

Green Tea (*Camellia sinensis*) acts as immunomodulatory therapy to elevate CD8 T- lymphocytes against *H. pylori*-associated gastric cancer with IL-2 cytokines through autophagy induction

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Research Article

Keywords: *Helicobacter pylori* (*H. pylori*), Green tea (*Camellia sinensis*), CD8, CD4, Gastric diseases, type-I cytokines, whole cell vaccine, autophagy, hemolytic plaque assay, immune response

Posted Date: January 8th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8457488/v1>

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Additional Declarations: No competing interests reported.

Abstract

Helicobacter pylori (*H. pylori*) is a causative agent of gastritis, gastroduodenal ulcers and gastric adenocarcinoma. Long duration chemotherapy results in the emergence of resistance in *H. pylori* and persists for life. Immunomodulation might provide an alternate means of reducing *H. pylori*-mediated disease through qualitative changes to the inflammation induced by chronic infection. Camellia sinensis (CS) is reported as a significant antimicrobial dietary agent. Here, we analyzed the immunomodulatory effect of CS against *H. pylori* infection by using CS immunomodulatory *H. pylori* associated-gastritis mice model and then determining the molecular mechanism by flow cytometry, qPCR, ELISA in the mouse and T-cell lines. Hemolytic plaque assay is a method which helps to *in-vitro* enumeration of cells producing antibodies against *H. pylori* surface and sonicate whole cell antigens. The PFU were calculated as PFU/spleen. Mice treated with *H. pylori* sonicate antigen with CS showed significant high immunomodulatory effect against *H. pylori* infection. In the center of each plaque antibody secreting plasma B cell was present. CS consumption, together with *H. pylori* sonicate vaccine, causes upregulation of CD8 lymphocytes with high secretion of type-I cytokines. CS induces autophagy with the upregulation of ATG5, Beclin, ATG7, ULK1, LC3a, FIP200, LC3b and BNIP3I. This study suggests a significant immunomodulatory effect of CS and helps to understand the bacterial and host drivers of chronic inflammation and cellular infiltration in presence of *H. pylori*. Hence, CS is helpful in the eradication of *H. pylori* from patients with gastroduodenal ulcer disease. A hemolytic plaque assay is a simple, sensitive assay which shows the kinetics of the immune response as observed in *in-vivo* assays of serum antibodies. CS can be used as an alternative means for eradicating *H. pylori* by stimulating the immune response.

Layman Summary

H. pylori infect more than half the world's population and is now known to be a major cause of gastritis and is the strongest known risk factor for the development of gastric adenocarcinoma. *Helicobacter pylori* (*H. pylori*) is a causative agent of gastritis, gastroduodenal ulcers and gastric adenocarcinoma. *H. pylori* infection is closely correlated with the immune response and microenvironment in the gastrointestinal tract. Long duration of traditional therapy leads to the emergence of antibiotic resistance and persistence of *H. pylori* infection. Hence, Immunomodulation through dietary factors is a critical way to prevent from *H. pylori* infection and its resistance. Green tea showed a significant immunomodulatory effect in the prevention of *H. pylori* infection by increasing immunity against it.

Introductions

Helicobacter pylori (*H. pylori*) is a causative agent of gastritis, ulcer diseases and development of gastric cancer (1, 2, 3). SC risk appears to evolve over time as a possible result of changes in dietary and lifestyle factors, including *H. pylori* infection, diet, alcohol consumption, and stress; however, the etiology of SC is complex, comprising bacterial variability, host genetic and environmental factors and thus has not been clarified (4, 5, 6). It is gram negative microaerophilic, curved, flagellated and spiral shaped bacilli (7, 8, 9). More than 50% world population is colonized by *H. pylori* but few of them suffer from active disease because of poor socio-economic status, age, gender, crowding, hyperacidity, alcohol abuse, dietary risk, smoking habit, unhygienic and poor living conditions (10, 11, 12, 13, 14). Developing countries had a prevalence of 80% while the developed countries had a prevalence of 20–50% (15, 16). The gastric mucosa has been regarded as an active site of humoral immunity since the discovery of *H. pylori* (17, 18). *H. pylori* colonizes the human stomach which results in triggering of a humoral and cell mediated immune response of gastric to clear it (19, 20). *H. pylori* infection results in the development of chronic local inflammatory and systemic antibody response and sheds a large number of extracellular products which diffuse into the mucosa (21, 22). Epithelial cells secrete cytokines, chemokines and eicosanoids which result in increase of neutrophils and macrophages (23). It appears that the gastric mucosa may be one of the most active sites of B-cell function in human lymphoid tissue, which tissue may also play an etiological role in cancer development. Although the humoral immune response may be a major protective mechanism of the gastric mucosa, thus far, information on the *in vivo* activity of mucosal B cells is sparse. Analysis of this activity will provide important information on this mucosal immunity (24).

Major humoral and cellular responses develop against *H. pylori* infection, but these immune responses are unable to eliminate the organism from the host. (Amjad). Immunomodulation might provide an alternate means of *H. pylori* by stimulating the immune response against *H. pylori* mediated diseases. In order to be immunomodulatory there will be a complete understanding of bacterial and host drivers of chronic inflammation and cellular infiltration in presence of *H. pylori*. Hemolytic plaque assay is one of the ways to understand the bacterial and host drivers (25). Hemolytic plaque forming assay (PFC) was initially developed by Jerne in 1961. In this the B cell specific for the *H. pylori* antigen are secrete antibody to antigen determinants present on the surface of erythrocytes and result in creation of antibody-erythrocyte binding and complement presence allow to complement mediated lysis of antibody coated erythrocytes and form plaques in the agarose. In the center of each plaque antibody secreting plasma B cell is present. A single IgM can produce plaque with the help of complement fixing on it; it shows a high sensitivity of PFC (26).

CS extract contains polyphenols, tannin, and caffeine, and catechins have been most widely studied to prove the pharmacological action of CS. They have demonstrated significant immunological, antioxidant, anticarcinogenic, anti-inflammatory, thermogenic, probiotic, and antimicrobial properties. Recently, an acidic polysaccharide isolated from *C. sinensis* leaves has shown remarkable inhibitory effects against the adherence of *H. pylori* to human gastric epithelial cells (27, 28, 29). CS has anti-inflammatory properties because of abundant catechin of GRE, (-) epigallocatechin-3-gallate (EGCG) which inhibit neutrophils elastase, repress reactive oxygen species, inhibits apoptosis of activated neutrophils and inhibits chemokine-induced neutrophil chemotaxis. Epigallocatechin-3-gallate of CS is a potent anti-inflammatory compound (30). It also has a role in inhibition of TNF-alpha through inhibition of NF-κB and AP-1 activation; TNF-alpha is known as an endogenous tumor promoter so in this way it has a role in cancer therapy and

prevention in chronic inflammatory diseases (31). Here, we explore the combined effect of *H. pylori* immunization and CS consumption in the stimulation of immune response against *H. pylori*-associated gastric diseases and subsequently gastric cancer in mouse models. We further investigate the molecular mechanisms of the immunomodulatory effect of CS.

Materials & Method

Preparation of *H. pylori* surface and sonicate whole cell antigen

Tissue biopsies were processed and cultured on Colombia agar (CA) plates containing 7% lysed horse blood and antibiotics (amphotericin B, trimethoprim, cefsulodin and vancomycin). The CA plates were incubated for 4–5 days under microaerophilic conditions at 37 °C, and identification was carried out by different conventional and molecular methods. Multiple *H. pylori* strains were inoculated in the BHI broth, incubated at 37 °C for 5 days in microaerophilic environment. *H. pylori* heavy growth was treated with 0.5% formalized saline and kept overnight at 4 °C. Centrifuged at 10,000 rpm for 10 min and washed 3 times with 0.2% formalizes PBS (pH 7.2). Finally, the concentration with carbonate buffer (pH 9.8) was adjusted and stored at 4 °C. Furthermore, *H. pylori* whole cell sonicate antigen were prepared by centrifuged *H. pylori* cell growth at 10,000 rpm for 10 min, followed by 3 times washing with 20 mM Tris HCl buffer (pH 7.5) and suspended in the same buffer. Cells were sonicated on ice with 6 times for 30 s with a 60 s interval between each shock. The sonicated samples were centrifuged at 5000 rpm for 10 min to remove the cell debris. Then supernatant treated with 0.5% formalized saline and makes the aliquots and kept frozen at -20 °C. Protein concentration was measured by Coomassie blue assay (Bio-Rad, Hercules, CA, USA), bovine serum albumin used as a standard (32, 33).

Washing and sensitization of sheep RBCs:

Sheep blood was diluted with fresh Alsever's solution (pH 6.1) in 1:1 ratio and incubated at 4 °C for a week. Furthermore, RBCs, treated with tannic acid (pH 7.0; 1:20,000) and incubated for 10 min at 37 °C. Centrifuge at 15000 rpm for five min. Sensitized the RBCs with *H. pylori* surface whole cell (1.0 mg/ml) and *H. pylori* sonicate whole cell antigen (1.0 mg/ml) with the 1h incubation at room temperature., washed and resuspended in the buffer (34).

Animal model experimental design and immunization with *H. pylori* antigen and CS

Male mice were eight-week-old C57-BL/6J-219 mice (18– 20 g). The experiments were performed after protocol approval by the ethics committee of China-US Hormel Cancer Institute, Henan, China and were conducted in accordance with the current guidelines for laboratory animal care. C57-BL/6J-219 were randomly assigned to five groups of 6. Adult female rabbits were immunized intraperitoneally with the increasing amount of antigen (3×10^8 cells/ml) sheep RBC coated on the days of 1, 3, 5, 7 and 17. Moreover, mice were also treated with 50 mg/Kg/day CS extract. Control mice inoculated with only antigen or CS extract. Blood was collected from a marginal ear vein and by cardiac puncture after the slide agglutination test.

Splenectomy and preparation of splenocyte cell suspension

Mice spleens tissue were aseptically removed and minced into 3–4 pieces with firmly force into the fragments. Cells were allowed to pass through a wire mesh (70 μ M Nylon; Corning, Durham, USA, Cat number 431751) and prepared as a single cell suspension in DMEM Eagle's medium. The cell suspension was centrifuged at 1000rpm for 10 min at room temperature. The pellet was washed with PBS, and the cells were gently resuspended into Corning DMEM media.

Separation of B-Cell

Percoll in Hanks' balanced salt solution (HBSS) solution of different concentration 70%, 66%, 60% and 50% were gently added (2.5ml each) in a tube and incubated for 15 min on ice, respectively. Later, pour spleen suspension (approximately 5×10^7 /ml) on the top of the gradient and centrifuge for 15 min at 1900xG at 4 °C. we collected the cells at interface between the gradient of 50%. Washed the cells with HBSS and count the B cells up to 60% of total lymphocytes.

Antibody secreting cell assay

Spleens were removed aseptically, the cells teased from the capsule and prepared as a single cell suspension in Eagle's medium. Appropriate concentrations were then added to the overlay medium prepared by mixing equal volumes of 0.8% agar and DMEM medium. Mixture of *H. pylori* antigen coated with sheep RBCs, 0.1 ml spleen cell and 1 ml of complement (guinea pig serum) were added to the overlay medium of slides. The glass slide chambers are sealed with paraffin wax and incubated at 37 °C for 45–60 minutes. We make slides with different concentrations of spleen cells (1:100 or 1:10) in DMEM. Calculate the PFU/spleen and PFU/ 10^6 spleen cells.

ELISA to detect *H. pylori* antibody titer.

A 96-well plate (Sero-Wel, UK) was coated with 20 ug/ml (100 ul) of *H. pylori* surface whole cell antigen in coating buffer (Na_2CO_3 , NaHCO_3 , pH 9.6) with overnight incubation at 4 °C. Next day, it was washed five times with PBS-Tween20 (0.05%) (pH 7.2) and blocked free active sites by 3% BSA. After washing, 100 ul (1:100 diluted) of patient serum was added into consecutive wells along with negative and positive controls and incubated at 37 °C for

1 h. After extensive washing, HRP-conjugated goat antihuman IgG antibody (1:2,500) was added and incubated for 1 h at 37 °C. Later, 100 µl of substrates (TMB One solution G7431, Promega, USA) was added and kept for 30 min at room temperature in dark place. The reaction was stopped by adding 0.3 M H₂SO₄, and color intensity was measured at 450 nm by an ELISA reader (STAT FAX-2100) (32, 33).

Real-time RT-PCR

Real-time RT-PCR

Total RNA was extracted using the commercial RNA extraction kit (Ambion by Life Technologies, Van Allen Way Carsbad, USA, 92008) and cDNA synthesized by amfiRivert cDNA synthesis platinum master mix (GenDEPOT, Katy, TX, USA, Cat number R5600-200). Real-time PCR (qRTPCR) was conducted using a 7500 FastDX (Applied Biosystems, MA, USA) and the power SYBR green PCR master mix (Applied biosystem, Warrington WA1 4SR, UK, Cat number 4367659). Primer IDs and sequences are shown in Supplementary Table S1, available at Carcinogenesis Online (35).

Cell culture

Jurkat T-lymphocytes and HEK-293T cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM, RPMI-1640 media supplemented with 10% FBS (Invitrogen, Camarillo, CA, US), 100 U/ml penicillin and 100 µg/ml streptomycin. All the cell lines were authenticated for mycoplasma; with the passage number of three to five times. The cell cultures were maintained at 37°C under 5% CO₂. Cells were seeded in 100. 20 mm dish with DMEM or F12 medium. Upon 70% confluence, cells were treated with CS (5 mg/ml) or infected with *H. pylori* strain (ATCC-43504) (1 x10³ bacteria/ml).

Tissue harvesting for cell sorting and fluorescence activated cell sorting analysis

Mice stomach tissue was minced into 3–4 pieces and pressed firmly to force the fragments apart; this allowed cells to pass through a wire mesh (70 µM Nylon; Corning, Durham, USA, Cat number 431751). The cell suspension was centrifuged at 250g for 5 min at room temperature. Each cell pellet was treated with ammonium chloride (0.17 M per 1 ml cell pellet) and incubated at room temperature for 5 min, followed by centrifugation at 250g for 5 min. The pellet was washed with PBS, and the cells were gently resuspended into Corning DMEM media. After washing with PBS containing 2% BSA, cells were incubated with a lineage cocktail containing biotinylated antibodies against CD4 (Biolegend, London, UK, Cat number 557307), CD8 (Biolegend; Cat number 553030), CD19 (Biolegend; Cat number 557398), CD11b (Biolegend; Cat number 561688), CD11c (Biolegend; Cat number 557400) and CD49b (Biolegend; Cat number 561067) at 4°C for 30 min in the dark. After washing twice with PBS containing 1% FBS and 0.1% sodium azide, cell sorting was performed using a BD FACSAria II, San Jose, CA, SN: P69500099 cell sorter with BD FACSDiva Software version 8.0.2. Data were analyzed with BD FlowJo version 10 software.

RESULTS

Hemolytic plaque assay to determine the effect of CS on immune response against *H. pylori*.

A hemolytic plaque assay was performed to determine the immunomodulatory effect of CS with immunization of *H. pylori* antigens. We found considerable immunomodulatory effects of CS against *H. pylori* infection. We measured the number of plaque forming units (PFU/spleen). We found an increase in the PFU/spleen in mouse immunized with *H. pylori* surface (6410/spleen) or sonicated whole cell antigen (85388/spleen) along with CS as compared to mice immunized alone (2129 & 11050/spleen) at 1:10 dilution, respectively ($P < 0.0001$; Figure 1A-C and Table 1; Supplementary Table. S2-S6). Control mouse, which was not immunized and treated with, and CS showed negligible number of PFU/spleen (210/spleen). On the other hand, *H. pylori* sonicate whole cell antigen showed a significant high number of PFU as compared to *H. pylori* whole cell antigen, which proved the significant differences between the nature of antigens. Furthermore, determined the antibody titer in mice before and after immunization of CS along with respective antigen by ELISA. High titer was observed in the mice immunized with CS along with *H. pylori* whole cell (1: 10⁵) or sonicate (1: 10¹⁰) antigen as compared to mice immunized with alone (1: 10⁴ & 1: 10⁶) (P -value<0.05), respectively ($P < 0.0001$; Figure 1D-E and Table 2; Supplementary Table. S7-S14). Our study demonstrates that efficacy of antigen affects the immune response against *H. pylori* and CS that have immunomodulatory effects.

CS stimulated the numbers of CD8+ T-Lymphocytes in *H. pylori* induced gastritis mice.

We analyzed the effect CS extract on immune cells by using flow cytometric analysis, the results showed that mouse treated with CS (50 mg/kg) along with *H. pylori* surface and sonicate immunization increased CD8+ (cytotoxic) T-cell percentage from ($P = 0.006$; 17 to 21% & $P = 0.011$; 22 to 30%; Figure 2A), CD4+ helper T-cell percentage ($P = 0.006$; 11 to 13% & $P = 0.006$; 11 to 14%; Figure 2B). However, CD49b+ ($P = 0.266$; 6 to 7% & $P = 0.944$; 6 to 6%; Figure 2C), CD11b+ ($P = 0.582$; 6 to 7% & $P = 0.758$; 6 to 6%; Figure 2D), CD11c+ ($P = 0.509$; 5 to 5% & $P = 0.651$; 6 to 6%; Figure 2E) and CD19+ ($P = 0.884$; 7 to 7% & $P = 0.232$; 7 to 8%; Figure 2F), respectively. Untreated mice show negligible PFU. We further investigated the dose dependent effect of CS treatment on CD8+ stimulation and *H. pylori* vaccine immunization. We found a gradual increase of CD8+ lymphocytes ($P < 0.0001$, Figure. 2G) with the increasing dose of CS in *H. pylori* immunized mice. These results confirmed the significant effect of CS in the stimulation of immune response against *H. pylori* (Figure 2H).

CS with *H. pylori* immunization reduced *H. pylori* Induced cytokine levels:

To determine the effect of catechins on immune response, we investigated the cytokines production in mRNA level in the mice. Total RNA was extracted for the quantitative PCR to evaluate type I and type II cytokines expression. We found that catechins increases type I cytokines level, such as IL-2, TNF- α , IL-12 and IFN- γ compared to type II cytokines level (IL10, IL13, IL4 and IL5) ($P < 0.0001$; Figure 3A-H). Moreover, we assessed the profile of cytokine levels by qPCR in specific CD8⁺ cells separated from the mice spleen. We found consecutive results of high expression level of type I cytokines and type II cytokines show no change in the expression ($P < 0.0001$; Figure 3I). We further investigated the dose dependent effect of CS and *H. pylori* vaccine treatment on cytokines profile. We found gradual increase of type I cytokines level treated with continuing increase of CS dose and *H. pylori* vaccine ($P < 0.0001$; Figure 3J & K) while type II shows no change and decreasing with the higher concentration of CS and *H. pylori* vaccine ($P < 0.0001$; Figure 3L & M). To further explore the effect of CS on inflammation, we determined the neutrophils (MPO), macrophages (F4/80) and ROS level. Our data demonstrated that *H. pylori* infection increased the stomach neutrophils and macrophages as well as increased the level of ROS production ($P < 0.0001$; Figure 3N, O & P). However, pretreatment by CS significantly depressed the levels of macrophages, neutrophils, and ROS. The above results strongly indicating that CS decreased inflammation. As a result, we hypothesized that CS would selectively enhance type-I immune responses and weaken type-II immune responses simultaneously. Overall, these data illustrate that the immune microenvironment is influenced by *H. pylori* immunization with CS and Type I overexpression cytokines, ultimately leading to increased CD8⁺ cell count and stimulation of *H. pylori* titer.

Catechins reduced gastric inflammation through activation of autophagy:

CS inhibits gastric inflammation through the induction of autophagy in *H. pylori*-induced gastritis model. Autophagy plays a crucial role in regulating inflammatory response by eliminating inflammasome, cytokines, and cellular components, which provides an important mechanism to inhibit inflammation. In order to investigate the role of CS in the inhibition of *H. pylori*-induced gastric inflammation through autophagy activation in vivo, we developed a *H. pylori* induced gastritis model. We found that CS significantly reduced gastric inflammation through induction of autophagy. Analysis of autophagy gene expression indicated that the mRNA level of ATG5 (Figure. 4A), Beclin (Figure. 4B), ATG7 (Figure. 4C), ULK1 (Figure. 4D), LC3a (Figure. 4E), FIP200 (Figure. 4F), LC3b (Figure. 4G) and BNIP3I (Figure. 4H) were significantly induced in response to CS treatment with *H. pylori* immunization. These results proved the role of CS in the induction of autophagy to reduce *H. pylori*-associated gastric inflammation.

Discussion

H. pylori as a causative agent for gastritis, gastric ulcer (36), chronic nonspecific gastritis and duodenal ulcer diseases (37). Colonization of *H. pylori* in the human stomach results in production of a humoral and cellular immune response but does not always result in clearance of *H. pylori* and persists for a long time in absence of antibiotic therapy (38). Eradication of *H. pylori* occurs by both therapeutic and by stimulating the immune response against *H. pylori* (39). Researchers are trying to find alternative antimicrobial agents and plant extract as an alternative source of treatment, which is cheapest, easily available, and has less side effects. Plant extract is used in many old civilizations against many infectious agents (40, 41, 42)

Besides its antibacterial activity, CS is thought to have a potentiating effect on the immune system of humans and various animals (43). CS is used as both antimicrobial and immunomodulator. It contains polyphenols (especially catechins) which act as strong antioxidants and antibacterial activity. It contains minerals, vitamins and Catechins (44). Using mouse models, this study provides evidence of CS as an immunomodulator against *H. pylori* associated gastric disease and gastric cancer (Supplementary Figs. 1 & 2).

This study also reveals the critical role of TNF- α , IFN- γ , IL-2 and IL-12 cytokines in stimulating the host immunity via CD8⁺ cell upregulation and autophagy induction. The major importance of the present work is that this system offers an in vitro method for primary stimulation and measurement of cells responsive to specific *H. pylori* antigenic stimulation by sheep erythrocytes in whole spleen cell populations. The sensitivity of this method is seen from the fact that antibody-producing cells were observed as early as day 1–4 after primary immunization. The specificity of the plaque assay system is indicated by the fact that the number of plaques forming cells against the unrelated antigen is a small fraction of that against the specific antigen (45). Hemolytic plaque assay is a sensitive assay for the detection of antibody-forming cells (46). We developed a hemolytic plaque assay to study the immunological response of *H. pylori* surface and sonicated whole cell antigen along with the effect of CS on immune response against *H. pylori*. In which sheep RBCs, used as indicators for lyses because of the presence of *H. pylori* antibody-forming cells. The lyses of *H. pylori* coupling sheep RBC; s help to develop the plaque assay which is specific for the *H. pylori* plaque forming unit. We observed the high number of plaques in mice immunized with *H. pylori* sonicate antigen as compared to surface whole cell antigen which proved the production of high immune response specific for *H. pylori* sonicate antigen. Moreover, mice immunized with sonicate whole cell antigen and CS showed high numbers of plaques as compared to the mice immunized with antigen only (Fig. 1). These results proved that CS acts as an immunomodulator and helps to protect from gastritis infection caused by *H. pylori* infection as well as helps to avoid the use of antibiotics and reduce antibiotic resistance.

In mice model of plaque cell assay, immunization of *H. pylori* surface or sonicate antigen upregulates immune cell populations and downregulates the anti-inflammatory cytokines by autophagy induction. Here, we provide evidence for CS regulation of CD8 lymphocytes stimulation and inflammatory responses in activated CD8⁺ cells (Fig. 2). These data suggested that CS acts as an immunomodulator of *H. pylori* immunization to stimulate CD8⁺ cells. As a result, *H. pylori* infection is prevented by stimulating host immunity. Furthermore, *H. pylori*-associated inflammation is coupled with decreased type-I cytokines secretion in CD8⁺ cells with autophagy reduction. This chronic inflammation predisposes gastric cells towards carcinogenesis (Fig. 3). Hence, higher host immunity prevents *H. pylori* infection of gastric cells towards carcinogenesis.

H. pylori infection increases peroxidation processes and leads to the production of superoxide radicals and aldehydes, such as MDA, MPO, and hydrogen peroxide, that can form adducts with DNA and proteins. However, A combination of CS and *H. pylori* vaccination decreases the peroxidation

process. Subsequently, we proposed that *H. pylori* infection and other metabolites, including acetaldehydes, aldehydes, and peroxides, damage DNA, DNA repair enzymes, and immune cell protein cytokines, resulting in gastric tissue damage (11). We evaluated ROS levels through activation of the electron transport chain (ETC). ROS levels were decreased in T-lymphocytes cells with combinatorial treatment of CS and *H. pylori* vaccine compared to single treatment or *H. pylori* infection mice (Figure: 3). In our mice model, the number of pro-inflammatory cell types was significantly increased (i.e., leukocyte infiltration), as observed by the numbers of activated macrophages (F4/80 positive) and neutrophils (myeloperoxidase, MPO). Due to increased immunocyte infiltration, we analyzed several inflammatory-related chemokines and cytokines at the mRNA and protein (secretion) levels by qPCR and a customized multiplex magnetic bead array. Our data suggested that CS intake combined with *H. pylori* immunization causes marked increased in type-I cytokine levels (Fig. 2) Overall, the *H. pylori* and CS consumption showed great sensitivity and specificity towards immune T-lymphocytes (CD8); especially this combination could be used to prevent gastric diseases, including gastritis, gastric ulcer and gastric cancer (11).

Recently, autophagy has attracted attention due to the role of antimicrobial capacity in which autophagy can kill pathogens and protect from pathogens as well as inflammation secretions factors (47, 48, 49). In our study, we found that *H. pylori* infected mice model significantly inhibits the autophagy related protein levels of Beclin, LC3a, LC3b, and others. In contrast, CS treatment significantly enhanced the autophagy-related protein levels and enhanced the immune response against *H. pylori*. Hence, our study proved the role of CS in the stimulation of immune response via induction of autophagy.

Conclusions

The management of *H. pylori* infections requires control of antibiotic resistance and most important increased host immunity to prevent *H. pylori* infection. Lack of understanding about the *H. pylori* mode of transmission facilitates the spreading of *H. pylori* infection to other individuals and leads to increasing resistance (Bonacorsi et al., 2009). In the future, antibiotic resistance will be the greatest obstacle in the treatment of *H. pylori* infections. Hence, preventive measures to limit the spread of the infections need to be developed by using alternate sources of treatment. Despite progress in conventional chemistry and pharmacology in producing effective drugs, medicinal plants might provide a useful source of treatment against *H. pylori* by stimulating immune response for the development of novel pharmaceutical entities or, alternatively, as simple dietary adjuncts to existing therapies (50, 51). Hence, medicinal plants e.g., CS will provide a useful source of immunomodulator agent against *H. pylori* infection as well as facilitate to prevent *H. pylori* antibiotic resistance.

Abbreviations

ELISA Enzyme-linked immunosorbent assay

H. pylori: *Helicobacter pylori*

OD Optical density

PCR Polymerase chain reaction

UBT Urea breath test

IARC, International Agency of Research on Cancer

IFN, interferon

IL, interleukin

MPO, myeloperoxidases

TNF, tumor necrosis factor

WHO, World Health Organization

Declarations

Author contributions

F.A. designed and performed the experiments, derived the models, and analyzed the data; F.A. conceived the idea. S.U.K. supervised the project. F.A. analyzed the data and interpretate the results. F.A. performed the pathological analysis of mouse tissue samples. F.A. and R.S. wrote and edited the manuscript.

Acknowledgement:

There are no other financial declarations by any of the authors. This research received no funding.

Ethics approval and Institutional Review Board Statement.

The study was approved by the Ethical Review Board of University of Karachi on 13 May 2009 (approval number: ERB/KU0002/2009SUK). The animal experiments were conducted in accordance with the current guidelines for laboratory animal care.

Conflict of interest

We have no financial relationships or interests to disclosure.

Funding:

This research received no external funding.

Informed Consent Statement:

Not applicable.

Data Availability Statement:

The data that support the findings of this study are available on reasonable request from the corresponding author.

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Table

Table 1: Table showing the comparison of number of plaques with and without immunization of CS.

MICE IMMUNIZE TREATMENT	SPLEEN CELL DILUTIONS									
	1:10		1:20		1:50		1:100		1:1000	
	PFU/spleen	PFU/10 ⁶ spleen cells	PFU/spleen	PFU/10 ⁶ spleen cells	PFU/spleen	PFU/10 ⁶ spleen cells	PFU/spleen	PFU/10 ⁶ spleen cells	PFU/spleen	PFU/10 ⁶ spleen cells
CONTROL MICE	210	7	118	4	73	2	0	0	0	0
WHOLE CELL IMMUNIZE MICE	2129	55	1157	30	917	24	702	18	395	10
WHOLE CELL WITH CS IMMUNIZA MICE	6410	88	5223	72	3194	44	2138	29	1090	15
SONICATE ANTIGEN IMMUNIZE MICE	11050	213	10032	193	9161	176	8334	160	7102	137
SONICATE ANTIGEN IMMUNIZE MICE WITH CS	85388	938	69594	768	54792	602	22650	249	14530	160

Figures

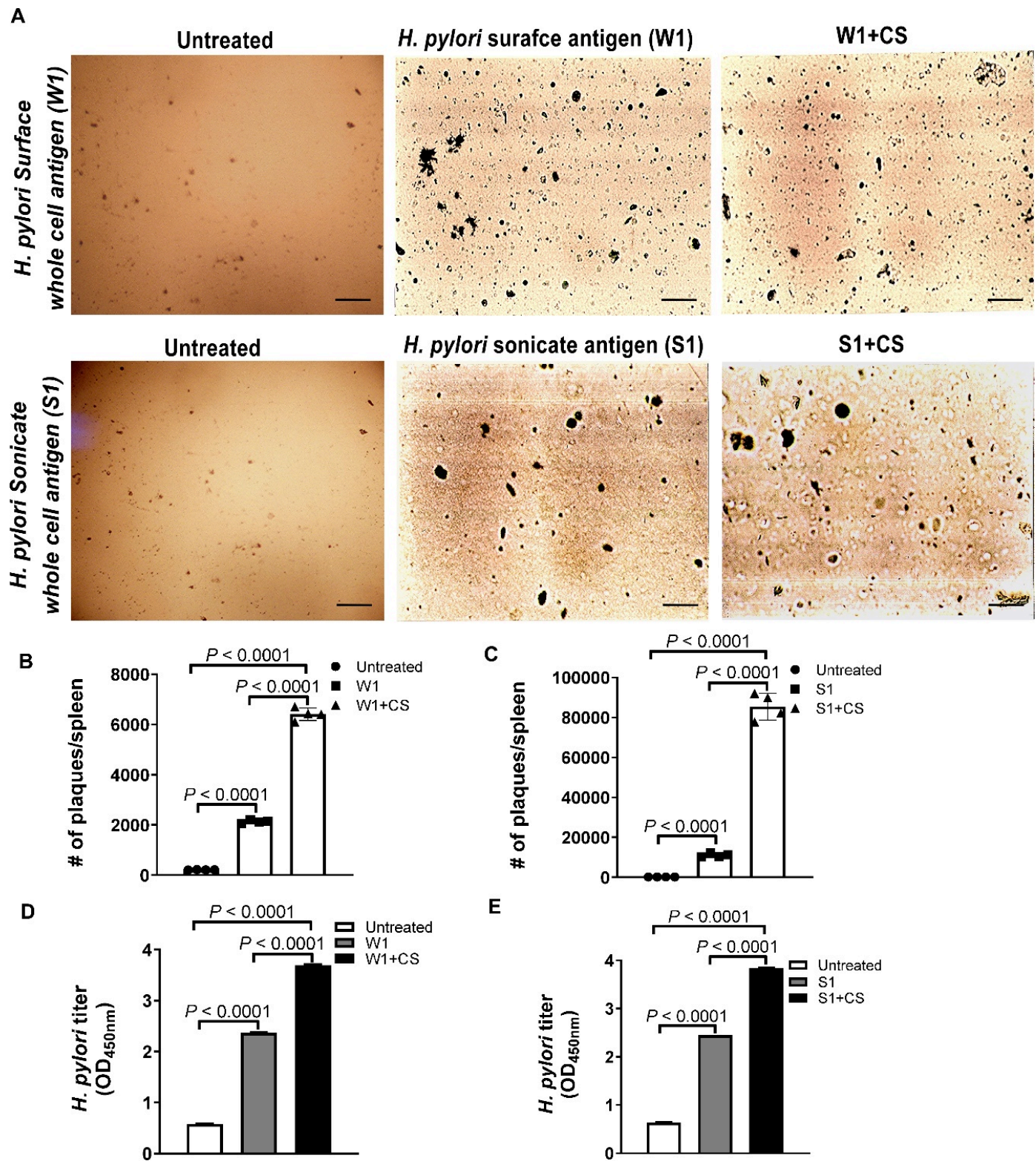


Figure 1

Hemolytic plaque cells assay of mice to determine the effect of CS in the stimulation of immune response against *H. pylori* infection. Quantification of Hemolytic plaque of *H. pylori* surface A-B) and sonicate antigen A & C) with CS (n = 5/cohort). The scale bars represent 100 μ m (10x). Bar diagram showing titer of mice immunize with *H. pylori* surface antigen D) and sonicate antigen E) with CS. Data represent mean $\pm$ SE. *, $P < 0.05$.

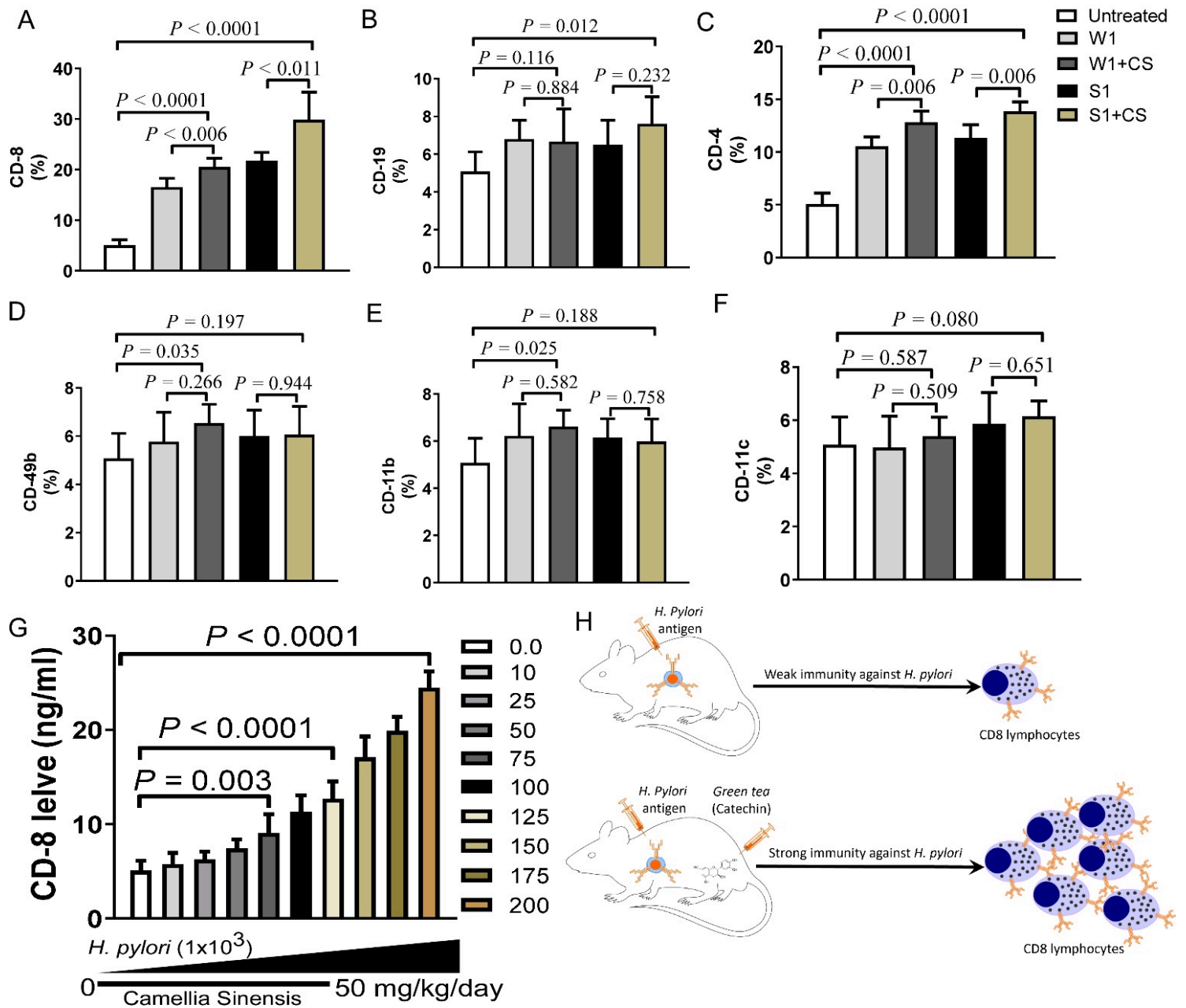


Figure 2

Immune cell populations in stomach tissue from CS immunomodulatory mouse models. Expression profiles of A) CD-8, B) CD-19, C) CD4, D) CD-49b, E) CD11b, and F) CD-11c cells in mouse stomach tissue samples measured by flow cytometry (n = 5/cohort). Representative samples showing relative levels of $\Delta\psi_m$ and cell percentages. G) Dose-dependent response of *H. pylori* vaccine with CS intake on CD8 level as measured by ELSIA. Data represent mean $\pm$ SE. *, P < 0.05.

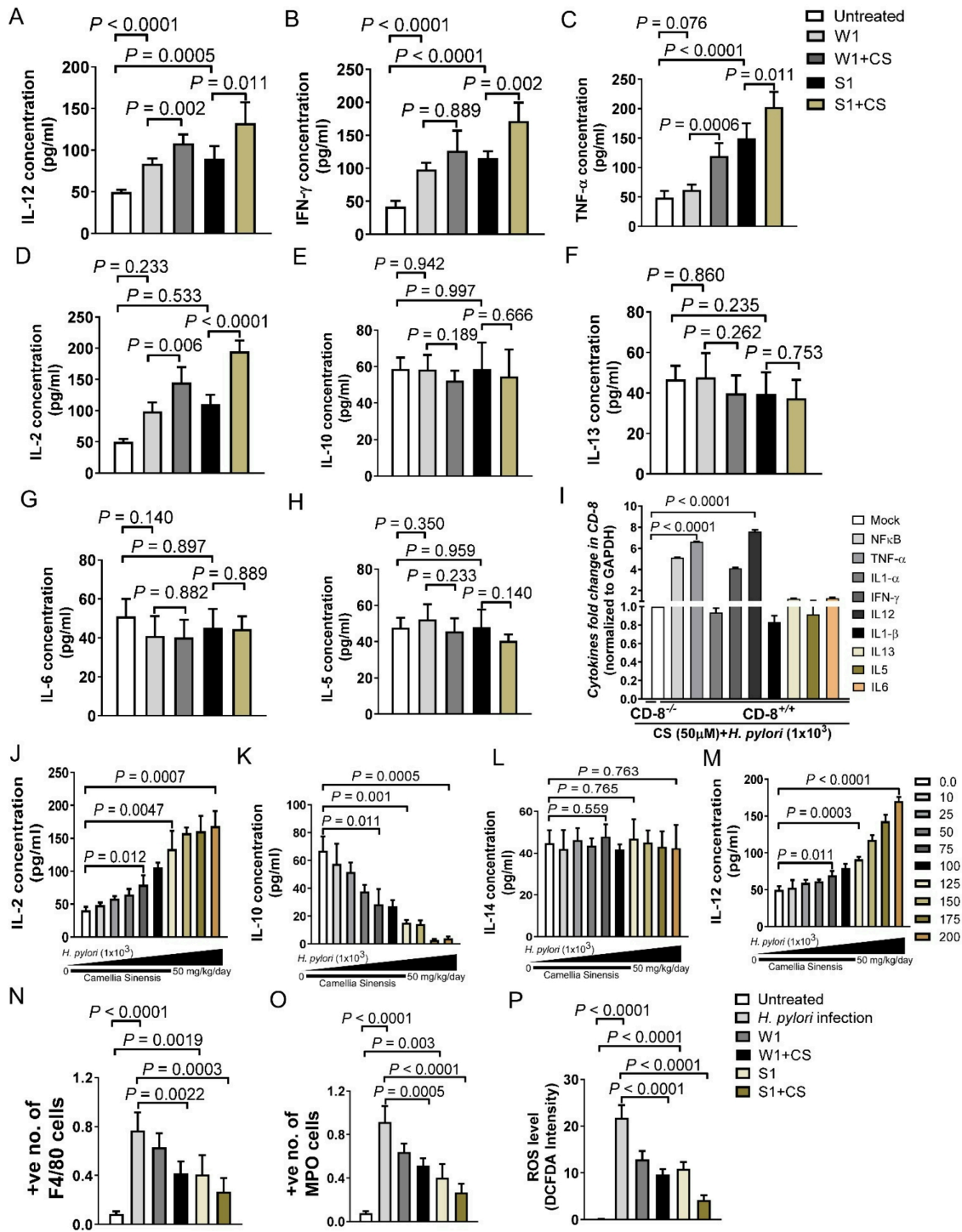


Figure 3

Analysis of inflammatory mediators in tissues from the CS immunomodulatory mouse models. Analysis of cytokines in serum from mouse models. Secretory profiles of pro-inflammatory cytokines A) IL-12, B) IFN- γ , C) TNF- α , D) IL-2, E) IL-10, F) IL-13, G) IL-6 and H) IL-5 in mouse serum samples measured by multiplex magnetic bead array. Graphs show secreted protein expression relative to serum samples from untreated mice. (n = 5/cohort). I) mRNA profiles of pro-inflammatory cytokines in CD8 cells measured by qPCR. Graphs show mRNA expression relative to cell lysate from untreated CD8 immune cells. Dose-dependent response of CS intake on J) IL-2, K) IL-10, L) IL-14 and M) IL-12 production as measured by ELSIA. Quantitative immunohistochemistry to detect N) MPO and O) F4/80 expression revealed a higher score in *H. pylori* infection compared to tissues from mice treated with *H. pylori* surface or sonicate vaccine or with CS intake. Staining intensity was weaker in untreated gastric tissue compared to gastric tumor tissue.

A score of 1 denotes the highest staining or expression levels, whereas a score of 0 indicates negative staining. P) Representative bar chart shows the percentages of cells with ROS production.

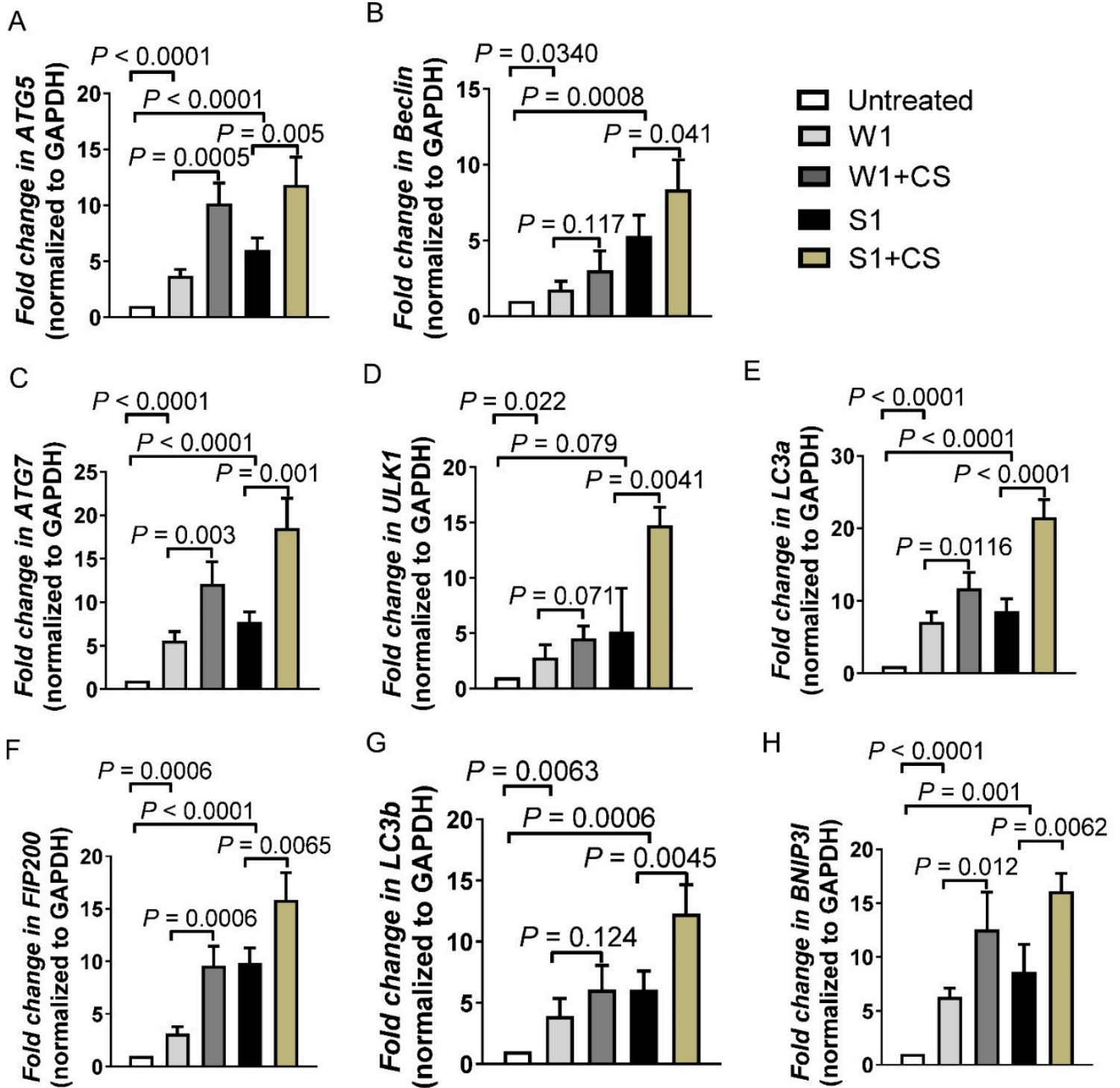


Figure 4

Transcriptome analysis of gastric disease mouse models. Total mRNA analysis of A) ATG5, B) Beclin, C) ATG7, D) ULK1 E) *LC3a*, F) *FIP200*, G) *LC3b* and H) *BNIP3* in mice treated with *H. pylori* surface and sonicate vaccine and CS by qPCR. Bar graphs show the average fold change in mRNA relative to untreated mice (n = 5/cohort).

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