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Vitamin C supplementation does not protect L-gulono- γ -lactone oxidase-deficient mice from *Helicobacter pylori*-induced gastritis and gastric premalignancy

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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^{tm1Umc/mmed} (gulo^{-/-}) mice lacking L-gulono- γ -lactone oxidase, we compared gastric lesions and Th1 immune responses in *H. pylori*-infected gulo^{-/-} mice supplemented with low (33 mg/L) or high (3,300 mg/L) vitamin C in drinking water for 16 or 32 weeks. Vitamin C levels in plasma and gastric tissue correlated with the vitamin C supplementation levels in gulo^{-/-} mice. *H. pylori* infection resulted in comparable gastritis and premalignant lesions in wildtype C57BL/6 and gulo^{-/-} mice supplemented with high vitamin C, but lesions were less severe in gulo^{-/-} mice supplemented with low vitamin C at 32 weeks post infection. The reduced gastric lesions in infected gulo^{-/-} 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. These results in the *H. pylori*-infected gulo^{-/-} 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^{-/-} mice from *H. pylori*-induced premalignant gastric lesions. In addition, less severe gastric lesions in *H. pylori* infected gulo^{-/-} mice supplemented with low vitamin C correlated with an attenuated Th1 inflammatory response.

Keywords

Helicobacter pylori; vitamin C; gulo^{-/-} mouse model

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Helicobacter pylori infects the human stomach¹ 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 species 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.^{22,23} Mice and Mongolian gerbils have been used to evaluate *H. pylori* gastritis and vitamin C oral intake.^{24–26} 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*^{tm1Umc/mmc}) lacks L-gulonolactone 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.

Material 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*^{+/-} mice (B6.129P2-*Gulo*^{tm1Umc/mmc}, 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*^{-/-} mice were weaned at 3 weeks of age and fed *ad libitum* with regular mouse chow (Prolab RMH 3,000 PMI Nutrition International, Richmond, IN) 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 euthanized with CO₂ at 16- or 32-weeks post infection (WPI) (Tables I and II). 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 II).

Experimental infection with *H. pylori*

H. pylori Sydney strain (SS1) was used for oral inoculation as described previously.^{28,30} After incubation for 24 hr 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 OD₆₀₀ = 1.000 in PBS. This was ~10⁹ organisms/mL. Mice were dosed with 0.2 mL of the *H. pylori* suspension in PBS by gavage every other day for 3 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°C. Vitamin C was extracted from plasma by adding an equal volume of cold perchloric acid (PCA) solution (1 L contained 50 mL of PCA and 95 mg of EDTA in ddH₂O), followed by vortexing and centrifugation at 2,500g at 4°C for 10 min. The supernatant was frozen at -70°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°C. Frozen tissue was added to cold PCA solution at a ratio of 1:9 (weight/weight) followed by homogenization and centrifugation at 2,500g at 4°C for 10 min, and then frozen at -70°C until analysis. Throughout the vitamin C extraction process, samples were kept on ice, protected from light, and measured within 1-week post processing.

Histological evaluation

At necropsy, the stomach and proximal duodenum were removed and opened 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). Using criteria described previously,³² gastric lesions were scored for inflammation, epithelial defects, oxyntic atrophy, foveolar hyperplasia, pseudopyloric metaplasia and dysplasia by board certified veterinary pathologists (BHR and ABR) blinded to sample identity. The sum of individual scores was used to define the gastric disease index.³⁰ Mucous metaplasia was scored separately from total gastric indices because this lesion is not clearly understood, having been observed to develop spontaneously in some strains of mice as well as result from a variety of pathological processes in humans and mice that are independent of *H. pylori* infection.³⁰

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, Foster 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 $\Delta\Delta CT$ 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 hr 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. Pathology scores between different genders were compared by the Mann-Whitney test. 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, 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 levels in plasma and gastric tissues correlated with vitamin C supplementation levels in *gulo*^{-/-} mice

Both uninfected and *H. pylori*-infected *gulo*^{-/-} 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*^{-/-} 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*^{-/-} 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*^{-/-} mice supplemented with low vitamin C ($p = 0.071$, $p = 0.069$, respectively). This reduction in gastric tissue vitamin C associated with *H. pylori* was significant in *gulo*^{-/-} 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*^{-/-} mice supplemented with low or high vitamin C. Consistent with previous reports,^{8,23} *H. pylori*-infected WT mice developed robust gastritis at 16 and 32 WPI characterized by corpus inflammation with lymphocytic and granulocytic infiltration, epithelial defects, foveolar hyperplasia, pseudopyloric metaplasia, and oxyntic atrophy (Figs. 2 and 3).

At 16 and 32 WPI, *H. pylori*-infected *gulo*^{-/-} 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*^{-/-} mice supplemented with low vitamin C to have fewer epithelial defects ($p = 0.094$), foveolar hyperplasia ($p = 0.069$), pseudopyloric metaplasia ($p = 0.15$), oxyntic atrophy ($p = 0.15$), and dysplasia ($p = 0.078$) relative to infected *gulo*^{-/-} mice supplemented with high vitamin C. These trends were also reflected in slightly lower gastric disease indices at 16 WPI in *H. pylori*-infected *gulo*^{-/-} mice supplemented with low vitamin C ($p = 0.095$).

Compared to *H. pylori*-infected *gulo*^{-/-} mice supplemented with high vitamin C, by 32 WPI *H. pylori*-infected *gulo*^{-/-} mice supplemented with low vitamin C demonstrated trends for less severe gastritis ($p = 0.11$), oxyntic atrophy ($p = 0.06$), and dysplasia ($p = 0.12$) and had significantly less extensive epithelial defects ($p < 0.01$), mucous metaplasia ($p < 0.05$), and foveolar hyperplasia ($p < 0.05$). When comparing gastric disease indices between low and high vitamin C supplementation levels, infected *gulo*^{-/-} mice supplemented with low vitamin C had statistically significant lower gastric disease indices compared to those mice supplemented with high vitamin C ($p < 0.05$). On the basis of differences identified in cytokine gene expression (below), one-way trend analysis of *H. pylori*-infected *gulo*^{-/-} mice with low vitamin C compared to *gulo*^{-/-} mice with high vitamin C followed by comparison with WT mice demonstrated a difference in oxyntic atrophy ($p = 0.053$) and significant differences in epithelial defects ($p < 0.001$), foveolar hyperplasia ($p < 0.05$), mucous metaplasia ($p < 0.05$), and gastric disease indices ($p < 0.05$).

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

Control *gulo*^{-/-} 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 upregulated the mRNA levels of IFN- γ and TNF- α in the stomachs of *gulo*^{-/-} 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*^{-/-} mice supplemented with high vitamin C ($p < 0.001$) and WT mice ($p < 0.001$) compared to *gulo*^{-/-} mice given low vitamin C supplementation (IFN- γ $p < 0.01$, TNF- α $p = 0.088$) (Fig. 4). Among *H. pylori*-infected mice, *gulo*^{-/-} mice supplemented with low vitamin C had lower gastric mRNA levels of IFN- γ and TNF- α compared to *gulo*^{-/-} 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 6 *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 did not 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*^{-/-} mice supplemented with low vitamin C maintained higher *H. pylori* colonization levels than *gulo*^{-/-} mice supplemented with high vitamin C ($p < 0.05$) or WT mice ($p = 0.082$) in which *H. pylori* colonization levels decreased over time in concert with development of more severe gastritis.

Gastrin amidation was not impaired in vitamin C-supplemented *gulo*^{-/-} mice

Amidation of the intermediate glycine-extended gastrin (G-gly) is vitamin C dependent^{11,37} and amidated gastrin levels are elevated in *H. pylori* infection in C57BL/6 mice.⁸ At 32 WPI, levels of the intermediate G-gly in plasma from most WT and *gulo*^{-/-} mice supplemented with low or high vitamin C were below the assay detection limit (data not shown). Uninfected WT mice and *gulo*^{-/-} mice supplemented with low or high vitamin C had similar amidated gastrin levels ($p = 0.13$) (Fig. 7). Uninfected and *H. pylori*-infected *gulo*^{-/-} 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 *H. pylori*-infected *gulo*^{-/-} mice supplemented with high vitamin C compared to uninfected counterparts ($p = 0.21$). In contrast, *H. pylori* 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 *H. pylori*-infected *gulo*^{-/-} and WT mice, *gulo*^{-/-} 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).

Male *gulo*^{-/-} mice supplemented with low vitamin C developed less severe pathology, lower gastric vitamin C and lower IFN- γ levels compared to female mice

We and others have previously reported on male- or female-predominant helicobacter-associated gastric disease in select strains of mice.^{38,39} In the current study, gender effect was not analyzed for 16 WPI because there were only 4 male and 2 female WT mice that were included as positive controls for confirming the pathogenicity of the *H. pylori* SS1 inoculum. Statistical analysis of the infected WT mice (5 males, 10 females) at 32 WPI revealed no gender effect on total gastric lesion indices. Only 1 subfeature, intestinal metaplasia, was less severe in infected female WT mice ($p < 0.05$). Consistent with the results from infected WT mice, analysis of pathology lesions in infected *gulo*^{-/-} mice supplemented with high vitamin C, which were comparable to WT mice in vitamin C status, revealed no gender effect on gastric disease indices at 16 weeks (4 males, 4 females) or at 32 WPI (15 males, 11 females) (data not shown). Only one feature, mucous metaplasia, was less severe in males ($p < 0.05$).

Analysis for gender effects in the *gulo*^{-/-} mice supplemented with low vitamin C revealed that at 16 WPI, gastric disease indices were equivalent between 5 males and 8 females. In contrast, the features of mucous metaplasia, and of more significance to helicobacter-associated disease in humans, oxyntic atrophy, were less severe in male mice ($p < 0.05$). At 32 WPI, the additional features of intestinal metaplasia and the gastric disease indices were also less severe in male *gulo*^{-/-} mice on the low vitamin C intake compared to females ($p < 0.05$). Notably, at 16 WPI, lower vitamin C levels in gastric tissues were observed in *gulo*^{-/-} males on low vitamin C compared to females ($p < 0.05$) with a similar trend in the high vitamin C *gulo*^{-/-} mice ($p = 0.06$) (data not shown). This correlation between gender effects and vitamin C content of gastric tissues was not observed at 32 WPI in the low vitamin C supplemented *gulo*^{-/-} mice. The high vitamin C supplemented male *gulo*^{-/-} mice had lower gastric vitamin C content than females, but this was in the absence of a corresponding gender effect on pathology, as previously stated. Additionally, plasma levels of vitamin C were not impacted by gender in any experimental group.

In support of potential female-predominant disease in *H. pylori*-infected *gulo*^{-/-} mice on low vitamin C supplementation, IFN- γ expression levels in gastric tissues were lower in males at 32 WPI ($p < 0.05$). IFN- γ levels were also lower in the male *gulo*^{-/-} mice supplemented with high vitamin C ($p < 0.05$) despite an equivalent severity of pathology to female mice. However, there were no differences in *H. pylori*-specific IgG2c and IgG1 levels between male and female mice in any experimental group (data not shown).

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.^{19,40} *Gulo*^{-/-} mice supplemented with high vitamin C and chronically infected with *H. pylori* over a period of 32 weeks developed chronic gastritis and oxyntic atrophy comparable to WT mice, indicating high vitamin C supplementation did not protect infected *gulo*^{-/-} mice from gastritis nor the development of premalignant lesions.

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*^{-/-} mice at 16 WPI which is consistent with reduced vitamin C levels in gastric juice during acute *H. pylori* infection in humans.⁴¹ However, gastric

tissue vitamin C levels were increased in *H. pylori*-infected *gulo*^{-/-} mice at 32 WPI. The increased gastric tissue vitamin C levels may have been associated with enhanced infiltration of lymphocytes and granulocytes, which have high intracellular vitamin C levels.⁴² 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. In humans, intravenous dosing with vitamin C was shown to block further secretion of vitamin C into gastric juice during acute *H. pylori* infection,⁴¹ but it has not been conclusively shown that naturally acquired *H. pylori* infection affects vitamin C levels in human gastric mucosa.^{20,43} Therefore, additional studies are necessary to evaluate the impact of *H. pylori* gastritis on vitamin C levels in the gastric tissues of humans.

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.^{18,21,44,45} 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.^{18,21,44-46} To obviate these variables, in the *gulo*^{-/-} 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 development of *H. pylori*-associated gastric lesions.

Despite the limitation of using rodents that endogenously synthesize vitamin C, rodent studies have reported that 7–10 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/day²⁵ and decreased *H. pylori* colonization levels in Mongolian gerbils given 10 mg vitamin C/day.²⁴ 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 levels of 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, in *H. pylori*-infected gerbils.²⁶ Supplementation with vitamin C in the diet at 10-fold higher levels than a maintenance dose (3,102 mg/kg) for 6 weeks in guinea pigs, which lack L-gulonolactone oxidase like humans and *gulo*^{-/-} mice, did not impact *H. pylori* colonization levels nor severity of gastritis.⁴⁷ Similar to the guinea pig study, *H. pylori*-infected *gulo*^{-/-} 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*^{-/-} mouse model as well as in guinea pigs did not prevent the development of *H. pylori* gastric disease or impact disease progression.

It is interesting, in the present study, that low vitamin C-supplemented *gulo*^{-/-} mice tended to have lower degrees of inflammation, oxyntic atrophy and dysplasia and significantly less severe epithelial defects, pseudopyloric metaplasia and foveolar hyperplasia relative to high vitamin C-supplemented *gulo*^{-/-} and WT mice. Helicobacter-induced gastric disease in humans and mice is mediated by a Th1-predominant host immune response.⁴⁸⁻⁵⁰ *Gulo*^{-/-} mice supplemented with high vitamin C had a similar Th1-promoted inflammatory response to *H. pylori* as infected WT mice. We observed that low vitamin C-supplemented, *H. pylori*-infected *gulo*^{-/-} mice had less severe gastric lesions 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*^{-/-} mice. This was manifested by less severe pneumonia and suppressed pulmonary expression of proinflammatory IL-1 β and

TNF- α mRNA in vitamin C-deprived *gulo*^{-/-} mice during the first few days of acute viral influenza.⁵¹ In hosts with low or no vitamin C intake, attenuated Th1 responses to other types of infection, such as tuberculosis in humans or *Klebsiella pneumoniae* sepsis in *gulo*^{-/-} mice, may also be important in predicting survival.^{52,53}

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.⁵⁴ In C57BL/6 mice coinfecting 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.⁵⁴ 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.⁵⁵ 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.⁵⁶ 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 because of attenuated gastric immune responses or inflammation. Although this hypothesis is consistent with a report of diminished mitogen responses of peripheral blood mononuclear cells from pigs affected by heritable vitamin C deficiency,⁵⁷ the biological significance of these responses is unclear.

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.³⁹ In a guinea pig model, vitamin C-deprivation impaired the amidation of gastrin and levels of intermediate G-gly were 30-fold higher than normal in the gastric antra.³⁷ In our study, uninfected *gulo*^{-/-} mice supplemented with low vitamin C had undetectable G-gly but comparable levels of amidated gastrin relative to uninfected *gulo*^{-/-} mice supplemented with high vitamin C. Consistent with a previous report,⁸ *H. pylori* infection significantly increased plasma amidated gastrin levels in wt mice ($p < 0.01$). However, *H. pylori* infection only slightly increased amidated gastrin levels in *gulo*^{-/-} mice supplemented with high vitamin C, with no increase in those supplemented with low vitamin C. These results suggest that the dose used for low vitamin C supplementation was sufficient for gastrin amidation in uninfected animals but not sufficient to sustain increased amidated gastrin levels during *H. pylori* infection. Higher levels of amidated gastrin in *H. pylori*-infected *gulo*^{-/-} 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*^{-/-} mice.

In the current study, *H. pylori*-infected *gulo*^{-/-} mice developed marked mucous metaplasia in the corpus, which was more prominent in high vitamin C-supplemented mice that developed more severe gastric pathology. In humans, *H. pylori*-associated gastric carcinogenesis has been associated with atrophy and intestinal and mucous metaplasia.¹⁸ Helicobacter-associated mucous metaplasia has been associated with increased expression of trefoil factor 2 (TFF2) by gastric mucous neck cells in mice^{58,59} and has been suggested to be a precursor lesion of gastric cancer in mice⁶⁰ and humans.⁶¹ In contrast, TFF2 has been reported to be a negative regulator of helicobacter-associated gastritis.^{62,63} Additionally, mucous metaplasia has been observed to develop spontaneously in some strains of mice⁶⁴ as well as in T cell-reconstituted *Rag2*^{-/-} mice irrespective of *H. pylori* infection status. The significance of mucous metaplasia is unclear and requires further study.

In summary, our study indicates that *gulo*^{-/-} mice, unlike other rodent models, provide a reliable model to study the role of dietary vitamin C in *H. pylori*-associated gastric disease. High vitamin C supplementation in this model, similar to previous epidemiological studies in humans,^{18–21} did not prevent progression of *H. pylori*-induced gastritis and development of premalignant gastric lesions. In contrast, low dietary vitamin C resulted in less severe gastric disease by downregulating gastric and systemic Th1 immune responses to *H. pylori* infection. We and others have previously reported on male- or female-predominant helicobacter-associated gastric disease in select strains of mice^{38,39} and the data presented here also support gender effects on gastric disease when vitamin C status is low. Additional studies are needed to confirm these observations in concert with determining a mechanism for suppressed inflammatory or immune responses when vitamin C supplementation is low.

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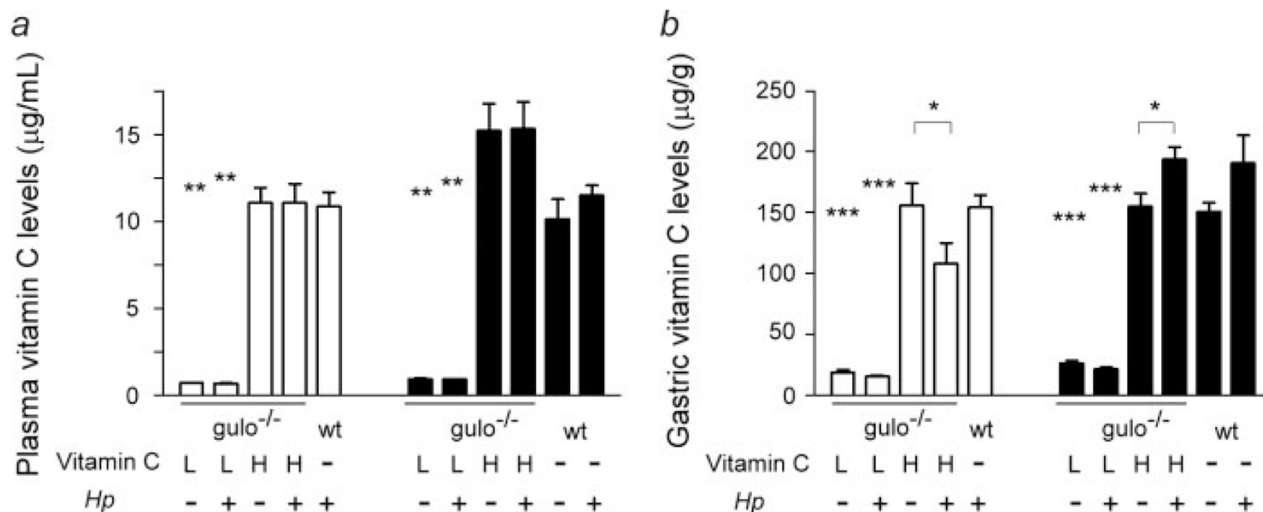


Figure 1.

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 C57BL/6 (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 (*, $p < 0.05$).

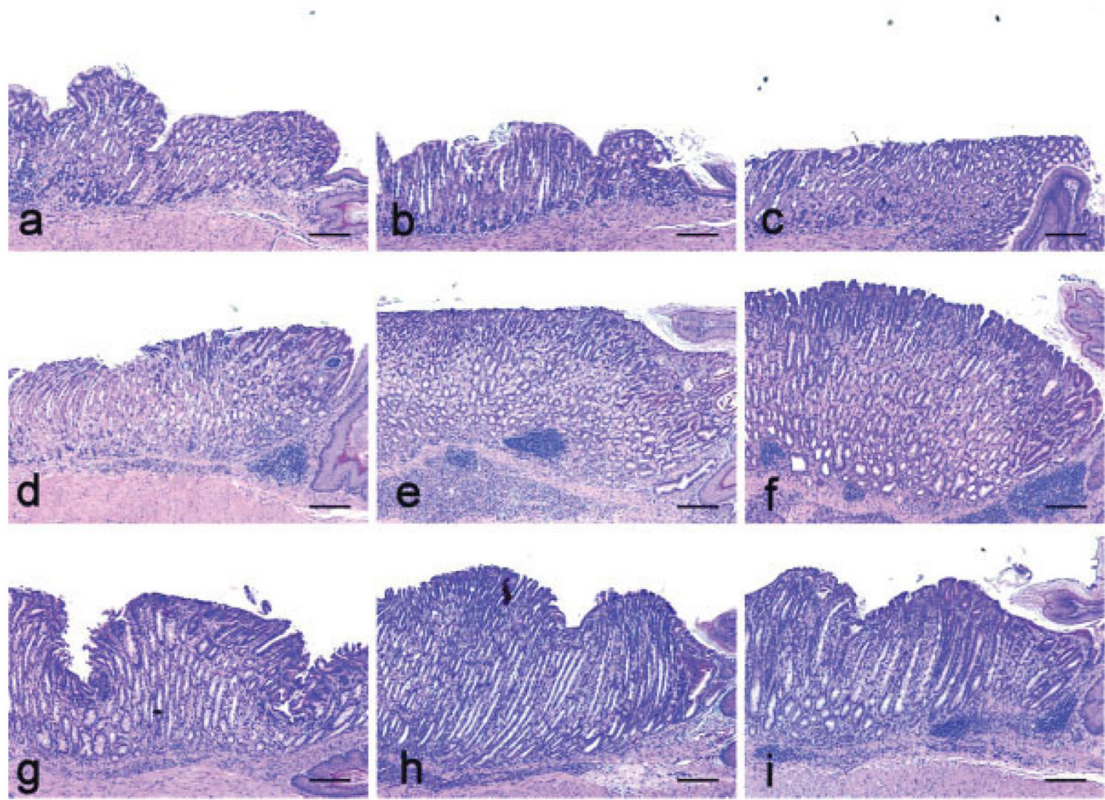
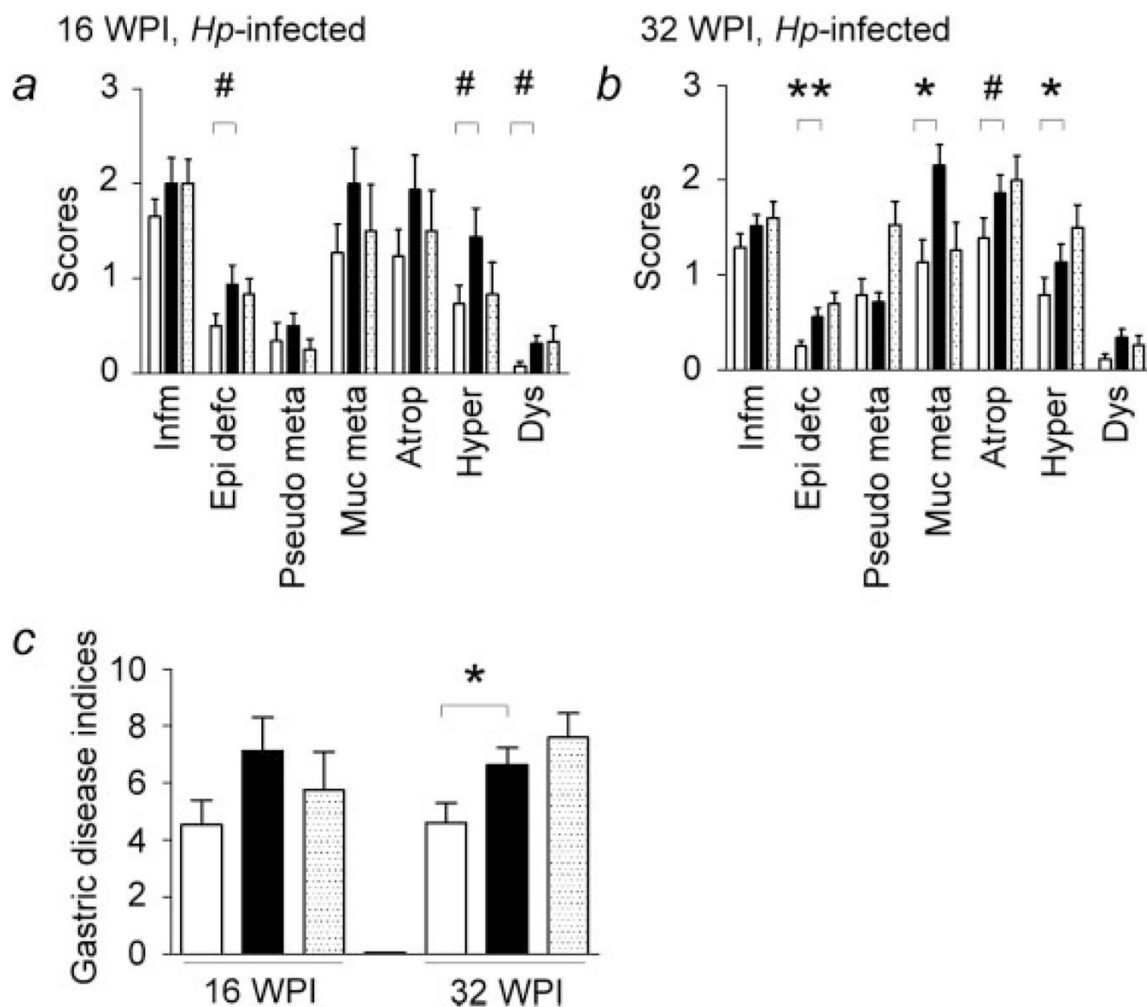


Figure 2.

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 C57BL/6 (WT) 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 WPI, although *gulo*^{-/-} mice with low vitamin C supplementation (d) exhibited a trend for less severe epithelial defects, foveolar 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 foveolar hyperplasia.

**Figure 3.**

Histological scores and gastric disease indices in *H. pylori* (*Hp*)-infected mice. *Gulo*^{-/-} mice supplemented with high vitamin C had similar gastric lesions and gastric disease indices compared to C57BL/6 (WT) (dotted bars) 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, foveolar 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 foveolar hyperplasia relative to those supplemented with high vitamin C (*, *p* < 0.05; **, *p* < 0.01). There was a trend toward less severe oxyntic 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, gastric disease indices were significantly lower in *gulo*^{-/-} mice that received low vitamin C than those mice receiving high vitamin C (*, *p* < 0.05). Infm, inflammation; Epi defc, epithelial defects; Pseudo meta, pseudopyloric metaplasia; Muc Meta, mucous metaplasia; Atroph, oxyntic atrophy; Hyper, foveolar hyperplasia; Dys, dysplasia.

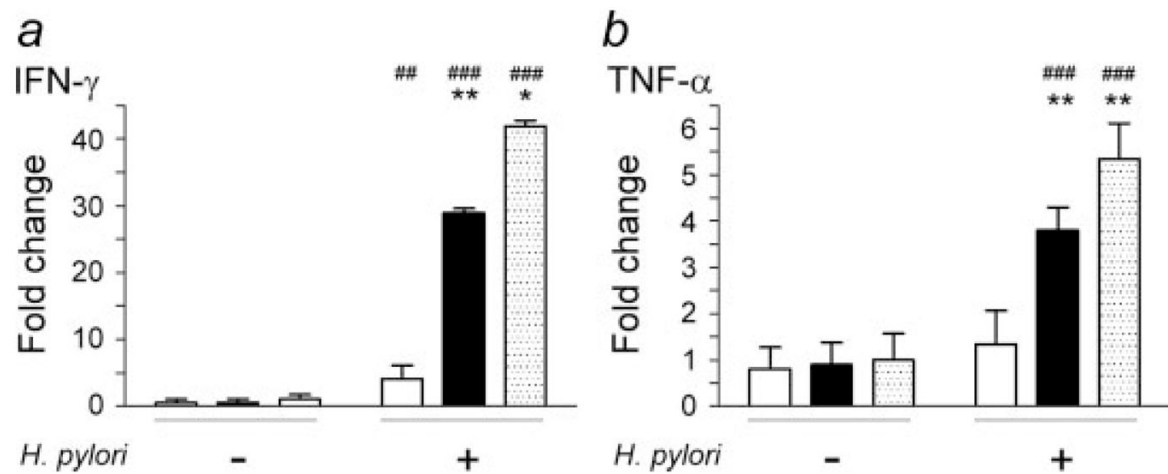


Figure 4.

Gastric mRNA levels of IFN- γ (a) and TNF- α (b) in *gulo*^{-/-} and C57BL/6 (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 upregulated 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 upregulated 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, *, $p < 0.05$; **, $p < 0.01$).

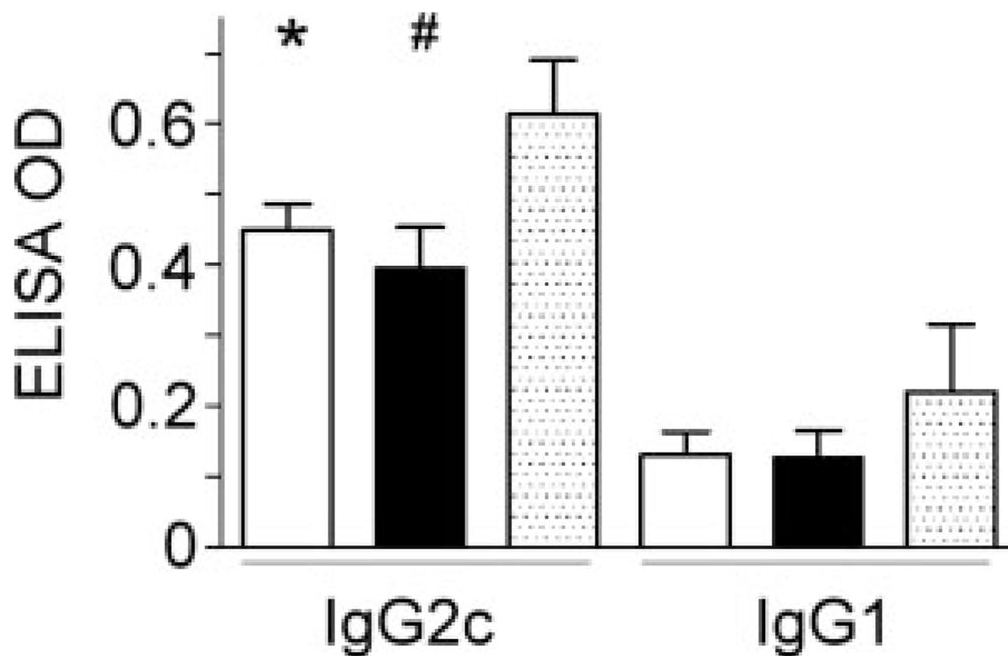


Figure 5.

H. pylori-specific IgG2c and IgG1 levels in *H. pylori*-infected mice. At 32 WPI, there was no difference in IgG2c levels among *gulo*^{-/-} mice supplemented with low vitamin C (white bar) and high vitamin C (black bar). *Gulo*^{-/-} mice supplemented with low or high vitamin C had lower IgG2c levels than did C57BL/6 (WT) mice (dotted bar) but differences were significant only for *gulo*^{-/-} mice supplemented with low vitamin C (*, $p < 0.05$). IgG1 levels among these 3 groups of mice were low and similar at 32 WPI ($p = 0.44$).

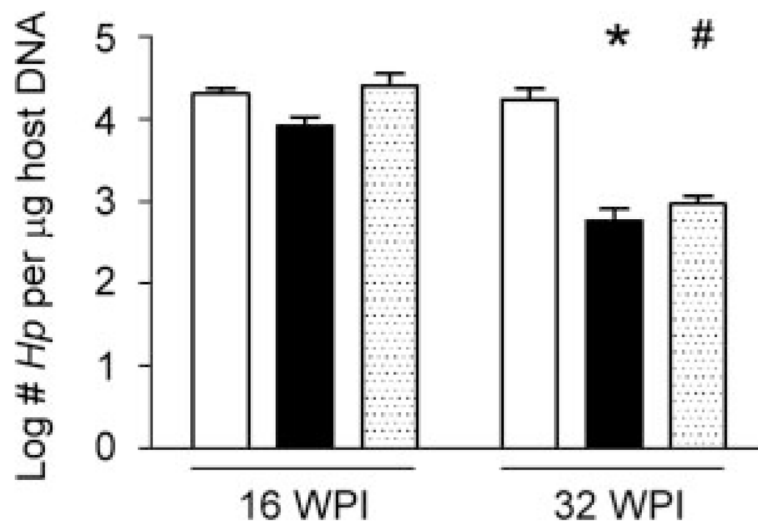


Figure 6. *H. pylori*(*Hp*) colonization levels (log CFU/μg host DNA). At 16 WPI, there were no differences in *H. pylori* colonization levels across groups. At 32 WPI, *gulo*^{-/-} mice supplemented with low vitamin C (white bars) had higher *H. pylori* colonization levels than *gulo*^{-/-} mice supplemented with high vitamin C (black bars) and C57BL/6 (WT) mice (dotted bars). (*, $p < 0.05$; #, $0.05 < p < 0.10$).

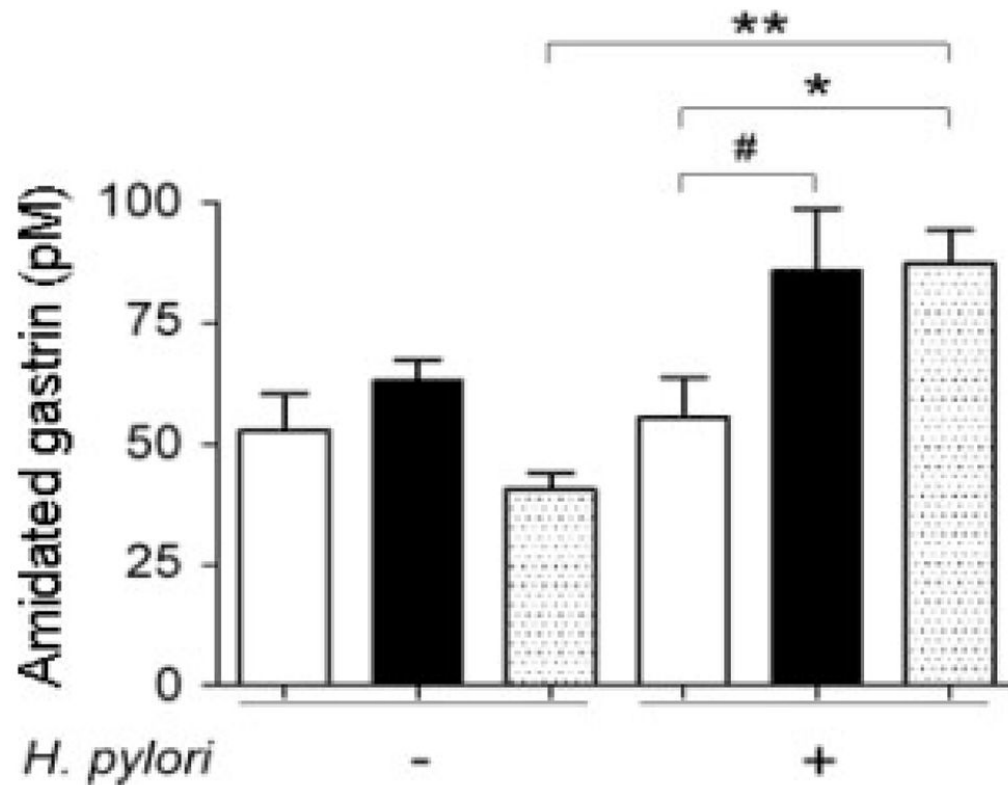


Figure 7.

Plasma levels of amidated gastrin at 32 WPI. Among uninfected mice, C57BL/6 (WT) mice (dotted bar) and *gulo*^{-/-} 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 upregulated amidated gastrin levels in wt mice ($p < 0.01$), but not in *gulo*^{-/-} mice that received low or high vitamin C. Among *H. pylori*-infected mice, *gulo*^{-/-} mice supplemented with low vitamin C had amidated gastrin levels lower than *gulo*^{-/-} mice supplemented with high vitamin C and wt mice ($p = 0.065$ and <0.05 , respectively). (*, $p < 0.05$; **, $p < 0.01$; #, $p = 0.065$).

TABLE I

H. pylori INFECTION AT 16 WPI

Genotype	<i>H. pylori</i>	Vitamin C	Number of mice
Gulo ^{-/-}	-	Low	5
Gulo ^{-/-}	+	Low	13
Gulo ^{-/-}	-	High	5
Gulo ^{-/-}	+	High	8
WT	+	None	6

TABLE II

H. pylori INFECTION AT 32 WPI

Genotype	<i>H. pylori</i>	Vitamin C	Number of mice ¹
Gulo ^{-/-}	-	Low	7/13
Gulo ^{-/-}	+	Low	11/15
Gulo ^{-/-}	-	High	7/13
Gulo ^{-/-}	+	High	12/14
WT	-	None	3/9
WT	+	None	5/10
WT	+	High	0/6

¹First experiment/second experiment.