation produced an M2 response in BMmacs, but there were no differences between the genotypes. Moreover, ARG2 knockout did not have a profound effect on Mreg macrophage activation. There was a modest increase in *Il-10* expression in *Arg2*$^{-/-}$ mice that was not found in *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice. The lack of *Il-10* expression in *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ BMmacs may partially explain why histologic gastritis in *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice is statistically similar to that in *Arg2*$^{-/-}$ mice; lack of an anti-inflammatory response may allow for pro-inflammatory M1 macrophage response to go unchecked. Together, these data reinforce that the role of ARG2 is specifically important in M1 macrophage activation. The T-cell phenotypes herein provide partial support for our initial hypothesis that effects of ARG2 deletion were dependent on NOS2. There is a significant Th1/Th17 response in *Arg2*$^{-/-}$ mice, that is ablated in *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice. These data indicate that either expression of Th1/Th17 markers and/or that recruitment and differentiation of Th1/Th17 cells is directly dependent on NOS2.

Our most novel finding was the dramatic effect of ARG2 deletion on the polyamine pathway. Both *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice demonstrated upregulated expression of various polyamine metabolism enzymes, as well as significantly increased spermine levels within gastric tissue. These data suggest that ARG2 has an important role in regulation of polyamine synthesis and catabolism, which would have broad effects on polyamine flux within tissues. *Arg1* and *Odc* expression was similarly increased in primary macrophages as well, indicating an important role for ARG2 in polyamine synthesis within macrophages. Our laboratory has demonstrated that polyamines and the enzymes involved in their metabolism are important in macrophage function, specifically in response to *H. pylori* infection (Barry et al. 2011; Chaturvedi et al. 2004, 2007, 2010, 2012, 2014; Gobert et al. 2002a, b, 2001, 2011; Lewis et al. 2010, 2011). The current study specifically highlights the importance of ARG2 as a regulator of polyamine metabolism in gastric tissues and in macrophage function and thus, the immune response to chronic *H. pylori* infection. Potential explanations for how upregulation of polyamine metabolism within *Arg2*$^{-/-}$ and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice could contribute to increased gastritis in a NOS2-independent manner, include alterations in macrophage viability allowing for enhanced function (Gobert et al. 2002a; Chaturvedi et al. 2004) or direct alteration of macrophage phenotypes (Van den Bossche et al. 2012).

Polyamine metabolism has been implicated in the regulation of immune responses to various pathogens, in addition to *H. pylori* (Bansal and Ochoa 2003; Bronte and Zanovello 2005; Chaturvedi et al. 2010). We have now identified ARG2 as a critical player in polyamine metabolism in response to a major human pathogen. Future studies investigating the role of ARG2 in polyamine metabolism during other bacterial infections, particularly in the gastrointestinal system, could provide additional insights into their immunopathogenesis. ARG2 could potentially serve as a target for intervention in mucosal infections, allowing for an enhanced immune response to prevent bacterial survival within the host.

Methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All reagents used for cell culture were from Invitrogen (Carlsbad, CA, USA). RNA extraction reagents were from Qiagen (Valencia, CA, USA), and reagents utilized for cDNA synthesis and RT-PCR were from Bio-Rad (Hercules, CA, USA). Recombinant macrophage colony stimulating factor (M-CSF) was purchased from Peprotech (Rocky Hill, NJ, USA). Protease Inhibitor Cocktail, Set III and Phosphatase Inhibitor Cocktail, Set I were purchased from Calbiochem (Darmstadt, Germany). BCA protein assay was from Pierce Biotechnology (Rockford, IL, USA).

Bacteria, cells, cell culture conditions and infections

Helicobacter pylori strains PMSS1 and SS1 were grown as previously described (Chaturvedi et al. 2010; Lewis et al. 2011). French-pressed lysates were prepared as described (Asim et al. 2010).

Bone marrow-derived macrophages (BMmacs) were isolated from all mouse genotypes utilized in this study. BMmacs were differentiated as previously described (Weischenfeldt and Porse 2008), but with the following exceptions: red blood cells were lysed with ammonium-chloride-potassium (ACK) lysing buffer for 3–5 min, and recombinant murine M-CSF was utilized at a concentration of 20 ng/mL over the course of 7 days.

All co-culture experiments were performed in an antibiotic-free Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), 2 mM l-glutamine, 25 mM HEPES, and 10 mM sodium pyruvate. BMmacs were infected at a multiplicity of infection (MOI) of 100, for all *H. pylori* experiments in vitro.

Animal studies

WT and *Arg2*$^{-/-}$ mice were utilized as previously described (Lewis et al. 2011). *Nos2*$^{-/-}$ mice were initially purchased from Jackson Laboratories (Farmington, CT, USA) and then bred in-house for all animal experiments. *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice were generated by crossing the *Arg2*$^{-/-}$ and *Nos2*$^{-/-}$ mice. See Supplemental Table 1 for primers used for genotyping. Male mice between the ages of 6–12 weeks at the time of infection were used for all studies, regardless of genotype, and mice were not moved from the cages into which they were weaned. Male mice were selected based on previous data indicating that female mice were protected from *H. pylori*-induced histologic gastritis (Sheh et al. 2011). Sample sizes were based on previous studies from our laboratory (Chaturvedi et al. 2010; Gobert et al. 2014; Lewis et al. 2011). All mice were randomly selected for control and experimental groups.

Mice were orogastrically infected with 5×10^8 CFUs of *H. pylori* SS1, every other day for a total of three inoculations. Mice were killed 4 months post-inoculation and their stomachs divided longitudinally into four sections for additional studies. Colonization was assessed by serial dilution and culture. Histologic gastritis was assessed by a gastrointestinal pathologist

(M.B.P.), using the Sydney System (Gobert et al. 2014). All scoring was performed in a blinded manner.

Real-time polymerase chain reaction

RNA was isolated from tissue and BMmacs using the RNeasy kit. cDNA was prepared using the iScript cDNA synthesis kit with 1 μg of RNA. RT-PCR was performed as described (Gobert et al. 2014). See Supplemental Table 1 for all primers utilized in this study.

Western blot analysis

Tissue and BMmacs were lysed in CellLytic MT Reagent supplemented with Protease Inhibitor Cocktail (Set III) and Phosphatase Inhibitor Cocktail (Set I). Protein concentration was determined by BCA protein assay. The following antibodies were utilized in this study: Rabbit polyclonal anti-NOS2 (1:5000; EMD Millipore), Rabbit polyclonal anti-ARG2 (1:1000; Santa Cruz Biotechnology), Mouse monoclonal anti-β-actin (1:10,000; Sigma-Aldrich), Goat anti-mouse IgG, HRP labeled (1:30,000; Sigma-Aldrich), and Goat anti-rabbit IgG, HRP labeled (1:3000–1:6000; Sigma-Aldrich).

Luminex assay

A 25-plex assay (EMD Millipore, Cat. MCYTOMAG-70K-PMX, Billerica, MA, USA) was performed on gastric tissues from control and *H. pylori* SS1-infected mice from all genotypes. A 25-plex assay was also performed on supernatants from splenic T-cell cultures. Protein isolation, quantification and Luminex assay were performed as previously described (Coburn et al. 2013).

Measurement of nitric oxide

The concentration of nitrite (NO_2^-), the oxidized metabolite of NO, was assessed by the Griess reaction as previously described (Chaturvedi et al. 2010).

Flow cytometry

Flow cytometry was performed as previously described (Lewis et al. 2011). The following antibodies were utilized in this study: Anti-mouse CD4-PerCp-Cy5.5 (1:200; Biolegend), and Anti-mouse IFN-γ-FITC (1:100; BD Biosciences).

Measurement of polyamines

Polyamines were measured from gastric tissue lysates by high-performance liquid chromatography as previously described (Chaturvedi et al. 2010).

Splenocyte isolation

Splenocytes were isolated from the spleens of control and infected WT and *Arg2*$^{-/-}$ mice. Red blood cells were lysed with ACK for 3–5 min. Splenocytes utilized for T-cell studies were plated in 96-well plates coated with anti-CD3 antibody and stimulated with anti-CD28 for 24 h. Cells were then stimulated with *H. pylori* SS1 French-pressed lysate for 4 days. Luminex assay and flow cytometry were then performed. Splenocytes utilized for

macrophage studies were plated in 96-well plates without anti-CD3. Adherent cells were then stimulated with *H. pylori* French-pressed lysate for 24 h.

Statistical analysis

All data shown in this study represent the mean. The n for each experiment is listed in the figure legends. Where data were normally distributed, Student's t test was used to determine significance in studies with only two groups, and one-way ANOVA with a NewmanKeuls post-test was used to determine significance in studies with multiple groups. Where data were not normally distributed, one-way ANOVA with a Kruskal–Wallis test, followed by a Mann–Whitney U test was performed. All statistical tests for each experiment are listed in the figure legends. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). A p value of <0.05 was considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Compliance with ethical standards

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Abbreviations

AMD1	Adenosylmethionine decarboxylase 1
ARG1	Arginase 1; arginase, type I
ARG2	Arginase 2; arginase, type II
CCL	C–C chemokine ligand
CD	Cluster of differentiation
CHIA1	Chitinase, acidic 1
CXCL	C–X–C chemokine ligand
FPL	French-pressed lysate
IFN-γ	Interferon gamma

IL	Interleukin
M-CSF	Macrophage colony stimulating factor
MOI	Multiplicity of infection
NO	Nitric oxide
NOS2	Nitric oxide synthase 2; inducible nitric oxide synthase
ODC	Ornithine decarboxylase
PMSS1	Pre-mouse Sydney Strain 1
RT-PCR	Real-time polymerase chain reaction
RETNLA	Resistin like molecule alpha
SAT1	Spermidine/spermine N^1-acetyltransferase 1
SMOX	Spermine oxidase
SS1	Sydney Strain 1
TGF	Transforming growth factor
Th	T helper
TNF	Tumor necrosis factor
TNFSF14	Tumor necrosis factor superfamily member 14

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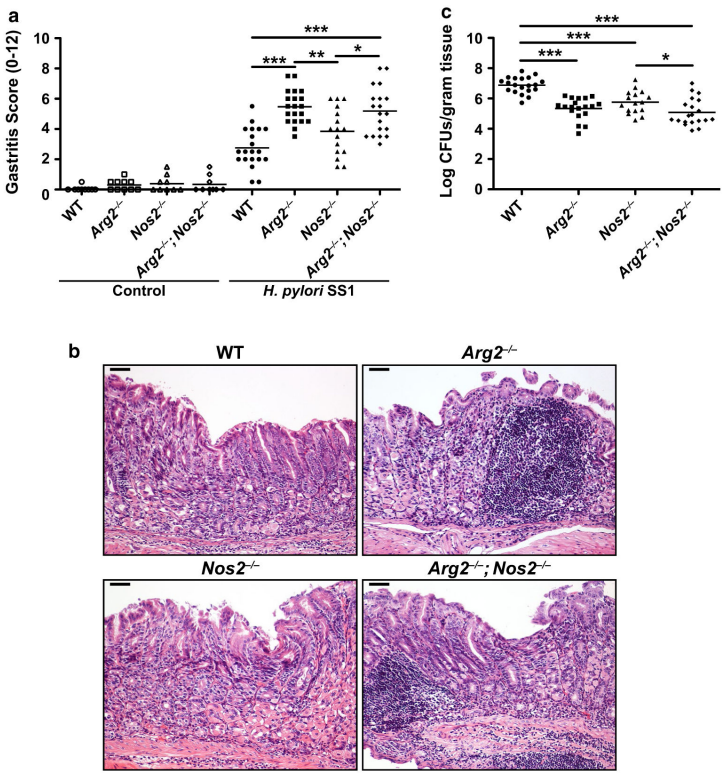

Fig. 1.

Effect of *Arg2* and/or *Nos2* knockout on *H. pylori* pathogenesis after chronic infection. WT, $Arg2^{-/-}$, $Nos2^{-/-}$, and $Arg2^{-/-};Nos2^{-/-}$ mice were infected with *H. pylori* Sydney Strain 1 (SS1) for 4 months. Mice were killed and their stomachs were isolated for analysis. **a** Histologic gastritis was assessed in a blinded manner according to the Sydney System by a gastrointestinal pathologist. $n = 9–10$ uninfected and 16–20 *H. pylori* SS1-infected mice per genotype. Statistical significance was determined by one-way ANOVA with Kruskal–Wallis test, followed by Mann–Whitney *U* test. **b** Representative images of hematoxylin and eosin-stained tissues, from *H. pylori*-infected mice of each genotype. *Scale bars* 100 μM. **c** *H. pylori* colonization was assessed by serial dilution and culture. $n = 16–20$ *H. pylori* SS1-infected mice per genotype. Statistical significance was determined by one-way ANOVA with NewmanKeuls post-test. In **a** and **c**, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

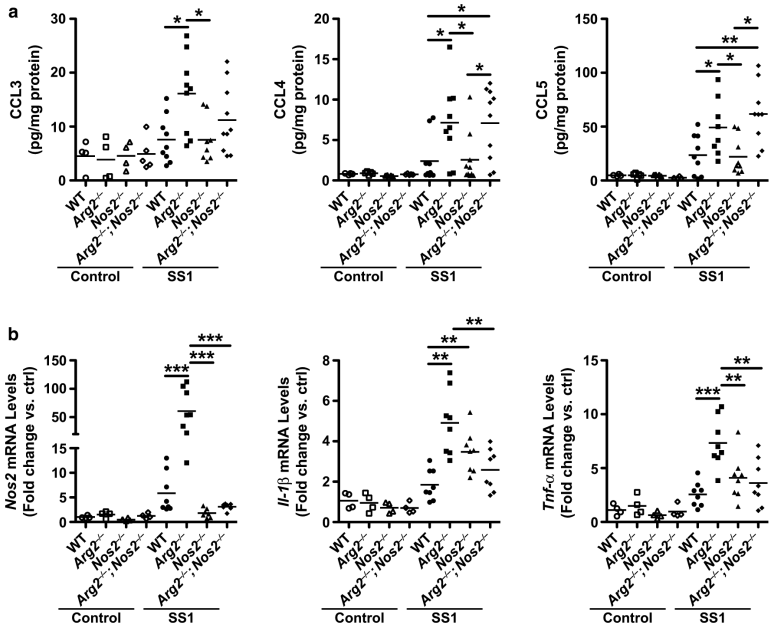

Fig. 2.

Loss of ARG2 leads to enhanced pro-inflammatory innate immune responses to *H. pylori*. **a** Protein was isolated from gastric tissues from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice 4 months post-inoculation (p.i.). with *H. pylori* SS1. Protein levels of the immune cell-derived chemokines, CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) were assessed by Luminex assay. $n = 4$–5 uninfected and 8–10 *H. pylori* SS1-infected mice per genotype. Statistical significance was determined by one-way ANOVA with Kruskal–Wallis test, followed by Mann–Whitney *U* test. **b** RNA was isolated from gastric tissues from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice 4 months p.i. with *H. pylori* SS1. The proinflammatory markers, *Nos2*, *Il-1β*, and *Tnf-α*, were assessed by RT-PCR. $n = 4$ uninfected and 7–8 *H. pylori* SS1-infected mice per genotype. Statistical significance was determined by one-way ANOVA with Kruskal–Wallis test, followed by Mann–Whitney *U* test. In **a** and **b**, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

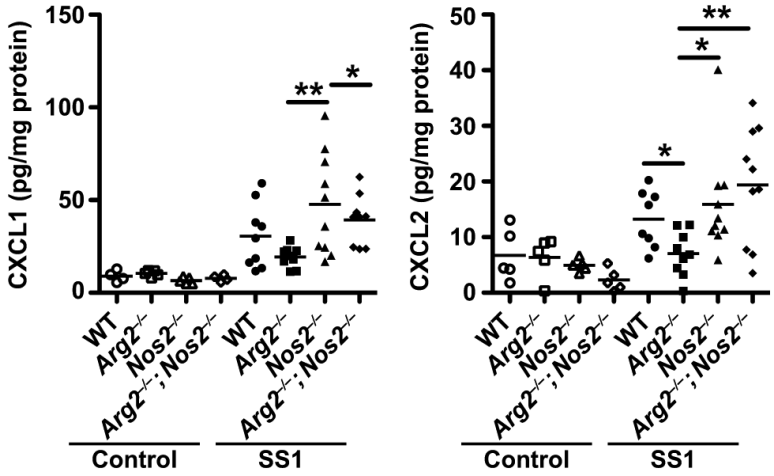

Fig. 3.

Loss of NOS2 leads to enhanced epithelial cell-derived chemokine production. Protein was isolated from gastric tissues from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice 4 months p.i. with *H. pylori* SS1. Levels of the epithelial cell-derived chemokines, CXCL1 (KC) and CXCL2 (MIP-2), were assessed by Luminex assay. $n = 4–5$ uninfected and 9–10 *H. pylori* SS1-infected mice per genotype. Statistical significance was determined by one-way ANOVA with Kruskal–Wallis test, followed by Mann–Whitney *U* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

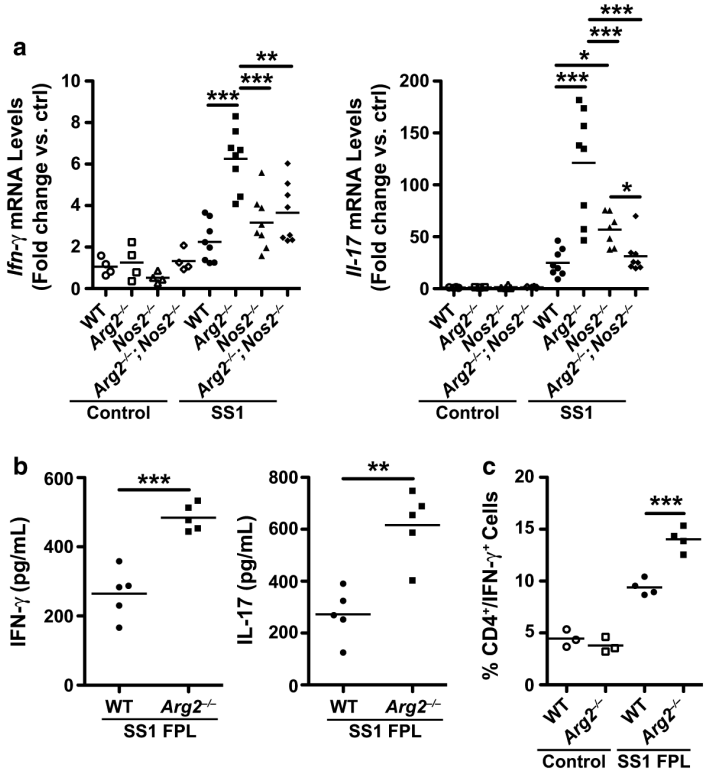

Fig. 4.

Loss of ARG2 results in diminished Th1/Th17 T-cell differentiation in response to *H. pylori*. **a** RNA was isolated from gastric tissues from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$;*Nos2*$^{-/-}$ mice 4 months p.i. Expression of the Th1 marker, *Ifn-γ*, and the Th17 marker, *Il-17a*, was assessed by RT-PCR. $n = 4$ uninfected and 8 *H. pylori* SS1-infected mice per genotype. Statistical significance was determined by one-way ANOVA with Kruskal–Wallis test, followed by Mann–Whitney *U* test. **b** Splenocytes were isolated from WT and *Arg2*$^{-/-}$ mice 4 months p.i. and cultured in the presence of CD3 and CD28. The splenic T-cells were then stimulated with *H. pylori* SS1 French-pressed lysate (FPL) for 24 h. Protein levels of secreted IFN-γ (Th1) and IL-17 (Th17) were assessed by Luminex assay on the splenic T-cell supernatants. $n = 5$ mice per genotype. Statistical significance was determined by Student's *t* test. **c** Splenic T-cells were generated as in **b**. The CD4$^+$IFN-γ$^+$ population of Th1 cells was assessed by flow cytometry. $n = 3$ uninfected and 4 *H. pylori* SS1-infected mice per genotype. Statistical significance was determined by one-way ANOVA with NewmanKeuls post-test. In **a**–**c**, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

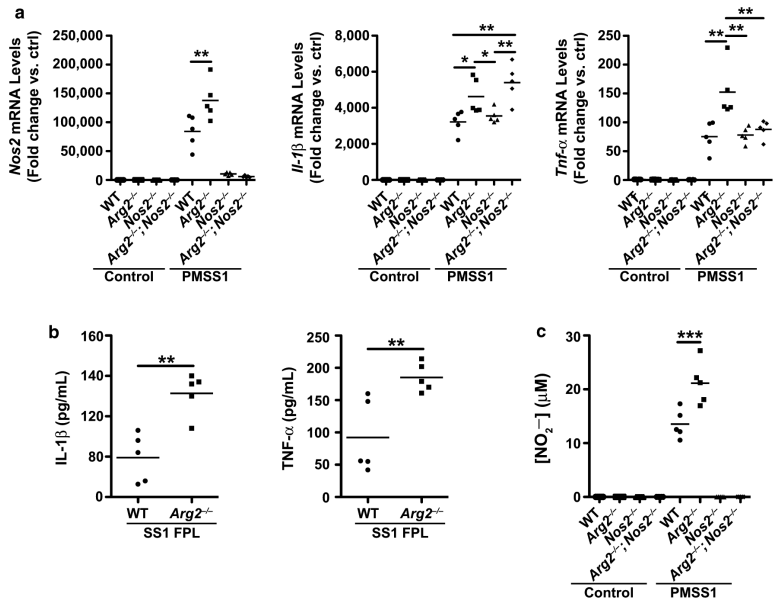

Fig. 5.

Arg2 knockout leads to super-induction of M1 macrophage activation in response to *H. pylori*. **a** Bone marrow-derived macrophages (BMmacs) were isolated from WT, $Arg2^{-/-}$, $Nos2^{-/-}$, and $Arg2^{-/-};Nos2^{-/-}$ mice and stimulated with *H. pylori* Pre-Mouse Sydney Strain 1 (PMSS1) ex vivo for 24 h. Markers of M1 macrophage activation, *Nos2*, *Il-1β*, and *Tnf-α*, were assessed by RT-PCR. $n = 5$ biological replicates per genotype. Statistical significance was determined by one-way ANOVA with NewmanKeuls post-test. **b** Splenic macrophages were isolated from WT and $Arg2^{-/-}$ mice 4 months p.i. and stimulated ex vivo with SS1 FPL. Protein levels of secreted IL-1β and TNF-α, M1 macrophage effector proteins, were assessed by Luminex assay on the splenic macrophage supernatants. $n = 5$ mice per genotype. Statistical significance was determined by Student's *t* test. **c** BMmacs were isolated as in **a**. Levels of the oxidized NO metabolite, NO_2^-, was assessed by the Griess reaction. $n = 5$ biological replicates per genotype. Statistical significance was determined by one-way ANOVA with NewmanKeuls post-test. In **a**–**c**, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

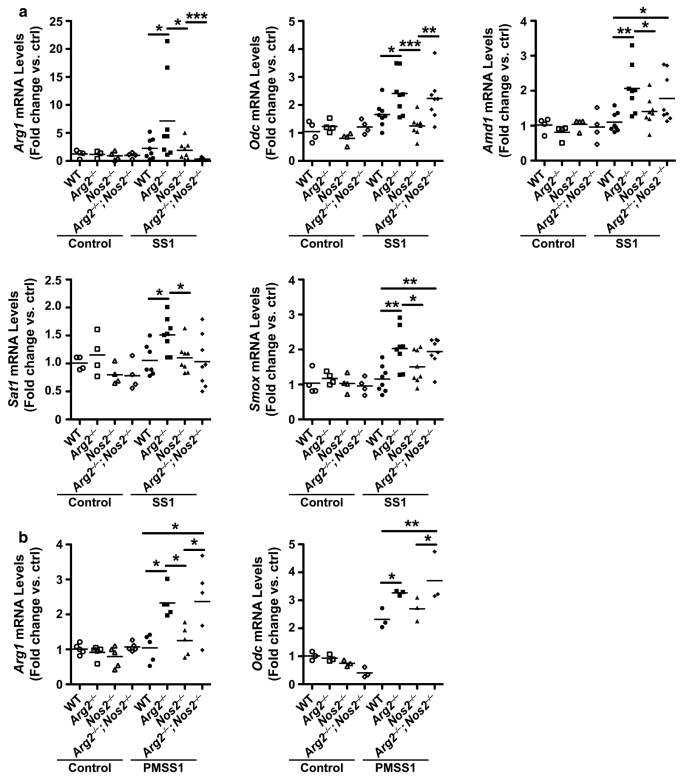

Fig. 6.

Loss of ARG2 leads to a compensatory upregulation in polyamine metabolic enzyme expression in response to *H. pylori*. **a** RNA was isolated from gastric tissues from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice 4 months p.i. mRNA levels of *Arg1, Odc*, *Amd1*, *Sat1*, and *Smox* were assessed by RT-PCR. n = 3–4 uninfected and 7–8 *H. pylori* SS1-infected mice per genotype. Statistical significance was determined by one-way ANOVA with Kruskal–Wallis test, followed by Mann–Whitney *U* test. **b** RNA was isolated from BMmacs from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice 24 h (*Arg1*) and 6 h (*Odc*) p.i. mRNA levels of *Arg1* and *Odc* were assessed by RT-PCR. n = 3–5 biological replicates per genotype. Statistical significance was determined by one-way ANOVA with NewmanKeuls post-test. In **a** and **b**, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

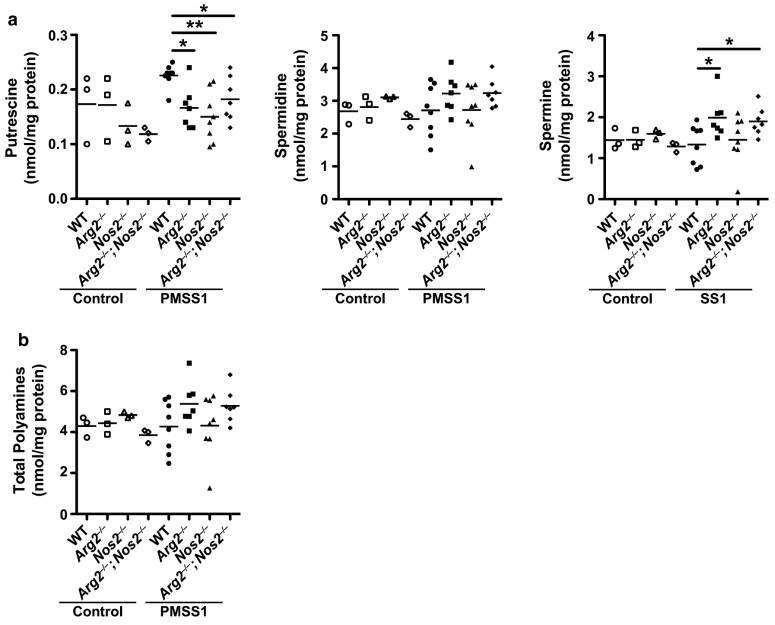

Fig. 7.

Loss of ARG2 increases polyamine levels in response to *H. pylori*. **a** Levels of putrescine, spermidine, and spermine were assessed by high-performance liquid chromatography in gastric tissues 4 months p.i. from WT, *Arg2*$^{-/-}$, *Nos2*$^{-/-}$, and *Arg2*$^{-/-}$*;Nos2*$^{-/-}$ mice. $n = 3$ uninfected and 7–8 *H. pylori* SS1-infected mice per genotype. Statistical significance was determined by one-way ANOVA with Kruskal–Wallis test, followed by Mann–Whitney *U* test. *$p < 0.05$, **$p < 0.01$. **b** Total polyamine levels were calculated from the sum of putrescine, spermidine, and spermine in **a**