Pathogenesis of the Carcinogenic Bacterium, *Helicobacter pylori*

by

Chung-Wei Lee

M.D., Medicine (1998)

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Submitted to the Division of Biological Engineering

in Partial Fulfillment of the Requirement for the Degree of

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Pathogenesis of the Carcinogenic Bacterium, *Helicobacter pylori*

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Submitted to the Division of Biological Engineering on May 25, 2007 in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy Degree in Molecular and Systems Bacterial Pathogenesis

ABSTRACT

Gastric cancer is the second most common malignancy in the digestive system and the second leading cause of cancer-related death worldwide. Epidemiological data and experimental studies have identified several risk factors for gastric cancer, including *Helicobacter pylori* infection, low fruit and vegetable intake, N-nitrosoamine exposure, high salt diet, and smoking. Among these risk factors, *H. pylori* infection is the major cause of gastric cancer. Therefore, *H. pylori* has been classified as a type 1 (definite) carcinogen for gastric cancer by the World Health Organization (WHO) in 1994.

*H. pylori* colonizes the human stomach and has been definitively linked to chronic gastritis. Infection in some susceptible individuals results in serious gastric disease such as peptic ulcer or gastric cancer. The first aim of this thesis was to examine the role of different T cell subpopulations in *H. pylori* gastritis. Using a murine adoptive transfer model, adoptive transfer of wildtype (wt) effector T cells (T_E) into *H. pylori*-infected lymphopenic Rag2^{-/-} recipient mice resulted in *H. pylori*-associated corpus gastritis superimposed with non-specific gastroduodenitis. Cotransfer with T_E and regulatory T cells (T_R) from wt or IL10^{-/-} mice reduced gastroduodenitis, but only wt T_R cells reduced corpus gastritis. The second aim of this thesis was to evaluate the effect of vitamin C on *H. pylori* gastritis in vitamin C-deficient gulo^{-/-} mice. It was found that a high vitamin C supplementation (3300 mg/L) in drinking water did not protect *H. pylori* gastritis, while a low vitamin C supplementation (33 mg/L) reduced the severity of *H. pylori* gastritis via an attenuated cellular immune response to *H. pylori*. The third aim of this thesis was to examine the role of DNA repair in *H. pylori*-associated gastric disease. We found that *H. pylori*-associated premalignant gastric atrophy was more severe in infected mice lacking DNA repair protein 3-alkyladenine DNA glycosylase or O^6^-methylguanine DNA methyltransferase in comparison to
infected wt control mice. The forth aim of this thesis was to examine whether antimicrobial *H. pylori* eradication therapy could prevent gastric cancer development in INS-GAS mice, a model of gastric cancer. We found that antimicrobial *H. pylori* eradication therapy prevented the progression to gastric cancer in *H. pylori*-infected INS-GAS mice when treatment was instituted at an early stage of *H. pylori* infection.

In conclusion, these studies provide further insight into the role of host immune responses in *H. pylori* pathogenesis. Additionally, information was garnered regarding the roles of vitamin C supplementation, DNA repair proteins, and *H. pylori* eradication therapy in *H. pylori*-associated gastric disease using genetically manipulated mice.

Thesis Supervisor: James G. Fox
Title: Professor, Division of Biological Engineering
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§ Gastric cancer

Pathology

Adenocarcinoma (referred to as gastric cancer in this paper) accounts for about 85% of gastric malignancies (Harrison's Principles of Internal Medicine, 16th ed, McGraw-Hill). Gastric cancers are classified as cardiac and noncardiac according to anatomical sites. Histologically, gastric cancers are subdivided into two types: an intestinal type characterized by well-differentiated neoplasitic cells that form a tubular, glandular structure mimicking the intestinal glands and a diffuse type characterized by profound cell infiltration and poorly-differentiated cells lacking any glandular structure [1, 2]. The intestinal type is more common in males and older age groups. In contrast, the diffuse type is the same in both genders and more common in younger age groups. There has been no germline mutation linked to the intestinal type of gastric cancer. The diffuse type of gastric cancer is sometimes familial in distribution and has been associated with germline mutations of E-cadherin (CDH1) [3]. Thus, different etiologic factor(s) may be involved in each type of gastric cancer [4]. Histologically, the intestinal type of gastric cancer is usually found in areas of tissue with chronic inflammation and atrophy. Correa proposed a human model of gastric carcinogenesis with sequential changes beginning with superficial gastritis and progressing to chronic inflammation, atrophic gastritis, intestinal metaplasia, dysplasia, and finally gastric cancer [5].

Epidemiology of gastric cancer

Gastric cancer is the second most common malignancy in the digestive system and the 14th leading cause of death worldwide [6]. The incidence and mortality of gastric cancer have declined sharply in the past few decades. For example, there was an approximate 50% decrease
in the incidence of gastric cancer and gastric cancer-related mortality in the United States between 1975 to 2002 (http://crchd.cancer.gov/definitions/statistics.html). Despite this, gastric cancer remains the second leading cause of cancer-related death worldwide and is still a serious epidemiological problem in the 21st century [7]. It has been estimated that there were more than 870,000 deaths from gastric cancer in the year 2000, accounting for approximately 12% of all cancer-related mortality [4]. The survival rate for gastric cancer is poor and depends on the stage of disease at the time of diagnosis. Because early gastric cancer usually does not present clinically, screening and treatment are difficult. Thus, the stage of gastric cancer at diagnosis is usually advanced, making the prognosis poor with an overall 5-year survival rate less than 25% [8].

There is a marked geographic variation in the incidence of gastric cancer. Gastric cancer incidence is relatively low in Western industrialized countries such as the United States and the United Kingdom. Despite the decrease in gastric cancer incidence worldwide, the incidence of gastric cancer remains high in Japan, China, and Chile [4]. The International Agency for Research of Cancer (IARC) reported in 1997 that the age-adjusted incidence of gastric cancer death in males ranged from the lowest in Caucasians in the United States (7.5 per 100,000) to the highest in Yamagata, Japan (95.5 per 100,000) [7].

There are significant differences in the risks of gastric cancer in different ethnic groups in the same regions [7]. In the United States, gastric cancer incidence in blacks was approximately twice that in whites between 1998-2002 (Ries LAG, Eisner MP, Kosary CL, Hankey BF, Miller BA, Clegg L, Mariotto A, Feuer EJ, Edwards BK (eds). SEER Cancer Statistics Review, 1975-
Gender variations in the incidence of gastric cancer have also been observed; both cardia and noncardia gastric cancers are more common in males than females [7].

**Risk factors**

Epidemiological data and experimental studies have identified risk factors for gastric cancer, including *Helicobacter pylori* infection, N-nitrosoamine exposure, high salt diet, low fruit and vegetable intake, and smoking [9]. Since *H. pylori* was first reported in 1983 [10], the role of this microorganism in gastric inflammation and carcinogenesis has been extensively studied. *Helicobacter pylori*, which infects approximating 50% of the population worldwide [11], is highly associated with gastric carcinogenesis. It has been demonstrated in an international study that there is a direct correlation between high incidence of gastric cancer and high prevalence of *H. pylori* infection [12]. Therefore, *H. pylori* has been classified as a type 1 (definite) carcinogen for gastric cancer by the World Health Organization (WHO) in 1994 [13]. Interestingly, the incidence of gastric cancer is low in some areas of Africa despite a high prevalence of *H. pylori* infection. This has been referred to as the "African enigma" [14].

§ *Helicobacter pylori*

**History**

Spiral-shaped microorganisms have been observed in gastric tissue for more than a century [15, 16]. Doenges reported gastric spiral bacteria in 43% of human samples and 100% of rhesus macaque samples in 1939 [16]. In a 1975 report, spiral-shaped bacteria were observed in
inflamed human gastric mucosa and were associated with polymorphonuclear leukocyte infiltration [17]. A gram-negative, spiral-shaped, microaerophilic, and flagellate microorganism was first isolated and identified in the antrum of patients with chronic gastritis, gastric ulceration and duodenal ulceration by Marshall and Warren in 1982 [10]. This bacterium was initially named *Camphylobacter pyloridis* but was later renamed *Camphylobacter pylori* [18]. Due to its differing 16S rRNA gene sequence and sheathed flagella, nomenclature was further changed in 1989 to *Helicobacter pylori* to reflect a new genus [19]. *H. pylori* experimental infection in human volunteer, who had histologically normal gastric mucosa before infection, resulted in acute pyloric gastritis within 10 days post infection [20]. These findings were subsequently reproduced when an individual dosed himself with *H. pylori* and developed chronic gastritis as a result of the bacterial infection [21]. Thus, *H. pylori* satisfies Koch's postulates as a causative microbe of human gastritis.

*H. pylori* infection is mainly acquired in childhood and usually persists for life within the gastric mucosa [22]. Many gastric diseases, including gastritis, peptic ulcer, atrophic gastritis, intestinal metaplasia, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (MALToma), have been linked to *H. pylori* infection epidemiologically in humans and experimentally in mice and other laboratory animal models [9]. Gastric cancer develops predominantly in *H. pylori*-infected humans. Among these infected individuals, those with duodenal ulcer are not at risk to develop gastric cancer; those with gastric ulcer or corpus-predominant gastritis, severe gastric atrophy, or intestinal metaplasia are at increased risk of gastric cancer [23]. Most infected humans remain asymptomatic; only 15% will develop peptic ulcers and less than 1% will develop gastric cancer [24].
Animal models of *H. pylori* infection

Neonatal gnotobiotic piglets were the first reported animal model of *H. pylori* infection [25]. *H. pylori* was also reported to colonize and cause gastritis in Japanese monkeys [26] and Mongolian gerbils [27]. In early experiments, *H. pylori* did not consistently colonize mice and other laboratory animals [27, 28]. As a result, *Helicobacter felis*, a gastric helicobacter closely related to *H. pylori* that reliably colonized mice, was used and continues to be used to study helicobacter-associated gastric disease in mouse models [29]. *H. felis* causes oxyntic gland atrophy, and gastric cancer in C57BL/6 mice by 15 months of infection, [30, 31]. A standardized mouse model of *H. pylori* experimental infection was introduced in 1997 [32]. *H. pylori* Sydney strain (SS1) contains the entire cytotoxin associated gene pathogenicity island (cag-PAI), reproducibly colonizes the inbred mouse strains C57BL/6, BALB/c, DBA/2, and C3H/He, and causes chronic active gastritis in C57BL/6 mice [32]. Additionally, guinea pigs colonized with *H. pylori* Sydney strain SS1 developed antral gastritis and mucosa associated lymphoid tissue (MALT) [33]. Experimental infection with another rodent-adapted, cag-PAI positive *H. pylori* strain, B128, resulted in gastritis, ulceration, and atrophy in Mongolian gerbils [34] and our substrain of B128 caused rapid onset of gastric cancer in male Mongolian gerbils [35]. This strain of *H. pylori* also and promoted gastric carcinogenesis in INS-GAS mice [36]. Recently, a third mouse-adapted *H. pylori* strain, Sydney 2000, that lacks the entire cag-PAI was reported to induce chronic gastritis in mice [37]. (Table 1)

Due to the difficulty of longitudinally following the carcinogenic process of gastric cancer in humans, various rodent models have been used to study the pathogenesis of helicobacter-
mediated gastric disease [38, 39]. *H. pylori* infection in Mongolian gerbils recapitulated gastric inflammation in humans and resulted in the sequential changes proposed by Correa [5, 40-42]. *H. pylori* (SS1) infection in C57BL/6 mice results in chronic and atrophic gastritis, but *H. pylori* infection alone does not reliably induce gastric cancer in these mice [37]. In contrast, *H. pylori* experimental infection has been used to study helicobacter-associated gastric carcinogenesis in the susceptible INS-GAS transgenic mice (on a FVB background) [43] and in B6129 mice [44].

(Table 1)
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</table>
Virulence factors and pathogenesis of *H. pylori*

The *H. pylori* genome (1.65 Mbp) encodes about 1500 proteins [45, 46]. The genome changes continuously during chronic colonization in an individual host by importing small pieces of foreign DNA from other *H. pylori* strains by natural transformation [47] during persistent or transient mixed infection [48-51], theoretically facilitating adaptation of the organism to the gastric mucosa. The acidic environment, secretory antimicrobial peptides, and mucosal barrier provide protection for gastric tissue against bacterial infection [52]. However, after being ingested, *H. pylori* can evade the acidic, bactericidal environment of the gastric lumen by entering the mucosal layer. It does this by using urease to hydrolyze urea into carbon dioxide and ammonia, thereby neutralizing the acidic milieu and allowing colonization [27, 53]. In colonizing the gastric mucosa, multiple bacterial surface proteins aid the organism’s ability to attach tightly to epithelial cells [52]. For example, the well characterized adhesins, BabA and SabA, bind to the fucosylated Lewis B blood group antigen and sialyl-dimeric-Lewis x glycosphingolipid, respectively, on the gastric epithelial surface [54, 55]. Additionally, flagella of *H. pylori* are essential for motility and facilitate colonization of the gastric mucosa [56].

Most strains of *H. pylori* produce the vacuolating cytotoxin VacA, a secreted exotoxin [52]. The toxin is targeted to the cell membrane as well as to mitochondrial membranes, where it forms an anion-selective, voltage-gated channel [57]. Cytosolic bicarbonate, organic anions and mitochondrial cytochrome c can be released through the VacA channel to induce apoptosis and subsequently increase the turnover rate of gastric epithelium [58-60]. The role of VacA in the pathogenesis of *H. pylori* is unclear. Although a VacA-positive strain of *H. pylori* was reported to have some growth advantage in initial host colonization over a VacA-negative isogenic strain,
VacA was not essential for *H. pylori* survival and had no effect on gastric inflammation [61]. Additionally, both VacA-negative and -positive *H. pylori* strains have been isolated from humans [62]. Some VacA-positive *H. pylori* strains are associated with more severe disease in Western countries, but this association has not been noted in Asian countries [63]. These reports suggest that VacA may facilitate the transmission of *H. pylori* by promoting the initial colonization in the host. Additionally, VacA blocks T cell proliferation by inducing a G1/S cell cycle arrest and results in down-regulation of IL-2 transcription [64], suggesting VacA-positive *H. pylori* strains evade host immune response and may reduce *H. pylori*-associated gastric inflammation.

Some strains of *H. pylori* have a pathogenicity island containing a cytotoxin-associated antigen A (cag-PAI). Pathogenicity islands are a distinct class of linked genes on the bacterial chromosome that correlate with pathogenicity [65]. The *H. pylori* cag-PAI is a 37-kb genomic fragment containing 29 genes and encoding a type IV secretion system [66]. Cytotoxin-associated antigen A (CagA) is a marker for the cag-PAI [66]. Most *H. pylori* strains are classified into two types: type I strains possess an entire cag-PAI and have been associated with more severe gastric disease; type II strains do not contain a complete cag-PAI and have been associated with milder gastric lesions [52].

*H. pylori* delivers the CagA protein into gastric epithelial cells through the type IV secretion system encoded by cag-PAI [67]. Translocation of CagA is essential for the induction of IL-8 up-regulation, formation of stress fibers, and a growth change phenotypically similar to that induced by hepatocyte growth factor [68, 69]. Additionally, CagA is tyrosine phosphorylated within the epithelium at the EPIYA domains by Src family kinases. Phosphorylated CagA binds to SHP-2
tyrosine phosphatase and provokes abnormal activation of SHP-2 and Erk kinase [70, 71]. Perturbation of the SHP-2 oncoprotein and other signaling molecules by CagA may predispose epithelial cells to gastric cancer progression [72]. Polymorphisms in the EPIYA domain of CagA are associated with differences in the magnitude and duration of CagA activities [73]. CagA polymorphisms may be useful markers to classify *H. pylori* strains into “benign” and “malignant” strains [74]. Due to the *in vitro* data showing that *H. pylori* CagA is essential for bacteria-mediated transformation of host cells [68, 72], the understanding of CagA polymorphisms may help to determine clinical guidelines for *H. pylori* treatment [73].

**Host immune responses to *H. pylori***

*H. pylori* induces expression of proinflammatory cytokines including IL-8 through activation of NF-κB in epithelial cells and macrophages [75, 76]. *H. pylori*-induced NF-κB activation in epithelial cells is mediated by NOD1 and dependent on a functional cag-PAI and bacteria-epithelia contact. In contrast, *H. pylori*-induced NF-κB activation in macrophages is mediated by the TLR4 pathway and independent of cag PAI or contact [77, 78] (Figure 1). In the acute phase of *H. pylori* infection, bacterial antigens stimulate maturation of antigen presenting cells like macrophages and dendritic cells, which then induce the differentiation of naive T lymphocytes into Th1 cells [79].
Figure 1. *H. pylori* activates NF-κB in epithelial cells and macrophages via different mechanisms.

*H. pylori* activates epithelial cells depending on direct contact and through NOD1 pathway. *H. pylori* activates macrophages in a contact-independent manner and through TLR4 pathway [77, 78]. NF-κB activation is the common down-stream pathway that mediates *H. pylori* activation of epithelial cells and macrophages.

![Diagram](image)

NF-κB activation

Though innate immunity is activated during *H. pylori* infection, the innate immune system alone is not sufficient to induce *H. pylori*-associated gastritis. *H. pylori*-infected wildtype (wt) C57BL/6 mice, that have functional B and T cells, develop robust helicobacter gastritis [32]. Lymphopenic B6.129S7-Rag1<sup>tm1Mom</sup> and B6.CB17-Prkdc<sup>scid</sup>/SzJ (SCID) mice, and T cell-deficient B6.129P2-Tcrb<sup>tm1Mom</sup>-<sup>tm1Mom</sup> mice are colonized at a high density with *H. pylori* and *H. felis*, but developed only minimal gastritis [80, 81]. Conversely, *H. pylori*-infected, B cell-deficient C57BL/6-Igh-6<sup>tm1Cgn</sup> mice developed identical gastric lesions observed in infected wt
C57BL/6 mice [81]. These data indicate that T lymphocytes are critical in inducing helicobacter-associated gastric inflammation.

*H. pylori* induces a Th1-predominant cellular immune response in humans and mice characterized by activation of CD4+ T helper lymphocytes, production of pro-inflammatory Th1 cytokines such as IFN-γ, and down-regulated Th2 cytokine expression such as IL-4 [82, 83]. Reconstitution of T lymphocytes in helicobacter-infected SCID mice with Th1-predominant lymphocytes induced more severe gastritis than those reconstituted with Th2-predominant lymphocytes [84, 85]. (Figure 2)

**Figure 2. Pathogen–Host Interactions in the Pathogenesis of Helicobacter pylori Infection.**

The host response to *H. pylori* participates in the induction of damage to the gastric epithelium and therefore has an integral role in *H. pylori* pathogenesis. During the early phase of the infection, binding of *H. pylori* to gastric epithelial cells, in particular through BabA and by strains harboring the cag pathogenicity island, results in the production of interleukin-8 and other chemokines, such as epithelial-cell-derived neutrophil-activating peptide 78 (ENA-78) and growth-related oncogene α(GRO-α), by epithelial cells. Nuclear factor-κB (NF-κB) and the early-response transcription-factor activator protein 1 (AP-1) are the intracellular messengers involved in this process. The chemokines secreted by epithelial cells bind to the proteoglycan scaffolding, generating a gradient along which polymorphonuclear cells (PMN) are recruited. The chronic phase of *H. pylori* gastritis combines an adaptive lymphocyte response with the initial innate immune response. Lymphocyte recruitment is facilitated by chemokine-mediated expression of vascular addressins such as vascular-cell adhesion molecule 1 (VCAM-1) and intercellular...
adhesion molecule 1 (ICAM-1) that are required for lymphocyte extravasation. Macrophages that participate in interleukin-8 production produce proinflammatory cytokines involved in the activation of the recruited cells, in particular T helper cells (Th0, Th1, Th2), that respond with a biased Th1 response to *H. pylori*. In turn, Th1-type cytokines such as interferon-γ (INF-γ) induce the expression of class II major histocompatibility complexes (MHC) and accessory molecules B7-1 and B7-2 by epithelial cells, making them competent for antigen presentation. The cytotoxin VacA- and Fas-mediated apoptosis induced by tumor necrosis factor α (TNF-α) leads to disruption of the epithelial barrier, facilitating translocation of bacterial antigens and leading to further activation of macrophages. Cytokines produced by macrophages can also alter the secretion of mucus, contributing to *H. pylori*-mediated disruption of the mucous layer. Cytokines produced in the gastric mucosa induce changes in gastric-acid secretion and homeostasis (dashed lines). TNF-α, interleukin-1β, and interferon-γ increase gastrin release, stimulating parietal and enterochromaffin cells and thus acid secretion. TNF-α also induces a decrease in the number of antral D cells, leading to decreased somatostatin production and indirectly enhancing acid production. LPS denotes lipopolysaccharide [52].
Figure 1. Pathogen–Host Interactions in the Pathogenesis of *Helicobacter pylori* Infection. (Suerbaum and Michetti, N Engl J Med, 2002)
Recent data has demonstrated that different subpopulations of CD4\(^+\) T lymphocytes play diverse roles in mediating and regulating \textit{H. pylori}-induced gastritis. In SCID mice, adoptive transfer of wt CD4\(^+\)CD45RB\(^{Hi}\) T effector (T\(E\)) cells from naïve donors causes severe gastritis in \textit{H. pylori}-infected recipients, while co-transfer of wt CD4\(^+\)CD45RB\(^{Lo}\) T regulatory (T\(R\)) cells protects against development of gastritis [84]. T\(R\) cells have also been defined by expression of the IL-2 receptor \(\alpha\) chain and Foxp3 [86], the forkhead transcription factor critical for thymic selection of CD4\(^+\)CD25\(^+\) T\(R\) cells [87]. Depletion of CD25\(^+\)Foxp3\(^+\) T\(R\) cells in \textit{H. pylori}-infected C57BL/6 mice is reported to cause loss of immune regulation and more severe gastritis [88] as does adoptive transfer of lymphocytes depleted of CD4\(^+\)CD25\(^+\) cells into \textit{H. pylori}-infected B6.Cg-Foxn1\(^{nu}\) (nu/nu) recipients [89]. Of the many T cell subsets with ascribed regulatory function [90], cell sorting experiments commonly use CD4\(^+\)CD25\(^+\)CD45RB\(^{Lo}\) as naturally occurring T\(R\) cells. However, the mechanism(s) for regulation by this type of T\(R\) cell are not fully understood.

Reconstitution of CD4\(^+\)CD25\(^-\)CD45RB\(^{Hi}\) T\(E\) cells and CD4\(^+\)CD25\(^-\)CD45RB\(^{Lo}\) T\(R\) cells in \textit{H. pylori}-infected B6.129S6-Rag2\(^{tm1Fwa}\) (Rag2\(^{-/-}\)) mice demonstrates that wt T\(E\) cells mediate a gastroduodenitis in Rag2\(^{-/-}\) mice independently of \textit{H. pylori} infection and that co-transfer of wt T\(R\) cells suppresses this lesion to a greater extent than IL10\(^-/-\) T\(R\) cells. Only wt T\(R\) cells suppress additive corpus gastritis attributable to \textit{H. pylori} infection [91]. These results indicate that IL10-competent T\(R\) cells are pivotal to contain immune response to \textit{H. pylori}-associated gastritis (discussed in Chapter 2).

**Persistent infection, chronic inflammation, and cancer**

The link between inflammation and cancer has been recognized since 1863 [92]. The paradigm of persistent infection leading to chronic inflammation and subsequent carcinogenesis is...
supported by some chronic infection-associated malignancies, including *Schistosoma*-induced squamous cell carcinoma of the urinary bladder, liver fluke-induced cholangiocarcinoma or liver cancer, viral hepatitis-induced hepatocellular carcinoma, and *H. pylori*-induced gastric cancer [13, 93].

Chronic inflammation results in persistent damage to tissue and biomolecules of all types including DNA, protein, lipid, and carbohydrate due to the reactive oxygen and nitrogen species (RONS) from inflammatory cells [94]. The DNA damage, cell proliferation, and tissue remodeling in an inflammatory microenvironment may increase the risk of neoplasia due to tumor initiation. Moreover, inflammatory cells also secrete proinflammatory cytokines, chemokines, and growth factors that may affect survival, growth, proliferation, and differentiation of cells [92]. Thus, persistent infection leads to a chronic inflammatory milieu affording promotion and progression of initiated cells [95].

The mechanism of *H. pylori*-associated carcinogenesis has not been well characterized. Since *H. pylori* infection usually persists lifelong and causes chronic active inflammation, the missing link between *H. pylori* infection and gastric cancer may reside in the helicobacter-induced inflammation. The paradigm of persistent infection, chronic inflammation, and cancer may extend to *H. pylori*-associated gastric carcinogenesis and provide another approach for gastric cancer prevention. Since host Th1, but not Th2, immune responses are responsible for *H. pylori*-associated gastritis [82-85], Fox *et al* examined the effect of modulating the Th1 response of mice to *H. felis* infection using a mouse model of parasitic infection that causes a Th2 immune response [96]. In C57BL/6 mice coinfected with helminths and *H. felis*, reduced systemic Th1
later observed that acute *H. pylori* infection resulted in transient hypochlorhydria and a persistent decrease of vitamin C in gastric juice. Furthermore, the total vitamin C concentration in gastric juice became elevated after successful *H. pylori* antimicrobial eradication therapy in patients with intestinal metaplasia or duodenal ulcer [102, 103]. It is not clear whether the decreased levels of ascorbic acid in gastric juice is due to dietary vitamin C, impaired secretion of vitamin C by gastric mucosa, hypochlorhydria, increased oxidative stress in the stomach, or *H. pylori* infection *per se*. It appears that a very complex interaction exists between *H. pylori* infection, severity of gastric lesions, and ascorbic acid metabolism [104].

Using the vitamin C-deficient B6.129P2-Gulo<sup>+/+</sup> mice lacking L-gulono-γ-lactone oxidase, we observed that high vitamin C supplementation (3300 mg/L) in drinking water correlated with physiologically high vitamin C levels in plasma and gastric tissue. These levels of vitamin C did not protect *H. pylori*-infected gulo<sup>−/−</sup> mice from helicobacter-associated gastric disease. Low vitamin C (33 mg/ml)-supplemented *H. pylori*-infected gulo<sup>−/−</sup> mice had physiologically low vitamin C levels in plasma and gastric tissue, and less severe gastric lesions compared to high vitamin C-supplemented infected gulo<sup>−/−</sup> mice (discussed in Chapter 3). The systemic *H. pylori*-specific IgG isotype responses and gastric proinflammatory cytokine responses directly correlated with vitamin C levels in plasma and gastric tissue, indicating that a physiologically low vitamin C supplementation may impair the immune response against *H. pylori* infection and thus reduce severity of gastritis.

In a human epidemiological study comparing populations in New Orleans, LA and Colombia, S.A., populations in Colombia had higher *H. pylori* prevalence, gastric cancer incidence, and
immune responses and lower levels of Th1-mediated gastric cytokines were associated with increased *H. felis* colonization levels, attenuated gastric inflammation, and less severe premalignant lesions [96]. The findings strongly suggest that *H. pylori* infection results in chronic gastric inflammation that is largely responsible for the early stages of disease progression and may promote gastric carcinogenesis [97]. These results in the C57BL/6 mice coinfected with helminths and *H. felis* may also in part explain the "African enigma" where the incidence of gastric cancer is low in some African counties where parasitic infections are common despite a high prevalence of *H. pylori* infection [14].

§ Diet and environmental factors

Ascorbic acid

Vitamin C, the water soluble ascorbic acid, is essential for maintaining health. Deficiency in vitamin C causes scurvy which presents as spongy gums, subcutaneous and mucosal bleeding, and eventually death [98]. Dietary ascorbic acid is usually oxidized to dehydroascorbic acid (DHAA) in the stomach, absorbed in the small intestine, and reduced to ascorbic acid in the circulation. Circulating ascorbic acid is accumulated in leukocytes and actively secreted into gastric juice; excessive ascorbic acid is excreted into urine [99].

It has been speculated for a long time that vitamin C might be important in preventing progression from gastritis to gastric cancer because vitamin C is an antioxidant that inhibits formation of gastric carcinogenic N-nitroso compounds *in vitro* (discussed below) [100]. Sobala GM and *et al* reported that levels of ascorbic acid in gastric juice were significantly reduced in patients with chronic gastritis and directly correlated with hypochlorhydria [101]. This group
vitamin C levels in serum, but significantly lower vitamin C levels in gastric juice compared to populations in New Orleans. Vitamin C levels in gastric juice were inversely correlated with severity of gastritis, atrophy, and gastric juice pH [104]. Our results in the gulo<sup>−/−</sup> mouse model may provide an explanation for the findings in Colombia [104]. High serum vitamin C levels afford robust immune and inflammatory responses to <i>H. pylori</i> and result in more severe gastric lesions and hypochlorhydria. Severe gastric mucosal damage may impair secretion of vitamin C into gastric juice; hypochlorhydria allows overgrowth of bacteria that catalyzes formation of more N-nitroso compounds that further consume vitamin C. Our results in the gulo<sup>−/−</sup> mouse model may also explain why protection against gastric cancer development was not observed in several clinical trials of vitamin C supplementation alone or in combination with other micronutrients in humans [105-107].

**N-nitroso compounds**

More than one hundred N-nitroso compounds are carcinogenic in laboratory animals [108-111]. N-nitroso compound-associated tumors have been observed in at least 39 animal species [112-114]. Although the causal link between N-nitroso compound exposure and human cancer has not been established [115, 116], these findings in laboratory animals strongly suggest that N-nitroso compounds may be carcinogenic to humans. Thus, N-nitroso compounds have been classified as carcinogenic for humans by IARC in 1987 [117-119].

N-nitroso compounds in the Swedish variant of moist oral smokeless tobacco (snus) have been directly associated with gastric cancer development in mouse models [120]. Exposure to snus alone increased the risk of gastric carcinoma in situ (CIS) in INS-GAS mice on a FVB
background. Exposure to snus and H. pylori infection synergistically increased the risk of CIS in wt FVB and INS-GAS mice [120]. These results suggest that snus is a gastric carcinogen in mice.

N-nitroso compounds are mainly from two sources: exogenous and endogenous. Exogenous sources are from intake of preformed N-nitroso compounds in the diet or environment. Endogenous sources are resulted from the formation of N-nitroso compounds in vivo from nitrosatable precursors or nitrosating agents [114]. The main site for endogenous N-nitroso compound formation is the stomach. N-nitroso compounds in the stomach are formed by two mechanisms: non-enzymatic and enzymatic [114]. Non-enzymatic formation of N-nitroso compounds requires the presence of nitrite, nitrosatable substrates, and acid (pH 1-3) [121]. In the United States, the average dietary intake of nitrate is 75 mg/day, mainly from vegetables or drinking water [122]. About 25% of the ingested nitrate is secreted into saliva, reduced to nitrite by oral bacteria, and then enters the stomach [122, 123]. The remainder of ingested nitrate is reduced to nitrite in the stomach by gastric bacteria [114, 124]. Nitrite and most secondary amines can form N-nitroso compounds in the presence of gastric acid [114]. Bacterial catalysis of N-nitrosation is another means of forming N-nitroso compounds. Bacteria, such as H. pylori, that possess nitrate reductase or nitrosating enzymes such as cytochrome cd1-nitrite reductase contribute to formation of N-nitroso compounds in the achlorhydric stomach [125, 126]. Additionally, nitrosating compounds can also be synthesized by enzymatic reaction in LPS-activated macrophages [127, 128]. Upregulated inducible nitric oxide synthase (iNOS) in activated macrophages mediates the generation of nitric oxide (NO) that reacts with superoxide (O_2) to form peroxynitrite (ONOO') that could be converted to nitrous anhydride (N_2O_3), a
potent nitrosating agent [129]. It has been demonstrated that *H. pylori* extract stimulates production of NO [130]. *H. pylori* infection leads to up-regulation of iNOS in the antrum accompanied with increased levels of nitrotyrosine, a marker for peroxynitrite [131]. These *in vivo* and *in vitro* findings strongly suggest that *H. pylori* infection may increase the production of N-nitroso compounds in the stomach. Due to continual exposure to N-nitroso compounds in food and water and synthesis of N-nitroso compounds by *H. pylori* catalysis or production of NO from inflammatory cells in the gastric mucosa, N-nitroso compounds have been proposed to be gastric carcinogenic in humans [132].

§ Host genetic factors

Although *H. pylori* is the major etiology of gastric cancer, most evidence suggests the importance of host genetic factors in the progression of gastric cancer [133]. Approximately 10% of gastric cancer cases show familial clustering [134, 135], and relatives of gastric cancer patients have a two- to three-fold increased risk of developing gastric cancer in both genders after controlling for *H. pylori* infection [136]. E-cadherin germline mutations were identified in familial, early-onset, poorly-differentiated diffuse-type gastric cancer [3]. Nevertheless, few specific genetic abnormalities responsible for the increased risk of intestinal-type gastric cancer have been identified. Some polymorphisms in inflammation-related or DNA repair genes have been associated with a high risk of gastric cancer in many populations throughout the world [133]. The association between gastric cancer and these polymorphisms is discussed below.

Polymorphisms of inflammation-related genes

Antrum-predominant gastritis or duodenitis retains normal or higher acid secretion, while corpus-
predominant gastritis is associated with gastric atrophy and hypochlorhydria. Since IL-1β is a pro-inflammatory cytokine and a powerful inhibitor of gastric acid secretion [137], these factors were examined for their association with gastric cancer. In Scotland and Poland, polymorphisms of IL-1β (IL-1β -31*C or -511*T) and its receptor antagonist IL-1RN (IL-1RN*2/*2) were first identified in populations with increased risk of gastric atrophy and cancer [138]. A higher risk of gastric cancer from populations in China, Japan and Taiwan has been associated with certain polymorphisms of genes in the IL-1 pathway, including IL-1, IL-1RN, and type 1 IL-1 receptor (IL-1RI) [139-141]. Polymorphisms of other pro-inflammatory cytokines, such as IL-2 (IL-2 -330 T to G), IL-4 (IL-4 -33 C to T), IL-8 (IL-8 -251 T to A), and TNF-α (TNF-α -308 G to A), also have been associated with a higher risk of gastric cancer [142-147]. Studies also identified the association between polymorphisms of IL-10 (low IL-10 haplotype base on polymorphisms -592, -819, or -1082), an anti-inflammatory cytokine, and a higher incidence of gastric cancer in Taiwan and other countries [142, 145, 147-150]. A combination of different genetic polymorphisms may have synergistic effects on gastric cancer risk. A recent study observed that polymorphisms of TNF-α and IL-10, when combined with polymorphisms of genes in IL-1 pathway, lead to a 27-fold or greater risk of gastric cancer [142]. These data indicate that polymorphisms of inflammation-related genes are important determinants of susceptibility to and severity of \textit{H. pylori}-associated gastritis and gastric cancer [151].

**Polymorphisms of DNA repair genes**

\textit{H. pylori} gastritis may lead to increased oxidative DNA damage due to the production of RONS during chronic inflammation [94]. RONS cause DNA damage via (i) direct base oxidation and deamination and (ii) indirect alkylation to form ε-base lesions by 4-hydroxyalkenals through
lipid peroxidation [152-154]. Increased levels of oxidative DNA damage, double-strand DNA breaks, and DNA fragmentation have been observed in human and mouse gastric mucosa with *H. pylori* infection [131, 155-160]. Meanwhile, proteins related to DNA damage and repair, such as Ku, poly (ADP-ribosyl) polymerase, 8-hydroxyguanine glycosylase (OGG1), and MSH2 were selectively up-regulated in human gastric mucosa with acute gastritis [158, 159]. These results suggest the importance of DNA repair enzymes in *H. pylori*-related carcinogenesis by removing oxidative DNA damage.

Recent studies have identified several DNA repair proteins associated with gastric cancer, including O⁶-methylguanine DNA methyltransferase (Mgmt) and 8-oxoguanine DNA glycosylase (Ogg1) [161, 162]. Mgmt is the DNA repair protein capable of repairing specific types of mutagenic DNA adducts including O⁶-methylguanine and O⁴-methylthymine [163, 164]. Transcription of Mgmt is frequently inactivated by promoter hypermethylation in precancerous intestinal metaplasia and gastric cancer [165, 166]. Additionally, Mgmt Ile143Val polymorphism results in reduced enzyme activity [167] and has been associated with a higher risk of gastric cancer in patients with low fruit and vegetable intake [161]. Ogg1 gene encodes the enzyme to remove 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) from DNA. Ogg1 Ser326Cys polymorphism results in impaired enzyme activity [168, 169] and has been associated with accumulation of 8-oxo-dG in *H. pylori*-infected gastric mucosa and a higher risk of gastric cancer [170, 171]. These observations suggest that activities of DNA repair proteins may affect the natural course and outcome of chronic *H. pylori* gastritis. The roles of DNA repair proteins Mgmt and 3-alkyladenine DNA glycosylase (Aag) in *H. pylori*-associated gastric disease are the subject of Chapter 4.
§ Intervention of *H. pylori* infection and *H. pylori*-related disease

**Antimicrobial *H. pylori* eradication therapy**

Since *H. pylori* was identified in patients with chronic gastritis and peptic ulcers in 1982 [10], antimicrobial *H. pylori* eradication therapy has been the first-line therapy for peptic ulcer disease [97]. Additionally, *H. pylori* is the major etiology of gastric cancer. According to Correa’s model of gastric cancer, *H. pylori* infection results in gastritis and initiates the progression to atrophy, intestinal metaplasia, dysplasia, and finally gastric cancer [97, 133]. However, whether antimicrobial *H. pylori* eradication therapy reduces the risk of gastric cancer is not conclusive.

Eradication of *H. pylori* in humans has been associated with preventing the progression of preneoplastic lesions [105, 107, 172-174]. The optimal effect of antibiotic eradication therapy in preventing gastric cancer has been observed in *H. pylori* infected patients who did not have precancerous lesions prior to intervention with antimicrobial *H. pylori* eradication (p<0.05) [175]. Once the preneoplastic lesions developed, antimicrobial *H. pylori* eradication therapy failed to reduce the combined prevalence of dysplasia or gastric cancer [107]. These clinical trials continue to pose key questions; in which patients would *H. pylori* eradication be beneficial in preventing gastric cancer and at what stage in the progression of gastric disease would *H. pylori* antimicrobial eradication prevent progression of gastric lesions.

In human antimicrobial *H. pylori* eradication studies, confounding variables include duration of *H. pylori* infection, stage of *H. pylori* gastritis, development of preneoplastic lesions prior to *H. pylori* eradication therapy, diet, host genetic and environmental factors. Therefore, animal models have been used to analyze the effect of antimicrobial *H. pylori* eradication therapy on
gastric cancer development. Antimicrobial \textit{H. pylori} eradication therapy reverses the histologic progression of dysplasia in \textit{H. pylori}-infected Mongolian gerbils [176, 177]. In \textit{H. felis}-infected C57BL/6 mice, antibiotics given at early time points of helicobacter infection prevents development of dysplasia; eradication of \textit{H. felis} during late stage of disease arrests progression of dysplasia and prevents gastric cancer-related deaths [31]. These \textit{in vivo} data support the hypothesis that eradication therapy, depending on the timing of antibiotic treatment, may be effective in preventing helicobacter gastritis from progressing to gastric cancer. Efficacy of antimicrobial \textit{H. pylori} eradication therapy in the development of \textit{H. pylori}-associated gastric cancer using INS-GAS mice is the subject of Chapter 5.

**Other chemopreventive drugs**

Cancer chemoprevention targeting the malignant cells or tumor microenvironment has been extensively examined [178]. Some molecules or oncoproteins, including cyclooxygenase 1 or 2 (COX1 or COX2), epidermal growth factor receptor (EGFR), Akt, NF-kB, and histone deacetylase, have been identified as potential targets for chemopreventive treatment [178].

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin E2 synthesis of COX-1 and COX-2 and have been widely used as anti-inflammatory and antipyretic agents. The safety of NSAIDs has been confirmed in the long-term use. NSAIDs are known to reduce the risk of colon cancer and breast cancer [179-182]. Despite the increased cardiovascular risk associated with long-term use, selective COX-2 inhibitors have a chemopreventive effect on colon cancer and do not have the gastrointestinal complications of non-selective NSAIDs [182]. Moreover, short-term (2 weeks) treatment with selective COX-2 inhibitor, rofecoxib, enhances proapoptotic
proteins-dependent apoptosis and reduces gastrin-mediated cell growth in *H. pylori*-infected patients, suggesting that inhibition of COX-2 may prevent the development of gastric cancer [183]. Additionally, a retrospective study demonstrated that long-term use (> 3 years) of non-aspirin NSAIDs, but not aspirin, correlates with a reduced risk of gastric cancer [184]. Several laboratory animal models have been used to examine the effect of NSAIDs on gastric cancer. The selective COX-2 inhibitor, celecoxib, reduced the multiplicity and size of gastric tumors in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-treated Wistar rats [185]. Other selective COX-2 inhibitors were also reported to reduced gastric cancer incidence in *H. pylori*-infected, *N*-methyl-*N*-nitrosourea (MNU)-treated C57BL/6 mice or Mongolian gerbils [186, 187]. The efficacy of longterm NSAIDs treatment as chemopreventive drugs merits further studies.

Gastric cancer prevention with other chemopreventive drugs has also been examined in animal models. For example, the combination of gastrin- and histamine H2-receptor antagonists (YF476 and loxitidine) has a synergistic, inhibitory effect on gastric cancer in *H. felis*-infected INS-GAS mice [188]. Curcumin, extracted from the root of *Curcuma longa Linn*, inhibits the PI3K/Akt pathway and induces apoptosis in vitro [189, 190] and reduces the multiplicity of gastric cancer in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-treated Wistar rats [191]. Further human studies are necessary to verify the chemopreventive effects of these compounds.
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Chapter 2: Wildtype and IL10⁻/⁻ T regulatory cells reduced T effector cell-mediated gastroduodenitis in Rag2⁻/⁻ mice but only wildtype T regulatory cells suppressed Helicobacter pylori gastritis

Abstract

Introduction

Materials and Methods

Experimental groups

Helicobacter pylori infection

Cell sorting and adoptive transfer

Histological evaluation

Special stains and immunohistochemistry

RNA extraction and quantitative PCR for cytokine expression and Foxp3

Quantitative culture of H. pylori

Statistical analysis

Results

Rag2⁻/⁻ Tₑ-recipient mice developed morbidity and gastroduodenitis independent of H. pylori infection

Adaptive immunity is required to develop Hp-associated gastritis

Hp infection exacerbated corpus gastritis in Rag2⁻/⁻ Tₑ-recipient mice

Adoptive transfer of wt or IL10⁻/⁻ Tᵣ cells reduced morbidity and gastroduodenitis but only wt Tᵣ cells reduced severity of Hp corpus gastritis

Th₁ cytokine responses were down-regulated to a greater extent by wt than IL10⁻/⁻ Tᵣ cells

Foxp3 expression in gastric tissues was promoted to a greater extent by wt than IL₁₀⁻/⁻ Tᵣ cells

Hp colonization levels were reduced by Tₑ cells and maintained when wt Tᵣ cells were co-transferred

Discussion

Acknowledgements

References

Legends

Figures

Table

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ABSTRACT

CD4⁺CD45RB⁺CD25⁻ T effector cells (Tₐ) promote *Helicobacter pylori* (Hp) gastritis in mice and CD4⁺CD45RB⁻CD25⁺ T regulatory cells (Tₐ) are anti-inflammatory. Using adoptive transfer into Hp-infected Rag2⁻⁻ mice, we evaluated effects of wildtype C57BL/6 (wt) or congenic IL10⁻⁻ Tₐ cells on gastritis, gastric cytokines and Hp colonization. Infected Rag2⁻⁻ mice colonized in the corpus and antrum with 10⁵ to 10⁶ Hp CFU/gram without associated gastritis. Tₐ cell transfer caused morbidity and a Hp-independent pangastritis and duodenitis (gastroduodenitis) associated with increased expression of IFN-γ and TNF-α. Tₐ cell transfer to Hp-infected mice led to additive corpus gastritis associated with inflammatory cytokine expression and reduced colonization. wt Tₐ cells reduced morbidity, Hp corpus gastritis, gastroduodenitis, inflammatory cytokine expression and reversed the decline in Hp colonization attributable to Tₐ cells. Although less effective than wt Tₐ cells, IL10⁻⁻ Tₐ cells also reduced morbidity and gastroduodenitis but did not reduce Hp corpus gastritis or impact Tₐ cell inhibition of colonization. Gastric tissues from mice receiving wt Tₐ cells expressed higher levels of Foxp3 compared to recipients of IL10⁻⁻ Tₐ cells, consistent with lower regulatory activity of IL10⁻⁻ Tₐ cells. These results demonstrate that wt Tₐ cells suppressed Tₐ cell-mediated Hp-independent gastroduodenitis and Hp-dependent corpus gastritis more effectively than IL10⁻⁻ Tₐ cells. Compartmental differences in Tₐ cell- and Hp-mediated inflammation and the differences in regulatory T cell effects between wt Tₐ and IL10⁻⁻ Tₐ cells suggest that IL10 expression by wt Tₐ cells is important to regulatory suppression of gastric inflammation.
INTRODUCTION

*Helicobacter pylori* (*Hp*) infects the human stomach and causes gastritis, with a subset of patients developing peptic ulcer disease, gastric carcinoma and gastric mucosa-associated lymphoid tissue lymphoma [11, 186]. Infection of C57BL/6 mice with *Hp* or *H. felis* results in chronic active gastritis [28, 31] and has been used to study the immune basis of *Hp*-induced gastritis. Helicobacter infections in humans and mice induce a Th1-predominant immune response with activation of CD4+ T lymphocytes and expression of pro-inflammatory cytokines such as IFN-γ [79, 80]. This cell-mediated immunity results in gastritis characterized by mononuclear cell infiltrates, mucosal hyperplasia and intestinal metaplasia. In contrast, immunodeficient B6.129S7-Rag1<sup>1m1Mom</sup> mice that lack mature B and T cells colonized at high density with *H. felis* but developed only minimal gastritis [78], indicating the importance of the adaptive immune response to helicobacter-associated gastric disease.

Recent data have demonstrated that different subpopulations of CD4+ T lymphocytes play diverse roles in mediating and regulating *Hp*-induced gastritis. In B6.CB17-Prkdc<sup>scid</sup> (SCID) mice, adoptive transfer of wildtype (*wt*) CD4+CD45RB<sup>Hi</sup> T effector (*T<sub>E</sub>*) cells from naïve donors caused severe gastritis in *Hp*-infected recipients, while co-transfer of *wt* CD4+CD45RB<sup>Lo</sup> T regulatory (*T<sub>R</sub>*) cells protected against development of gastritis [81]. *T<sub>R</sub>* cells have also been defined by expression of the IL-2 receptor α chain and Foxp3 [83], the forkhead transcription factor critical to thymic selection of CD4+CD25+ T cells [84]. Depletion of CD25+Foxp3+ *T<sub>R</sub>* cells in *Hp*-infected C57BL/6 mice led to loss of immune regulation and more severe gastritis [85] as did adoptive transfer of lymphocytes depleted of CD4+CD25+ cells into *Hp*-infected B6.Cg- Foxn1<sup>nu</sup> (nu/nu) recipients [86]. Of the many T cell subsets with ascribed regulatory
function [87], cell sorting experiments commonly use CD4*CD25*CD45RBLo as naturally occurring TR cells. However, the mechanism(s) for regulation by this type of TR cell are not fully understood.

In adoptive transfer models using co-administration of wt TE cells and wt or IL10-deficient (IL10−/) TR cells, IL10 has been shown to be essential for the function of TR cells in suppressing inflammatory bowel disease, dysplasia, and cancer of the colon [187, 188]. Based on this evidence, we evaluated Hp gastritis in B6.129S6-Rag2tm1Fwa (Rag2−/) mice that had received wt CD4*CD25*CD45RBlow TR cells from either wt C57BL/6 or congenic IL10−/− mice. The results demonstrate that TE cells mediated a gastroduodenitis in Rag2−/− mice independently of H. pylori infection and that wt TR cells suppressed this lesion to a greater extent than IL10−/− TR cells. Only wt TR cells suppressed additive corpus gastritis attributable to Hp infection. The data support compartmental differences in TE and H. pylori-mediated inflammation of the stomach and the differences in regulatory T cell effects between wt and IL10−/− TR cells suggest that IL10 expression by wt TR cells is important to regulatory suppression of gastric inflammation.

MATERIALS and METHODS

Experimental groups

C57BL/6 wildtype (wt) cell donor mice and adoptive transfer recipient Rag2 gene knockout mice (B6.129S6-Rag2tm1Fwa or Rag2−/−), backcrossed 12 generations to B6 wt mice, were originally from Taconic Farms (Germantown, NY). IL10 knockout cell donor mice (B6.129P2-Il10tm1Cgn/J or IL10−/−), backcrossed 10 generations to B6 wt mice, were originally from the Jackson
Laboratory (Bar Harbor, ME). Mice were bred and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility in static microisolator cages under specific-pathogen-free conditions as previously described [41]. Male and female, 6 to 8 week old, helicobacter-free Rag2\(^{-/-}\) mice were randomly assigned to uninfected or \(Hp\)-infected groups that were further subdivided 4 weeks later into groups that received either no T cells, \(wt\) T\(_E\) cells, or \(wt\) T\(_E\) cells in combination with \(wt\) or IL10\(^{-/-}\) T\(_R\) cells. Helicobacter-free C57BL/6 mice were also dosed with the same inoculum of \(Hp\) to confirm the mouse-adapted strain induced robust gastritis in \(wt\) mice. Some mice died acutely or were euthanized at earlier time points due to declining body condition. Only data from mice surviving to the predetermined necropsy time points of 16 and 20 weeks post \(Hp\) infection (12 and 16 weeks post adoptive transfer, respectively) were analyzed. The protocol was approved by the Committee on Animal Care of the Massachusetts Institute of Technology.

**Helicobacter pylori infection**

*Helicobacter pylori* Sydney strain 1 (SS1) was used for oral inoculation as described previously [34, 41]. \(Hp\) was grown for 24 hours at 37°C under microaerobic conditions in Brucella broth with 10% fetal bovine serum. The inoculum was suspended in PBS to an \(OD_{600}=1.000\) and then assessed by Gram stain and phase microscopy for purity, morphology, and motility as well as for urease, catalase, and oxidase activity. Mice were gavaged with 0.2 ml every other day for three doses. Control groups were given 0.2 ml of PBS.

**Cell sorting and adoptive transfer**

Single cell suspensions from spleens and mesenteric lymph nodes of \(wt\) or IL10\(^{-/-}\) mice were
prepared as described previously [189]. In brief, CD4+ cells were isolated by using CD4 Dynabeads and CD4 DETACHaBEAD (Dynal, Oslo, Norway). Cells were then labeled with anti-CD4-Cy, anti-CD45RB-FITC and anti-CD25-PE antibodies (Pharmingen, La Jolla, CA) and then sorted by flow cytometry (Model Mo-flo, Cytomation Inc., Fort Collins, CO) to a purity of >95% for wt T_E cells (CD4+CD25−CD45RB^hi) and wt or IL10^−/− T_R cells (CD4+CD25−CD45RB^lo). Anesthetized Rag2^−/− mice were injected in the retro-orbital sinus with 3 x 10^5 T_E cells alone or in combination with 3 x 10^5 wt or IL10^−/− T_R cells suspended in 200 µl HBSS.

**Histological evaluation**

At necropsy, the stomach and proximal duodenum were removed and cut along the greater curvature. Linear gastric strips from the lesser curvature were fixed overnight in 10% neutral-buffered formalin, embedded, cut at 4 µm, and stained with hematoxylin and eosin (H & E). Lesions were scored by a veterinary pathologist blinded to sample identity as described previously [42]. Total lesion indices were calculated by the addition of individual scores for each assessment described in Table 1 except for mucous metaplasia and hyalinosis, which have been observed to develop spontaneously in mice [190] as well as from *H. felis* infection [29, 191].

**Special stains and immunohistochemistry**

Selective tissues were characterized using special stains and immunohistochemistry. Acidic (intestinal type) mucins were demonstrated using pH 2.5 Alcian blue followed by periodic acid-Schiff (AB-PAS) to stain remaining neutral (gastric type) mucins [42]. Macrophages were stained with monoclonal antibody F4/80 (Caltag Laboratories, Burlingame, CA, 1:150) and with
an avidin-biotin-peroxidase complex kit (Vector Laboratories, Burlingame, CA) according to the manufacturer instructions.

**RNA extraction and quantitative PCR for cytokine expression and Foxp3**

Stomach tissue was harvested and snap-frozen in liquid nitrogen. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 5 μg of total RNA using the High Capacity cDNA Archive kit (Applied Biosystems, Forster City, CA). Levels of IFN-γ, TNF-α, IL4, IL10 and Foxp3 were quantified with SYBR Green PCR reagent (Qiagen, Valencia, CA) in an ABI Prism Sequence Detection System 7700 (Applied Biosystems) per the manufacturer instructions. Primers were designed by Lasergene software (DNASTAR, Madison, WI). Sequences of primers were as follows: INFγ: Forward (F): CATggCTgTTTCTggCTgTTACTg, Reverse (R): gTTgCTgATggCCTgATTgTCTTT, Tm: 56° C; TNFα: F: CATCTTTCTCAAAATTCgAgTgACAA, R: TgggAgTAgACAAggTACAACCC, Tm: 60° C; IL4: F: ACAggAgAAgggACgCCAT, R: gAAgCCCTACAgACgAgCTCA, Tm: 60° C; IL10: F: ggTTgCCAAAgCCTTATCggA, R: ACCTgCTCCACTG CCTTgCT, Tm: 60° C; Foxp3: F: CCCAggAAAgACAgCAACCTT, R: CTCACAACCAggCCACTTgCA, Tm: 60° C; GAPDH: F: TCCATgACAACTTTggCATTg, R: TCACgCCACAgC TTTCCA, Tm: 60° C. The final concentration of each primer was 0.3 μM. Ten-fold dilutions (10^7 to 10^1 copies) of each cytokine cDNA plasmid were used to generate standard curves. Expression levels were calculated as mRNA copies of each cytokine per 10^5 copies of GAPDH or as ratio of Foxp3 to the internal control of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (User Bulletin #2, Applied Biosystems).
Quantitative culture of *H. pylori*

Colonization of *Hp* in the stomach was assessed by quantitative culture as described previously [41]. Briefly, tissues from corpus and antrum were weighed and homogenized in 250 μL of Brucella broth using a sterile glass tissue grinder. The homogenate was serially diluted 10- and 100-fold in Brucella broth and plated onto selection plates containing 5% horse blood, 250 μg/ml amphotericin B, 7 μg/ml bacitracin, 10.7 μg/ml nalidixic acid, 3.3 μg/ml polymyxin, and 100 μg/ml vancomycin. Plates were incubated microaerobically at 37°C for 7 days. Bacterial colonies were counted and the CFU per gram of tissue calculated.

Statistical analysis

Morbidity between groups was compared by the Logrank test. Lesion scores were compared by the Mann-Whitney U test or by the Kruskal-Wallis one-way analysis of variance with Dunnett’s test. Cytokine and Foxp3 expression levels and *H. pylori* colonization data (post log transformation) were compared using the Newman-Keuls test. Statistical analysis was performed using commercial software (Graphpad Prism 4.0, GraphPad Software, Inc., San Diego, CA) with significance at p<0.05.

RESULTS

Rag2−/− T<sub>E</sub>-recipient mice developed morbidity and gastroduodenitis independent of *H. pylori* infection

*Hp*-infected and helicobacter-free control Rag2−/− mice that did not receive cell transfers were clinically normal throughout the 20 week study period. In contrast, 7 of 10 uninfected T<sub>E</sub>-recipient and 3 of 12 *Hp*-infected T<sub>E</sub>-recipient Rag2−/− mice died acutely or developed diarrhea
and declining body condition, necessitating early euthanasia between 4 and 12 weeks post T cell transfer (Table 1). All remaining uninfected and Hp-infected Rag2<sup>-/-</sup> mice that had received T<sub>E</sub> cells alone were necropsied at 12 weeks post T cell transfer which was also 16 weeks post Hp infection (WPI).

In all uninfected T<sub>E</sub>-recipient Rag2<sup>-/-</sup> mice, the entire alimentary tract including the stomach through the colon was grossly edematous. The stomach and proximal duodenum from all mice were evaluated histologically and there was severe pangastritis that involved the squamous and glandular compartments of the stomach with inflammation extending into the proximal duodenum (Table 1 and Fig. 1). This T<sub>E</sub> cell-mediated gastroduodenitis was characterized by extensive infiltration of the mucosa and submucosa with lymphocytes, macrophages, eosinophils and neutrophils. Other histological changes included hypertrophy and orthokeratotic hyperkeratosis of the squamous portion of the stomach, as well as epithelial defects, hyalinosis and mucous metaplasia of the corpus, along with mild blunting, atrophy and fusion of duodenal villi (Table 1 and Fig. 1). A consistent feature of the gastroduodenitis was loss of Brunner’s glands through a combination of atrophy and dysplasia, accompanied by metaplasia to a tubuloductular phenotype (Fig. 1). The bowel distal to the proximal duodenum was examined histologically in 3 of these mice and had inflammatory infiltrates in the cecum and colon (data not shown) which is consistent with a previous report of T<sub>E</sub> cell transfer-associated colitis in Rag2<sup>-/-</sup> mice [192].

**Adaptive immunity is required to develop Hp-associated gastritis**

When evaluated at 20 WPI, the stomach and intestinal tract of Hp-infected Rag2<sup>-/-</sup> mice appeared
grossly normal and were similar to those from uninfected Rag2\(^{-/-}\) mice. Histologically, \(Hp\)-infected Rag2\(^{-/-}\) mice developed only minimal corpus gastritis with scattered neutrophils (Table 1, Fig. 2) and F4/80\(^+\) macrophages in the submucosa (not shown). \(Hp\)-infected wt mice were evaluated to confirm robust gastritis from infection with \(Hp\) SS1. These mice had moderate gross thickening of the stomach, accompanied by histological lesions consisting of significant infiltration with mononuclear cells and neutrophils, epithelial defects, oxyntic atrophy, intestinal metaplasia, and hyperplasia (Table 1, \(p<0.001\)), as previously reported [31]. These findings indicated that \(Hp\)-associated gastritis in mice was promoted by adaptive immunity and that inflammation observed in \(Hp\)-infected, T\(_E\) recipient Rag2\(^{-/-}\) mice was attributable to the inflammatory activity of donor T\(_E\) cells.

**Hp infection exacerbated corpus gastritis in Rag2\(^{-/-}\) T\(_E\)-recipient mice**

\(Hp\)-infected Rag2\(^{-/-}\) mice that received T\(_E\) cells at 4 WPI and were necropsied at 16 WPI were clinically affected similar to that observed in uninfected Rag2\(^{-/-}\) mice that received T\(_E\) cells alone. Unexpectedly, \(Hp\)-infected, T\(_E\)-recipient Rag2\(^{-/-}\) mice had less mortality than uninfected T\(_E\)-recipients, with 9 of 12 mice surviving to the time point of 16 WPI (Table 1, \(p<0.05\)). The infected T\(_E\)-recipient mice developed similar gross changes in the stomach and intestinal tract with similar histological evidence of gastroduodenitis (Table 1, Fig. 2). However, corpus gastritis was notably more severe in the \(Hp\)-infected T\(_E\)-recipient mice compared to the uninfected T\(_E\)-recipients (Table 1, \(p<0.05\)). Otherwise, total lesion indices were similar (Table 1, \(p=0.20\)) between \(Hp\)-infected and uninfected T\(_E\) cell-recipient Rag2\(^{-/-}\) mice, suggesting that T\(_E\) cells mediated the antral gastritis, duodenitis and destruction of Brunner's glands. These results also indicate that \(Hp\) infection further promoted the corpus gastritis that was superimposed on \(Hp\)-
independent gastroduodenitis.

Adoptive transfer of wt or IL10−/− TR cells reduced morbidity and gastroduodenitis but only wt TR cells reduced severity of Hp corpus gastritis

Clinical morbidity post transfer of TE cells into Hp-infected Rag2−/− mice was less severe when either wt (p<0.001) or IL10−/− TR cells (p<0.01) were co-transferred (Table 1). Total lesion indices for TE cell-associated gastroduodenitis that developed independently of Hp infection were lower in Rag2−/− mice co-transferred with either wt TR (p<0.001) or IL10−/− TR cells (p<0.01) (Table 1). wt TR cells were more efficacious than IL10−/− TR cells in ameliorating antral gastritis (p<0.01, p<0.10, respectively), Brunner’s gland destruction (p<0.01, p<0.05, respectively) and antral dysplasia (p<0.01, p<0.05, respectively). Comparable to Hp-infected wt mice, gastritis in the Hp-infected Rag2−/− mice that received TE and either type of TR cells was concentrated in the corpus (Table 1, Fig. 2), with mild inflammation in adjacent compartments. Notably, the TE cell-mediated corpus pathology in Hp-infected Rag2−/− mice was diminished to the greatest extent in mice that were co-transferred with wt TR cells compared to recipients of IL10−/− TR cells (corpus gastritis p<0.001, epithelial defects p<0.05, hyperplasia p<0.05; total lesion indices p<0.001). None of the individual pathology parameters characterizing corpus gastritis were significantly different in Hp infected TE-recipient mice given IL10−/− TR cells from infected mice that received TE cells alone (p=0.26 and higher, Table 1, Fig. 2). Interestingly, co-transfer of wt TR cells resulted in significantly greater mucous metaplasia of parietal cells than the Hp-infected Rag2−/− mice that received TE cells alone (p<0.01, Fig. 2).

Th1 cytokine responses were down-regulated to a greater extent by wt than IL10−/− TR cells
Consistent with the absence of gastritis, low levels of mRNA expression for IFN-γ, TNF-α, IL4 and IL10 were observed in gastric samples from uninfected and \( Hp \)-infected \( \text{Rag}^{2^{-/-}} \) mice that had not received T cells (Fig. 3). \( \text{T}_E \) cell-associated gastroduodenitis was accompanied by a significant transcriptional up-regulation of IFN-γ, TNF-α, IL4 and IL10 mRNA (\( p<0.001 \)) regardless of \( Hp \) infection status (\( p=0.2 \) and higher); indicating that \( \text{T}_E \) cells were the main stimulus for cytokine expression. Notably, Th1-associated IFN-γ and TNF-α expression levels in gastric samples from \( \text{T}_E \)-recipient, \( Hp \)-infected \( \text{Rag}^{2^{-/-}} \) mice were two logs higher than Th2-associated IL4 and IL10 mRNA levels, consistent with the proinflammatory response to gastric helicobacters [81, 93]. \( Hp \)-infected \( \text{Rag}^{2^{-/-}} \) mice that received \( \text{T}_E \) plus \( \text{wt} \) \( \text{T}_R \) cells had significantly lower expression levels of IFN-γ, TNF-α, IL4 and IL10 (\( p<0.01 \) and lower) compared to infected \( \text{Rag}^{2^{-/-}} \) mice that received \( \text{T}_E \) cells only. Consistent with amelioration of \( \text{T}_E \) cell-mediated gastroduodenitis, co-transfer of IL10~−~\( \text{T}_R \) cells also lowered mRNA expression for IFN-γ, TNF-α and IL4 (\( p<0.005 \) and lower) but a decrease in IL10 was not observed (\( p=0.51 \)).

The ability of \( \text{wt} \) \( \text{T}_R \) cells, and not IL10~−~\( \text{T}_R \) cells, to suppress corpus gastritis was consistent with higher expression levels for IFN-γ (\( p<0.05 \), Fig. 3a) in recipients of IL10~−~\( \text{T}_R \) cells. Additionally, suppression of TNF-α mRNA levels was more significant in mice receiving \( \text{wt} \) compared to IL10~−~\( \text{T}_R \) cells (\( p<0.01 \), \( p<0.05 \), respectively, Fig. 3b). Thus, Th1-predominant cytokine responses in \( Hp \)-infected \( \text{T}_E \) cell-recipients were suppressed by both \( \text{wt} \) and IL10~−~\( \text{T}_R \) cells but suppression was greatest in recipients of \( \text{wt} \) \( \text{T}_R \) cells.

**Foxp3 expression in gastric tissues was promoted to a greater extent by \( \text{wt} \) than IL10~−~\( \text{T}_R \) cells**

Uninfected and \( Hp \)-infected \( \text{Rag}^{2^{-/-}} \) mice that did not receive \( \text{T}_E \) cells had no detectable mRNA
of Foxp3 in gastric tissues (Fig. 3e). Foxp3 expression levels were elevated in mice that received TE cells (p<0.05) and Hp infection did not further stimulate expression. Levels of Foxp3 were significantly higher in Hp-infected mice that received wt TR cells (p<0.001). Foxp3 expression in tissues from recipients of IL10\(^{-/-}\) TR cells was not further elevated over levels observed for Hp-infected mice that received TE cells alone.

**Hp colonization levels were reduced by TE cells and maintained when wt TR cells were co-transferred**

Hp colonization levels have been reported to be inversely related to the severity of associated chronic gastritis in mice [193]. Consistent with the absence of an inflammatory response, Hp-infected Rag2\(^{-/-}\) mice that did not receive T cells were colonized at levels 3 to 5 logs higher in the corpus and antrum, respectively, than in infected, TE cell-recipient Rag2\(^{-/-}\) mice (Fig. 4, p<0.001). Hp-infected mice that received TE and wt TR cells maintained colonization in the corpus at levels similar to Hp-infected mice that did not receive TE cells (Fig. 4a, p=0.18). In contrast, Hp-infected mice that received TE plus IL10\(^{-/-}\) TR cells had reduced corpus colonization similar to infected mice that received TE cells alone (p=0.43), suggesting IL10\(^{-/-}\) TR cells failed to influence a TE cell-mediated reduction of Hp colonization. In the antrum, wt TR cells maintained higher colonization levels than in infected mice that received TE cells alone (p<0.05), whereas colonization in mice receiving TE and IL10\(^{-/-}\) TR cells was similar to infected mice receiving TE without TR cells (p=0.19, Fig. 4b).

**DISCUSSION**

Using T cell transfer into Hp-infected Rag2\(^{-/-}\) mice, this study demonstrated that wt TR cells
reduced T E cell-mediated morbidity, Hp-dependent corpus gastritis and Hp-independent gastroduodenitis. The Hp-independent gastroduodenitis was characterized by antral gastritis, duodenitis, Brunner cell loss, and epithelial dysplasia. These lesions have not been previously described in mice with helicobacter infections and are consistent with autoimmune-like disease [87]. IL10−/− T R cells also reduced T E cell-mediated morbidity and to a lesser extent, Hp-independent gastroduodenitis. Notably, IL10−/− T R cells did not reduce corpus gastritis exacerbated by Hp infection, nor did IL10−/− T R cells reverse the T E cell-mediated reduction in Hp colonization levels. T E cells mediated a sufficiently robust pro-inflammatory cytokine response that IFN-γ and TNF-α expression levels were similar irrespective of Hp infection status. This Th1-predominant response was suppressed by both wt and IL10−/− T R cells but reduction in proinflammatory cytokine levels were greatest in recipients of wt T R cells. Gastric tissues from mice receiving wt T R cells expressed higher levels of Foxp3 compared to recipients of IL10−/− T R cells, consistent with lower regulatory activity of IL10−/− T R cells. These results suggest compartmental differences in T E cell and Hp-mediated gastritis and that IL10 expression by wt T R cells is important to regulatory suppression of gastric inflammation.

Similar to H. felis infection in Rag1−/− mice [78] and despite high Hp colonization levels, Rag2−/− mice that did not receive T E cells did not develop morbidity or gastrointestinal lesions. T E-recipient mice developed morbidity and gastroduodenitis independently of Hp infection, consistent with previous studies of T E cell transfer into Rag2−/− mice [192, 194] and mouse models of T R deficiency [195, 196]. Observation of T E cell-mediated inflammation in the upper gastrointestinal tract has been reported [81, 197] but not as frequently as colitis [195, 196] and may be impacted by the number of cells transferred (VP Rao, personal communication). Extra-
intestinal inflammation attributable to T_E cell infiltrates in sites such as the liver and Harderian gland have also been observed [198] and may contribute to morbidity. At these sites, T_E cells likely proliferate in response to host, or more likely, bacterial antigens either absorbed through the local epithelium or distributed systemically by vascular and lymphatic circulation. T_E cell-mediated morbidity may also be attributable to systemic effects of up-regulation of IFN-γ, TNF-α, IL4 and IL10 and other mediators released from gastrointestinal and extra-intestinal tissues. Interestingly, pre-existing Hp infection resulted in a higher survival rate in mice that subsequently received T_E cells, potentially by promoting homing of T_E cells to mucosal sites, resulting in less extra-intestinal inflammation.

In uninfected T_E cell-recipient Rag2⁻/⁻ mice, reactive T_E cells in the gastrointestinal mucosa most likely expanded in response to dietary antigens and antigens of normal microbiota known to colonize all regions of the bowel, including the upper gastrointestinal tract of mice [199]. T_E-mediated pangastritis involved the squamous and glandular compartments of the stomach, extending into the duodenum. Lymphocytic infiltration of the antrum and duodenum promoted antral dysplasia, villus atrophy, and destruction of Brunner glands and was an overlapping but distinct disease pattern compared to the Hp corpus gastritis as described in this study and by others [192, 194, 200]. Severity of corpus gastritis was inversely correlated with Hp colonization levels which is indirect evidence that T_E cells were responding to Hp antigens. Both wt and IL10⁻/⁻ T_R cells reduced the Hp-independent gastroduodenitis but only wt T_R cells reduced the Hp corpus gastritis and maintained higher levels of Hp colonization, suggesting compartmental or other differences in the stomach that may favor a protective role for IL10. The anti-inflammatory effect of IL10 has been implicated by observation that IL10⁻/⁻ mice developed more severe H.
*Felis* [201] and *Hp* gastritis [202, 203] than congenic wildtype mice, as did *Hp*-infected SCID mice injected with IL10<sup>−/−</sup> splenocytes [81]. Additionally, a requirement for IL10-competency of T<sub>R</sub> cells has been shown in a mouse model of inflammatory bowel disease using *H. hepaticus* infection. In this model only *wt* T<sub>R</sub> but not IL10<sup>−/−</sup> T<sub>R</sub> cells reduced inflammation, dysplasia and cancer lesions [188].

IL10<sup>−/−</sup> T<sub>R</sub> cells reduced morbidity and attenuated T<sub>E</sub> cell-mediated gastroduodenitis along with lower IFN-γ and TNF-α expression. This observation is also consistent with the absence of this distinct gastroduodenitis in uninfected or *Hp*-infected IL10<sup>−/−</sup> mice [203] that lack IL10-competent T<sub>R</sub> cells. Interestingly, IL10 expression in the Rag2<sup>−/−</sup> gastric tissues was not reduced in IL10<sup>−/−</sup> T<sub>R</sub> recipient mice as observed in mice that received *wt* T<sub>R</sub> cells. This result suggests that IL10 mRNA was expressed by donor T<sub>E</sub> or host epithelial or other inflammatory cells [204], possibly as a compensatory response of inflamed gastric tissue. Similarly, IL10 protein levels in the *Hp*-infected gastric mucosa of humans have been positively associated with the severity of gastritis [204]. Although the gastric IL10 level was not sufficient to dampen *Hp*-mediated corpus gastritis, our findings suggest that IL10-competency of T<sub>R</sub> cells, but not local IL10 *per se*, directly or indirectly reduces severity of *Hp* gastritis. Importantly, IL10 competence appears important for T<sub>R</sub> cell suppression of *Hp* gastritis but our results do not rule out other mechanisms. For example, CTLA-4 engagement induced and maintained anergy of *Hp*-specific T cells in an T<sub>R</sub>-independent manner [205]. Our data also show that Foxp3 expression in gastric tissues was promoted by T<sub>E</sub> cells, suggesting differentiation of T<sub>E</sub> cells into T<sub>R</sub> cells as reported by others [206]. Lastly, Foxp3 expression was promoted only by *wt* and not IL10<sup>−/−</sup> T<sub>R</sub> cells, consistent with the more potent regulatory activity of *wt* compared to IL10<sup>−/−</sup> T<sub>R</sub> cells. Further
studies are necessary to clarify these issues.

In humans, *Hp* gastritis and subsequent progression to carcinoma has been associated with atrophy and intestinal metaplasia of fundic glands [102], consistent with the hypothesis that gastritis, gastric atrophy, and gastric cancer represent a continuum of progressive disease [127]. Irrespective of *Hp* infection, Rag2<sup>−/−</sup> mice that received T<sub>E</sub> cells developed marked mucous metaplasia in the corpus which was more prominent in mice that received co-transfer of wt T<sub>R</sub> cells. Fundic atrophy from chronic *H. felis* infection in mice has been associated with mucous metaplasia characterized by increased expression of trefoil factor 2 (TFF2), also known as spasmolytic polypeptide, by gastric mucous neck cells [29, 191]. This lesion appears to be a replacement of parietal and chief cells with TFF2-secreting mucous cells that are similar in morphology to antral or Brunner's glands and has been suggested to be a precursor lesion of gastric cancer in mice [34] and in humans [207, 208]. Alternatively, because TFF2<sup>−/−</sup> mice infected with *H. pylori* [209] or *H. felis* [210] developed more severe gastritis and hyper-responsiveness to IL-1β compared to wildtype mice, TFF2 may be a negative regulator of gastritis and the promotion of mucous metaplasia by wt T<sub>R</sub> cells may reflect a protective TFF2-associated response through an unknown mechanism.

In summary, our data demonstrate the role of T<sub>E</sub> cells as one component of adaptive immunity that can trigger and sustain gastroduodenal inflammation in the absence of T<sub>R</sub> cells. *Hp* infection coupled with T<sub>E</sub> cell transfer resulted in a more severe corpus gastritis that was compartmentally distinct from T<sub>E</sub> cell-mediated gastroduodenitis. Further, our results demonstrate that IL10-competence of T<sub>R</sub> cells appeared to be a requirement for suppression of *Hp*-induced gastritis, but
was not as critical for amelioration of $T_E$-mediated gastroduodenitis or protection against associated morbidity. These results are compatible with previous reports that *Hp*-induced gastritis in mice is regulated by an IL10-dependent, Th1-type immune response [201, 203], likely via the function of CD25$^+$Foxp3$^+$ $T_R$ cells [85] and is consistent with increased Foxp3 expression in *wt* $T_R$-recipients. Compartmental differences in $T_E$ and *Hp*-mediated inflammation and differential regulation by *wt* or IL10$^{-/-}$ $T_R$ cells should prove valuable for study of bacterial and immune-mediated gastritis.

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REFERENCES


Legends

Figure 1. Transfer of wildtype (wt) T effector (T_E) cells into Rag2^-^ mice resulted in immune-mediated pangastritis and gastroduodenitis. (a) Normal squamous gastric compartment of Rag2^-^- mouse that did not receive T_E cells. (b) T_E cell transfer resulted in inflammation of the squamous stomach with reactive epithelial cell hypertrophy and plication of the surface mucosa. (c) T_E cell transfer also caused inflammation in the cardia and corpus. (d) T_E cell-mediated gastritis of the glandular and oxyntic mucosa resulted in oxyntic atrophy characterized by loss of parietal and chief cells. (e) Normal Brunner’s glands (arrow) in the proximal duodenum. (f) T_E cell transfer produced duodenitis with near complete loss of Brunner’s glands due to atrophy and tubuloductular metaplasia (arrow). H&E; bar = 200 μm.

Figure 2. H. pylori (Hp) infection in Rag2^-^- mice with or without subsequent transfer of wildtype (wt) T effector (T_E) cells alone or in combination with wildtype (wt) or IL10^-^- T regulatory (T_R) cells. (a) In the absence of T cells, Hp infection produced minimal to no gastritis. (b) Hp infection followed by transfer of T_E cells produced moderate to severe gastritis. (c) Co-transfer of wt T_R cells ameliorated severity of gastric inflammation in Hp infected, T_E-recipient mice, but for unknown reasons, marked mucous metaplasia of parietal cells developed (arrow). (d) Co-transfer of IL10^-^- T_R cells into Hp-infected T_E-recipient mice decreased T_E cell-mediated antral gastritis, Brunner cell loss and epithelial dysplasia but did not reduce Hp-induced corpus gastritis. H&E; bar = 200 μm.

Figure 3. Mean expression levels of mRNA for IFN-γ (a), TNF-α (b), IL4 (c), IL10 (d), and Foxp3 (e) were determined by quantitative PCR in gastric tissues from uninfected and H. pylori
(Hp)-infected Rag2−/− mice subsequently transferred with wildtype (wt) T effector (TE) cells alone or in combination with wildtype (wt) or IL10−/− T regulatory (TR) cells. TE cells up-regulated the expression of Th1 cytokines (IFN-γ, TNF-α) and Th2 cytokine (IL4) with mRNA for Th1 cytokines expressed 1-2 logs higher than for Th2 cytokines. Co-transfer of TE plus wt or IL10−/− TR cells suppressed expression of IFN-γ, TNF-α, and IL4. IL10 expression was up-regulated in TE-recipient mice while concurrent transfer of wt TR but not IL10−/− TR cells down-regulated IL10 expression. Foxp3 expression was significantly higher in mice that received TE cells. Co-transfer of wt TR cells resulted in 4-fold up-regulation of Foxp3 expression which was significantly higher than induced by IL10−/− TR cells (p<0.05) which was unchanged from mice receiving TE cells alone. Bars represent standard error. (* p<0.05, ** p< 0.01; *** p< 0.001).

**Figure 4.** Mean *H. pylori* (Hp) colonization in the corpus (a) and antrum (b) of Rag2−/− mice receiving either no T cells, wildtype (wt) T effector (TE) cells or TE cells in combination with T regulatory (TR) cells from wildtype (wt) or IL10−/− mice. Hp colonization in the corpus was lower in mice receiving TE or TE plus IL10−/− TR cells. Co-transfer of wt TR cells maintained colonization levels in the corpus (only) whereas IL10−/− TR cells significantly diminished Hp colonization in the corpus and antrum. Bars represent standard error. (* p<0.05, ** p< 0.01; *** p< 0.001).
Fig. 4

(a) H. pylori CFU/g corpus

(b) H. pylori CFU/g antrum

Log H. pylori + + + +

wt IL10/-

Log H. pylori + + + +

wt IL10/-

Log H. pylori + + + +

wt IL10/-
### Table 1. Gastric lesions at 16 and 20 weeks post *H. pylori (Hp)* infection (WPI)

#### Median (Range) of Lesion Indices:

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>WPI Hp</th>
<th>T_E</th>
<th>T_R</th>
<th>Corpus</th>
<th>Antrum</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td><strong>Range</strong></td>
<td><strong>Median</strong></td>
<td><strong>Range</strong></td>
<td><strong>Median</strong></td>
<td><strong>Range</strong></td>
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<tr>
<td>WT (2/2)</td>
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</tr>
<tr>
<td></td>
<td>1/2</td>
<td>+</td>
<td>-</td>
<td>(1-2)</td>
<td>(0.5-1)</td>
</tr>
<tr>
<td>WT (3/3)</td>
<td>20</td>
<td>+</td>
<td>-</td>
<td>(2-2.5)</td>
<td>(0.5-1)</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>+</td>
<td>-</td>
<td>(1-2)</td>
<td>(0.5-1)</td>
</tr>
<tr>
<td>Rag (7/7)</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>(0)</td>
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<tr>
<td></td>
<td>1/2</td>
<td>+</td>
<td>-</td>
<td>(0-1.5)</td>
<td>(0-0.5)</td>
</tr>
<tr>
<td>Hp (11/11)</td>
<td>20</td>
<td>+</td>
<td>-</td>
<td>0</td>
<td>(0)</td>
</tr>
<tr>
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<td>1/2</td>
<td>+</td>
<td>-</td>
<td>(0-1.5)</td>
<td>(0-0.5)</td>
</tr>
<tr>
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<td>(1.5-2)</td>
<td>(0.5-1)</td>
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<tr>
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<td>1/2</td>
<td>+</td>
<td>-</td>
<td>(2-3)</td>
<td>(0.5-2)</td>
</tr>
<tr>
<td>Hp+T_E (9/12)</td>
<td>16</td>
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<td>+</td>
<td>(1.5-2)</td>
<td>(0.5-2)</td>
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<tr>
<td>T_R (6/7)</td>
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<td>+</td>
<td>(0-1.5)</td>
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<tr>
<td></td>
<td>1/2</td>
<td>+</td>
<td>-</td>
<td>(0.5-1)</td>
<td>(0.5-1)</td>
</tr>
<tr>
<td>Hp+T_E+IL10^T_R (4/6)</td>
<td>16</td>
<td>+</td>
<td>+</td>
<td>(1.5-3)</td>
<td>(0.5-1)</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>+</td>
<td>+</td>
<td>(1.5-3)</td>
<td>(0.5-1)</td>
</tr>
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<td>Abbreviation: T_E: T effector cells; T_R: T regulatory cells; Infm: inflammation; Ep Defc: epithelial defect; Hyper: hyperplasia; Int: intestinal metaplasia; Atrop: atrophy; Muc: mucous metaplasia; Brnr: Bruner's gland destruction; Dys: dysplasia.</td>
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<td><em>Number necropsied/Group size. Differences represent early morbidity</em></td>
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<td><em>Significant influence of T_R cells at a p of &lt;0.05</em></td>
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<tr>
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<tr>
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<tr>
<td><em>Significant difference between wt and IL10^-T_R cells at a p of &lt;0.05</em></td>
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</table>
Chapter 3: High vitamin C intake and normal vitamin C levels in plasma and gastric tissue do not protect vitamin C-deficient L-gulono-γ-lactone oxidase knockout mice from *Helicobacter pylori*-induced premalignant gastric lesions

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ABSTRACT

In human studies, low vitamin C intake has been associated with more severe Helicobacter pylori gastritis and a higher incidence of gastric cancer. However, vitamin C supplementation has not been definitively shown to protect against gastric cancer. Using vitamin C-deficient B6.129P2-Gulo<sup>−/−</sup> mice lacking L-gulono-γ-lactone oxidase, we compared gastric lesions and Th1 immune responses in H. pylori-infected gulo<sup>−/−</sup> mice supplemented with low (33mg/L) or high (3300mg/L) vitamin C in drinking water for 16 or 32 weeks. Vitamin C content in plasma and gastric tissue correlated with the vitamin C supplementation levels in gulo<sup>−/−</sup> mice. H. pylori infection resulted in comparable gastritis and premalignant lesions in wildtype C57BL/6 and gulo<sup>−/−</sup> mice supplemented with high vitamin C, but lesions were less severe in gulo<sup>−/−</sup> mice supplemented with low vitamin C at 32 weeks post infection. The reduced gastric lesions in infected gulo<sup>−/−</sup> mice supplemented with low vitamin C correlated with reduced Th1-associated IgG2c, gastric IFN-γ and TNF-α mRNA and higher H. pylori colonization levels. Additionally, a trend toward higher amidated gastrin levels may have contributed to more severe premalignant lesions in H. pylori-infected, high vitamin C-supplemented gulo<sup>−/−</sup> mice. These results in the H. pylori-infected gulo<sup>−/−</sup> mouse model suggest that although supplementation with a high level of vitamin C achieved physiologically normal vitamin C levels in plasma and gastric tissue, this dose of vitamin C did not protect gulo<sup>−/−</sup> mice from H. pylori-induced premalignant
gastric lesions. However, low vitamin C supplementation correlated with an attenuated Th1 inflammatory response and less severe gastric lesions.

INTRODUCTION

*Helicobacter pylori* infects the human stomach [1] and has been definitively linked to chronic gastritis, which in some individuals results in serious gastric disease such as peptic ulcer, gastric adenocarcinoma or gastric MALToma [2]. Multiple factors have been evaluated for impact on helicobacter-associated gastric disease [2, 3]. Dietary factors, including nitrosamines, high salt, and low dietary vitamin C (ascorbic acid), have been proposed to negatively influence the clinical outcome of *H. pylori* infection in epidemiological and animal studies [4-8].

Vitamin C, a water-soluble antioxidant, reduces the formation of carcinogenic N-nitroso compounds in gastric juice and scavenges reactive oxygen metabolites in the gastric mucosa [9, 10]. Vitamin C is also important for carboxyamidation of gastrin and cross linkage of collagen and elastin [11, 12]. Epidemiological studies in humans have linked vitamin C deficiency to more severe *H. pylori*-associated gastritis and a higher risk for gastric cancer [10, 13]. It has also been reported that reduced vitamin C levels in gastric juice and plasma in *H. pylori*-infected patients returned to normal levels after *H. pylori* eradication [10, 13-16]. Supplementation of
vitamin C has been associated with reduced gastric cancer risk in some human studies [7, 13].

Despite initial promising results in a prospective trial in a very high-risk population supplemented with vitamin C at 2 g per day for 6 years [17], a followup study on this population indicated that vitamin C supplementation over a 12 year period did not provide any lasting protection against gastric cancer [18]. These results are consistent with other studies that did not observe a correlation between severity of chronic gastritis or gastric cancer risk and vitamin C levels [19-21].

In human vitamin C intervention studies, confounding variables include diet, vitamin C status, genetic polymorphisms, duration of infection with specific or unknown H. pylori strains, and degree of gastritis. Therefore, animal models have been used to analyze the effects of vitamin C on H. pylori gastritis and gastric cancer [22-24]. Mice and Mongolian gerbils have been used to evaluate H. pylori gastritis and vitamin C oral intake [25, 26]. However, like most laboratory rodents, a major limitation for using these animal models for vitamin C studies is their ability to endogenously synthesize vitamin C. Thus results in these rodents are difficult to interpret. In contrast, the vitamin C-deficient gulo−/− mouse on a C57BL/6 background (B6.129P2-Gulo<sup>tm1Umc<sup>mmcd</sup>) lacks L-gulono-γ-lactone oxidase, and thus cannot endogenously synthesize vitamin C [27]. Using this model, we were able to modulate low and high vitamin C levels in
plasma and gastric tissue during *H. pylori* infection to specifically analyze whether dietary vitamin C would influence the outcome of *H. pylori* infection. We hypothesized that high dietary levels of vitamin C would reduce the severity of *H. pylori* gastritis, while physiologically lower levels of vitamin C would exacerbate disease.

**MATERIALS and METHODS**

**Mice**

Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility in static microisolator cages under specific-pathogen-free (SPF) status including free of *Helicobacter* spp. as previously described [28]. Wildtype (wt) helicobacter-free C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). Gulo<sup>−/−</sup> mice (B6.129P2-Gulo<sup>−/−</sup>om/om, back-crossed to C57BL/6 for 10 generations) were obtained from the Mutant Mouse Regional Resource Center (University of California at Davis, CA), rederived to SPF status, and bred to maintain a homozygous state [27]. For breeding and maintenance, gulo<sup>−/−</sup> mice were weaned at 3 weeks of age and fed *ad libitum* with regular mouse chow (Prolab RMH 3000, PMI Nutrition International, Richmond, Indiana) and water supplemented with 330 mg/L of L-ascorbic acid (Sigma-Aldrich Co., St. Louis, MO) and 0.01 mM EDTA (Sigma-Aldrich Co.). Supplemented water was changed weekly [27]. Animal experiments were approved by the
Committee on Animal Care of the Massachusetts Institute of Technology.

Experimental design

Male and female, 6-8 week old gulo−/− mice supplemented with vitamin C (330 mg/L) were experimentally infected with *H. pylori* and then randomly subdivided into low and high vitamin C supplemented groups. The low vitamin C group was supplemented with vitamin C in water at 33 mg/L and the high vitamin C group was supplemented with vitamin C in water at 3,300 mg/L [29]. Control uninfected gulo−/− mice were supplemented with low or high vitamin C. Age-matched control uninfected wt mice and wt mice dosed with the same inoculum of *H. pylori* were used to confirm the mouse-adapted strain induced robust gastritis in wt mice. Approximately half of the mice of each group were euthanatized with CO₂ at 16 or 32 weeks post infection (WPI) (Table 1 and 2). To confirm the results observed at 32 WPI, a second experiment evaluated the same vitamin C treatment groups of uninfected and *H. pylori*-infected gulo−/− mice along with uninfected and *H. pylori*-infected wt mice. In addition, a group of *H. pylori*-infected wt mice were supplemented with high vitamin C in the water (Table 2).

Experimental infection with *H. pylori*

*H. pylori* Sydney strain (SS1) was used for oral inoculation as described previously [28, 30]. After incubation for 24 hours at 37°C while shaking under microaerobic conditions in *Brucella* broth with 10% fetal bovine serum, *H. pylori* was harvested, resuspended in PBS and assessed by
Gram stain and phase microscopy for purity, morphology, and motility. The bacterial concentration was adjusted to $\text{OD}_{600}=1.000$ in PBS. This is approximately $10^9$ organisms/ml.

Mice were dosed with 0.2 ml of the $H. pylori$ suspension in PBS by gavage every other day for three doses. Control mice were dosed with PBS.

**Vitamin C measurements**

Total vitamin C levels were measured by high performance liquid chromatography and UV detection as described previously with some modifications [31]. In brief, blood was collected at necropsy in EDTA and centrifuged immediately at $4^\circ\text{C}$. Vitamin C was extracted from plasma by adding an equal volume of cold perchloric acid (PCA) solution (1 L contained 50 ml of perchloric acid and 95 mg of EDTA in ddH$_2$O), followed by vortexing and centrifugation at 2,500 g at $4^\circ\text{C}$ for 10 minutes. The supernatant was frozen at $-70^\circ\text{C}$ pending analysis. To measure vitamin C levels in tissue, a longitudinal strip of the gastric greater curvature was weighed, frozen in liquid nitrogen, and stored at $-70^\circ\text{C}$. Frozen tissue was added to cold PCA solution at a ratio of 1:9 (weight/weight) followed by homogenization and centrifugation at 2,500 g at $4^\circ\text{C}$ for 10 minutes, and then frozen at $-70^\circ\text{C}$ until analysis. Throughout the vitamin C extraction process, samples were kept on ice, protected from light, and measured within one week post processing.

**Histological evaluation**

At necropsy, the stomach and proximal duodenum were removed and opened along the greater
Linear gastric strips from the lesser curvature were fixed overnight in 10% neutral-buffered formalin, embedded, cut at 4 μm, and then stained with hematoxylin and eosin. Using criteria described previously [32], gastric lesions were scored for inflammation, epithelial defects, mucous metaplasia, atrophy, hyperplasia, intestinal metaplasia, and dysplasia by board certified veterinary pathologists (BR and ABR) blinded to sample identity. Total gastric indices were calculated by the addition of individual scores for each type of gastric lesion.

RNA extraction and quantitative PCR for cytokine mRNA

A longitudinal strip of gastric tissue from the anterior wall was harvested and snap-frozen in liquid nitrogen. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 5 μg of total RNA with the High Capacity cDNA Archive kit (Applied Biosystems, Forster City, CA). mRNA levels of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were quantified with TaqMan gene expression assays and TaqMan Fast Universal PCR Master Mix in a 7500 Fast Real-Time PCR system (Applied Biosystems) per manufacturer’s instructions. mRNA levels of each cytokine were normalized to the mRNA level of internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared to data from uninfected wt mice using the ΔΔCT method (Bulletin 2, Applied Biosystems).

Plasma IgG isotypes measurement

Plasma was evaluated for *H. pylori*-specific IgG2c and IgG1 by ELISA using an outer membrane
protein preparation from *H. pylori* (SS1 strain) as described previously [33]. In brief, 96 well flat-bottom plates were coated with 100 μl of antigen (10 μg/ml) overnight at 4°C, and sera were diluted 1:100. Biotinylated secondary antibodies for detecting IgG2c and IgG1 were from clone 5.7 and A85-1 (BD Pharmingen, San Jose, CA). Incubation with extravidin peroxidase (Sigma-Aldrich) was followed by treatment with 2,2'-azinobis (3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for color development. The optical density was recorded by a plate reader per manufacturer’s protocol (Power WaveX Select, Biotek Instruments, Winooski, VT).

**Quantitative PCR for *H. pylori* colonization**

A longitudinal strip of gastric tissue from the greater curvature was proteinase K digested at 55°C for 8 hours followed by DNA extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation. *H. pylori* colonization levels (DNA copy numbers) were quantified by a fluorogenic quantitative PCR assay using urease B primers and probe [34]. *H. pylori* copy numbers were normalized to the amount of murine genomic DNA as determined by quantitative PCR using a eukaryotic 18S endogenous control (Applied Biosystems) (Bulletin 2, Applied Biosystems). Data is presented and compared as log-transformed copy numbers per μg host DNA.

**Quantification of plasma levels of gastrin**
Plasma concentrations of glycine-extended gastrin (G-gly) and amidated gastrin were determined by radioimmunoassay using the antibodies 109-21 and L2 specific to G-gly and amidated gastrin, respectively, as described previously [35].

Statistical Analysis

Histological scores from both 32 WPI experiments were pooled for analysis because they were performed under identical conditions. Pathology scores were analyzed by variance (ANOVA) followed by post-hoc comparison when ANOVA results were significant. Because cytokine mRNA levels increased progressively across the *H. pylori*-infected groups from low vitamin C to high vitamin C to wt, we assigned a numerical value from 1 to 3 to each group in the model and estimated the significance level of gastric lesions using a general linear model that tests for trends. Levels of gastric cytokine, plasma IgG isotypes, *H. pylori* colonization, and amidated gastrin were compared by the Student t test. All statistical analyses were two-sided tests at a significance level of 0.05 performed with SAS software version 9.1 (SAS Institute Inc., Cary, NC). The mean and standard error of all data are presented in the figures using Graphpad Prism 4.0 (Graphpad software, San Diego, CA).

RESULTS

Vitamin C content in plasma and gastric tissues correlated with vitamin C supplementation
levels in gulo<sup>−</sup> mice

Both uninfected and H. pylori-infected gulo<sup>−</sup> mice supplemented with low vitamin C had significantly lower plasma (p<0.01) and gastric tissue (p<0.001) vitamin C levels relative to the mice supplemented with high vitamin C at 16 and 32 weeks (Fig. 1). Uninfected and H. pylori-infected gulo<sup>−</sup> mice supplemented with high vitamin C had plasma and gastric tissue vitamin C levels comparable to those in unsupplemented wt mice at the same time points. H. pylori infection had no significant effect on plasma vitamin C levels in gulo<sup>−</sup> mice supplemented with low or high vitamin C or in wt mice (p=0.37 and higher). At 16 and 32 WPI, there was a trend for H. pylori infection to reduce gastric tissue vitamin C levels in gulo<sup>−</sup> mice supplemented with low vitamin C (p=0.071, =0.069, respectively). This reduction in gastric tissue vitamin C associated with H. pylori was significant in gulo<sup>−</sup> mice supplemented with high vitamin C at 16 WPI (p<0.05), however, paradoxically, H. pylori infection was associated with increased gastric tissue vitamin C levels at 32 WPI (p<0.05). Infection in wt mice did not significantly affect gastric tissue vitamin C levels at 32 WPI (p=0.24).

High vitamin C supplementation did not reduce H. pylori gastritis

Gastritis was not observed in uninfected wt mice and gulo<sup>−</sup> mice supplemented with low or high vitamin C. Consistent with previous reports [8, 26], H. pylori-infected wt mice developed robust
chronic atrophic gastritis at 16 and 32 WPI characterized by corpus inflammation with lymphocytic and granulocytic infiltration, epithelial defects, hyperplasia, intestinal metaplasia, and atrophy (Fig. 2, 3).

At 16 and 32 WPI, *H. pylori*-infected gulo<sup>−/−</sup> mice supplemented with high vitamin C developed gastric lesions of comparable severity relative to infected wt mice. At 16 WPI, there was a trend for *H. pylori*-infected gulo<sup>−/−</sup> mice supplemented with low vitamin C to have fewer epithelial defects (p=0.094), hyperplasia (p=0.069), intestinal metaplasia (p=0.15), atrophy (p=0.15), and dysplasia (p=0.078) relative to infected gulo<sup>−/−</sup> mice supplemented with high vitamin C. These trends were also reflected in slightly lower total gastric indices at 16 WPI in *H. pylori*-infected gulo<sup>−/−</sup> mice supplemented with low vitamin C (p=0.15).

Compared to *H. pylori*-infected gulo<sup>−/−</sup> mice supplemented with high vitamin C, by 32 WPI *H. pylori*-infected gulo<sup>−/−</sup> mice supplemented with low vitamin C had developed less severe gastritis (p=0.11), atrophy (p=0.06), and dysplasia (p=0.12) accompanied by significantly less extensive epithelial defects (p<0.01), mucous metaplasia (p<0.05), and hyperplasia (p<0.05). When comparing total gastric indices of disease between low and high vitamin C supplementation levels, infected gulo<sup>−/−</sup> mice supplemented with low vitamin C had statistically significant lower
total gastric indices compared to those mice supplemented with high vitamin C (p<0.05). Based on differences identified in cytokine gene expression (below), one-way trend analysis of *H. pylori*-infected gulo<sup>−/−</sup> mice with low vitamin C compared to gulo<sup>−/−</sup> mice with high vitamin C followed by comparison with wt mice demonstrated a difference in atrophy (p=0.053) and significant differences in epithelial defects (p<0.001), hyperplasia (p<0.05), mucous metaplasia (p<0.05), and total gastric indices (p<0.05).

Less severe gastritis in *H. pylori*-infected gulo<sup>−/−</sup> mice supplemented with low vitamin C was associated with lower gastric mRNA levels of IFN-γ and TNF-α.

Control gulo<sup>−/−</sup> mice supplemented with low or high vitamin C and uninfected wt mice had similar background gastric mRNA levels of IFN-γ and TNF-α at 32 WPI. *H. pylori* infection significantly up-regulated the mRNA levels of IFN-γ and TNF-α in the stomachs of gulo<sup>−/−</sup> and wt mice at 32 WPI. IFN-γ and TNF-α expression levels were elevated by *H. pylori* infection to a greater extent in gulo<sup>−/−</sup> mice supplemented with high vitamin C (p<0.001) and wt mice (p<0.001) compared to gulo<sup>−/−</sup> mice given low vitamin C supplementation (IFN-γ p<0.01, TNF-α p=0.088) (Fig. 4). The *H. pylori*-infected gulo<sup>−/−</sup> mice supplemented with low vitamin C had lower gastric mRNA levels of IFN-γ and TNF-α compared to infected gulo<sup>−/−</sup> mice supplemented with high vitamin C or wt mice (p<0.05 or lower). There were no significant differences in
gastric mRNA levels of IFN-γ and TNF-α between H. pylori-infected gulo^- mice supplemented with high vitamin C and infected wt mice (p=0.31, p=0.23, respectively).

Gulo^- mice supplemented with low vitamin C had lower H. pylori-specific IgG2c responses

H. pylori infection resulted in a Th1-predominant IgG2c response in wt and gulo^- mice as previously reported [33, 36] (Fig. 5). At 32 WPI, H. pylori-specific IgG2c levels were significantly higher in H. pylori-infected wt mice than in infected gulo^- mice supplemented with low vitamin C (p<0.05) but IgG2c responses were similar between gulo^- mice given low or high vitamin C (p=0.44). There was a trend for infected wt mice to have higher H. pylori-specific IgG2c levels than gulo^- mice supplemented with high vitamin C (p=0.051). However, in the H. pylori-infected wt mice given vitamin C supplementation, there was no difference in H. pylori-specific IgG2c responses compared to the 10 infected wt mice that didn’t receive oral vitamin C supplementation (p=0.51, data not shown). H. pylori infection induced a low IgG1 response in all infected groups.

H. pylori colonization levels were higher in gulo^- mice supplemented with low vitamin C at 32 WPI

H. pylori colonization levels in the stomach were comparable among infected gulo^- mice
supplemented with low or high vitamin C and wt mice at 16 WPI (Fig. 6). By 32 WPI, infected
gulo<sup>−/−</sup> mice supplemented with low vitamin C maintained higher <i>H. pylori</i> colonization levels
than gulo<sup>−/−</sup> mice supplemented with high vitamin C (p<0.05) or wt mice (p=0.082) where <i>H.
pylori</i> colonization levels decreased over time in concert with development of more severe
gastritis.

**Gastrin amidation was not impaired in vitamin C-supplemented gulo<sup>−/−</sup> mice**

Amidation of the intermediate glycine-extended gastrin (G-gly) is vitamin C dependent [11, 37]
and amidated gastrin levels are elevated in <i>H. pylori</i> infection in C57BL/6 mice [8]. At 32 WPI,
levels of the intermediate G-gly in plasma from most wt and gulo<sup>−/−</sup> mice supplemented with low
or high vitamin C were below the assay detection limit (data not shown). Uninfected wt mice and
gulo<sup>−/−</sup> mice supplemented with low or high vitamin C had similar amidated gastrin levels
(p=0.13) (Fig. 7). Uninfected and <i>H. pylori</i>-infected gulo<sup>−/−</sup> mice supplemented with low vitamin
C had comparable plasma amidated gastrin levels (p=0.82). There was a trend toward higher
plasma amidated gastrin levels in <i>H. pylori</i>-infected gulo<sup>−/−</sup> mice supplemented with high vitamin
C compared to uninfected counterparts (p=0.21). In contrast, <i>H. pylori</i> infection was associated
with a significant increase in plasma amidated gastrin levels in wt mice compared to uninfected
wt controls (p<0.01). Among the <i>H. pylori</i> infected gulo<sup>−/−</sup> and wt mice, gulo<sup>−/−</sup> mice
supplemented with low vitamin C had lower amidated gastrin levels compared to infected gulo* mice supplemented with high vitamin C and wt mice (p=0.065 and <0.05, respectively).

4 DISCUSSION

Using the gulo* mouse model, we were able to accurately assess and correlate low and high vitamin C supplementation with corresponding levels in plasma and gastric tissue, thus enabling in vivo evaluation of the role of dietary vitamin C in H. pylori-associated gastric disease. We observed that plasma and gastric tissue vitamin C levels in gulo* mice directly correlated with vitamin C intake, consistent with human studies [19, 38]. Gulo* mice supplemented with high vitamin C and chronically infected with H. pylori over a period of 32 weeks developed chronic gastritis and atrophy comparable to wt mice, indicating high vitamin C supplementation did not protect infected gulo* mice from gastritis nor the development of premalignant lesions. In contrast, low vitamin C-supplemented gulo* mice tended to have lower degrees of inflammation, atrophy and dysplasia and significantly less severe epithelial defects, intestinal metaplasia and hyperplasia relative to high vitamin C-supplemented gulo* and wt mice.

Plasma vitamin C levels in gulo* mice directly correlated with the level of dietary vitamin C and were not affected by H. pylori infection. Gastric tissue vitamin C levels also correlated with
dietary vitamin C intake but in contrast to plasma levels, gastric tissue vitamin C levels were reduced in *H. pylori*-infected gulo<sup>−/−</sup> mice at 16 WPI which is consistent with reduced vitamin C levels in gastric juice during acute *H. pylori* infection in humans [39]. However, for unknown reasons gastric tissue vitamin C levels were increased in *H. pylori*-infected gulo<sup>−/−</sup> mice at 32 WPI. Notably, gastric tissue vitamin C levels were not impacted by *H. pylori* infection in wt mice which may be confounded by endogenous synthesis of vitamin C in normal mice.

Acknowledging that the mechanism of vitamin C secretion into gastric juice is unclear [40], and that it has not been conclusively shown whether *H. pylori* infection affects vitamin C levels in human gastric mucosa [20, 41], additional studies are necessary to delineate the influence of *H. pylori* gastritis on vitamin C levels in the gastric compartment.

Several clinical trials have examined nutritional interventions using vitamin C alone or in combination with *H. pylori* eradication or other nutrients in preventing gastric carcinogenesis in humans; none of these studies demonstrated a reproducible benefit of vitamin C supplementation [18, 21, 42, 43]. However, the results of these studies are difficult to interpret given the probability of preexisting *H. pylori*-associated premalignant lesions in the target populations when vitamin C intervention or *H. pylori* eradication therapies were initiated [18, 21, 42-44]. To obviate these variables, in the gulo<sup>−/−</sup> mouse model vitamin C supplementation was administered...
orally at lower and higher levels than the recommended level for maintenance, allowing us to
establish dietary levels of vitamin C prior to dosing with *H. pylori* and subsequent development
of *H. pylori*-associated gastric lesions.

Despite the limited interpretation of data using rodents that endogenously synthesize vitamin C,
rodent studies have reported that 7 to 10 days of vitamin C supplementation reduced gastric
inflammation, *H. pylori* colonization levels, and lipid peroxidation in BALB/c mice given 400
mg vitamin C / kg / day [23] and decreased *H. pylori* colonization levels in Mongolian gerbils
given 10 mg vitamin C / day [22]. However, long-term vitamin C supplementation at 50 mg / kg
/ day over a period of 52 weeks had no protective effect on severity of gastritis, bacterial
colonization levels, and mucosal 8-hydroxydeoxyguanosine levels in *H. pylori*-infected gerbils
[24]. Supplementation with vitamin C in the diet at 10-fold higher levels than a maintenance
dose (3102 mg / kg) for 6 weeks in guinea pigs, which lack L-gulono-γ-lactone oxidase like
humans and gulo<sup>−</sup> mice, did not impact *H. pylori* colonization levels nor severity of gastritis
[45]. Similar to the guinea pig study, *H. pylori*-infected gulo<sup>+</sup> mice supplemented with high
vitamin C had comparable gastric inflammation and premalignant lesions compared to the
infected wt mice. Thus, high level supplementation with vitamin C in the gulo<sup>−</sup> mouse model as
well as in guinea pigs did not prevent the development of *H. pylori* gastric disease or impact
disease progression.

Helicobacter-induced gastric disease in humans and mice is mediated by a Th1-predominant host immune response [46-48]. Gulo<sup>+</sup> mice supplemented with high vitamin C had a similar Th1-promoted inflammatory response to *H. pylori* as infected wt mice. We unexpectedly observed that low vitamin C-supplemented, *H. pylori*-infected gulo<sup>+</sup> mice had attenuated gastric disease associated with suppressed Th1 responses as evidenced by reduced levels of plasma IgG2c, reduced expression of gastric IFN-γ and TNF-α mRNA, and increased *H. pylori* colonization levels. These data suggest that low dietary vitamin C impairs the host’s ability to sustain an inflammatory response and conversely, that adequate vitamin C levels in the infected host may be important for maintaining a robust Th1 immune response to chronic *H. pylori* infection. Consistent with our data, others have reported attenuated Th1 immune responses in vitamin C-deprived gulo<sup>+</sup> mice. This was manifested with less severe pneumonia and suppressed pulmonary expression of proinflammatory IL-1β and TNF-α mRNA in vitamin C-deprived gulo<sup>+</sup> mice during the first few days of acute viral influenza [49]. In hosts with low or no vitamin C intake, attenuated Th1 responses to other types of infection, such as tuberculosis in humans or *Klebsiella pneumonia* sepsis in gulo<sup>+</sup> mice, may also be important in predicting survival [50, 51].
Using a mouse model of intestinal parasitic infection that causes a Th2 immune response, Fox et al. examined the effect of modulating the Th1-associated response to H. felis infection [52]. In the C57BL/6 mice coinfected with helminths and H. felis, reduced systemic Th1 immune responses and lower levels of Th1-mediated gastric cytokines were associated with increased H. felis colonization levels and less severe premalignant lesions [52]. These results may in part explain the "African enigma" where the incidence of gastric cancer is low in some African countries, where parasitic infections are common, despite a high prevalence of H. pylori infection [53]. Our findings using the H. pylori-infected, low vitamin C-supplemented gulo−/− mouse model may offer another possible explanation to the African enigma. In Gambia, due to the impact of drought on the food supply, mean daily intake of vitamin C approaches zero for 7 months of the year, and is accompanied by low plasma vitamin C levels [54]. It is tempting to speculate based on our animal studies, that minimal vitamin C intake during H. pylori infection may be one of the factors contributing to a lower incidence of gastric cancer in some populations in Africa due to attenuated gastric inflammation. This hypothesis is also consistent with a report of diminished mitogen responses of peripheral blood mononuclear cells from pigs affected by heritable vitamin C deficiency [55].
Plasma amidated gastrin levels are increased in *H. pylori*-infected C57BL/6 mice [8]. Overexpression of amidated gastrin promotes the progression of *H. pylori*-associated gastritis and gastric cancer [28]. In a guinea pig model, vitamin C-deprivation impaired the amidation of gastrin and levels of G-gly were 30-fold higher than normal in the gastric antra [37]. In our study, uninfected gulo<sup>−/−</sup> mice supplemented with low vitamin C had undetectable G-gly but comparable levels of amidated gastrin relative to uninfected gulo<sup>−/−</sup> mice supplemented with high vitamin C. Consistent with previous report [8], *H. pylori* infection significantly increased plasma amidated gastrin levels in wt mice. *H. pylori* slightly increased amidated gastrin levels in gulo<sup>−/−</sup> mice supplemented with high vitamin C but not in those supplemented with low vitamin C. Additionally, amidated gastrin levels in *H. pylori*-infected gulo<sup>−/−</sup> mice supplemented with low vitamin C were lower than in infected wt mice and gulo<sup>−/−</sup> mice supplemented with high vitamin C. These results suggests that the dose used for low vitamin C supplementation is sufficient for gastrin amidation in uninfected animals but not sufficient to sustain increased amidated gastrin levels during *H. pylori* infection. Higher levels of admidated gastrin in *H. pylori*-infected gulo<sup>−/−</sup> mice supplemented with high vitamin C, compared to those with low vitamin C, may in part explain the higher degree of premalignant lesions in the high vitamin C supplemented gulo<sup>−/−</sup> mice.
In summary, our study indicates that gulo<sup>−/−</sup> mice, unlike other rodent models, provide a reliable model to study the role of dietary vitamin C in <i>H. pylori</i>-associated gastric disease. High vitamin C supplementation in this model, similar to previous epidemiological studies in humans, did not prevent progression of <i>H. pylori</i>-induced gastritis and development of premalignant gastric lesions. In contrast, low dietary vitamin C resulted in less severe gastric disease by down-regulating gastric and systemic Th1 immune responses to <i>H. pylori</i> infection. Additional studies to examine more chronic phases of <i>H. pylori</i> gastritis in gulo<sup>−/−</sup> mice and investigation of how low vitamin C intake impacts immune function in this model are warranted.

ACKNOWLEDGEMENTS

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LEGENDS

Figure 1. Vitamin C levels in plasma (a) and gastric tissue (b). White bars are 16 WPI; Black bars are 32 WPI. Compared to gulo$^{-/-}$ mice supplemented with high vitamin C (H), gulo$^{-/-}$ mice supplemented with low vitamin C (L) had significantly lower plasma and gastric tissue vitamin C levels (**, p<0.01; ***, p<0.001). Vitamin C levels in plasma and gastric tissue in gulo$^{-/-}$ mice supplemented with high vitamin C were comparable to those in wt mice. H. pylori (Hp) infection had no significant effect on plasma vitamin C levels or gastric vitamin C levels in gulo$^{-/-}$ mice supplemented with low vitamin C or in wt mice. However, gastric tissue vitamin C levels were reduced at 16 WPI but increased at 32 WPI by H. pylori infection in gulo$^{-/-}$ mice supplemented with high vitamin C (*, p<0.05).

Figure 2. Gastric histopathology of H. pylori infection in Gulo$^{-/-}$ with low vitamin C supplementation (a, d, g) or high vitamin C supplementation (b, e, h) versus wt C57BL/6 controls (c, f, i). (a-c) No significant lesions developed in uninfected mice in any group at 32 weeks. (d-f) Equivalent gastric lesions developed in all H. pylori-infected groups at 16 weeks post infection (WPI), although gulo$^{-/-}$ mice with low vitamin C supplementation (d) exhibited a trend for less severe epithelial defects, hyperplasia and dysplasia. (g-i) Equivalent gastric inflammation developed in all groups at 32 WPI, although gulo$^{-/-}$ mice with low vitamin C supplementation (g) had less severe epithelial defects, mucous metaplasia and hyperplasia.

Figure 3. Histological scores and total gastric indices in H. pylori (Hp)-infected mice. Gulo$^{-/-}$ mice supplemented with high vitamin C had similar gastric lesions and total gastric indices compared to wt mice at 16 and 32 WPI. (a) At 16 WPI, there was a trend for gulo$^{-/-}$ mice
supplemented with low vitamin C (white bars) compared to those supplemented with high vitamin C (black bars) for less severe epithelial defects, hyperplasia, and dysplasia (#, 0.05<p<0.10). (b) At 32 WPI, H. pylori-infected gulo⁻ mice supplemented with low vitamin C had significantly lower degrees of epithelial defects, mucous metaplasia, and hyperplasia relative to those supplemented with high vitamin C (*, p<0.05; **, p<0.01). There was a trend toward less severe atrophy in gulo⁻ mice supplemented with low vitamin C compared to those mice supplemented with high vitamin C (#, 0.05<p<0.10). (c) At 32 WPI, total gastric indices were significantly lower in gulo⁻ mice that received low vitamin C than those mice receiving high vitamin C (*, p<0.05).

Figure 4. Gastric mRNA levels of IFN-γ (a) and TNF-α (b) in gulo⁻ and wt mice at 32 WPI. Fold changes in expression levels were normalized using data from uninfected wt mice. There were no differences in IFN-γ and TNF-α mRNA levels among uninfected wt or gulo⁻ mice that received low or high vitamin C. H. pylori infection up-regulated IFN-γ mRNA levels in gulo⁻ mice supplemented with low vitamin C (white bar, p<0.01) and to the greatest extent in gulo⁻ mice supplemented with high vitamin C (black bar) and wt mice (dotted bar) (p<0.001). H. pylori-infected gulo⁻ mice supplemented with low vitamin C had significantly lower IFN-γ mRNA levels compared to gulo⁻ mice supplemented with high vitamin C or wt mice (p<0.01 and 0.05, respectively). H. pylori infection significantly up-regulated TNF-α mRNA levels in gulo⁻ mice supplemented with high vitamin C and wt mice (p<0.001), but not in gulo⁻ mice supplemented with low vitamin C. Therefore, TNF-α mRNA levels in gulo⁻ mice supplemented with low vitamin C were significantly lower than the other two groups (p<0.01). (Compared to uninfected wt mice, ##, p<0.01; ###, p<0.001. Compared to H. pylori-infected gulo⁻ mice
supplemented with low vitamin C, *, p<0.05; **, p<0.01).

Figure 5. *H. pylori*-specific IgG2c and IgG1 levels in *H. pylori*-infected mice. At 32 WPI, there was no difference in IgG2c levels among gulo<sup>−</sup> mice supplemented with low vitamin C (white bar) and high vitamin C (black bar). Gulo<sup>−</sup> mice supplemented with low or high vitamin C had lower IgG2c levels than did wt mice (dotted bar) but differences were significant only for gulo<sup>−</sup> mice supplemented with low vitamin C (*, p<0.05). IgG1 levels among these three groups of mice were low and similar at 32 WPI (p=0.44).

Figure 6. *H. pylori* (Hp) colonization levels (log CFU / μg host DNA). At 16 WPI, there were no differences in *H. pylori* colonization levels across groups. At 32 WPI, gulo<sup>−</sup> mice supplemented with low vitamin C had higher *H. pylori* colonization levels than gulo<sup>+</sup> mice supplemented with high vitamin C and wt mice. (*, p<0.05; #, 0.05<p<0.10).

Figure 7.

Plasma levels of amidated gastrin at 32 WPI. Among uninfected mice, wt mice (dotted bar) and gulo<sup>−</sup> mice supplemented with low (white bar) or high (black bar) vitamin C had similar levels of amidated gastrin (p=0.13) *H. pylori* infection significantly up-regulated amidated gastrin levels in wt mice (p<0.01), but not in gulo<sup>−</sup> mice that received low or high vitamin C. Among *H. pylori*-infected mice, gulo<sup>−</sup> mice supplemented with low vitamin C had amidated gastrin levels lower than gulo<sup>+</sup> mice supplemented with high vitamin C and wt mice (p=0.065 and <0.05, respectively). (*, p<0.05; **, p<0.01; #, p=0.65).
Fig. 3

a) 16 WPI, *H.p.-infected*

Scores

- Infl
- Epi defc
- Int meta
- Muc meta
- Atrop
- Hyper
- Dys

b) 32 WPI, *H.p.-infected*

Scores

- Infl
- Epi defc
- Int meta
- Muc meta
- Atrop
- Hyper
- Dys

C) Total gastric indices

- 16 WPI
- 32 WPI
Fig. 4

(a) IFN-γ

Fold change

H. pylori  
-  
+  

(b) TNF-α

Fold change

H. pylori  
-  
+  

Fig. 5

ELISA OD

IgG2c  
IgG1  

*  
#  

**  
***  

116
Table 1. *H. pylori* infection at 16 weeks post infection (WPI)

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<th><em>H. pylori</em></th>
<th>Vitamin C</th>
<th>Number of mice</th>
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<td>Low</td>
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<td>High</td>
<td>8</td>
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<tr>
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<td>None</td>
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Table 2. *H. pylori* infection at 32 WPI

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<th>Vitamin C</th>
<th>Number of mice(^a)</th>
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<td>High</td>
<td>0/6</td>
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\(^a\)First experiment/second experiment
Chapter 4: Dysfunction of DNA repair genes 3-alkyladenine DNA glycosylase and O\textsuperscript{6}-methylguanine DNA methyltransferase enhanced development of preneoplastic oxyntic gland atrophy in the stomach of chronically *Helicobacter pylori*-infected mice.

Abstract

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ABSTRACT

Dysfunction and polymorphisms of DNA repair enzymes have been associated with a higher risk of gastric cancer. Due to the fact that Helicobacter pylori causes gastritis and promotes progression to gastric cancer, we designed studies to examine whether defects in the 3-alkyladenine DNA glycosylase (Aag) or O6-methylguanine DNA methyltransferase (Mgmt) genes would have an effect on H. pylori-associated gastritis and gastric cancer. To access this question, Aag or Mgmt knockout mice on a C57BL/6 background were used. Aag−/− mice infected with H. pylori Sydney strain for 32 weeks developed significantly more severe gastric lesions including foveolar hyperplasia (p<0.05), mucous metaplasia (p<0.001), and precancerous oxyntic gland atrophy (p<0.01) relative to wild type (wt) mice. H. pylori-infected Mgmt−/− mice developed higher degrees of oxyntic atrophy (p<0.05) and mucous metaplasia (p<0.05) than infected wt mice. No significant differences in inflammation, epithelial defects, intestinal metaplasia, and dysplasia were observed between H. pylori-infected wt and Aag−/− or Mgmt−/− mice. Both H. pylori-infected Aag−/− and Mgmt−/− mice had similar levels of H. pylori-specific, Th1-associated IgG2c response and H. pylori colonization compared to infected wt mice. Levels of etheno-forms of DNA adducts were statistically similar between H. pylori-infected wt and Aag−/− mice. Dysfunction of Aag or Mgmt may potentiate H. pylori-associated gastric carcinogenesis by promoting onset of premalignant gastric atrophy.
INTRODUCTION

*Helicobacter pylori* infects the human stomach [1] and has been strongly associated with gastritis, peptic ulcer, atrophic gastritis, gastric cancer, and gastric MALToma [2]. *H. pylori* was classified as a group I carcinogen by a Working Group of the International Agency for Research on Cancer, a branch of the World Health Organization in 1994 based on accumulated epidemiologic studies [3]. Infection of C57BL/6 mice with *H. pylori* or *H. felis* results in chronic active gastritis which can progress to severe dysplasia or *in situ* gastric adenocarcinoma [4, 5]. In addition to *H. pylori* infection, host and environmental factors contribute to the multi-factorial gastric carcinogenesis [2, 3]. Recent studies have identified several genetic factors associated with gastric cancer, including DNA repair proteins O\(^6\)-methylguanine DNA methyltransferase (Mgmt) and 8-oxoguanine DNA glycosylase (Ogg1) [6, 7]. Leukocytes and other phagocytic cells generate reactive oxygen and nitrogen species (RONS) that damage DNA and other macromolecules during inflammation [8]. RONS cause DNA damage via (i) direct base oxidation and deamination and (ii) indirect alkylation to form ε-base lesions by 4-hydroxyalkenals through lipid peroxidation [9-11]. It has been demonstrated that gastric helicobacter infection resulted in chronic inflammation accompanied with higher oxidative stress, and increased levels of 8-hydroxy-deoxyguanine and some alkylated bases were found in the DNA from *H. pylori*-infected gastric tissues *in vitro* and *in vivo* [12, 13]. The role of oxidative DNA damage and DNA repair proteins in chronic *H. pylori* infection has not been studied.

*Aag* gene encodes 3-alkyladenine DNA glycosylase (Aag), the major DNA glycosylase that removes 3-methyladenine (3-MeA), deaminated and etheno adducts and hypoxanthine from DNA to initiate the base excision repair pathway [14]. Mouse embryonic stem cells defective for Aag are more sensitive to alkylating agents [14] and mitomycin-C [15]. In contrast, bone marrow cells are more
resistant to alkylation agents [16]. Aag activity is higher in stomach compared to other organs in mice, suggesting Aag is important in the gastric compartment [17]. Higher 3-MeA levels were observed in gastric mucosa with chronic *H. pylori* infection [13]. However, the role of Aag in chronic *H. pylori* gastritis and development of gastric cancer has not been examined. Since Aag overexpression was associated with chronic active inflammation in patients suffering from ulcerative colitis and correlated with increased microsatellite instability [18], it is possible that Aag may play an important role in *H. pylori*-associated chronic gastritis and gastric cancer.

Mgmt is the DNA repair protein capable of repairing specific types of mutagenic DNA adducts including O\(^6\)-methylguanine and O\(^4\)-methylthymine [19, 20]. Transcription of Mgmt is frequently inactivated by promoter hypermethylation in gastric cancer [21]. However, mice lacking Mgmt [22] do not develop gastric cancer spontaneously, and there is no evidence that O\(^6\)-alkylguanine-induced DNA adducts contribute to the spontaneous mutational spectrum in liver and small intestinal epithelium from 6-8 week-old and 12 month-old Mgmt\(^{-/-}\) mice [20]. There is no increase in spontaneous malignancy in the aged Mgmt\(^{-/-}\) mice up to 24 months old (unpublished observation). Therefore, it is not clear whether transcriptional inactivation of Mgmt is the cause or consequence of gastric carcinogenesis in humans.

To investigate the possible roles of the DNA repair proteins Aag and Mgmt in *H. pylori*-associated gastric cancer, C57BL/6 mice lacking functional Aag [16] or Mgmt [22] were infected with *H. pylori*. We hypothesized that dysfunction of DNA repair proteins Aag or Mgmt would promote progression of *H. pylori*-associated gastric cancer.

**MATERIALS AND METHODS**
Mice

Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility in static microisolator cages as previously described [23]. *Aag*<sup>+</sup> mice on a C57BL/6 background were rederived to barrier specific-pathogen-free (SPF) status; *Mgmt*<sup>+</sup> mice on a C57BL/6 background were housed in a non-barrier SPF facility which is free of human *Helicobacter spp* with the exception of *H. rodentium*. Mice were weaned at 3 weeks of age and fed *ad libitum* with regular mouse chow and water. Wild-type (wt) C57BL/6 mice were obtained from Taconic Farms (Germantown, NY) and divided equally into two groups as controls. Dirty bedding material from cages of *Mgmt*<sup>+</sup> or *Aag*<sup>+</sup> mice was mixed with that from cages of corresponding wt mice to ensure all groups of mice had similar commensal microbial flora. The protocol was approved by Committee on Animal Care of the Massachusetts Institute of Technology.

Bacteria and experimental infection

*H. pylori* Sydney strain (SS1) was used for oral inoculation as described previously [23, 24]. The bacteria were grown for 24 hours at 37°C under microaerobic conditions in *Brucella* broth with 10% fetal bovine serum, harvested, and resuspended in PBS. *H. pylori* was assessed by Gram's stain and phase microscopy for purity, morphology, and motility. The bacterial concentration was adjusted to OD<sub>600</sub>=1.000 in PBS. Mice were dosed with 0.2 ml of *H. pylori* suspension in PBS by gavage every other day for three doses. Control mice were dosed with PBS.

Experimental design

Both genders of 6-8 week old *Mgmt*<sup>+</sup>, *Aag*<sup>+</sup>, and wt mice were experimentally infected with *H. pylori*. Mice were euthanatized with CO<sub>2</sub> at 32 weeks post infection (WPI) (Table 1 and 2).
Histological evaluation

At necropsy, the stomach and proximal duodenum were removed and cut along the greater curvature. Linear gastric strips from the lesser curvature were fixed overnight in 10% neutral-buffered formalin or 70% ethanol, embedded, cut at 4 μm, and stained with hematoxylin and eosin. Selected tissues from infected and control mice were characterized with special stains and immunohistochemistry. Acidic (intestinal type) mucins were shown with Alcian blue at pH 2.5 and neutral (gastric type) mucins were stained by periodic acid-Schiff [25]. Expression of trefoil factor 2 (TFF2) protein was stained by immunohistochemistry [26]. Lesions were scored by a veterinary pathologist (BR) blinded to sample identity as described previously [25].

Serum IgG isotype measurement

Serum collected at necropsy was evaluated for H. pylori-specific IgG1 (Th2-associated isotype) and IgG2c (Th1-associated isotype) by enzyme-linked immunosorbent assay (ELISA) using an outer membrane protein (OMP) preparation from H. pylori (SS1 strain) as described previously [27]. In brief, 96 well flat-bottom plates were coated with 100 μl OMP (10 μg/ml) overnight at 4°C, and sera were diluted to a ratio of 1:100. Biotinylated secondary antibodies (clones A85-1 and 5.7, BD Pharmingen, San Jose, CA) were used for detecting IgG1 and IgG2c. Incubation with extravidin peroxidase (Sigma-Aldrich) followed by treatment with ABTS [2,2'-azinobis(3-ethylbenzthiazolesulfonic acid)] substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD.) was used for color development. The optical density was recorded by a plate reader per manufacturer’s protocol (Power WaveX select, Biotek Instruments, Winooski, VT).

Quantitative PCR for H. pylori colonization

H. pylori colonization levels were quantified using a fluorogenic quantitative PCR assay. In brief,
frozen gastric tissues were first digested by proteinase K at 55°C for 8 hours followed by DNA extraction with phenol: chloroform: isoamyl alcohol (25:24:1) followed by ethanol precipitation. Genomic DNA was subjected to a quantitative PCR assay using UreB primers [24]. Copy numbers of gastric *H. pylori* DNA (colonization forming units, CFU) were normalized to the amount of murine chromosomal DNA determined by quantitative PCR using a eukaryotic 18S endogenous control (Applied Biosystems) [24].

**Measurements of DNA adducts**

Etheno adducts of DNA, 1, N⁶-ethenodeoxyadenosine (etheno-dA) and 3, N⁴-ethenodeoxycytosine (etheno-dC), were quantified from selected tissues of *H. pylori*-infected or control *Aag*⁺⁻ and wt mice. DNA was isolated from stomach using a Genomic DNA isolation Kit for Cells and Tissues (Roche, Indianapolis, IN) per manufacturer’s protocol with some modification. In brief, an antioxidant (0.1 mM desferrioxamine) and a combination of deaminase inhibitors (5 μg/ml cofomycin, 50 μg/ml tetrahydouridine) were added into the lysis buffer to reduce artifactual oxidative DNA damage during DNA isolation. Etheno adducts were analyzed using an LC-MS/MS method described previously [28].

**Statistical Analysis**

Direct comparison of mean histological grades was made by Mann-Whitney *U* test. Group means of log-transformed bacterial colonization and serum antibody titers were compared by *t* test. Graphpad Prism 4.0 (Graphpad software, San Diego, CA) software was used for all statistic analysis. Statistical significance was set at *p* < 0.05.

**RESULTS**
*H. pylori* infection resulted in more severe oxyntic gland atrophy and hyperplasia in *Aag*" and *Mgmt*" mice than in wt mice

There were no significant gross or microscopic gastric lesions in *H. pylori*-free *Aag*"*, Mgmt"*, or wt mice (Table 1, Fig. 1). *H. pylori* infection in wt mice at 32 weeks post infection (WPI) resulted in chronic atrophic gastritis of the corpus characterized by infiltration of lymphocytes and granulocytes, parietal and chief cell loss, and foveolar hyperplasia (Fig. 1). Both *H. pylori*-infected male and female wt mice developed similar degrees of gastric lesions (Table 2).

*H. pylori*-infected *Aag*" mice developed more severe oxyntic gland atrophy (p<0.01), foveolar hyperplasia (p<0.05), and mucous metaplasia (p<0.001) in the corpus than did wt mice with *H. pylori* infection at 32 WPI (Table 1, Fig. 1). *H. pylori* infection resulted in similar degrees of inflammation, epithelial defects, intestinal metaplasia, and dysplasia in both *Aag*" and wt mice. *H. pylori*-associated gastric lesions were comparable between infected male and female *Aag*" mice or wt mice, and both genders of *Aag*" mice with *H. pylori* infection developed more severe oxyntic gland atrophy and mucous metaplasia than did their infected wt counterparts (p<0.05 for all comparisions) (Table 2). Higher degrees of foveolar hyperplasia were observed in *H. pylori*-infected male *Aag*" mice than in infected male wt mice (p<0.05), but differences in foveolar hyperplasia were not observed between *H. pylori*-infected female *Aag*" and wt mice (Table 2).

*H. pylori*-infected *Mgmt*" mice developed more severe oxyntic gland atrophy (p<0.05) and mucous metaplasia (p<0.05) in the corpus than did wt mice with *H. pylori* infection at 32 WPI (Table 1). *H. pylori* infection resulted in similar degrees of inflammation, epithelial defects, foveolar hyperplasia, intestinal metaplasia, and dysplasia in both *Mgmt*" and wt mice. However, *H. pylori*-infected female *Mgmt*" mice developed more severe oxyntic atrophy (p<0.05) and mucous metaplasia (p<0.05) than
did infected female wt mice, irrespective of comparable degrees of other histological parameters including corpus inflammation, epithelial defects, epithelial hyperplasia, intestinal metaplasia, and dysplasia (Table 2). Due to the limited number (n=2) of *H. pylori*-infected male *Mgmt*−− mice, gastric lesions in these mice were not compared with wt male mice.

**Aag**−− and **Mgmt**−− mice developed *H. pylori*-specific, Th1-predominant IgG2c responses comparable to infected wt mice

At 32 WPI, *H. pylori* infection resulted in robust Th1-associated IgG2c responses in infected wt (p<0.01), Aag−− (p<0.01) and Mgmt−− (p<0.001) mice relative to uninfected mice (Fig. 2). Although *H. pylori* induced less robust Th2-associated IgG1 responses than IgG2c responses in wt, Aag−− and Mgmt−− mice (p<0.001), *H. pylori*-specific IgG1 antibody responses were significantly elevated in infected Mgmt−− mice (p<0.05) but not in infected Aag−− mice (p= 0.13) or wt mice (p=0.10). Additionally, there were no significant differences in *H. pylori*-specific IgG2c or IgG1 responses between Aag−− or Mgmt−− mice and their wt counterparts.

**H. pylori** colonization levels were comparable between Aag−− or Mgmt−− mice and their wt counterparts

The mean densities of *H. pylori* (log CFU/μg genomic DNA) at 32 WPI were comparable between Aag−− or Mgmt−− mice and their wt counterparts (p=0.99 and 0.31, respectively) (Fig. 3).

**DNA adducts in gastric tissues**

Levels of etheno-dA in *H. pylori*-uninfected Aag−− and wt mice were comparable (Fig. 4). *H. pylori* infection resulted in slight but not significant elevation in levels of etheno-dA (wt mice, p=0.28; Aag−−−− mice, p=0.15). Etheno-dC levels were slightly higher in *H. pylori*-infected wt mice than in infected
Aag<sup>−/−</sup> mice (p=0.08). Etheno-dC levels were not compared in H. pylori-uninfected mice due to the limited numbers of DNA samples.

DISCUSSION

The present study investigated the roles of DNA repair proteins Aag and Mgmt in the immune response and gastric lesions in mice with chronic H. pylori infection. Aag<sup>−/−</sup> and Mgmt<sup>−/−</sup> mice infected with H. pylori developed more severe oxyntic gland atrophy, mucous metaplasia, and/or foveolar hyperplasia, but had similar degrees of inflammation and epithelial defects compared to the infected wt mice at 32 WPI. These results suggest that Aag and Mgmt play a role in attenuating the progression of H. pylori-induced gastric gland atrophy, a premalignant lesion in humans and mice [29, 30], but these DNA repair proteins have no protective effect on gastric inflammation.

It has been demonstrated that the Th1 proinflammatory response to H. pylori or H. felis is required for the development of corpus gastritis and progression to parietal cell loss and oxyntic gland atrophy [31, 32]. Modulation of the balance between Th1 and Th2 immune responses elicited by gastric helicobacter infection varying by host background or by concurrent parasite infection can dramatically affect the progression of H. pylori-induced gastric diseases [29, 33]. In this study, the degrees of corpus inflammation, epithelial defects, intestinal metaplasia and dysplasia were comparable between Aag<sup>−/−</sup> or Mgmt<sup>−/−</sup> mice and their wt controls, whereas more severe gastric atrophy was noted in mice lacking functional Aag or Mgmt. H. pylori colonization levels are documented to be inversely correlate with the intensity of H. pylori- and Th1-mediated inflammation [23, 33]. This observation is consistent with comparable degrees of corpus inflammation, similar levels of H. pylori colonization and of H. pylori-specific IgG2c responses in Aag<sup>−/−</sup> or Mgmt<sup>−/−</sup> mice and their wt control mice. As a result, the more severe gastric atrophy in Aag<sup>−/−</sup> or Mgmt<sup>−/−</sup> mice
suggests that dysfunction of DNA repair proteins Aag or Mgmt may be partially responsible for the progression of preneoplastic gastric gland atrophy associated with chronic *H. pylori* infection.

Several previous observations demonstrated that gastric helicobacter infection resulted in chronic inflammation accompanied with increased oxidative stress and oxidative DNA damage *in vitro* and *in vivo* [12, 13], which may contribute to gastric mutagenic effects of *H. pylori* and *H. felis* [34]. It is unclear, however, how these gastric mutations contribute to gastric carcinogenesis. Whether the helicobacter-associated oxidative DNA damage is repaired by DNA repair proteins and whether these DNA mutations reside in epithelial or inflammatory cells is not known. Mice defective in Ogg1 DNA glycosylase developed less severe *H. pylori*-associated gastritis and had lower gastric mutant frequency than the *H. pylori*-infected wt mice [35]. Our current study demonstrates that *H. pylori* infection resulted in more severe preneoplastic gastric atrophy in mice lacking functional Aag or Mgmt, the proteins that can repair *H. pylori*-associated DNA adducts including 3-methyladenine [13] or O\(^6\)-alkylguanine [36]. Importantly, transcriptional inactivation of Mgmt by promoter hypermethylation is observed in the presence of severe chronic gastric inflammation and intestinal metaplasia, and has been associated with gastric cancer in humans [7, 21, 37]. Transcriptional inactivation of gastric Mgmt by DNA hypermethylation in *H. pylori*-associated gastritis results in incapability to repair certain mutagenic oxidative DNA adducts, accumulation of gastric mutations, and progression to gastric cancer. In addition, certain polymorphisms of Mgmt gene have been associated with a higher risk of gastric and other malignancies [6, 38, 39], which may be related to differences in Mgmt activity. The role of Aag in human gastritis and gastric cancer may be similar, since the CpG island within its promoter region [40] may be important for epigenetic regulation in *H. pylori* gastritis that predisposes to inactivation of Aag. In this study, we observed slightly higher gastric levels of etheno-dC in *H. pylori*-infected wt mice than infected *Aag\(^{-}\)* mice. The elevated
etheno-dC might bind to Aag protein, “hijack” its enzyme activity to excise etheno-dA, and resulted in accumulation of etheno-dA observed in *H. pylori*-infected wt mice [41]. Further studies are necessary to examine the roles of DNA repair proteins in the progression to gastric cancer.

TFF2, also known as spasmodic polypeptide (SP), is mainly secreted by gastric mucous neck cells [42]. TFF2 is upregulated during acute parietal cell loss induced in rodents treated with DMP-777 and in mice chronically infected with gastric helicobacter [26, 43-46]. These helicobacter-infected mice developed atrophic gastritis and mucous metaplastic cells that are TFF2-positive. The spasmodic polypeptide expressing metaplasia (SPEM) lineages are associated with a strong Th1-predominant immune response and upregulation of IFN-γ during chronic helicobacter infection [47, 48]. This TFF2-expressing SPEM lineages are noted in helicobacter-infected mice with severe dysplasia and in the dysplastic glands of gastric adenocarcinoma in humans [24, 49, 50]. In fact, it has been documented that SPEM cells are recruited from bone marrow-derived cells (BMDCs) during chronic *H. felis* infection. BMDCs recruitment to gastric mucosa is not observed in acute helicobacter infection or transient mucosal damage [51]. Therefore, SPEM lineages appear to be the precursor of gastric cancer; chronic inflammation is an essential factor to create a milieu for recruitment and repopulation of BDCMs in the stomach and for progression to metaplasia, dysplasia, and cancer. A higher degree of preneoplastic oxyntic gland atrophy in *H. pylori*-infected Aag<sup>−/−</sup> and Mgmt<sup>−/−</sup> mice, consistent with more TFF2-expressing cells, suggests that dysfunction of Aag and Mgmt potentiates *H. pylori*-associated gastric carcinogenesis.

In summary, our data demonstrate the roles of DNA repair enzymes Aag and Mgmt in *H. pylori*-associated gastric diseases. Compared to wt mice, *H. pylori* infection in Aag<sup>−/−</sup> and Mgmt<sup>−/−</sup> mice resulted in similar degrees of corpus gastritis and host immune responses against *H. pylori*
manifested as comparable levels of anti-\textit{H. pylori} IgG2c and \textit{H. pylori} colonization. Inactivation of Aag or Mgmt promoted the development of more severe mucous metaplasia and preneoplastic gastric gland atrophy in the corpus, and may potentiate \textit{H. pylori}-associated gastric tumorogenicity through the accumulation of un-repaired mutagenic DNA damage associated with chronic \textit{H. pylori} gastritis. Importantly, these results in \textit{H. pylori}-infected Mgmt\(^{\text{-}}\) mice are compatible with previous reports in humans that transcriptional inactivation of Mgmt in chronic gastritis may predispose to development of gastric cancer [21, 37]. Aag\(^{\text{-}}\) and Mgmt\(^{\text{-}}\) mice provide useful models to dissect the mechanisms of oxidative DNA damage and DNA repair in carcinogenicity of chronic \textit{H. pylori} infection in humans.

**ACKNOWLEDGEMENTS**

We thank Lisiane B. Meira and Leona D. Samson for providing Aag\(^{\text{-}}\) and Mgmt\(^{\text{-}}\) mice; Kathy Cormier for providing histology expertise; Barry Rickman for pathological expertise; Bo Pang and Peter C. Dedon for expert assistance with DNA adduct measurements.

**REFERENCES**


LEGENDS

Fig. 1.
Histopathology of gastric disease and mucous metaplasia induced by *H. pylori* infection in the fundic mucosa of C57BL/6 wt or Aag<sup>−/−</sup> mice. Representative H&E, AB/PAS-stained, and anti-TFF2 immunohistochemistry (DAB) of stomachs from 38-week old wt (a,b,c), Aag<sup>−/−</sup> infected (d,e,f) and wt infected (g,h,i) mice. Uninfected mouse stomach showing normal microscopic architecture on H&E stain (a) and a thin surface lining of gastric-type neutral mucins (red) by AB/PAS (b). Anti-TFF2 antibody stained mucous neck cells in the fundic mucosa with high specificity (c). Tissue from *H*. *pylori*-infected Aag<sup>−/−</sup> mice (32 WPI) exhibited loss of oxyntic cell mass due to mucous metaplasia characterized by foamy change of oxyntic cell cytoplasm on H&E stain (d) and prominent coexpression of both intestinal-type acid mucins (blue) and gastric type mucins (red) in mucous metaplastic glands shown by AB/PAS stain (e). Anti-TFF2 antibody showed similar staining pattern in the mucous metaplastic glands (f) as shown in (e). Tissue from *H*. *pylori*-infected wt mice (32 WPI) exhibited mild loss of oxyntic cell mass with minimal mucous metaplasia of oxyntic cell cytoplasm on H&E stain (g) and mild coexpression of both intestinal-type acid mucins (blue) and gastric type mucins (red) in mucous metaplastic glands shown by AB/PAS stain (h). Anti-TFF2 antibody showed fewer metaplastic glands but similar staining pattern in the mucous metaplastic glands (i) as shown in (h).

Fig. 2.
Serum IgG1 and IgG2c responses to *H. pylori* (*H. pylori*) in Aag<sup>−/−</sup> (a) or Mgmt<sup>−/−</sup> (b) mice and their wt controls at 32 weeks post infection. Data were presented as mean values ± standard errors. *H. pylori* infection resulted in more robust Th1-associated IgG2c responses than Th2-associated IgG1 responses in Aag<sup>−/−</sup>, Mgmt<sup>−/−</sup>, and wt mice (p<0.001). Both mutant mice developed comparable IgG2c
responses against *H. pylori* compared to infected wt mice, suggesting that dysfunction of Aag or Mgmt had no effect on *H. pylori*-specific immunity.

**Fig. 3.**

*H. pylori* colonization levels in the stomach. Data were presented as log transformed colony formatting unit (CFU) per µg of genomic DNA. *H. pylori* colonization levels were not different between Aag−/− and wt mice or Mgmt−/− and wt mice. ns, non-significant.

**Fig. 4.**

Levels of etheno-dA and etheno-dC in gastric DNA. Data were presented as number of adducts per 10^7 nucleoside. There were trends toward elevated levels of etheno-dA in *H. pylori*-infected wt and Aag−/− mice, but the differences were not significant. *H. pylori*-infected wt mice had slightly higher levels of etheno-dC than infected Aag−/− mice (p=0.08).
Fig. 1

H&E

wt

Aag^-/- infected

wt infected

AB/PAS

Anti TFF2

a
d

b
e

c
f

g
h

i
Fig. 2

(a) ELISA Optical Density

IgG1  IgG2c
Aag^{+/—} wt  Aag^{+/—} wt

(b) ELISA Optical Density

IgG1  IgG2c
Mgmt^{+/—} wt  Mgmt^{+/—} wt

Fig. 3

Log CFU/μg genomic DNA

Aag^{+/—} wt  Mgmt^{+/—} wt

ns  ns
Fig. 4

a

etheno-dA (per $10^7$)

H. pylori  wt Aag$^{-/-}$

b

etheno-dC (per $10^7$)

H. pylori  wt Aag$^{-/-}$

p=0.08
## TABLE 1. Gastric lesions in *Helicobacter pylori* (*H. pylori*)-infected wild type (wt), Aag⁻/⁻ and Mgmt⁻/⁻ mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>H. pylori</th>
<th>no.</th>
<th>Infm</th>
<th>Epi defc</th>
<th>Int meta</th>
<th>Atroph</th>
<th>Muc meta</th>
<th>Hyper</th>
<th>Dys</th>
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<tbody>
<tr>
<td>wt</td>
<td>None</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>wt</td>
<td>+</td>
<td>11</td>
<td>1 (0-2)</td>
<td>0 (0-1)</td>
<td>0</td>
<td>1 (0-2)</td>
<td>0.5 (0-3)</td>
<td>0.5 (0-1)</td>
<td>0</td>
</tr>
<tr>
<td>Aag⁻/⁻</td>
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<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aag⁻/⁻</td>
<td>+</td>
<td>13</td>
<td>1.5 (1-2.5)</td>
<td>1 (0-2)</td>
<td>0 (0-0.5)</td>
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<td>2.5 (2-3.5)</td>
<td>1 (0-2)</td>
<td>0</td>
</tr>
<tr>
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<td>5</td>
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<td>0</td>
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<td>0</td>
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</tr>
<tr>
<td>wt</td>
<td>+</td>
<td>7</td>
<td>1 (0.5-2.5)</td>
<td>0.5 (0-1)</td>
<td>0 (0-1)</td>
<td>1.5 (0.5-3)</td>
<td>2 (1-3)</td>
<td>1 (0-1)</td>
<td>0</td>
</tr>
<tr>
<td>Mgmt⁻/⁻</td>
<td>None</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Mgmt⁻/⁻</td>
<td>+</td>
<td>11</td>
<td>1 (0.5-1.5)</td>
<td>0.5 (0-2)</td>
<td>0 (0-0.5)</td>
<td>2.5 (1.5-3)</td>
<td>3 (2.5-3)</td>
<td>1 (0.5-1.5)</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: Infm, inflammation; Epi defc, epithelial defects; Int meta, intestinal metaplasia; Atroph, oxyntic atrophy; Muc meta, mucous metaplasia; Hyper, foveolar hyperplasia; and Dys, dysplasia.

*Comparison was made between Aag⁻/⁻ or Mgmt⁻/⁻ mice and wt mice by Mann-Whitney U test.*

- Significant influence of genotype (Aag⁻/⁻ vs wt) at a *p*<0.05.
- Significant influence of genotype (Aag⁻/⁻ vs wt) at a *p*<0.01.
- Significant influence of genotype (Aag⁻/⁻ vs wt) at a *p*<0.001.
- Significant influence of genotype (Mgmt⁻/⁻ vs wt) at a *p*<0.05.
TABLE 2. Gastric lesions in *Helicobacter pylori* (*H. pylori*)-infected wild type (wt), Aag<sup>−/−</sup> and Mgmt<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th><em>H. pylori</em></th>
<th>Sex&lt;sup&gt;e&lt;/sup&gt; (no.)</th>
<th>Inflm</th>
<th>Epi defc</th>
<th>Int meta</th>
<th>Atroph</th>
<th>Muc meta</th>
<th>Hyper</th>
<th>Dys</th>
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<tbody>
<tr>
<td>wt</td>
<td>+</td>
<td>M (6)</td>
<td>1 (0-2)</td>
<td>0 (0-1)</td>
<td>0</td>
<td>0.75 (0-2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 (0-3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 (0-1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F (5)</td>
<td>1.5 (1-2)</td>
<td>0.5 (0-1)</td>
<td>0</td>
<td>1 (0-1.5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5 (0-0.5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5 (0-1)</td>
<td>0</td>
</tr>
<tr>
<td>Aag&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>+</td>
<td>M (9)</td>
<td>1.5 (1-2.5)</td>
<td>0.5 (0-2)</td>
<td>0 (0-0.5)</td>
<td>2 (1.5-3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5 (2-3.5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 (0-2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F (4)</td>
<td>1.25 (1-2)</td>
<td>1.5 (0-1.5)</td>
<td>0 (0-0.5)</td>
<td>1.75 (1.5-2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (2-2.5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.75 (0-1)</td>
<td>0 (0-0.5)</td>
</tr>
<tr>
<td>wt</td>
<td>+</td>
<td>M (4)</td>
<td>1 (0.5-2.5)</td>
<td>0.75 (0-1)</td>
<td>0.25 (0-0.5)</td>
<td>2 (1-3)</td>
<td>2.5 (1.5-3)</td>
<td>1</td>
<td>0 (0-0.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F (3)</td>
<td>1 (0.5-1.5)</td>
<td>0.5 (0-1)</td>
<td>0 (0-1)</td>
<td>1 (0.5-2)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.5 (1-2)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0 (0-1)</td>
<td>0</td>
</tr>
<tr>
<td>Mgmt&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>+</td>
<td>M (2)</td>
<td>1.25 (1-1.5)</td>
<td>0.75 (0.5-1)</td>
<td>0</td>
<td>2.25 (2-2.5)</td>
<td>3</td>
<td>0.75 (0.5-1)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F (9)</td>
<td>1 (0.5-1.5)</td>
<td>0.5 (0-2)</td>
<td>0.5 (0-0.5)</td>
<td>3 (1.5-3)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3 (2.5-3)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1 (0.5-1.5)</td>
<td>0 (0-1)</td>
</tr>
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</table>

Abbreviations: Inflm, inflammation; Epi defc, epithelial defects; Int meta, intestinal metaplasia; Atroph, oxyntic atrophy; Muc meta, mucous metaplasia; Hyper, foveolar hyperplasia; and Dys, dysplasia.

<sup>a</sup>Comparison was made between same gender of Aag<sup>−/−</sup> or Mgmt<sup>−/−</sup> mice and wt mice by Mann-Whitney U test.

<sup>b</sup>Significant influence of genotype (Aag<sup>−/−</sup> vs wt) at a p<0.05.

<sup>c</sup>Significant influence of genotype (Mgmt<sup>−/−</sup> vs wt) at a p<0.05.

<sup>d</sup>Significant influence of genotype (Mgmt<sup>−/−</sup> vs wt) at a p<0.01.

<sup>e</sup>M, male; F, female.
Chapter 5: *Helicobacter pylori* Eradication Prevents Progression of Gastric Cancer in Hypergastrinemic INS-GAS Mice

Abstract

Introduction

Materials and Methods

Mice

Experimental design

Tissue collection and histological analysis

Confirmation of *H. pylori* eradication by quantitative PCR

Serum *H. pylori*-specific antibodies

Quantitative analysis of mRNA expression

Measurements of DNA adducts

Analysis of lipids in serum

Statistical analysis

Results

Eradication of *H. pylori* was confirmed in INS-GAS mice that received eradication therapy

*H. pylori* infection promoted developed of gastric cancer in INS-GAS mice

*H. pylori* eradication at 8 WPI reduced gastritis and premalignant lesions to the greatest extent

*H. pylori* eradication at 8 WPI prevented progression to low-grade and high-grade gastrointestinal intraepithelial neoplasia

*H. pylori*-specific antibody responses

Gastric IFN-γ, TNF-α, and iNOS mRNA levels were reduced in all mice that received *H. pylori* eradication therapy

Gastric mucosal cell proliferation was also reduced in all mice that received *H. pylori* eradication therapy

DNA adducts in gastric tissues

*H. pylori*-infected INS-GAS mice had significantly higher serum levels of triglyceride, total cholesterol, and elevated fraction of very-low-density lipoprotein

Discussion

Acknowledgements

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Legends

Figures

Tables
ABSTRACT

*Helicobacter pylori* infection results in chronic gastritis which may progress to gastric cancer. In the *H. pylori*-infected INS-GAS mouse, a model of gastric cancer, omeprazole, metronidazole, and clarithromycin were administered at 8, 12, or 22 weeks post *H. pylori* infection (WPI) to evaluate the efficacy of eradication in preventing the development of gastric cancer. *H. pylori* infection resulted in severe dysplasia classified as high- and low-grade gastrointestinal intraepithelial neoplasia (GIN) in INS-GAS mice at 28 WPI. *H. pylori* antimicrobial therapy at 8, 12 and 22 WPI significantly reduced the severity of dysplasia (p<0.01). Moreover, *H. pylori* eradication at 8 WPI completely prevented development of GIN (p<0.001) with the return of mucosal architecture to that of uninfected mice. Although not as effective as early antimicrobial treatment, prevention of progression to high-grade GIN was achieved by *H. pylori* eradication at 12 and 22 WPI (p<0.05 or less). Consistent with reduced gastric pathology, *H. pylori* eradication at all time points significantly down-regulated gastric IFNγ, TNFα, and iNOS mRNA levels (p<0.05) and reduced epithelial proliferation in the corpus (p<0.01). Additionally, elevated triglyceride and total cholesterol concentrations in serum were observed in infected INS-GAS mice compared to other treatment groups. We conclude that *H. pylori* eradication may prevent progression to gastric cancer to the greatest extent when antibiotics are given at an early point of *H. pylori* infection. Mice with severe dysplastic lesions may benefit from *H. pylori* eradication therapy given at a later time point although it is not as effective as early *H. pylori* eradication.
INTRODUCTION

*Helicobacter pylori* was first identified in the antrum of patients with active chronic gastritis and peptic ulcers [1]. Based on epidemiological evidence, *H. pylori* was identified as the major cause of gastric cancer and has been classified as a group I carcinogen of gastric cancer by a Working Group of the International Agency for Research on Cancer, a branch of the World Health Organization in 1994 [2, 3]. *H. pylori* infection causes a persistent chronic gastritis, which in susceptible individuals may progress to atrophy, intestinal metaplasia, dysplasia, and finally intestinal-type gastric cancer [3, 4]. Although a clinical trial failed to demonstrate a protective effect of *H. pylori* eradication against progression of preneoplastic lesions such as gastric atrophy and intestinal metaplasia [5], eradication of *H. pylori* in humans has been associated with prevention or regression of preneoplastic lesions [6-10]. Some epidemiological studies observed that antibiotic treatment during knee or hip replacement surgery reduced the incidence of gastric cancer in the following years, probably due to eradication of *H. pylori* [11, 12]. In intervention studies, the optimal effect of antibiotic eradication therapy in preventing gastric cancer has been observed in *H. pylori* infected patients who did not have precancerous lesions prior to antimicrobial *H. pylori* eradication therapy (p<0.05) [13]. However, *H. pylori* eradication did not reduce the overall prevalence of dysplasia or gastric cancer in another studies [7, 10, 13, 14]. Since it takes decades for gastric cancer to develop in susceptible hosts acquiring *H. pylori* infection at an early age [4], these *H. pylori* eradication trials continue to pose key questions; in which patients would *H. pylori* eradication be beneficial in preventing gastric cancer and at what stage of gastric disease would *H. pylori* antimicrobial eradication prevent the progression of gastric lesions to gastric cancer.
Several animal models have been used to examine whether *H. pylori* eradication is effective in reversal of preneoplastic gastric lesions and preventing the progression of these preneoplastic lesions to gastric cancer. Antibiotic eradication therapy reversed the histologic progression of dysplasia in *H. pylori*-infected Mongolian gerbils [15, 16]. In *Helicobacter felis*-infected C57BL/6 mice that developed gastric cancer within 24 months post infection, antibiotic therapy given at 2 or 6 months post infection (MPI) led to a regression of inflammation and reversion of premalignant lesions. Antibiotics given at 12 MPI arrested progression of dysplasia and reduced the risk of gastric cancer [17]. These *in vivo* data support the hypothesis that eradication therapy, depending on the timing of antibiotic treatment, may be effective in preventing helicobacter gastritis from progressing to gastric cancer.

Recent studies suggest an association with hypergastrinemia, helicobacter infection and gastric cancer in humans and mice [18-21]. In the absence of helicobacter infection, transgenic INS-GAS mice that over-express amidated gastrin have a higher gastric acid secretion and an increased parietal cell number when they are 1-3 months old. As the mice age, they lose parietal cell mass and develop hypochlorhydria, gastric atrophy, metaplasia, and dysplasia. At 20 months of age, helicobacter-uninfected INS-GAS mice develop invasive gastric cancer [20, 22]. The development of gastric cancer is accelerated by helicobacter infection and lesion severity is more profound in male INS-GAS mice [18, 20, 21]. The purpose of this study was to examine the effect of *H. pylori* eradication at different stages of progression from gastritis to gastric cancer in INS-GAS mice.
MATERIALS AND METHODS

Mice

The animal protocol was reviewed and approved by the Massachusetts Institute of Technology Committee on Animal Care. Specific pathogen-free (including Helicobacter spp.) male INS-GAS mice on a FVB/N background were used in this study [22]. Mice were maintained in an AAALAC accredited facility and housed on hard wood bedding in microisolator, solid-bottomed polycarbonate cages, and given a normal rodent diet (Prolab RMH 3000, PMI Nutrition International, Richmond, Indiana) and water ad libitum. Age-match male wt FVB/N mice were from retired breeding colony.

Experimental design

Fifty-four 6-8 week old mice were infected by oral gavage with H. pylori (SS1 strain) on alternate days for a total of 3 doses [23, 24]. The H. pylori inoculum for oral gavage was adjusted with phosphate-buffered saline (PBS) to an optical density of 1.0 at 600 nm [23]. Helicobacter-uninfected mice were sham-dosed with PBS. Infected mice were orally dosed with omeprazole (400 μmol/kg/day, Sigma-Aldrich, St. Louis, MO), metronidazole (14.2 mg/kg/day, Sigma-Aldrich), and clarithromycin (7.15 mg/kg/day, Abbott Laboratory, North Chicago, IL) in 0.2 ml twice a day for 7 days [25]. This antimicrobial therapy was previously demonstrated to eradicate 100% of mice infected with H. pylori was instituted [25]. Treatment was administered at 8, 12, or 22 weeks post H. pylori infection (WPI).

Tissue collection and histological analysis

Mice were euthanized at 28 WPI. After CO2 asphyxia, blood was collected by cardiac puncture.
The stomach and proximal duodenum were removed and cut along the greater curvature. Linear gastric strips from the lesser curvature were fixed overnight in 10% neutral-buffered formalin, embedded, cut at 4 μm, and stained with hematoxylin and eosin (H&E). Tissue sections were scored for gastric lesions using previously published criteria by veterinary pathologists (B.R and A.B.R) blinded to sample identities [26]. A dysplasia score of 3.0 is considered carcinoma in situ or low-grade gastrointestinal intraepithelial neoplasia (GIN). Dysplasia scores equal or higher than 3.5 represent intramucosal carcinoma or herniated, high-grade GIN [27]. Both low- and high-grade GIN in mouse stomachs are diagnosed as gastric cancer. Ki67 immunostaining (BD Biosciences, San Diego, CA) was used to measure epithelial proliferation of gastric mucosa. The ratio of positive to total epithelial nuclei in glands occupying the full length of the proximal corpus was quantified manually for the Ki67 labeling index (LI), and results were averaged. The remainder of the gastric tissue was snap-frozen in liquid nitrogen and stored in -70 °C for DNA and RNA analysis.

**Confirmation of H. pylori eradication by quantitative PCR**

A longitudinal strip of gastric tissue from the greater curvature was digested with proteinase K at 55°C overnight followed by DNA extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation. *H. pylori* copy numbers were quantified using a fluorogenic quantitative PCR assay with urease B primers [28]. *H. pylori* copy numbers were normalized to microgram of murine genomic DNA as determined by quantitative PCR using a eukaryotic 18S endogenous control (Applied Biosystems) (User Bulletin #2, Applied Biosystems, Foster City, CA).
Serum *H. pylori*-specific antibodies

Sera collected at necropsy were evaluated for *H. pylori*-specific, Th2-associated IgG1 and Th1-associated IgG2a by enzyme-linked immunosorbent assay (ELISA) using an outer membrane protein preparation from *H. pylori* (SS1 strain) as described previously [29]. In brief, 96 well flat-bottom plates were coated with 100 μl antigen (10 μg/ml) overnight at 4°C; sera were diluted to a ratio of 1:100 and added to the wells. Biotinylated secondary antibodies included goat anti-mouse antibodies were used for detecting IgG1 and IgG2a (clones A85-1 and 5.7, BD Pharmingen, San Jose, CA). Incubation with extravidin peroxidase (Sigma-Aldrich) was followed by treatment with ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD.) for color development. The optical density was recorded at OD405 and OD595 by a plate reader per manufacturer's protocol (Power WaveX select, Biotek Instruments, Winooski, VT)

Quantitative analysis of mRNA expression

Two mice in which *H. pylori* eradication was unsuccessful were excluded from further analysis (Table 1). A longitudinal strip of gastric tissue from the anterior wall was harvested and snap-frozen in liquid nitrogen. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 5 μg of total RNA with High Capacity cDNA Archive kit (Applied Biosystems). IFN-γ and TNF-α mRNA levels were quantified with SYBR Green PCR reagent (Qiagen, Valencia, CA) using primers for INFγ: CATGGCTGTTTCTGGCTGTTACTG (Forward [F]) and GTTGCTGATGGCCTGATTGTCTTT (Reverse [R]) annealing at 56°C; for TNFα: CATCTTCTCAAAATTCGAGTGACA (F) and TGGGAGTAGACAAGGTACCAAC (R) annealing at 60°C. The final concentration of each primer was 0.3 μM. iNOS and
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were quantified with TaqMan gene expression assays (Applied Biosystems) in an ABI Prism Sequence Detection System 7700. mRNA levels of each cytokine were normalized to the mRNA level of internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared to data of uninfected mice using the ΔΔCT method (User Bulletin #2, Applied Biosystems).

Measurements of DNA adducts
Adducts of DNA, 2'-deoxyinosine (dI), N6-ethenodeoxyadenosine (etheno-dA), and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), were quantified from selected tissues of *H. pylori*-infected or control INS-GAS and wt mice. DNA was isolated from stomach using a Genomic DNA isolation Kit for Cells and Tissues (Roche, Indianapolis, IN) per manufacturer’s protocol with some modification. In brief, an antioxidant (0.1 mM desferrioxamine) and a combination of deaminase inhibitors (5 μg/ml coformycin, 50 μg/ml tetrahydrouridine) were added into the lysis buffer to reduce artifactual oxidative DNA damage during DNA isolation. DNA adducts were analyzed using an LC-MS/MS method described previously [30].

Analysis of lipids in serum
Sera collected from unfasted animals at necropsy were evaluated for triglyceride and total cholesterol concentrations enzymatically using reagents from Roche (Indianapolis, IN) and Sigma, respectively [31]. Lipoproteins were separated by fast-protein liquid chromatography after equal volumes of serum from 5 mice per group were pooled. Cholesterol concentrations in individual fractions were determined enzymatically [32].
Statistical analysis

Infected mice with successful *H. pylori* eradication were included in the analysis. Gastric lesion scores and Ki67 LI for proliferation indices were compared by the Mann-Whitney U test. Cytokines and iNOS expression levels were compared using the student t test. Incidences of no, low-grade, and high-grade GIN were compared by Fisher's exact t test. DNA adduct levels and triglyceride and total cholesterol concentrations were compared by Student t test. Statistical analysis was performed using a commercial software (Graphpad Prism 4.0, GraphPad Software, Inc., San Diego, CA) with significance at p<0.05.
RESULTS

Eradication of *H. pylori* was confirmed in INS-GAS mice that received eradication therapy

To assess the effect of *H. pylori* eradication on progression of gastric cancer in INS-GAS mice, eradication therapy using the combination of omeprazole, metronidazole, and clazithromycin was administered orally to mice at 8, 12, or 22 WPI. Eradication of *H. pylori* was confirmed at necropsy using quantitative PCR. *H. pylori* was successfully eradicated in all mice treated at 8 or 12 weeks post infection (WPI). Twelve of fourteen (85.7%) animals that underwent eradication therapy at 22 WPI were successfully *H. pylori* eradicated (Table 1).

*H. pylori* infection promoted developed of gastric cancer in INS-GAS mice

Helicobacter infection promotes gastric carcinogenesis in INS-GAS mice particularly in male animals [20, 21]. Consistent with a previous study [21], uninfected INS-GAS mice at 28 to 34 weeks of age developed progressive gastric lesions including atrophy, epithelial defect, and dysplasia (p<0.05) accompanied with minimal inflammation, severe hyperplasia and intestinal metaplasia (Table 2). Corpus hypertrophy in infected INS-GAS mice at 22 and 28 WPI was noted at necropsy as thickened mucosal folds. The *H. pylori*-infected INS-GAS mice had chronic atrophic gastritis with profound changes in mucosal architecture. These histologic changes were mainly restricted to the corpus and were characterized by loss of parietal and chief cells (Fig 1). Compared to age-matched uninfected mice, infected INS-GAS mice had more severe inflammation (p<0.001), oxyntic atrophy (p<0.05), hyperplasia (p<0.05), epithelial defects (p<0.001), intestinal metaplasia (p<0.05) and dysplasia (p<0.001) at 22 WPI, and higher degrees of inflammation (p<0.001), dysplasia (p<0.001), and hyperplasia (p<0.01) at 28 WPI (Fig. 2). Compared to infected mice at 22 WPI, infected mice at 28 WPI had more severe dysplasia and
atrophy (p<0.05).

**H. pylori eradication at 8 WPI reduced gastritis and premalignant lesions to the greatest extent**

*H. pylori*-infected INS-GAS mice received antimicrobial eradication therapy at 8, 12, or 22 WPI and were euthanized at 28 WPI. Infected mice that received *H. pylori* eradication therapy at 8 WPI had a gastric architecture indistinguishable from that of uninfected age-matched mice (Fig. 1). Compared to *H. pylori*-infected INS-GAS mice that did not receive antimicrobial eradication therapy, *H. pylori* antimicrobial eradication therapy at 8 WPI inhibited corpus dysplasia, inflammation, atrophy, hyperplasia, epithelial defects (all p<0.001), and intestinal metaplasia (p<0.05) (Fig. 2). Additionally, these mice had milder corpus inflammation, atrophy, and epithelial defects when compared to uninfected mice (p<0.05, <0.01 and <0.01, respectively) (Fig. 3).

*H. pylori*-infected male INS-GAS mice that received *H. pylori* antimicrobial eradication therapy at 12 WPI and were euthanized 16 weeks later had corpus hyperplasia and thickened mucosal folds. Microscopically, these mice had distorted mucosal architecture with dilated glands and glandular dysplasia (Fig. 1). Compared to untreated *H. pylori*-infected INS-GAS mice, infected male INS-GAS mice that received *H. pylori* antimicrobial eradication therapy at 12 WPI had statistically less severe dysplasia, inflammation, and intestinal metaplasia (p<0.01, <0.05, and <0.05, respectively) but similar degrees of atrophy, hyperplasia, and epithelial defects (Fig. 2). Compared to uninfected mice, infected male INS-GAS mice that received *H. pylori* antimicrobial eradication therapy at 12 WPI had more severe dysplasia and inflammation (p<0.001) but
comparable atrophy, hyperplasia, epithelial defects, and intestinal metaplasia.

*H. pylori*-infected INS-GAS mice that received *H. pylori* antimicrobial eradication at 22 WPI and were euthanized 8 weeks later had thickened mucosal folds and developed corpus hyperplasia and dysplasia (Fig. 1). Microscopically, these mice developed statistically less severe dysplasia, inflammation, atrophy, and epithelial defects compared to untreated *H. pylori*-infected INS-GAS mice (p<0.01 or less) (Fig. 2). Compared to uninfected mice, infected INS-GAS mice that received *H. pylori* antimicrobial eradication therapy at 22 WPI had more severe dysplasia, hyperplasia, and epithelial defects (p<0.05 and <0.01).

Among the infected INS-GAS mice that received *H. pylori* antimicrobial eradication therapy, the mice receiving antimicrobial therapy at 8 WPI had significantly lower scores of dysplasia, inflammation, atrophy, and hyperplasia compared to those mice receiving antimicrobial therapy at 12 or 22 WPI (p<0.05 or less), and less severe epithelial defects compared to the mice receiving antimicrobial therapy at 12 WPI (p<0.001). Most gastric lesions in infected mice that received eradication therapy at 12 and 22 WPI were comparable, except for epithelial defects that were more severe in the 12 WPI group (p<0.01).

**H. pylori** eradication at 8 WPI prevented progression to low-grade and high-grade gastrointestinal intraepithelial neoplasia

Uninfected INS-GAS mice develop spontaneous gastric cancer at 20 months of age [20]; none of the uninfected INS-GAS mice between 34 to 36 weeks old developed gastrointestinal intraepithelial neoplasia (GIN). In contrast, all untreated *H. pylori*-infected INS-GAS mice
(n=10) at 28 WPI developed gastric cancer; two with low-grade GIN (20%) and eight with high-grade GIN (80%) (Fig. 3). In the infected mice that received eradication therapy at 8 WPI (n=11), ten of them did not have GIN (91%) and one had low-grade GIN (9%). In the infected mice that received eradication therapy at 12 WPI (n=9), one did not have GIN (11%), seven had low-grade and one had high-grade GIN (78 and 11%, respectively). Six of the 12 infected mice that received eradication therapy at 22 WPI did not have GIN (50%), and the remainder had low-grade GIN (50%).

Compared to uninfected mice, infected mice that received eradication therapy at 8 WPI had a similar incidence of GIN (p=0.38). In contrast, infected mice that received eradication therapy at 12 or 22 WPI and those untreated *H. pylori*-infected mice with had a higher incidence of low- and high-grade GIN (p<0.05 or less). Compared to *H. pylori*-infected INS-GAS mice that did not receive eradication therapy, the incidences of low-grade and high-grade GIN were statistically lower in the infected mice receiving eradication therapy at 8, 12, or 22 WPI (p<0.05 or less). Among the infected mice receiving eradication therapy, eradication therapy at 8 WPI resulted in significantly lower incidences of GIN compared to eradication therapy at 12 or 22 WPI (p<0.05). The incidences of low-grade and high-grade GIN were statistically similar between infected mice receiving eradication therapy at 12 and 22 WPI (p=0.12).

**H. pylori-specific antibody responses**

The *H. pylori*-specific, Th1-associated IgG2a levels were significantly induced in all infected mice that received eradication therapy and untreated *H. pylori*-infected mice compared to uninfected mice (p<0.01) (Fig.). *H. pylori* eradication therapy at all time points had no effect on
H. pylori-specific IgG2a levels compared to untreated H. pylori-infected mice. However, infected mice that received eradication therapy at 22 WPI had statistically higher H. pylori-specific IgG2a levels compared to those receiving eradication therapy at 8 or 12 WPI (p<0.05 and <0.01). H. pylori-specific, Th2-associated IgG1 responses were induced in all infected mice that received eradication therapy and untreated H. pylori-infected mice compared to uninfected mice (p<0.05). Compared to untreated H. pylori-infected mice, H. pylori-specific IgG1 levels were reduced in mice that received eradication therapy at 8 WPI (p<0.01) but not affected by eradication therapy at 12 or 22 WPI (p=0.11 or higher). H. pylori-specific IgG1 levels in mice that received eradication therapy at 8 WPI were also lower than those in mice that received eradication therapy at 12 or 22 WPI (p<0.01 and <0.05)

Gastric IFN-γ, TNF-α, and iNOS mRNA levels were reduced in all mice that received H. pylori eradication therapy

Given the importance of the inflammatory response in the pathogenesis of H. pylori gastritis, we analyzed selected pro-inflammatory cytokines and iNOS mRNA levels in the gastric tissue at 28 WPI. Compared to uninfected mice, IFN-γ mRNA levels were down-regulated in H. pylori-infected mice receiving antimicrobial therapy at 8 WPI (p<0.05), but significantly up-regulated in the infected mice that received H. pylori antimicrobial therapy at 12 (p<0.001) or 22 WPI (p<0.05) as well as untreated H. pylori-infected INS-GAS mice (p<0.001) (Fig. 5). Compared to untreated H. pylori-infected mice, H. pylori antimicrobial eradication significantly reduced mRNA levels of IFN-γ in infected mice receiving eradication therapy at 8, 12 (p<0.001), and 22 WPI (p<0.05). Among the infected mice receiving eradication therapy, those receiving eradication therapy at 8 WPI had lower IFN-γ mRNA levels relative to those receiving
eradication therapy at 12 or 22 WPI (p<0.01).

Compared to uninfected mice, gastric TNF-α mRNA levels were down-regulated in H. pylori-infected mice that received H. pylori eradication therapy at 8 WPI, but up-regulated in infected mice that received H. pylori eradication therapy at 12 (p<0.01) and untreated H. pylori-infected mice (p<0.001). Compared to untreated H. pylori-infected mice, gastric TNF-α mRNA levels were significantly reduced in all mice that received eradication therapy (p<0.001). Among the infected mice receiving eradication therapy, infected mice that received eradication therapy at 8 WPI had significantly lower TNF-α mRNA levels compared to those mice that received eradication therapy at 12 or 22 WPI (p<0.001 and <0.05). Infected mice that received eradication therapy at 12 WPI had higher TNF-α mRNA levels compared to the mice that received eradication therapy at 12 WPI (p<0.05).

Compared to uninfected mice, gastric iNOS mRNA levels were down-regulated in infected mice that received eradication therapy at 8 WPI (p<0.01), but up-regulated in H. pylori-infected mice that received eradication therapy at 12 and 22 WPI and untreated H. pylori-infected INS-GAS mice (p<0.05 or less). Compared to untreated H. pylori-infected mice, gastric iNOS mRNA levels were significantly down-regulated by H. pylori eradication therapy at all time points (p<0.05 or less). Among the infected mice that received H. pylori eradication therapy, those receiving eradication therapy at 8 WPI had lower iNOS mRNA levels compared to the mice receiving eradication therapy at 12 and 22 WPI (p<0.001).

Gastric mucosal cell proliferation was also reduced in all mice that received H. pylori
eradication therapy

Epithelial proliferating cells detected by Ki67 immunohistological staining were mainly in the isthmus regions of corpus mucosa in *H. pylori*-uninfected INS-GAS mice and infected mice that received eradication therapy at 12 WPI (Fig. 5). Proliferating cells in the corpus expanded from isthmus regions to hypertrophic foveolar regions in INS-GAS mice infected by *H. pylori*. Compared to uninfected mice, corpus epithelial proliferation measured by Ki67 labeling indices (LI) were comparable in infected mice that received *H. pylori* eradication at 8 WPI (p=0.093), but higher in infected mice that received *H. pylori* eradication at 12 or 22 WPI, and untreated *H. pylori*-infected mice (p<0.05 or lower). *H. pylori* eradication at 8, 12 and 22 WPI significantly reduced Ki67 LI in the corpus compared to untreated *H. pylori*-infected mice (p<0.01 or lower). Among the infected mice receiving *H. pylori* eradication therapy, mice receiving eradication therapy at 8 WPI had lower corpus epithelial LI than those receiving eradication therapy at 12 or 22 WPI (p<0.05 and =0.058, respectively)

DNA adducts in gastric tissues

Levels of dI and etheno-dA in uninfected wt mice, INS-GAS, *H. pylori*-infected INS-GAS mice receiving eradication at 12 WPI, and untreated *H. pylori*-infected INS-GAS mice were comparable (Fig. 6). Levels of 8-oxodG were higher in uninfected INS-GAS mice than *H. pylori*-infected INS-GAS mice receiving eradication at 12 WPI and untreated infected INS-GAS mice (p<0.05).

*H. pylori*-infected INS-GAS mice had significantly higher serum levels of triglyceride, total cholesterol, and elevated fraction of very-low-density lipoprotein
Triglyceride concentrations in untreated *H. pylori*-infected INS-GAS mice were significantly higher than uninfected mice or *H. pylori*-infected mice that received eradication therapy at 12 WPI (*p*<0.001) (Fig. 7). There were no differences in triglyceride concentrations between uninfected mice and *H. pylori*-infected mice that received eradication therapy at 12 WPI (*p*=0.39). Total cholesterol concentrations in untreated in infected mice that received eradication therapy at 12 WPI were significantly low than in uninfected or *H. pylori*-infected INS-GAS mice (*p*<0.05). Uninfected or *H. pylori*-infected INS-GAS mice has similar concentrations of total cholesterol levels (*p*=0.20). *H. pylori* infection increased the proportion of very-low-density lipoprotein (VLDL) cholesterol in serum compared to uninfected or infected INS-GAS mice that received eradication therapy at 12 WPI.
DISCUSSION

In this study, we used the well-described INS-GAS mouse model to study the effect of *H. pylori* eradication at different stages of *H. pylori*-associated gastric pathology. All INS-GAS mice infected with *H. pylori* for 28 WPI developed gastric cancer accompanied by inflammation, loss of parietal and chief cells, and hypertrophy of foveolar glands. When eradication therapy was instituted at an early point of infection, these histopathologic changes were reversible and completely prevented progression to gastric cancer. Infected mice receiving *H. pylori* eradication therapy at 8 WPI had a gastric cancer risk comparable to that of uninfected mice. Additionally, reduced gastric inflammation in these mice was accompanied by lower proinflammatory cytokine mRNA levels and decreased epithelial cell proliferation, which may contribute to lower dysplasia and reduced gastric cancer risk. Eradication therapy at 12 and 22 WPI also resulted in a statistically lower degree of gastric dysplasia, inflammation, and prevented progression to high-grade GIN when compared to the data recorded in untreated *H. pylori*-infected mice. The attenuated inflammation was accompanied by reduced cytokine mRNA levels and epithelial cell proliferation. Eradication therapy at later time points of infection did not reverse some histopathologic changes such as atrophy, hyperplasia, and dysplasia. Thus, eradication at these time points did not completely prevent progression to gastric cancer compared to uninfected mice or infected mice that received treatment at 8 WPI.

*H. felis*-infected C57BL/6 mice developed invasive carcinoma at 15 MPI [33]. Helicobacter eradication therapy in *H. felis*-infected C57BL/6 mice at 2 or 6 MPI prevented development of gastric cancer; eradication therapy at 12 MPI reduced the risk of gastric cancer [17]. Consistent with the *H. felis* C57BL/6 model, our data demonstrate that *H. pylori* eradication therapy at 8
WPI in INS-GAS mice restores mucosal architecture and prevented development of GIN. Also eradication therapy at 12 or 22 WPI prevents progression to high-grade GIN compared to untreated *H. pylori*-infected INS-GAS mice. However, the incidence of low-grade and high-grade GIN was statistically similar between *H. pylori*-infected mice that received eradication therapy at 12 and 22 WPI. These results suggest that *H. pylori* infection in INS-GAS mice at 12 WPI results in certain irreversible changes in the gastric mucosa and that *H. pylori* eradication may have little effect on preventing the progression to gastric cancer after such a "point of no return". Mature parietal cells are necessary to maintain the integrity of gastric mucosa. Parietal cell loss results in the dysregulation of gastric stem cell homeostasis and migration-associated differentiation of pit and zymogenic cells [34]. The hypergastrinemic INS-GAS mice have increased parietal cell mass before 3 months of age, then start developing gastric atrophy, metaplasia, dysplasia, and finally cancer [20]. Independent studies from our laboratory confirmed parietal cell loss in uninfected INS-GAS mice after 5 months of age (approximately equivalent to 12-14 weeks post *H. pylori* infection) [20]. Therefore, *H. pylori* eradication therapy given at 8 WPI in infected INS-GAS mice might reverse *H. pylori*-associated parietal cell loss concomitant with the restoration of normal epithelial architecture. In contrast, eradication therapy given at 12 WPI or later might fail to restore parietal cell mass and subsequent epithelial differentiation.

*H. pylori* eradication in INS-GAS mice at all time points was associated with attenuated gastric inflammation manifested as lower degrees of inflammatory scores and down-regulated IFN-γ, TNF-α, and iNOS mRNA levels compared to untreated *H. pylori*-infected mice. Gastric inflammatory scores and IFN-γ mRNA levels in INS-GAS mice correlated with epithelial
proliferation that appears to be mediated by up-regulated inflammatory cytokines such as IFN-γ [35]. Increased cell proliferation is a biomarker of gastric cancer risk. Reversal to a normal epithelial proliferation has been associated with a reduce cancer risk [36, 37]. *H. pylori* eradication therapy at 8 WPI returned epithelial proliferation rates to the levels of uninfected mice. Eradication therapy at 12 or 22 WPI significantly reduced epithelial proliferation compared to untreated *H. pylori*-infected mice but did not completely restore proliferation rates to the levels of uninfected mice, suggesting that a higher gastric cancer risk existed. This notion is supported by the elevated incidence of low-grade and high-grade GIN despite a successful *H. pylori* eradication at 12 or 22 WPI. Another possibility is that with accumulated genetic damage in mice as a result of long-standing *H. pylori* infection, *H. pylori* infection may no longer be necessary for sustained dysplasia and progression to gastric cancer.

It has been proposed in human clinical trials that the regression of premalignant lesions in *H. pylori* eradication may follow a sigmoid curve, where statistically significant results may not be observed during the first a few years after treatment [9, 38]. The protective effect of *H. pylori* eradication at 12 or 22 WPI in INS-GAS mice may not be seen at necropsy at 28 WPI. Intervention at late stages of *H. pylori* infection may require a longer period post treatment to observe benefit.

Unexpectedly, *H. pylori*-infected mice receiving eradication therapy at 8 WPI had significantly lower degrees of gastric inflammation accompanied by less severe atrophy and down-regulated IFN-γ, TNF-α, and iNOS mRNA levels relative to those levels recorded in uninfected mice. The mechanism by which antimicrobial eradication therapy exerted a protective effect in the *H.
pylori-infected INS-GAS mice at 8 WPI is unknown. However, there is a complex ecosystem of bacterial microbiota in mouse and human stomachs, including Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria phyla, which may be associated with gastric inflammation particularly when they overgrow in elevated gastric pH [39, 40]. Experimental infection with Acinetobacter lwofii in C57BL/6 mice causes chronic gastritis as does H. pylori [41]. Moreover, studies in humans and in various animal models have shown that bacteria are important in triggering inflammation and epithelial damage in the stomach [42], suggesting the potential interaction between H. pylori and other bacteria in the induction of gastric disease. A triple antimicrobial therapy regimen with omeprazole, metronidazole and clarithromycin in H. pylori-infected dyspeptic human patients resulted in H. pylori eradication and a dynamic change of gastric microbiota; certain bacteria were eradicated (ex. Fusobacterium spp.) while some bacteria overgrew (ex. Haemophilus spp. and Neisseria spp) [43]. Thus, it is conceivable that part of the gastric phenotype in INS-GAS mice receiving antimicrobial therapy at 8 WPI could be attributed to eradication of H. pylori and other non-helicobacter bacteria from the stomach particularly in the hypochlorhydric state [40]. Our laboratory observed that among the 6 bacterial species of altered Schaedler flora in H. pylori-infected INS-GAS mice, antimicrobial agents eradicated gastric Mucispirillum schaederi (unpublished data) [40]. These findings in humans and mice suggest that antibiotic treatment potentially changes gastric microbiota, and this may impact gastric carcinogenesis. Further experimentation is necessary to investigate the role of microbiota on H. pylori-associated gastric disease.

H. pylori infection in INS-GAS mice induced both IgG2a and IgG1 responses [21]. Compared to untreated H. pylori-infected mice, antibiotic eradication therapy did not significantly reduce H.
pylori-specific, Th1-associated IgG2a levels. However, there was a trend toward lower levels of
H. pylori-specific, Th2-associated IgG1 in mice receiving eradication therapy. Our data suggest
that IgG subtypes may not be sensitive markers for H. pylori eradication.

Several previous observations demonstrated that gastric helicobacter infection resulted in chronic
inflammation accompanied with increased oxidative stress and oxidative DNA damage in vitro
and in vivo [44, 45]. Unexpectedly, no significant differences in the levels of dI and etheno-dA,
and 8-oxodG were observed among uninfected and H. pylori-infected INS-GAS mice with or
without antibiotic treatment. However, levels of 8-oxodG were higher in uninfected INS-GAS
mice than H. pylori-infected INS-GAS mice receiving eradication at 12 WPI and untreated
infected INS-GAS mice. These results are probably due to tissue specificity or detection limit.
Further efforts are necessary to examine DNA adducts in epithelial cells.

Although the association of H. pylori with hyperlipidemia has been speculated, no correlation
between H pylori seropositivity and hyperlipidemia has been observed [46]. However, elevated
concentrations of serum triglyceride and total cholesterol were observed in the untreated, H.
pylori-infected INS-GAS mice compared to uninfected mice and H. pylori-infected mice that
received eradication therapy at 12 WPI. Increased proportions of VLDL accompanied with serum
triglyceride and total cholesterol concentrations correlated with dysplasia and incidence of
gastric cancer in H. pylori-infected INS-GAS mice. One possible explanation for elevated VLDL
proportions in H. pylori-infected INS-GAS mice is that lipoprotein lipase activity is down-
regulated. Lipoprotein lipase deficiency results in hypertriglycemia and accumulation of VLDL
[47]. Elevated triglyceride levels were previously observed in aged APC1309 and APCmin mice,
animal model of human colon cancer [48, 49]. Down-regulated lipoprotein lipase mRNA levels in the liver and small intestine were observed in both $\text{APC}^{1309}$ and $\text{APC}^{\text{min}}$ mice [48], suggesting that reduced lipoprotein lipase activity may result in decreased clearance of VLDL from serum in mice with colon cancer.

Many clinical trials in humans demonstrated that $H. pylori$ eradication results in regression of gastric atrophy and inflammation [5-10, 13]. $H. pylori$ eradication has failed to reduce the incidence of gastric cancer in most intervention studies in humans except in subpopulations that did not develop premalignant lesions prior to antibiotic treatment [6-10, 13]. These results suggest that the stage of $H. pylori$ gastric disease is a determinant factor for the outcome of $H. pylori$ eradication therapy. Our results in $H. pylori$-infected INS-GAS mice support the premise that $H. pylori$ eradication therapy may be most beneficial in preventing gastric cancer when antibiotics are given at the early stages of $H. pylori$ infection. In the mice where metaplasia and dysplasia existed, $H. pylori$ eradication therapy substantially prevented progression to high-grade GIN. In conclusion, our study using INS-GAS mice strongly suggests that to prevent the development of gastric cancer $H. pylori$ infection should be treated, especially in patients with symptomatic gastritis or family history of gastric cancer. Those $H. pylori$-infected patients who have developed premalignant lesions might benefit from $H. pylori$ eradication to slow progression to advanced disease.

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REFERENCES

Histopathology of gastric disease induced by *H. pylori* infection in the corpus mucosa of INS-GAS mice with and without eradication therapy. Representative H&E of stomachs from uninfected INS-GAS mice (a), treated mice at 8, 12, and 22 weeks post-infection (b, c, d) and untreated infected mice (e, f). (a) Uninfected mouse stomach showing typical INS-GAS background lesions of mild to moderate dysplasia, mild inflammation, severe atrophy, and mild to moderate hyperplasia on H&E stain. (b) Tissue from a treated INS-GAS mouse 8 weeks post-infection exhibiting mild to moderate dysplasia, mild inflammation, moderate to severe atrophy, and mild to moderate hyperplasia on H&E stain. (c) Tissue from a treated INS-GAS mouse 12 weeks post-infection exhibiting moderate dysplasia, moderate inflammation, severe atrophy, and moderate hyperplasia on H&E stain. (d) Tissue from a treated INS-GAS mouse 22 weeks post-infection exhibiting moderate dysplasia, mild to moderate inflammation, severe atrophy, and moderate hyperplasia on H&E stain. (e) Tissue from an untreated INS-GAS mouse 22 weeks post-infection exhibiting severe dysplasia, moderate inflammation, severe atrophy, and moderate to severe hyperplasia on H&E stain. (f) Tissue from (e) at higher magnification demonstrating apoptotic bodies, dilated glands and glandular dysplasia characterized by loss of columnar orientation, elongation, branching, irregular cell shapes and sizes, and stratification.

Histological scores of (a) dysplasia, (b) inflammation, (c) atrophy, (d) hyperplasia, (e) epithelial defects, (f) intestinal metaplasia, and (g) incidences of non-gastrointestinal intraepithelial neoplasia (GIN), low-grade GIN (a dysplasia score of 3.0), and high-grade GIN (dysplasia scores equal or higher than 3.5). (Statistical significance is presented as *, p<0.05; **, p<0.01; ***, p<0.001 when
compared to uninfected mice; #, p<0.05; ##, p<0.01; ###, p<0.001 when compared to *H. pylori*-infected mice which did not received antimicrobial therapy; §, p<0.05; §§, p<0.01; §§§, p<0.001; comparison as indicated).

**Figure 3.**

Relative mRNA levels of IFN-γ (a), TNF-α (b), and iNOS (c) in the gastric tissue. Data are presented as fold change relative to uninfected INS-GAS mice. *H. pylori*-infected mice without eradication therapy had significantly higher levels of IFN-γ, TNF-α, and iNOS mRNA (p<0.001). *H. pylori* eradication at 8 WPI had statistically lower IFN-γ, TNF-α, and iNOS mRNA levels compared to uninfected mice and infected mice without eradication therapy. *H. pylori* eradication at 12 or 22 WPI had IFN-γ, TNF-α, and iNOS mRNA levels which were significantly higher than uninfected mice, but lower than infected control mice that did not receive eradication therapy. (Compared to uninfected mice: *, p<0.05; **, p<0.01; ***, p<0.001. Compared to infected mice which did not receive antimicrobial eradication: #, p<0.05; ##, p<0.01; ###, p<0.001).

**Figure 4.**

*H. pylori*-specific, Th1-associated IgG2a levels were significantly elevated in infected mice at 28 WPI irrespective of antimicrobial eradication when compared to uninfected controls (**, p<0.01) (a). *H. pylori* eradication did not significantly reduce serum levels of *H. pylori*-specific IgG2a. Comparing to untreated *H. pylori*-infected mice, mice received *H. pylori* eradication at 22 WPI had higher IgG2a responses than those received *H. pylori* eradication at 8 or 12 WPI (#, p<0.05; ##, p<0.01). *H. pylori*-specific, Th2-associated IgG1 levels were elevated in infected mice that received *H. pylori* eradication at 12 or 22 WPI and mice that actively infected with *H. pylori* (*, p<0.05; §,
0.05<p<0.10) compared to uninfected mice (b). Infected mice receiving eradication therapy at 8 WPI had significantly lower *H. pylori*-specific IgG1 levels compared to infected mice given eradication therapy at 12 or 22 WPI (#, p<0.05; ##, p<0.01) and infected mice which did not receive eradication therapy (p<0.01).

**Figure 5.**

Immunohistochemical staining of Ki67 in the corpus. Proliferating cells are positive stained for Ki67. In helicobacter-uninfected INS-GAS mice, proliferating cells were restricted to the isthmus regions (a). Proliferating cells were restricted to the isthmus regions irrespective of the hyperplastic foveolar glands in the infected mice that received *H. pylori* eradication at 12 WPI (b). Proliferating cells expanded from isthmus regions to foveolar regions in the infected INS-GAS mice that did not receive eradication therapy (c). Ki67 labeling indices (LI) of Ki67 were significantly increased in *H. pylori*-infected mice that received eradication therapy at 12 or 22 WPI, and infected control mice compared to uninfected mice (d). *H. pylori* eradication at 8, 12, and 22 WPI significantly reduced corpus LI compared to infected mice without eradication therapy. Additionally, infected mice receiving *H. pylori* eradication at 8 WPI had lower corpus LI compared to the infected mice receiving eradication therapy at 12 WPI. (Statistical significance is presented as *, p<0.05; **, p<0.01; ***, p<0.001 when compared to uninfected mice; #, p<0.05; ##, p<0.01; ###, p<0.001 when compared to *H. pylori*-infected mice which did not received antimicrobial therapy; §, p<0.05; comparison as indicated).

**Figure 6.**

DNA adducts of 2′-deoxyinosine (dI), N⁶-ethenodeoxyadenosine (etheno-dA), and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in the stomach. White bars are wt mice; black bars are INS-
GAS mice. Levels of (a) dI and (b) etheno-dA were similar among all groups. (c) Levels of 8-oxodG were significantly higher in uninfected INS-GAS mice than *H. pylori* infected mice that received eradication therapy at 12 WPI and untreated infected INS-GAS mice. (Statistical significance is presented as *, p<0.05).

**Figure 7.**
Triglyceride and total cholesterol concentrations and distribution of lipoprotein cholesterol in serum. (a) Triglyceride concentrations in untreated *H. pylori*-infected INS-GAS mice were significantly higher than uninfected mice and *H. pylori*-infected mice receiving eradication therapy at 12 WPI. (b) Total cholesterol levels in *H. pylori*-infected mice receiving eradication therapy at 12 WPI were lower than uninfected mice and untreated *H. pylori*-infected INS-GAS mice. Proportion of VLDL cholesterol in serum was increase in untreated *H. pylori*-infected INS-GAS mice (d) but not in uninfected INS-GAS mice (c).
Fig. 2

(a) Dysplasia score
(b) Inflammation score
(c) Atrophy score
(d) Hyperplasia score
(e) Epithelial defects score
(f) Intestinal metaplasia score
(g) Incidence, %

- Non-GIN
- Low-grade GIN
- High-grade GIN
Fig. 3

**Fig. 4**

**Fig. 5**
Table 1. Numbers of mice in each treatment group

<table>
<thead>
<tr>
<th>H. pylori&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Eradication (WPI)</th>
<th>Necropsy (WPI)</th>
<th>Nb&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>12/14</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>28</td>
<td>na/10</td>
</tr>
</tbody>
</table>

Abbreviation: WPI, weeks post infection; na, not-applicable

<sup>a</sup>H. pylori inoculation at the beginning of experiment.

<sup>b</sup>Numbers of mice with successful H. pylori eradication / Group size.

Table 2. Gastric lesions at 22 and 28 weeks post H. pylori infection (WPI)

<table>
<thead>
<tr>
<th>Weeks&lt;sup&gt;a&lt;/sup&gt;</th>
<th>H. pylori</th>
<th>N</th>
<th>D</th>
<th>I</th>
<th>A</th>
<th>H</th>
<th>ED</th>
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<td>-</td>
<td>9</td>
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<td>(1.5-3)&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>(1.5-3)&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td>+</td>
<td>10</td>
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<td>(3)&lt;sup&gt;c,d&lt;/sup&gt;</td>
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<td>(3-3.5)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(3)</td>
<td>(3-3.5)</td>
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</tbody>
</table>

Abbreviations: D, dysplasia; I, inflammation; A, Atrophy; H, hyperplasia; ED, epithelial defects; IM, intestinal metaplasia.

<sup>a</sup>Equivalent to WPI

<sup>b</sup>Significant difference between 22 and 28 WPI in uninfected mice, p<0.05.

<sup>c</sup>Significant difference between 22 and 28 WPI in infected mice, p<0.05.

<sup>d</sup>Significant difference between infected mice at 22 WPI, p<0.05.

<sup>e</sup>Significant difference between uninfected and infected mice at 22 WPI, p<0.001.

<sup>f</sup>Significant difference between uninfected and infected mice at 28 WPI, p<0.01.

<sup>g</sup>Significant difference between uninfected and infected mice at 28 WPI, p<0.001.
Chapter 6: Summary
This thesis reported studies on pathogenesis of the carcinogenic bacterium *Helicobacter pylori* and its inter-relationship with host and environmental factors contributing to gastric cancer. Using mouse models of experimental *H. pylori* infection, these studies focused on 4 areas: (1) IL10 requirement of CD4* regulatory T (T<sub>R</sub>) cells function in suppressing *H. pylori*-associated gastritis using adoptive transfer model; (2) whether vitamin C supplementation influences *H. pylori* gastritis; (3) the roles of DNA repair proteins Aag and Mgmt in *H. pylori* gastritis; and (4) whether *H. pylori* eradication prevents progression of *H. pylori* gastritis to gastric cancer.

The results of adoptive transfer model of *H. pylori* gastritis demonstrate that: (1) *H. pylori*-infected C57BL/6 Rag2<sup>−/−</sup> mice that do not have functional B and T cells do not develop gastritis; (2) transfer of wildtype (wt) CD4* effector T (T<sub>E</sub>) cells into Rag2<sup>−/−</sup> mice results in T<sub>E</sub>-mediated gastroduodenitis involving both stomach and proximal duodenum, while *H. pylori* infection superimposes severity of corpus gastritis; and (3) cotransfer of wt T<sub>E</sub> plus wt T<sub>R</sub> cells suppresses both T<sub>E</sub>-mediated and *H. pylori*-associated inflammation, while cotransfer of wt T<sub>E</sub> plus IL10<sup>−/−</sup> T<sub>R</sub> cells suppresses T<sub>E</sub>-mediated gastroduodenitis but not *H. pylori*-associated corpus gastritis, suggesting that IL10 is required for T<sub>R</sub> cells function in suppressing *H. pylori*-associated gastritis. These data are consistent with previous studies that T<sub>E</sub> cells are required to induce *H. pylori* gastritis [1, 2], while T<sub>R</sub> cells suppress *H. pylori* gastritis [3]. IL10 is critical for T<sub>R</sub> cell function in suppressing *H. pylori*-associated corpus gastritis [4], consistent with the IL10 requirement for T<sub>R</sub> cell suppression of inflammatory bowel disease [5]. Importantly, our data supports the findings in humans that polymorphisms of IL10 promoter are associated with a higher risk of gastric cancer [6].
Chronic *H. pylori* infection results in increased oxidative stress and oxidative DNA damage in human gastric mucosa [7, 8]. Since vitamin C is a strong antioxidant, it has been speculated whether vitamin C supplementation reduces *H. pylori*-induced oxidative damage and severity of gastritis. Consistent with human clinical trials of vitamin C [9-12], a vitamin C supplementation 10-fold higher than maintenance dose in gulo<sup>+</sup> mice does not reduce *H. pylori* gastritis. In contrast, a low vitamin C supplementation in gulo<sup>+</sup> mice results in less severe *H. pylori* gastritis due to reduced Th1 immune responses, consistent with impaired mitogen responses of peripheral blood mononuclear cells from pigs with heritable vitamin C deficiency [13]. Our data also supports the "African enigma" where the incidence of gastric cancer is low in some African countries despite a high prevalence of *H. pylori* infection [14]. In Gambia, people have very low dietary vitamin C and plasma vitamin C levels during raining season for 7 months/year [15]. The low vitamin C state may reduce Th1 responses to *H. pylori* and cause less severe *H. pylori* gastric lesions.

The molecular mechanism of *H. pylori*-induced gastric cancer remains unclear. Dysfunction and polymorphisms of DNA repair genes have been associated with a higher risk of gastric cancer [16, 17]. The roles of DNA repair proteins 3-alkyladenine DNA glycosylase (Aag) or O<sup>6</sup>-methylguanine DNA methyltransferase (Mgmt) in *H. pylori* gastritis were examined using Aag or Mgmt knockout mice on a C57BL/6 background. Both infected knockout mice developed more preneoplastic gastric atrophy, hyperplasia, and mucous metaplasia compared to infected wt mice, suggesting Aag and Mgmt may reduce the development of *H. pylori*-associated gastric cancer by preventing development of preneoplastic lesions.
*H. pylori* causes life-long infection and chronic gastritis. In a susceptible host, *H. pylori* chronic gastritis may progress to gastric cancer [18]. The natural course of *H. pylori* infection raises the possibility that eradication of *H. pylori* may prevent the development of gastric cancer. The key question is that at what stage of gastric disease would *H. pylori* eradication therapy prevent disease progression to cancer. Eradication of *H. pylori* in humans prevents development of gastric cancer in the patients without precancerous lesions [19], suggesting that disease stage when treatment is given may be important. Results of *H. pylori* eradication therapy in hypergastrinemic INS-GAS mice demonstrate that untreated *H. pylori*-infected mice develop gastric cancer at 28 WPI. *H. pylori* eradication at all time points (8, 12, and 22 WPI) reduces gastric inflammation, accompanied by down-regulated proinflammatory cytokine mRNA and lower cell proliferation which is associated with decrease dysplasia. Optimal effect on cancer prevention was observed when antibiotics are given at 8 WPI. These results support the Maastricht consensus III that *H. pylori* infection should be treated early, especially in patients with gastric ulcer or a family history of gastric cancer [20].

*H. pylori* infection is a serious problem for public health; more than 50% of population are infected worldwide [18]. *H. pylori*-associated gastric cancer is the 4th most common cancer and causes 2nd cancer-related mortality [21]. Although evidences support early *H. pylori* eradication reduces gastric cancer risk [19], it is impractical to treat all infected individuals. Preventive and therapeutic *H. pylori* vaccination looks promising [22], but large pharmaceutical firms to date have not been interested in pursuing this strategy. Data reported in diet studies and experiments conducted with parasitic infections demonstrate immune modulation from Th1 to Th2 or overall lower Th1 responses may reduce severity of *H. pylori* gastritis [23]. Chemoprevention trials to
date, however, have been disappointing in using this strategy to prevent gastric cancer. Our vitamin C study in gulo+ mice seems to support these results. It is well recognized those individuals with family history of gastric cancer should be treated for H. pylori infection [20]. Due to the genetic association, it is recommended that efforts be launched to develop rapid screening for polymorphisms in key cytokine genes that are associated with a higher risk of gastric cancer [6, 18]. Individuals with polymorphisms associated with increased risk for cancer should be identified and treated for H. pylori.

REFERENCES
