INFLUENCE OF CHRONIC HELICOBACTER PYLORI INFECTION ON SYSTEMIC AND NEUROINFLAMMATION AND ITS IMPACT ON COGNITION

BY

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DISSERTATION

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Abstract

Helicobacter pylori is the major bacterial colonizer of the human stomach. It is estimated that approximately 50% of humans worldwide are chronically infected with this bacterium. Epidemiologically, *H. pylori* is recognized as the major risk factor and etiological agent of peptic ulcer disease and gastric adenocarcinoma. Most animal models utilized for evaluating *H. pylori* infection have examined the gastric effects of chronic infection with regard to ulcer and cancer development. However, interest into the potential extra-gastric influences of chronic *H. pylori* infection has been growing in the field. Utilizing a Sprague-Dawley rat based model for chronic *H. pylori* infection, the role of chronic infection in the development of systemic and neuro-inflammation was evaluated. High sensitivity ELISA assays were utilized to determine the change in key inflammatory markers locally within the stomach, systemically in the plasma and within the liver and the spleen, and within the central nervous system in the hippocampus and cerebellum. Eradication studies were conducted to determine the causal relationship between *H. pylori* infection and observed levels of inflammation. Finally, cognitive behavioral studies were conducted to evaluate the potential influence of chronic *H. pylori* infection on cognitive function and health. Altogether, these studies illustrate a model by which chronic infection established during early life modulates the immune system response to generate a mild, but chronically sustained, systemic and neuro-inflammatory state which is associated with impairment of pre-frontal cortex mediated cognition. Furthermore, it is proposed that the development of these cognitive impairments arise during key developmental windows resulting in persistence of the impairment after successful eradication of *H. pylori*.

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It is with great pride and deep remorse that I dedicate this work to the memory of my father, Llewellyn Kenneth Reno. At the midpoint of my graduate study, my father was diagnosed with severe Stage IIIb multiple myeloma. He was advised that he likely only had weeks to live and that aggressive anti-cancer therapy may only offer him a little more time. My father underwent anti-cancer therapy and throughout all of the side effects and secondary illnesses that it brought, he never complained. My father was not fighting for himself but because he knew that we were not ready for him to leave. Through his fight, he gave us five more years of time to spend with him. In the summer of 2016, his cancer returned from remission and he prepared to fight it yet again. However, he was spared another long and difficult fight as a heart attack on the night of October 9th left him unresponsive. On October 10st in accordance with his final wishes, we said goodbye to my father and let him finally stop fighting for us. He raised three sons and was always a source of encouragement and guidance to us in both our good decisions as well as the bad. In the good, he was always content to proudly watch on from the background; in the bad, he was the first to step in and help us pick up the pieces and continue to move forward. In his final years, his desire to see me finish this Ph.D. became a major factor in his will to continue the fight against his cancer. While I regret he was unable to see the completion of this work, I know he would be proud that is at last completed. Thank you, Dad, for all of your sacrifices, those that we acknowledge and those we will never know, for the encouragement and the criticisms, the discussions and the arguments, the laughter and the tears, the good times and even the bad. Rest in peace knowing that you instilled strength, confidence, and independence in your three sons. Though you are gone from our sight, know that you will live on in our hearts and memories. Goodbye, Dad.

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Chapter 1: Introduction

1.1 Microbiota-Gut-Brain Axis

The gut-brain axis was initially proposed by psychologists and psychiatrists in the later nineteenth and early twentieth century[236,237] based on the empirical observations of emotions influencing gastrointestinal activity[57] as well as abdominal pain syndromes modulating emotional responses[180]. Modern examination into the interactions between the central nervous system (CNS) and the gastrointestinal tract began with the discovery of the enteric nervous system[126,127,138,149]. Despite the enteric nervous system's early characterization of independence from the CNS[115,391], progress in understanding its role in physiology has revealed aspects of the complexities of its intercommunication with the CNS[206]. Interactions along the gut-brain axis can be broadly grouped into three categories based on the physiological mode of communication: neuronal, hormonal, and immunological. The microbiota acts as both a moderator and a direct participant in these process by varied mechanisms including interactions with the vagus nerve[35,48,86,134], secretion of metabolites which have neuroactive properties[152,216,363], release of tryptophan metabolism intermediates which serve as precursors for the production of the neurotransmitter serotonin[61,321], and active stimulation of the innate immune response[82,97,350].

Animal studies evaluating the microbiota-gut-brain axis have largely focused on evaluation of behavioral disorders, such as anxiety and depression[120]. Physiologically, clinical major depressive episodes have been associated with pathogenic modulation of the hypothalamic-pituitary-adrenal (HPA) axis[29] and restoration of normal activity with resolution of the depressive episode[163,270]. Linkage to the microbiota-gut-brain axis was found in the form of endocrine studies comparing germ free (GF) mice with specific pathogen free (SPF) mice. GF mice possess no microbiota and have suppressed immunological function while their SPF counterparts possess both a normal microbiota

as well as normal functioning immune system[45,218,219,365]. GF mice were characterized to have an exaggerated stress response, similar to that seen during major depressive episodes[62,269,354]. Inoculating GF mice with normal microbiota from SPF mice led to the normalization of HPA axis regulation[34,62,160,268]. In disease models, administration of *Lactobacilli* and *Bifidobacteria* was associated with relief of pain due to stress and irritable bowel syndrome[5,130,241,320,382]. Additionally, oral administration of low levels of gut bacterial pathogen was associated with increased anxiety-like behavior[34,147,214]. The major modalities of interaction utilized by the microbiota-gut-brain axis are neuronal, hormonal, and immunological. While these processes are often considered as separate and distinct for the sake of clarity, physiology relies on the active integration of each of these processes in order to maintain stable homeostasis.

1.1.1 Influence of neuronal communication.

Neuronal communication within the gut-brain axis can be divided broadly into two categories based on the location of the cell body of the innervating neuron. Extrinsic neurons are those whose cell bodies reside within the central nervous system, principally the vagal and spinal afferents. The intrinsic primary afferent neurons (IPANs) reside within the gut[63]. IPANs form extensive self-reinforcing networks of reflex loops[199]. It is estimated that the IPANs account for an estimated 100 million connections to the human gut, in comparison to the 50 thousand connections from the extrinsic neurons[127]. Both the extrinsic neurons and IPANs contribute to maintaining the gastrointestinal homeostasis via modulation of complex reflex loops during internal perturbations[127]. While both extrinsic neurons and IPANs transmit information relating to chemical and mechanical stimuli, IPANs interact with enteric ganglia to regulate activity via intramural reflexes; extrinsic neurons integrate information into mesenteric, spinal, and supraspinal reflexes within the central nervous system[150,213,298].

Of the extrinsic neurons, those belonging to the vagus nerve are the most well studied and understood. The vagus nerve provides both efferent and afferent parasympathetic innervation to the majority of the digestive system, with the exception of the most distal aspects of the small intestine, descending colon and rectum which receive parasympathetic innervation from the sacral nerves. Subpopulations of vagal afferents terminate in close proximity to endocrine-like cells of the gastrointestinal tract[139]. Receptors on these neurons have been shown to be responsive to many of the small peptides with endocrine-like properties that are secreted by the gut, such as peptide YY[52,161] and ghrelin[83,84]. Expression of these receptors has been found to vary based on nutritional status[86] as well as by fasting[87,94]. It has been hypothesized that interaction between the vagus nerve and these small peptides allows for rapid signaling of satiety and energy status within the central nervous system[83,314,343].

Vagal afferents have also been shown to associate with immune cells of digestive system[28]. These neurons have been shown to a variety of immune cell secreted factors, including histamine and inflammatory cytokines[345]. Stimulation of the vagus nerve by cytokines followed by vagal suppression of cytokine production forms the basis for the inflammatory reflex[369]. Absence of this vital reflex results in excessive innate immune system activity and cytokine toxicity[47,384]. The vagus nerve and the inflammatory reflex play an important role in suppressing postprandial inflammation that naturally develops following high-fat or high-fat and high-carbohydrate meals[108,141,156]. Stimulation of vagal efferents resulted in inhibition of TNF-α production by the liver and prevented the development of shock in rats suffering from lethal endotoxemia[46]. While vagal efferents may act to suppress inflammation, the stimulation of vagal afferents has been shown to induce IL-1ß expression within the central nervous system as well as stimulate glucocorticoid production via the HPA axis[170]. Behaviorally, subdiaphragmatic vagotomy has been associated with attenuation of fever[225,337], food-motivated behavior[49],

and taste aversion learning in rodent[148]. Within the central nervous system, vagotomy has been associated with depletion of hypothalamic norepinephrine secreting neurons[117] and reduced IL-1ß production in response to systemic LPS administration[201].

1.1.2 Influence of hormonal communication.

Many small peptides with endocrine-like properties are secreted by the gut. In the treatment of type 2 diabetes mellitus, drugs mimicking glucagon like peptide-1 (GPL1) have been utilized as they increase glucose-induced insulin secretion[95,168]. The small peptides oxyntomodulin (XON)[64], ghrelin[185], and peptide YY (PYY)[74] plays varying roles in modulating satiety and regulating energy homeostasis. PYY and ghrelin are the most well studied of these gut secreted hormone-like peptides. PYY is released postprandially, crosses the blood-brain barrier into the accurate nucleus, and binds to the Y2 receptor of neuropeptide Y neurons in the hypothalamus, resulting in inhibition of food intake[30,312]. Intravenous administration of PYY in both humans and animals has been shown to reduce food intake[31]. Using functional MRI studies in humans, elevated serum PYY was associated with increased activity in corticolimbic and reduced activity in homeostatic regions[32]. In contrast, low circulating levels of PYY were associated with activation of hypothalamic homeostatic regions. This has led to the hypothesis that postprandial PYY level switch food intake regulation from homeostatic to hedonic, corticolimbic regions[32].

Ghrelin is secreted in response to emptying of the stomach, crosses the blood-brain barrier, and binds to the growth hormone secretagogue rector of the hypothalamic ventromedial nucleus and arcuate nucleus[27,191,368] where is increases food intake. Ghrelin levels increase in the preprandial state and decrease postprandially[12,75,374]. In humans, ghrelin levels have been observed to correlate with hunger scores in humans[73]. In addition to short-term satiety signaling, ghrelin has been implicated in long-term

energy balance. In rodents, daily administration of ghrelin led to decreased energy expenditures, leading to reduced fat utilization[373]. Finally, ghrelin is capable of regulating growth hormone release independently of its main regulation by pituitary growth-hormone-releasing hormone[188].

More broadly, the gut-brain axis interacts with the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis plays a key role in regulating stress, in addition to modulating a diverse set of somatic processes ranging from immune function to mood and emotion to energy storage and expenditure[317]. In brief, the hypothalamus releases corticotropin-releasing hormone and vasopressin. In turn, these stimulate the release of adrenocorticotropic hormone from the anterior pituitary which act at the adrenal cortex to stimulate the release of glucocorticoid hormones, principally cortisol. Both PYY and ghrelin are hypothesized to generate many of their associate physiological responses by influencing the actions of the HPA axis[145,169,175,196,347]. Empirically, many of the observations for the influence of the microbiota on the gut-brain axis have been via modulation of the HPA axis. For example, germ free animals were found to have exaggerated stress responses, in the form of elevated cortisol levels[62,269,354]. Reconstitution of the gut microbiota was associated with a normalization of cortisol release in response to stressors[34,62,160,268].

1.1.3 Influence of immunological communication.

It has been estimated that 70-80% of a human's immune cells reside within gut-associated lymphoid tissue. As the columnar intestinal epithelial cells for a barrier between the lumen of the gut and host, specialized lymphoid structures, Peyer's patches, allow for immunological sampling of the luminal space[14]. Dendritic cells, expressing a wide-range of pattern recognition receptors, are able to extend processes into the gut lumen and sample antigens and microorganisms present[244]. Vagal afferent

processes in close proximity are able to respond to the release of signaling molecules released by both mast cell and resident macrtophagers[28]. Similarly, the hormones secreted by enteroendrocrine cells of the gut are modulated by exposure inflammatory signaling molecules, such as cholecystokinin whose secretion is upregulated during gut inflammation[240]. Likewise, serotonin production by enterochromaffin cells is upregulated following exposure to inflammatory signals[235].

In mice, gastrointestinal inflammation due to *Campylobacter jejuni* infection has been associated with an increase in anxiety-like behavior, even in the absence of a systemic inflammatory response[215]. Low pathogenic load infections by *Citrobacter rodentium* and *C. jejuni* were still associated with increased anxiety-like behavior in mice despite no significant change in plasma cytokine levels or intestinal inflammation[147,214]. Unsurprisingly, increased gastrointestinal inflammation was associated with a notable increase in anxiety-like behavior[35,37]. Inoculation with *Bifidobacterium longum* has been shown to have to propensity to ameliorate gastrointestinal pathogen associated anxiety-like behavior[35,37].

1.1.4 The stomach as part of the gut-brain axis.

Classically, the “gut” aspect of the microbiota-gut-brain axis has referred to the small and large intestines. Direct neuronal communication between the stomach and the brain is primarily mediated via the vagus nerve. Gastric vagal afferent processes have been mapped to the dorsal motor nucleus with additional projections reaching the nucleus of the solitary tract[340]. Projections to the nucleus of the solitary tract allow for both vagovagal reflexes[247,313] as well as integration of chemosensory and mechanosensory information to the hypothalamus[172]. Studies in rats have indicated that suppression of vagal afferent signaling blocked ghrelin-induced feeding, secretion of growth hormone, and activation of growth hormone-releasing hormone-producing

neurons[83]. Vagal efferent process release acetylcholine in close proximity to parietal cells, stimulating the production and release of gastric acid[4]. Hormonally, the stomach acts as both a paracrine organ as well as an endocrine organ. As a paracrine organ, the G cells of the gastric epithelium secrete gastrin which acts locally on mast to trigger histamine release which acts on parietal cells to stimulate the production and secretion of gastric acid[325]. In turn, gastric acid stimulated D cells of the gastric epithelium to produce and secrete somatostatin, which acts to suppress gastric acid production[322]. As an endocrine organ, the stomach is the major site of ghrelin production and release. Ghrelin acts both to induce feeding as well as to promote growth hormone release[75,188,392]. Both major actions of ghrelin are mediated in the short-term by interaction with local receptors present on afferent processes of the vagus nerve[83,84]. In the long-term, circulating levels of ghrelin act a measure of global energy status for the maintenance of homeostasis via interaction with the hypothalamus[69,245]. Additionally, the stomach is a minor contributor to the production and secretion of leptin[20]. Leptin acts antagonistically to ghrelin in regards to satiety signaling via the vagus nerve[296,297]. Immunologically, the stomach is host to aspects of the gut-associated lymphoid tissue[205,238]. The gut-associated lymphoid tissue of the stomach is host predominantly to plasma cells and mast cells[129,198,397]. However, in the presence of prolonged local inflammation, the stomach becomes host to lymphoid follicles, allowing generation of new T and B cell populatiuons[137,351]. Therefore, it is wholly reasonable to consider the stomach an aspect of the gut-brain axis as it is capable of interacting with the brain by each of the major modalities of communication that make up the gut-brain axis.

1.2 Systemic and Neuro-Inflammatory Response to Chronic Infection

Classically, the hallmarks of the inflammatory process are pain, redness, heat swelling and loss of function. The inflammatory process is a generic, stereotyped, and

highly conserved process; as such, it is considered to be an aspect of the innate immune response[1]. Inflammation may be classified as acute or chronic. Acute inflammation is the initial response to tissue insult. It is characterized by the movement of plasma and primarily granulocytes into tissue and serves as one of the primary triggers for tissue regeneration[273]. Chronic inflammation is characterized by the shift in associated immune cells at the site of inflammation toward mononuclear cells and is associated with the simultaneous destruction and regeneration of tissues[128,338]. Traditionally, inflammation is considered a localized process occurring primarily at the foci of insult. However, in cases of widely disseminated insult or excessive chronic inflammation that is not properly suppressed, a systemic inflammatory response is generated[92]. The systemic inflammatory response is characterized by an increase in circulating inflammatory cytokines and is considered a risk factor in the development and progression of many pathological conditions[328].

1.2.1 Development of systemic inflammation.

The development of the systemic inflammatory response is complex, situational-specific process. Two general pathways have been proposed for the development of systemic inflammation: cytokine "spill-over" and dysregulation of immune suppression. The "spill-over" hypothesis was largely developed based on observations made during recovery from acute respiratory distress syndrome (ARDS)[209]. Epidemiologically, the ICU mortality of ARDS patients is 60.2%; however, the majority do not die from acute respiratory failure but rather from multiple organ failure[114,258,349,353]. It has been observed that ventilator-induced lung injury leads to localized production of inflammatory cytokines, increased alveolar-vascular permeability, and release of cytokines into circulation[88,179,303,342]. Similar observations have been made in COPD patients[123,324,360,380]. The presence of systemic inflammation in COPD patients has been

associated with the increased risk of developing further complications including weight loss[85,90], cachexia[3,334], osteoporosis[3,39], and cardiovascular disease[122,167,336]. Upon release into circulation, pulmonary derived cytokines were found to result in significant cytokine production and release from the liver in a Nf-κB-dependent manner[287]. A similar process has been proposed to explain the elevated levels of circulating cytokines in chronic periodontitis, juvenile idiopathic arthritis and rheumatoid arthritis[158]. "Spill-over" of localized cytokines into circulation driving a pro-inflammatory response from the liver has become a leading hypothesis for explaining the growing number of associations between chronic periodontitis with cardiovascular disease[210].

A second proposed mechanism for the development of systemic inflammation is the dysregulation of tolerogenic dendritic cells and T-regulatory cells[40,223]. Primarily, tolerogenic dendritic cells are responsible for maintaining central tolerance by deleting, render anergic, or rendering suppressive autoreactive T cells[2,10,11]. In peripheral circulation, tolerogenic dendritic cells have the additional mode of action to induce the differentiation and proliferation of T-regulatory cells[2,311]. Exposure to IL-10 is key to the development of tolerogenic dendritic cells, and suppression of IL-10 production has been associated with the development of systemic inflammatory disorders, such as Crohn's disease[359] and rheumatoid arthritis[72]. T-regulatory cells act to actively suppress activation of the immune system and particularly the inflammatory response[178,243]. Disruptions to the gene encoding FoxP3 are associated with a disruption to the differentiation and proliferation of T-regulatory cells[7,119]. Such genetic disruptions result in a rapidly fatal autoimmune disorder, immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX)[80,275,323]. IPEX syndrome is characterized by overwhelming systemic inflammation and autoimmunity within the first year of life[388]. It has been proposed that some pathogens, such as *Staphylococcus aureus*, attempt to

evade immune system by disrupting T-regulatory cell function, ultimately setting the stage for the development of toxic shock syndrome[364].

Ultimately, both of these processes likely contribute to the development of systemic inflammation. The release of inflammatory cytokines from a localized insult stimulates the liver to produce and release additional inflammatory cytokines and acute phase proteins into circulation. This inflammatory milieu is characterized by the release of potent mediators of inflammation, such as TNF-α, IL-6, and c reactive protein[358]. In turn, the cytokines drive differentiation and proliferation of immune cells in the spleen and lymph nodes[357]. Lack of proper suppression by tolerogenic dendritic cells and T-regulatory cells ultimately allows the development of hemodynamic, metabolic, and inflammatory associated changes that can compromise the function of uninvolved organs, leading to multiple organ dysfunction[300].

1.2.2 Influence of systemic inflammation on health.

Systemic inflammation has come to be recognized as a major risk factor in the development of many chronic organ dysfunction pathologies. Most well understood and studied is the association between systemic inflammation and cardiovascular disease[289,370]. In particular, liver-derived c reactive protein has been associated with a 7-fold increased risk of myocardial infarction[310]. Similarly, low-grade systemic inflammation, as measured by circulating IL-6 and c reactive protein levels, was found to be a predictive factor in the development of type 2 diabetes[98]. Additionally, circulating inflammatory cytokines were associated with elevated risk of development of diabetic complications[184,389]. Chronically elevated levels of IL-6 have been implicated to play a key role in the development of synovitis and bone erosions characteristic of autoimmune joint disease[118].

The relationship between inflammation and cancer has been long established[89]. It has estimated that approximately 15% of malignancies can be attributed to infections[44,200,333,338]. Chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease are associated with cancer development at similar frequencies to that associated with p53 mutations[395]. The strong associations between colon carcinogenesis and ulcerative colitis and Crohn's disease[100-102,387]. Likewise, chronic gastritis associated with infection by *Helicobacter pylori* is the leading cause of gastric adenocarcinoma[107]. Chronic infection is associated with induced DNA damage, which occurs as a result of continuous exposure to reactive oxygen and nitrogen species produced as part of the inflammatory response[220]. In fact, inflammation induced production of macrophage migration inhibitory factor suppresses the transcriptional activity of p53, allowing cell proliferation despite inflammation-induced DNA damage[171]. In melanoma, exposure to the chemokine cytokine CXCL1 enhances colony-forming activity and cell proliferation[21,272,281]. Similarly in breast cancer, exposure to the chemokine cytokine CXCL12 is hypothesized to govern the pattern of metastases[262]. The broader implication is that chronic inflammation and exposure to inflammatory cytokines plays roles in both the initiation of cancer and its development.

1.2.3 Development of neuro-inflammation.

Classically, the central nervous system has been considered an immune privileged site with the blood-brain barrier limiting the influence of the peripheral immune system on its function. However, several mechanisms have been proposed to explain the development of neuro-inflammation in response to systemic inflammation. These mechanisms include neuronal recognition of inflammatory cytokines by the vagus nerve[47,156,201] as well as the neurons of the sensory circumventricular organs[54,318,341], the

active transport of circulating cytokines across the blood-brain barrier[99,153,154], and the recent discovery of meningeal lymphatic vessels[15,211].

Vagotomy suppresses brain-mediated responses to systemic administration of bacterial endotoxin, particularly by intraperitoneal injection[49,133,337,386]. Peripheral administration of bacterial endotoxin activates vagal afferent processes, further supporting a role for the vagus nerve in signaling peripheral immune system activation in the brain[135]. Vagal afferent process have been found to express IL-1ra, the receptor for the inflammatory cytokine IL-1ß[148]. Furthermore, exposure to IL-1ß induces glutamate release by vagal afferent processes within the nucleus of the solitary tract[234]. The sensory circumventricular organs of the vascular organ of the lamina terminalis, the subfornical organ, and the area postrema are regions of the central nervous system that possess capillaries with a fenestrated epithelium. Traditionally, the sensory circumventricular organs play key roles in monitoring circulating hormones responsible for the maintenance of body fluid and cardiovascular homeostasis[113,242]. However, systemic administration of TNF-α has been observed to lead to pronounced increase in the expression of the TNF-receptor, p55, in each of the sensory circumventricular organs[265]. Similarly, IL-6 mediated signal transduction has been observed[379]. However, only the sensory circumventricular organ of the area postrema has been observed to be directly responsive to circulating IL-1ß[106]. Signal transduction from the nucleus of the solitary tract[76,77,106,329,330,356], in the case of vagal afferents, as well as from the circumventricular organs[251,326] proceeds to the medial parvocellular division of the paraventricular nucleus of the hypothalamus. Integration of these signaling pathways, leads to modulation of the hypothalamus-pituitary-adrenal axis with subsequent release of adrenocorticotropic hormone[53].

Additionally, it has been noted that circulating cytokines may be transported across the blood-brain barrier via carrier-mediated transport[23,25]. IL-1α and IL-1ß have

been found to cross the blood-brain barrier by the same saturatable transporter[24]. IL-6 uses a separate saturatable transporter[26]. Transport of TNF-α is mediated by trafficking of endothelial TNF receptors[283]. Upon entering the central nervous system, transported cytokines are able to interact with endogenous expressed receptors. Within the CNS cytokines are capable of acting in sequence, in which one cytokine triggers the synthesis and release of one or more additional cytokines, which may themselves further propagate the production and release of additional cytokines[301]. Additionally, cytokines may act in parallel, in which response patterns depend on the cooperative or antagonistic influence of multiple cytokines in response to neurophysiology[174,302].

The recent discovery of meningeal lymphatic vessels provides another potential route for the development of neuro-inflammation[15,211]. While simplified in comparison to lymphatic vessels found elsewhere in the body, these meningeal lymphatic vessels have been shown to be capable of transporting T lymphocytes and may provide a more conventional route for immune cells to access the central nervous system[211]. The meningeal lymphatic vessels have also been implicated in the draining of interstitial and cerebral spinal fluid into the deep cervical lymphatic vessels[15]. While the role remains largely unexplored, surgical removal of the deep cervical lymph nodes and ligation of the vessels is associated cognitive impairment in animal models due to impairment in the normal flow of meningeal associated T cells[307].

1.2.4 Influence of neuro-inflammation on cognitive health and function.

Neuro-inflammatory processes have been implicated to have broad impact on cognitive health and function. Expression of IL-1, IL-2, IL-6, and TNF-α have been shown to play various roles in disease-associated cognitive dysfunction[116,143,263,335]. The constellation of nonspecific behavioral changes that accompany illness, such as depression, lethargy, weakness, malaise, disinterest, and inability to concentrate, are

collectively referred to as “sickness behavior”. Together, with the fever response, these behavioral changes constitute a highly organized strategy to fight infection[157]. Cytokines with neuro-stimulatory effects have been associated with reduced memory performance[116,207] and have been suggested to play key roles in the development of neuropsychiatric disorders, such as schizophrenia and depression[221,222,263,335,346]. IL-1 and TNF-α are responsible for modulating sleep patterns during disease-associated sleep disturbances[194,195]. Production of IL-1ß in the nucleus of the solitary tract as mediated by the vagus nerve in response to peripheral LPS administration results in change to the hypothalamic thermal set point, inducing fever[316]. While vagotomy blocks development of the fever in response to LPS administration, it does not inhibit fever in response to peripheral IL-6 administration[189]. In animal models, intraperitoneal administration of LPS was found to in the upregulation of IL-1ß production within the central nervous system[376,379] with an associated effect on the inhibition of neurogenesis with the hippocampus[255]. It has been hypothesized that neurogenesis within the hippocampus plays a key role in the consolidation of memory. Therefore, neuro-inflammation has been proposed as a potential contributor to the development of Alzheimer’s Disease, Lewy Body Dementia, and AIDS Dementia Complex[50,91,217]. Low levels of neuro-inflammation due to IL-1α, TNF-α, and IL-6 production associated with obesity have been associated with the development of anxiety-like behavior and impairment of spatial memory in mice[93]. In humans, a growing body of evidence has associated neuro-inflammation with elevated risk of neurodegenerative disease and depression[173].

1.3 *Helicobacter pylori* and Human Health

The early history of scientific inquest into the nature of *Helicobacter pylori* is one of originating in antiquity and still unfolding in modern science. Hippocrates provided the

first written description of gastritis systems with Avicenna noting that relationship between onset of gastric pain and mealtime[183]. Gastric ulcers would be first formally observed by Marcellus Donatus of Mantua in the late 16th century[377] and described by Matthew Baillie in the late 18th century[183]. In 1812, François-Joseph-Victor Broussais was the first to note that acute gastritis, if untreated, had the potential to develop into a chronic condition[59]. Bottcher and Letulle found bacteria at the floor of ulcers and were the first to hypothesize that gastric ulcer were caused by bacteria in 1875[266]. In 1889, Walery Jaworski observed spiral shaped bacteria in gastric washings and suggested that they were responsible for the development of gastric ulcers and gastric cancer[190]. In 1939, Abraham Stone Freedberg pioneered the first studies into identifying a bacterial cause of gastric ulcers but ceased his work due to pressure from his superiors[352]. In 1954, Edward Pamler published his finding that no viable bacteria are found in the human stomach, which becomes the accepted dogma for gastroenterologists[282]. In 1983, Robin Warren and Barry Mashall first published their observation that half of patients undergoing gastroscopy showed gastric epithelial colonization by a previously undescribed curved Gram negative bacillus[173]. Later, Warren and Marshall positively associate bacterial colonization with gastritis and peptic ulceration[232]. In an attempt to address detractors, Marshall would publish the results of self-induced infection by cultured *H. pylori* with relief of gastritis brought about by antibiotic mediated eradication of the infection[96,233]. Brenden Drumm and colleagues published the first definitive evidence that *H. pylori* was a specific pathogen and causative agent of gastritis. In 1994, the World Health Organization's International Agency for Research on Cancer classified *H. pylori* as a Group I carcinogen based on the growing body of evidence for its role in gastric adenocarcinoma[56].

Similarly, the evolution history of *H. pylori* also originates in antiquity and continues today. Based on genetic data, the association between *H. pylori* and

humanity can be traced back to at least the last great migration of humanity out of Africa, roughly 65,000 years ago[111,112,208]. As a human specific pathogen, it is not surprising that the coevolution between *H. pylori* and its human host has blurred the distinction between pathogen and commensal[43]. It has been suggested that prior to the advent of widespread antibiotic usage that *H. pylori* was a universal inhabitant of the human stomach[41]. Such an intimate and ubiquitous association has led to the proposal that human gastric physiology evolved to maintain normal homeostasis in the presence of chronic *H. pylori* infection. The rise in gastroesophageal reflux disease, Barrett's esophagitis, esophageal cancer in populations where chronic *H. pylori* infection is decreasing in prevalence[104]. The protective relationship between chronic *H. pylori* infection and gastroesophageal reflux disease and its sequelae is hypothesized to result from gastric atrophy reducing acid production[187,394]. It has also been proposed that modulations of the human immune system chronic *H. pylori* infection may also provide some level of protection against the development of childhood asthma and diarrheal disease[319].

Finally, it is the ubiquitous nature of chronic *H. pylori* infection that grants it such a potential for impacting human health. It is estimated that approximately 50% of humans worldwide are chronically infected by *H. pylori*[51,228,362]. A clear disparity in the prevalence of infection has been noted in that the carriage rate of *H. pylori* between affluent and non-affluent nations. In "developing" nations the incidence rate of *H. pylori* infection is estimated at 10-20% of the population per year, resulting in carriages rates of 80-100%[51]. However, in "developed" nations, the incidence rate is estimated at 0.5% of the population per year with a total carriage rates of 10-30%[285]. Even within "developed" nations a disparity is observed in that prevalence rates among populations with lower socioeconomic standing approach those seen in "developing" nations[227-229]. As such, *H.*

pylori infection represents a potential additional burden on these already vulnerable populations.

1.3.1 *Helicobacter pylori* and gastric health.

The influence of *H. pylori* on long-term gastric health is perhaps the most well studied aspect of this pathogen. The hallmark of the interaction between *H. pylori* and its human host is the development of a superficial gastritis affecting the epithelial mucousa[162,288,306,371]. Neutrophilic infiltration of the lamina propria and epithelial glands is the most common histological finding during *H. pylori* infection[288]. However, the presence of other immune cells, such as lymphocytes, monocytes and macrophages, eosinophils, and plasma cell are commonly observed in chronically infected individuals[288]. Associated plasma cell populations in the gastric tissue have been found to secrete sIgA[309]. Of T cell populations present during chronic *H. pylori* infection, CD8+ cells are often overrepresented[284]. Active chronic infection is associated with a depletion of mucus from the epithelial cell surface[348], despite increased mucus production[70]. In a proportion of infected individuals, glandular atrophy develops due to loss of epithelial cell populations[110].

H. pylori does not invade the gastric epithelium but rather colonizes the overlaying mucus layer[159]. However, a fraction of *H. pylori* cells are able to directly adhere to the gastric epithelium via the use of adhesin-like protein[203]. Despite its lack of invasiveness, *H. pylori* is capable of inducing a robust inflammatory reaction, which is hypothesized to be related to its secretory activities. The urease of activity of *H. pylori* produces ammonia, which is a known pro-inflammatory insult to epithelial cells[383]. Additionally, *H. pylori* actively secrete cytotoxic factors, such as vacuolating cytotoxin A (VacA), which also produce an inflammatory response[66,203]. VacA induces massive vacuolation in epithelial cell lines[65]. There is no close homolog of *vacA* present in other

Helicobacter species or in other bacteria[68], suggesting a potential importance in specific interactions between *H. pylori* and the human stomach. Pores formed by VacA allow the egress of anions and urea from epithelial cell[177,366]. Release of urea from epithelial cells may be beneficial to *H. pylori* as urea hydrolysis by urease protects against gastric acidity. In mice, oral administration of VacA has been associated with ulcer formation[67]. Significant allelic variation has been observed in VacA, with certain allele types associated with elevated risk in development of gastric disease[16,17]. Likewise, the *cag* pathogenicity island is associated with increased risk for the development of both peptic ulcer disease[71,271] as well as gastric adenocarcinoma[375]. The *cag* pathogenicity island encodes for a type IV secretion system, by which bacterial cells in close association with host cells are able to inject macromolecules into the host cell[375]. The most well studied effector protein of the *cag* pathogenicity island, CagA, interacts with SHP-2[164] to influence spreading, migration, and adhesion of epithelial cells[396]. Additionally, CagA interacts with Grb2 to activate the Ras/MEK/ERK pathway, leading to cell scattering and proliferation[250]. Like *vacA,* no homologs are known for *cagA* in either other *Helicobacter* species or in other bacteria, indicating yet another evolved human specific interaction.

Chronic *H. pylori* infection is met with a robust innate and adaptive immune response, manifested by continuous epithelial cell cytokine release and gastric mucosal infiltration of neutrophils, macrophages, and lymphocytes[18,71,291]. The mucosal cytokine profile implies a predominantly Th1 mediated inflammatory response[22]. *H. pylori* gastritis reduces gastric somatostatin levels[260], which in turn results in hypergastrinemia as a result of the loss in negative regulation[204]. Elevated gastrin levels act on enterochromaffin-like and parietal cells, leading to greater acid secretion[103]. Chronically elevated gastrin levels have the additional effect of promoting proliferation of parietal cells[103,144]. Increased delivery of acid to the duodenum induces gastric metaplasia which is subsequently colonized by *H. pylori* with resultant inflammation and ulceration[155,182,277].

As chronic gastritis spreads from the gastric antrum to corpus, histamine release by enterochromaffin-like cells is inhibited which further inhibits secretion of gastric acid by parietal cells[33]. Reduced gastric acid secretion further enhancing gastrin production, which while failing to increase acid production continues to provide a proliferative stimulus[292,293]. Continued proliferation and inflammation lead to progressive loss of gastric glands, and such atrophic changes alongside continued cell proliferation lead to markedly increased risk of development of gastric ulceration and gastric adenocarcinoma[197,291].

1.3.2 *Helicobacter pylori* and extra-gastric health.

Much like the early studies seeking to link *H. pylori* infection to the development of gastric disease, the hypothesis that chronic *H. pylori* infection influences extra-gastric health has also generated controversy. A number of studies have reported associations between *H. pylori* infection and cardiovascular diseases. A higher prevalence of chronic *H. pylori* infection has been observed in patients suffering from ischemic heart disease[81,246]. Magnitude of anti-*H. pylori* antibody titer has been positively correlated with risk of death by myocardial infarction[6,294]. Interestingly, infection by *cag* pathogenicity island positive strains of *H. pylori* was found in significantly higher prevalence in ischemic stroke patients after controlling for other demographic variables[226,299]. However, large epidemiology studies, relying only on seropositivity data have found only minimal evidence for an association between *H. pylori* seropositivity and risk of myocardial infarction[38,315,393]. Seropositivity has been associated with a significant increase in adenomatous polyps and reduction in normal colonoscopy findings in human patients, suggesting a link between *H. pylori* infection and colorectal cancer[252]. Interestingly, hypergastrinemia has been hypothesized as a possible contributing factor in the development of colorectal cancer[124,252]. Hematologically, chronic *H. pylori*

infection has been well established as a major risk factor in the development of autoimmune idiopathic thrombocytopenic pupura[132,165,186,381]. Anti-CagA antibodies have been found to recognize two distinct platelet antigen in purpura patients but only one in normal subjects, suggesting that the intersection between molecular mimicry on the part of *H. pylori* and immune system predisposition determine the risk of developing this autoimmune disease[131]. Several studies have suggested a link between chronic *H. pylori* infection and idiopathic iron-deficiency anemia, though the mechanism remains unknown[125,355]. Likewise, *H. pylori* infection has been associated with elevated risk of developing preeclampsia during pregnancy; although, the mechanistic link remains highly specualtive[305,378]. Conversely, *H. pylori* infection appears to confer a degree of protection against the development of Th2 mediated diseases, such as atopy[55,239] and asthma[60,78]. Interestingly, the most commonly hypothesized link between chronic *H. pylori* infection and extra-gastric health is the interaction between the bacterium and the host's immune system.

1.3.3 Modulation of the immune response by *Helicobacter pylori*.

As *H. pylori* establishes a chronic infection that has the propensity to persist throughout the host's life, it is not unsurprising that *H. pylori* has evolved mechanisms by which it interacts with the immune system to allow it to evade clearance. Little is known about the acute phase of *H. pylori* infection, except that there is a rapid recognition by the host in the form of both an innate and adaptive immune response[290]. However, the chronic stages of *H. pylori* infection are characterized by continual activation of the immune response to the extent that antibody titers may remain consistently elevated for decades[295].

H. pylori evades the immune response by a wide range of adaptations. While the innate immune system naturally responds to bacterial flagella via recognition by TLR5,

the flagella of *H. pylori* is not stimulatory[9,140,202]. Similarly, TLR9 naturally recognizes the unmethylated DNA commonly found in bacterial pathogens but not the highly methylated DNA of *H. pylori*[280,331]. The Lipid A component of *H. pylori* LPS has mutated in such a way that it is anergenic and does not stimulate epithelial cell TLR4[19]. In cell culture studies, VacA has been shown the block phagosome maturation in macrophages[400], inhibit antigen presentation in T cells[254] and inhibit T cell proliferation[136]. Molecular mimicry of gastric epithelial fucosylated antigens further limits the adaptive host response[390].Perhaps, *H. pylori*'s most effective evasion of the immune system is its survival as a chronic pathogen without tissue invasion. The bulk of *H. pylori* cells resides in the mucus layer and lumen of the stomach[385], placing them beyond the reach of most immune effectors.

However, a proportion of *H. pylori* cells maintain an intimate association with the gastric epithelium and *H. pylori* derived products are able to cross the epithelial barrier[224]. This results in activation of both the innate and adaptive arms of the immune system[290]. *H. pylori* infection is met with a predominantly Th1 mediated inflammatory response[22]. While an extracellular, toxin-producing bacterial infection is more often met with a predominantly Th2 mediated B cell activation and antibody production, animal studies have suggested that a predominant Th1 mediated immune response is appropriate to control *H. pylori* infection[121,253]. While a Th1 mediated immune response is appropriate for control of *H. pylori* infection, the inflammatory component of the Th1 mediated response is a major contributing factor in the development of *H. pylori* associated gastric pathogenesis[121,151,253,344].

Systemically, chronic *H. pylori* infection is associated with several shifts in the composition of immune cell populations. Most well understood is the induction of a polarization of Th1 and Th2 cell populations toward a predominantly Th1 phenotype[22,79,367]. Production of INF-γ promotes T cell differentiation into the Th1

phenotype, which will, in turn, produce additional INF-γ establishing a Th1 dominant microenvironment while inhibiting IL-2 production and suppressing differentiation into the Th2 phenotype[249]. Based on its interference with calcineurin signaling, it has been hypothesized that VacA intoxication suppresses Th2 differentiation and activity by inhibiting IL-2 transcription[136]. Currently, a great deal of focus has been placed on the immune polarizing abilities of the neutrophil-activating protein (NAP) of *H. pylori*[256,327]. NAP is chemiotactic for neutrophils and stimulates a high production of oxygen radicals[8,109]. NAP has been shown to be able to cross the endothelial barrier efficiently and promote leukocyte adhesion and extravasation into infected tissue[109,304,327]. In tissue, NAP is able to activate mast cells to release TNF-α[257] as well as IL-12 and IL-23 release from neutrophils, monocytes, and dendritic cells[8]. This particular milieu of cytokines exerts a strong polarizing effect on the differentiation of naïve T cells toward the Th1 phenotype[267,278,372]. Conversely, impairment of TLR signaling has been associated with the generation of semimature dendritic cells[166,181,276]. These poorly immunogenic dendritic cells act to maintain peripheral immune tolerance through induction of anergy and deletion in autoreactive T cells[230]. Additionally, these semimature dendritic cells lead to the differentiation and proliferation of $CD4^+FoxP3^+$ T regulatory cells (Treg cells)[13]. The Treg cells act against T cell mediated immunity through the production of IL-10 as well as by direct suppression of memory T cell responses[212,308]. It is hypothesized that the generation of these Treg cell populations during *H. pylori* infection is responsible for the observed protective effect that chronic *H. pylori* infection has in regard to the risk of development of asthma[13,276].

1.3.4 *Helicobacter pylori* and cognitive health.

Very few studies have examined the influence of chronic *H. pylori* infection on cognitive health and development. In mice, chronic *H. pylori* infection has been

associated with changes in feeding behavior that persisted following eradication of the infection[36]. It was hypothesized that *H. pylori* associated changes in satiety signaling may not completely resolve following eradication of infection. In an elderly Greek cohort, *H. pylori* infection has been associated with reduced performance on the Mini Mental State Examination with successful eradication associated with improvement[192,193]. It was hypothesized that *H. pylori* associated atrophic gastritis led to the development of vitamin B12 deficiency which allowed the elevation of circulating homocysteine levels resulting in mild neuro-inflammation[193]. Finally, a negative association was observed between *H. pylori* seropositivity and IQ score in school aged children in an Israeli cohort[261]. The observed effect was found to be independent of socioeconomic and nutritional status, eliminating the two most commonly cited variables for detrimental cognitive effects.

1.3.5 *Helicobacter pylori* as part of the microbiota-gut-brain axis.

It has been proposed that *H. pylori* colonization and chronic infection of the human stomach was "universal" throughout most of human evolution[42,112,142]. As the stomach meets the criteria for consideration as a member of the gut-brain axis, it is reasonable to consider how *H. pylori* may modulate signaling along the gut-brain axis as a member of the resident microbiota. In mice, *H. pylori* infection has been associated with vagus nerve mediated alteration in feeding behavior[36]. Action by local secreted inflammatory cytokines in response to *H. pylori* on the vagus nerve result in alteration of sympathetic tone of the stomach as well as activate the hypothalamus-pituitary-adrenal axis[105], resulting in vagus nerve mediated suppression of inflammation[58,264]. These vagus nerve mediated alterations to homeostasis are hypothesized to be key plays in the protective aspect of chronic *H. pylori* infection in regard to the gastroesophageal reflux disease[339]. Hormonally, *H. pylori* infection is associated with impairment of ghrelin

production[176,279,361]. Eradication of *H. pylori* infection has been observed to result in an increase in ghrelin production and secretion by the gastric mucousa[176,274]. As ghrelin levels influence the production and secretion of growth hormone, *H. pylori* mediated alteration of ghrelin levels has been proposed as a potential mechanistic link in the association of *H. pylori* infection and height reduction in children[248,286]. Similarly, as reduced ghrelin levels are hypothesized to play an important role in the development of polycystic ovary syndrome[146,259,332], it has been proposed that reduction of ghrelin levels during *H. pylori* infection contribute to the finding of an association between this disorder of the reproduction system and gastric infection[231,398,399]. Immunologically, *H. pylori* engages in seemingly paradoxical activity. In generation of a Th1 polarizing response, chronic infection leads to the development of a pro-inflammatory state[22,79,367]. In turn, Th1 polarization leads to suppression of Th2 mediated immune activity[249]. Simultaneously, chronic *H. pylori* infection is associated with the generation and proliferation of Treg cell populations, which act to suppress the pro-inflammatory activity[181]. These modulations of the adaptive immune response are hypothesized to result in the observed protective effect that chronic *H. pylori* infection has against the development of asthma, atopy, and allergies[13,276].

1.4 Gaps in Knowledge

H. pylori has been well described as a gastric pathogen, capable of establishing a chronic infection, inducing a long-term superficial gastritis, and increasing the risk of developing gastric disease. However, the influence of *H. pylori* on extra-gastric health remains controversial and poorly understood. Seropositivity to *H. pylori* has been epidemiologically associated with increased levels of circulating inflammatory markers. However, such association studies are unable to address whether *H. pylori* is truly the causative agent of the observed changes in inflammatory markers, or, more importantly,

if curing of active *H. pylori* infection leads to a normalization of circulating inflammatory markers. A second gap in knowledge is the determination of whether chronic *H. pylori* infection is capable of inducing a neuro-inflammatory response. It has been shown that chronic systemic inflammation has the potential to generate an inflammatory response within the central nervous system. While a few researchers have speculated that chronic *H. pylori* infection may indeed induce a mild neuro-inflammatory state, such hypotheses are based solely on conjecture due to the limitations in gathering tissue samples from human subjects. The final gap in knowledge is whether chronic *H. pylori* infection is associated with detrimental changes in cognitive health. As chronic neuro-inflammation has been shown to negatively influence cognitive health, it is not unreasonable to propose that, if chronic *H. pylori* induces a neuro-inflammatory response, chronic H. pylori infection may negatively impact cognition. In order to evaluate these gaps in knowledge, a chronic animal model of *H. pylori* infection was developed in Sprague-Dawley rats, the influence of *H. pylori* infection on systemic inflammatory markers as well as neuro-inflammatory markers was monitored through time, the immunomodulatory influence of *H. pylori* was examined for its role in the development of extra-gastric inflammation, and cognitive behavioral studies were conducted to evaluate the influence of chronic *H. pylori* infection on cognition.

1.5 Significance of this Study

Altogether, these studies will address fundamental gaps in knowledge in the study of *Helicobacter pylori* and its influence on extra-gastric health. More broadly, these studies have to potential to increase the understanding of the influence that chronic infection has on the immune response and, in turn, how changes in the immune response are able to influence health at a systemic level. From a public health stand point, emphasis and focus has shifted toward attempting to address risks factors of late

life disease, rather than wait for the manifestation of symptoms. As systemic inflammation is a known risk factor in the development of many late life chronic illnesses, this work offers to shed light into the rate and mechanism by which systemic inflammation is established in response to a chronic bacterial infection. In the study of *H. pylori* infection, these studies offer insight into the earliest stages of chronic infection where data on the effects of chronic infection are incredibly scarce. These studies seek to address the long-standing question of whether chronic *H. pylori* infection has the potential to negatively impact the health of infected individuals who do not eventually develop gastric disease. Demonstrating that chronic *H. pylori* infection does indeed "set the stage" for the development of chronic systemic will provide long sought confirmation of large scale epidemiology studies. Furthermore, these studies provide a model for evaluating the influence of chronic *H. pylori* infection in a manner which more closely recapitulates the natural course of infection. It has been shown that most chronic *H. pylori* infections begin in early childhood and persist throughout life without direct medical intervention. The period of early childhood development presents a particularly vulnerable period in development where perturbations may leave behind lasting, if not permanent, negative effects. The influence of *H. pylori* infection on this period of life is only beginning to be evaluated by researchers, and the animal model developed as part of these studies offers great potential in gaining understanding into the influence of *H. pylori* on growth and development in juvenile populations.

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Chapter 2: Establishment of a rat based animal model of chronic *Helicobacter pylori* infection

2.1 Introduction

As *H. pylori* has evolved as a human specific pathogen, development of effective animal models that recapitulate the natural history of chronic *H. pylori* infection has been challenging. The first animal models for *H. pylori* infection relied on the use of germ-free piglets[14,26] as these animals possess gastric physiology similar to humans[13,18,28]. Ferrets, colonized by *Helicobacter mustelae*, were used as the first animal model to demonstrate gastric ulceration[19]. Most animal studies have utilized rodent, specifically mouse, models of infection as strains of *H. pylori* that have been identified which are capable of stable colonization[35,55]. However, much current research has utilized the Mongolian gerbil as infection results in the rapid development of ulceration and cancer in these animals[23,55]. Rat-based animal models have been utilized sparingly in *H. pylori* research. However, those few studies demonstrate several key benefits seen in the use of rats over other rodent models. Establishment of chronic infection in rats was possible with minimal adaptation of *H. pylori*[5,32,33,45]. Chronic infection in rats was observed to result in the colonization of the upper foveolar cells of gastric antrum[32], which more closely resembles the colonization pattern seen in humans[29] in comparison to the corpus-antrum transition colonization seen in other rodent models[30].

The early stages of natural infection by *H. pylori* are the least understood aspects of infection. It is hypothesized that chronic *H. pylori* infection is typically established during early childhood[56] and that young children (aged 0 to 5 years) appear to be particularly vulnerable to infection by *H. pylori*[24,50]. Decline in the prevalence of chronic *H. pylori* infection has been associated with the rise of gastric esophageal reflux disease[15,17,22,27,34,42,53], obesity[20,25,57] and even asthma[2,7,8,11]. In affluent nations, *H. pylori* prevalence is in decline, which may be at least partially explained by the absence *H.*

pylori associated modulation of physiology[6]. As many of these pathophysiologies have their root during early childhood development, it may be hypothesized that the establishment of chronic *H. pylori* during early development may influence extra-gastric health in ways not observed with infection established during adulthood.

2.2 Materials and Methods

Animal care. All experiments involving the use of live vertebrate animals were conducted with the approval of the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (IACUC). Rat populations were composed of male Sprague-Dawley rats (Envigo, formerly Harlan Laboratories, Indianapolis, IN). Mouse populations were composed of male C57BL\6J mice (Jackson Laboratory, Bar Harbor, ME). Animals were single housed with 12 h light-dark cycle, mean temperature of 22.1°C, humidity of 50%, and allowed food and water *ad libitum*.

Bacterial strains. *H. pylori* G27 (*cag* PAI+, *vacA* s1/m1; NCTC 13282), 26695 (*cag* PAI+, *vacA* s1/m1; ATCC 700392), and 60190 (*cag* PAI+, *vacA* s1/m1; ATCC 49503) were cultured in bisulfite-free *Brucella* broth (BSFB) supplemented with 5% fetal bovine serum (Sigma Aldrich, St. Louis, MO) and vancomycin 5 µg/ml (Sigma Aldrich), on a rotary platform shaker for 48 h at 37°C, under 5% CO_2 and 10% O_2. *H. pylori* transposon interruption mutants[47], G27 (*vacA*::Tn7), G27 (*cagA*::Tn7), G27 (*cagY*::Tn7), and G27 (*napA*::Tn7), were kind gifts from Nina Salama (Fred Hutchinson Cancer Research Center, Seattle, WA).

Determination of antibiotic susceptibility. All strains were tested for susceptibility to the pharmacological agents used in standard *H. pylori* eradication therapy. In brief, *H.*

pylori strains were plated onto Columbia Blood Agar (Thermo Fisher) supplemented with 7% defibrinated sheep's blood (Thermo Fisher). Additional Columbia Blood Agar plates were supplemented with either 50 µg/mL lansoprazole (Sigma Aldrich), 0.12 µg/mL amoxicillin (Sigma Aldrich), 0.25 µg/mL clarithromycin (Sigma Aldrich), or the combination of all three pharmaceutical agents. Thresholds for determining susceptibility for amoxicillin and clarithromycin were based on published standards for determination of antibiotic susceptibility in clinical settings[48].

Determination of mid-log phase growth time. Mid-log phase times for *H. pylori* strain G27, 26695, and 60190 were determined empirically by growth curve measurements. In brief, low passage frozen culture stocks were revitalized in BSFB media supplemented with 5% fetal bovine serum (Sigma Aldrich, St. Louis, MO) and vancomycin 5 µg/ml (Sigma Aldrich) 96 h at 37°C, under 5% CO_2 and 10% O_2. 500 mL flasks of BSFB media supplemented with 5% fetal bovine serum were inoculated with 1 mL of full grown stationary culture. Cultures were incubated on a rotary platform shaker for 36 h at 37°C, under 5% CO_2 and 10% O_2. OD_{600} was measured at 90 minute intervals and cell density confirmed by dilution plating on Columbia Blood Agar (Thermo Fisher, Hanover Park, IL) supplemented with 7% defibrinated sheep's blood (Thermo Fisher), 10 µg/ml vancomycin (Sigma Aldrich), 5 µg/ml trimethoprim (Sigma Aldrich), 5 µg/ml cefsulodin (Sigma Aldrich), and 5 µg/ml amphotericin B (Sigma Aldrich). Motility and cellular morphology were evaluated for each time point by phase contrast microscopy. Growth curves were plotted from resulting data and mid-log phase determined for each *H. pylori* strain.

Rodent adaptation of *H. pylori* strains. Male Sprague-Dawley rats (Envigo), aged 19-21 days, were orally fed 3.75 mg/kg lansoprazole (Sigma Aldrich) suspended in 20%

sucrose solution once daily for 7 days. On days 3 and 5 of treatment, animals received 0.5 ml of mid-log phase *H. pylori* culture diluted to $2x10^7$ cells/ml (active *H. pylori* infection) or 0.5 ml of sterile BSFB media (mock infected controls) by oral gavage using a 20 G disposable feeding needle (Thermo Fisher). Inoculum dosage was estimated by disposable hemocytometer (inCYTO, Seonggeo-gil, South Korea). Dilution was confirmed by serial dilution and spread plating on Columbia Blood Agar (Thermo Fisher) supplemented with 7% defibrinated sheep's blood (Thermo Fisher), 10 µg/ml vancomycin (Sigma Aldrich), 5 µg/ml trimethoprim (Sigma Aldrich), 5 µg/ml cefsulodin (Sigma Aldrich), and 5 µg/ml amphotericin B (Sigma Aldrich) incubated for 5 days at 37ºC under microaerophilic conditions (5% CO_2, 10% O_2). 7 days following competition of lansoprazole treatment, animals were euthanized and stomachs collected by necropsy. Stomach tissue was grossly chopped into ~1 cm^2 pieces using sterile razor blades and vigorously washed in 5 mL sterile BSFB media for 10 min. Resulting stomach wash was cultured on Columbia Agar (Thermo Fisher) supplemented with 7% defibrinated sheep's blood, vancomycin 10 mg/ml (Sigma Aldrich), trimethoprim 5 mg/ml (Sigma Aldrich), cefsulodin 5 mg/ml (Sigma Aldrich), and amphotericin B 5 mg/ml (Sigma Aldrich) for 5 days at 37ºC under microaerophilic conditions (5% CO_2, 10% O_2). Presumptive *H. pylori* colonies (oxidase +, catalase +, urease +, and motile curved bacilli under phase contrast microscopy) were colony purified 3 times. 10 purified isolates were combined and the adaptation process repeated. Final rodent adapted *H. pylori* strains were confirmed by 16S rRNA gene sequencing.

Establishment of *H. pylori* infections. Male Sprague-Dawley rats (Envigo), aged 19-21 days, were orally fed 3.75 mg/kg lansoprazole (Sigma Aldrich) suspended in 20% sucrose solution once daily for 7 days. On days 3 and 5 of treatment, animals received

0.5 ml of mid-log phase *H. pylori* G27 culture diluted to $2x10^7$ cells/ml (active *H. pylori* infection) or 0.5 ml of sterile BSFB media (mock infected controls) by oral gavage using a 20 G disposable feeding needle (Thermo Fisher). Inoculum dosage was estimated by hemocytometer and verified by CFU plating. Length of infection (2-, 8-, or 25-weeks) was defined as the number of weeks following competition of lansoprazole treatment prior to euthanasia and tissue collection. The weights of all animals were recorded on a weekly basis.

Inoculum influence on bacterial load. Male Sprague-Dawley rats (Envigo) aged 19-21 infected with rat-adapted *H. pylori* G27 culture diluted to $2x10^2$, $2x10^3$, $2x10^4$, $2x10^5$, $2x10^6$, $2x10^7$ cells/ml (active *H. pylori* infection) or 0.5 ml of sterile BSFB media (mock infected controls) by oral gavage as described previously. Animals were infected for 8 weeks prior to euthanasia and collection of tissues. Animal population consisted of a single animal for each inoculum dosage.

Determination of bacterial load. Stomach tissue was roughly chopped into 1 cm^2 pieces and washed vigorously in sterile BSFB media for 10 minutes. Resulting wash serially diluted and plated onto Columbia Agar (Thermo Fisher) supplemented with 7% defibrinated sheep's blood, 10 µg/mL vancomycin (Sigma Aldrich), 5 µg/mL trimethoprim (Sigma Aldrich), 5 µg/mL cefsulodin (Sigma Aldrich), and 5 µg/mL amphotericin B (Sigma Aldrich). Plates were incubated for 5 days at 37ºC under microaerophilic conditions (5% CO_2, 10% O_2). Stomach tissue was blotted dry using sterile gauze and weighed. Bacterial load was then determined as CFU per mg of stomach tissue.

Active *H. pylori* infection time series. Male Sprague-Dawley rats (Envigo) aged 19-21 days were orally fed lansoprazole 3.75 mg/kg (Sigma Aldrich) once daily for 7 days. On day 3 and 5 of treatment, animals received 0.5 ml of mid-log phase *H. pylori* G27 culture diluted to $2x10^7$ cells/ml (active *H. pylori* infection) or 0.5 ml of sterile BSFB media (mock infected controls). Inoculum dosage was estimated by hemocytometer and verified by CFU plating. Length of infection (2-, 8-, and 26-weeks) was defined as the number of weeks following competition of lansoprazole treatment prior to euthanasia and tissue collection. Each time point experiment consisted of 3 independent replicates composed of 6 animals (3 actively infected and 3 mock infected).

Eradication of active *H. pylori* infection studies. Male Sprague-Dawley rats (Envigo) aged 19-21 days were orally fed lansoprazole 3.75 mg/kg (Sigma Aldrich) once daily for 7 days. On day 3 and 5 of treatment, animals received 0.5 ml of mid-log phase *H. pylori* G27 culture diluted to $2x10^7$ cells/ml (active *H. pylori* infection) or 0.5 ml of sterile BSFB media (mock infected controls). Inoculum dosage was estimated by hemocytometer and verified by CFU plating. Animals underwent *H. pylori* eradication therapy 13 weeks after completion of lansoprazole treatment administered as part of the inoculation procedures. In brief, animals received eradication therapy consisting of 3.75 mg/kg lansoprazole (Sigma Aldrich), 2.25 mg/kg clarithromycin (Sigma Aldrich), and 4.5 mg/kg amoxicillin (Sigma Aldrich) suspended in 30% sucrose solution orally twice daily for 10 days. Animals were then allowed to remain uninfected for an additional 13 weeks after completing eradication therapy prior to euthanasia and collection of tissue. Prior infection by *H. pylori* was confirmed by Instant-View *H. pylori* Rapid Test seropositivity assay (Alpha Scientific, Poway, CA) on plasma samples. The experimental animal

population consisted of 3 independent replicates composed of 6 animals (3 actively infected and 3 mock infected).

Mouse infection studies. Male C57BL\6J mice (Jackson Laboratory, Bar Harbor, ME) aged 6 weeks were orally fed lansoprazole 3.75 mg/kg (Sigma Aldrich) once daily for 7 days. On day 3 and 5 of treatment, animals received 0.2 ml of mid-log phase *H. pylori* G27 culture diluted to $5x10^7$ cells/ml (active *H. pylori* infection) or 0.2 ml of sterile BSFB media (mock infected controls). Inoculum dosage was estimated by hemocytometer and verified by CFU plating. Animals were infected for 8 weeks prior to euthanasia and collection of tissues. The experimental animal population consisted of 3 actively infected animals and 3 mock infected animals.

***H. pylori* virulence factor mutant infection studies.** Male Sprague-Dawley rats (Envigo) aged 19-21 days were orally fed lansoprazole 3.75 mg/kg (Sigma Aldrich) once daily for 7 days. On day 3 and 5 of treatment, animals received 0.5 ml of mid-log phase rat-adapted *H. pylori* G27 (*vacA*::Tn7), *H. pylori* G27 (*cagA*::Tn7), *H. pylori* G27 (*cagY*::Tn7), or *H. pylori* G27 (*napA*::Tn7) culture diluted to $2x10^7$ cells/ml (active *H. pylori* infection) or 0.5 ml of sterile BSFB media (mock infected controls). Inoculum dosage was estimated by hemocytometer and verified by CFU plating. Each infection study using a mutant *H. pylori* strain was conducted a single independent block of 3 mock infected animals and 3 actively infected animals.

Terminal collection of tissue samples. At experimental end points, whole stomach tissue, liver, spleen and brain tissues along with large volume blood samples were

collected. In brief, animals were placed under surgical plane anesthesia by intraperitoneal administration of 100 mg/kg ketamine (Med Vet International, Mettawa, IL) and 10 mg/kg xylazine hydrochloride (Sigma Aldrich). Cardiovascular function was stabilized by intramuscular injection of 0.05 mg/ml atropine sulfate (Med Vet International). The animal's abdominal and thoracic cavities were opened and cardiac puncture blood draw conducted via the left ventricle using a 19 G needle. Collected blood was immediately transferred to EDTA anti-coagulated blood collection tubes (Becton, Dickinson, and Company, Franklin Lake, NJ). A 4-0 silk braided suture (MYCO Medical, Apex, NC) was used to tie a loop around the duodenum just inferior to the pyloric sphincter. The stomach was then cut free of the gastrointestinal tract just inferior to the suture and just superior to cardiac sphincter. The stomach was immediately placed in in cold PBS prior to processing. Whole liver, spleen and brain tissue were collected by necropsy and placed into cold PBS prior to processing.

Sample processing. Blood samples were centrifuged 10 min at 1500 x *g* at room temperature to separate plasma. Plasma was centrifuged a second time for 10 min at 2000 x *g* at room temperature to remove platelets. Plasma was aliquoted at 200 µL and stored at -20°C until assayed. 200 mg pieces of liver and spleen tissue as well as the cerebellum and hippocampus from brain tissue were sterilely collected from whole tissue and frozen at -20°C. Stomachs were opened along the lesser curvature and thin strips representing the greater curvature of the stomach were collected and set aside for histology. Remaining gastric tissue was processed for determining bacterial load. After washing for bacterial load determination, 200 mg of gastric tissue was collected and frozen at -20°C.

Preparation of whole tissue protein lysates. 200 mg of gastric tissue was mechanically homogenized using mortar and pestle with the aid of liquid nitrogen. Homogenized tissue was suspended in 2 mL of RIPA buffer (Thermo Fisher) supplemented proteinase inhibitor cocktail (Thermo Fisher). Cells were lysed on ice with gentle shaking for 3 hr. Unlysed tissues and lipid fraction were removed by centrifugation at 1500 x *g* for 15 min at 4°C. Total protein concentration for tissue lysates was determined by BCA assay (Thermo Fisher). Tissue lysates were aliquoted at 200 µL and frozen at -20°C until assayed.

Gastric histology. Strips of stomach tissue along the greater curvature from the gastric cardia through the pylorus were collected using sterile scalpel blade. Strips of tissue were fixed in Carnoy's fixative prior to embedding in paraffin wax. Paraffin embedded tissue was sectioned at 7 µm and stained by standard H&E procedures. Slides were digitized at 40X magnification using a Hamamatsu NanoZoomer slide scanner (Hamamatsu, Bridgewater, NJ).

Cytokine quantification. Tissue lysate was diluted to 500 µg/ml to standardize the amount of protein assayed across sample. Cytokine quantification was determined by quantitative ELISA for TNF-α (Eagle Bioscience, Nashua, NH), IL-6 (Eagle Biosciences), and CRP (Sigma Aldrich) according to manufacturer's directions.

Statistical analysis. Bacterial growth curves and animal weights were evaluated by non-linear regression analysis. Bacterial load was evaluated by one-way ANOVA with Tukey's correction for multiple comparisons. Cytokine levels from active infection time series were evaluated by two-way ANOVA of *H. pylori* infection status and length of

infection with Tukey's correction for multiple comparisons. Eradication studies and mouse studies were evaluated using a 2-tailed distribution, paired *t* test. Cytokine levels from transposon insertion *H. pylori* infection studies were evaluated by two-way ANOVA of *H. pylori* infection status and *H. pylori* strain. An alpha level of 0.05 was used as a threshold for statistical significance and all error bars represent standard error of the mean (SEM). Graphpad Prism (version 6.01) was used for all statistical analyses.

2.3 Results

2.3.1 Characterization of *H. pylori* growth in culture media.

Previous reports have shown that mid-log phase, actively growing *H. pylori* cultures have the highest potential to establish chronic infection during experimentally induced infection. To determine the appropriate conditions for culturing *H. pylori* for use in animal infections, growth curve experiments were conducted using three different strains of *H. pylori*: G27, 60190, and 26695. OD_{600} and CFU per mL were monitored at 90 minute intervals for a period of 72 hr. Resulting data were plotted and growth curves analyzed by non-linear regression using the Boltzmann sigmoidal formula (**Figure 2.1**). Evaluation of change in OD_{600} through time revealed that each of the three strains of *H. pylori* (G27, 26695, and 60190) possess similar growth rates. The "slope" variables for each curve were not significantly different ($p = 0.68$), indicating comparable rates of exponential growth among the strains of *H. pylori*. However, the "Top" variable for *H. pylori* 26695 was found to be significantly lower (ANOVA $p < 0.01$; Tukey's pairwise test $p = 0.02$) than that of strains G27 and 60190, indicating that strain 26695 produces a lower cell density upon reaching stationary phase.

As *H. pylori* is known to enter a non-culturable, non-infective coccoid state when under culturing stress (e.g. high cell density), viable cell counts were determined by

spread plating with cell density enumerated as CFU per mL. Again, the "slope" values were not found to vary significantly ($p = 0.45$) among the three strains of *H. pylori*. Additionally, the "Top" variable for *H. pylori* 26695 was found to be significantly lower (ANOVA $p < 0.01$; Tukey's pairwise test $p < 0.01$) than that of strains G27 and 60190, further supporting that strain 26695 produces a lower cell density upon reaching stationary phase.

Evaluation of motility and cellular morphology by phase contrast revealed an increasing proportion of non-motile, coccoid shaped *H. pylori* cells through time. Prior to 48 hrs, few to no coccoid shaped *H. pylori* were observed in collected samples. Between 48 and 60 hrs, the proportion of non-motile, coccoid *H. pylori* rises to approximately 10% of the collected samples. Between 60 and 72 hours, the proportion of non-motile, coccoid *H. pylori* increases to approximately 20% of the collected samples in strains G27 and 60190. In *H. pylori* 26695 the proportion of observed non-motile, coccoid cells rises to approximately 35% between 60 and 72 hours of growth. This elevation in proportion of cells undergoing transition into coccoid morphology potentially explains the difference in stationary phase cell density observed among the strains as coccoid cells are typically biochemically inactive and non-culturable. Based on these data, a period of growth of 36 hours was selected for all *H. pylori* cultures which would be used for infection studies.

2.3.2 Generation of a rat-adapted strain of *H. pylori*.

Rat-adapted strains of *H. pylori* were generated for every strain used in infection studies. To create more amenable conditions for colonization of the gastric epithelium, the proton pump inhibitor lansoprazole was administered orally to animals at 3.75 mg/kg for 7 days during the inoculation procedure. Animals were inoculated with *H. pylori* by oral gavage of approximately 1×10^7 cells on day 3 and 5 of lansoprazole treatment.

The dosage of *H. pylori* culture was selected based on previous studies of *H. pylori* infection in rats[32]. One week following the completion of lansoprazole treatment, animals were euthanized and their stomachs collected for culturing. *H. pylori* was cultured from the animals' stomachs using *H. pylori* selective media (Columbia agar supplemented with 7% sheep's blood, 10 µg/mL vancomycin, 5 µg/mL trimethoprim, 5 µg/mL cefsulodin, and 5 µg/mL amphotericin B). Resulting colonies were determined to be *H. pylori* by evaluation under phase contrast microscopy (motile, curved bacilli) and biochemical evaluation for catalase, oxidase, and urease activity. Ten independent *H. pylori* colonies were purified by repeated streak plating and pooled. A second adaptation passage was conducted using this culture. Following re-purification of *H. pylori* from the stomachs of the second set of animals, strain identity was confirmed by sequencing of the 16S rRNA gene. The *H. pylori* cultures resulting from this second passage were utilized as the rat-adapted strains for all subsequent infection studies.

2.3.3 Influence of oral gavage cell density on bacterial load.

To determine the influence of oral gavage cell density may have on the course of infection, bacterial load was determined following 8 weeks of active *H. pylori* G27 infection initiated by inoculums ranging from 1×10^2 to 1×10^7 cells. Following euthanasia, collected stomachs were roughly chopped using sterile razor blades and washed vigorously in sterile liquid media to free *H. pylori* cells from the tissue. The resulting stomach wash was serially diluted and plated for determination of CFU per mL. To account for variance in animal size, CFU numbers were normalized by mass of the stomach tissue utilized.

The resulting bacterial loads (**Figure 2.2**) revealed that bacterial load of *H. pylori* G27 after 8 weeks of active infection was not influenced by the cell density of the inoculum used in the oral gavages. Previous studies have estimated that the gastric

transit time in juvenile rat pups is approximately 30 minutes[43,54]. As such, it is reasonable to hypothesize that the majority of *H. pylori* delivered by oral gavage is unable to colonize the gastric epithelium prior to being lost to the lower gastrointestinal tract were it is unable to survive. The consistency of the bacterial loads suggests that *H. pylori* growth is limited by available niche space and likely exists in an equilibrium in which the natural motility of the stomach removes cells at a rate commensurate with replacement by active growth of the bacteria.

Based on these data, it may be preliminarily estimated that the ID_{50} for *H. pylori* G27 under these experimental conditions is 1×10^3 cells. Previous studies have suggested that the ID_{50} for rodents is 1×10^4 cells[32,33]. However, these studies did not utilize a strain of *H. pylori* that was adapted to rodents by passaging or proton pump inhibitor treatment to aid in successful colonization of the gastric epithelium.

2.3.4 *H. pylori* infection in Sprague-Dawley rats leads to establishment of reproducibly stable bacterial loads.

Active *H. pylori* infection studies were conducted in a time series system with infections lasting 2, 8, and 26 weeks. Infection length was determined to have begun at the completion of lansoprazole administration. Bacterial load was determined for each actively infected animal (**Figure 2.3**). To account for variance in size among animals, bacterial load numbers were normalized by the mass of the stomach tissue utilized in culturing. A significant trend of increasing bacterial load is observed through time (ANOVA: $p < 0.01$; Tukey's pairwise comparisons: $p < 0.01$ for all comparisons). It is hypothesized that this trend in increasing bacterial load results from changes in the available niche space that *H. pylori* is able to access as the animals grow.

To evaluate whether these observations regarding bacterial load during *H. pylori* infection were strain specific, the bacterial loads from 8-week infection studies utilizing

H. pylori strains 26695 and 60190 were determined and compared to those of *H. pylori* G27 (**Figure 2.4**). Consistent with prior growth curve analyses, *H. pylori* 26695 resulted in a significantly lower bacterial load (ANOVA: $p < 0.01$; Tukey's pairwise comparisons: $p < 0.01$) than strains G27 and 60190. The data indicate that the trajectory of chronic *H. pylori* infection may be subtlety influenced by the strain of bacteria despite each strain possessing the same major virulent factors.

2.3.5 Chronic *H. pylori* infection in Sprague-Dawley rats is associated with histological changes in gastric tissue consistent with those observed during human infection.

To determine if chronic *H. pylori* G27 infection in Sprague-Dawley rats resulted in comparable changes in gastric tissue to those observed during human infection, histological slides of gastric tissue were prepared for both mock and actively infected animals. Evaluation of gastric tissue from adult animals after 26 weeks of infection (**Figure 2.5A**) revealed the presence of a mild, superficial gastritis. Consistent with chronic *H. pylori* infection in humans prior to the onset of late-life gastric pathology, parietal cell hyperplasia is prominently observed in the mid-section of the gastric pits (**Figure 2.5B**). Expansion of chief, G and enterochromaffin-like cell population is observed in the basal region of the gastric pit (**Figure 2.5C**). Additionally, the infiltration of immune cell populations, predominantly mononuclear cells, is observed in the submucosa of actively-infected animals (**Figure 2.5D**).

These data demonstrate that chronic *H. pylori* in Sprague-Dawley rats recapitulates many of the key histological hallmark correlating with pathophysiological changes associated with chronic *H. pylori* infection in humans. Development of mild dysplasia of the luminal aspects of the gastric epithelium due to inflammatory insult is a common finding in gastric biopsies of humans that are chronically infected with *H. pylori*

but have not yet progressed to ulcer disease or gastric adenocarcinoma. Hyperplasia of parietal, chief, G, and enterochromaffin-like cell populations occurs partially as a consequence of *H. pylori* secreted urease raising the pH of the gastric lumen, resulting in physiological compensation by expansion of parietal cell populations. Additionally, inflammation associated with *H. pylori* results in physiologic suppression of acid secretion by parietal cells, resulting in the expansion of modulatory chief, G, and enterochromaffin-like cells which secrete additional factors leading to the proliferation of parietal cell populations.

2.3.6 *H. pylori* infection in Sprague-Dawley rats is associated with increased levels of inflammatory markers in gastric tissue.

Gastric levels of TNF-α, IL-6 and CRP were measured by high sensitivity ELISA from whole tissue lysates. Differences were recorded as the fold difference between mock infected control animals and actively infected animals (2-way ANOVA: H. pylori status p-value: <0.0001, 61.8% of total variation; Length of infection p-value: <0.0001, 18.9% of total variation; **Figure 2.6**). The greatest degree of elevated inflammatory markers was observed at the 2-week time point. It is hypothesized that this observation is the result of the acute phase of the immunological response to infection. The magnitude of elevated inflammatory markers is markedly reduced between the 2- and 8-week time points (Pairwise comparison by Tukey's p-value: <0.0001). Elevated levels of TNF-α and IL-6 remain consistent and non-significantly different between 8- and 26-week time points. However, CRP levels show a significant reduction between 8- and 26-week time points. Together these observations are indicative of the switch in the immunological response from the acute phase toward the chronic phase. As both TNF-α and IL-6 are hypothesized to be produced and secreted locally within the tissue, the maintenance of stably elevated levels between 8- and 26-week time points is supportive

of the transition from acute to chronic immunological response. As CRP is predominantly produced and secreted by the liver, it is hypothesized that the ongoing reduction of the magnitude of elevated CRP levels is related to the broader adaptation of the immune system toward a chronic inflammatory state.

To evaluate whether the observed elevation in gastric inflammatory markers was strain specific, 8-week infection studies were conducted using *H. pylori* 26695 and 60190. Similar to observed difference is bacterial load, *H. pylori* 26695 was associated with marginally lower levels of elevated inflammatory markers while *H. pylori* 60190 was associated with marginally higher level of elevated inflammatory markers in comparison to those observed during infection studies utilizing *H. pylori* G27 (**Figure 2.7**). These data indicate that strain specific variation in elevated gastric inflammatory markers does indeed exist. However, the degree of variation in elevation among these three type strains is marginal when considered from a biological context. As such, these data lend support to a "universal" inflammatory response to *H. pylori* strains which possess the s1/m1 allele of *vacA* as well as the *cag* pathogenicity island, which are associated with heightened risk of developing gastric disease[36,41,44,46].

2.3.7 Eradication of *H. pylori* infection in Sprague-Dawley rats leads to resolution of increased inflammatory markers.

To assess whether active *H. pylori* infection was the etiological agent responsible for the observed elevation in inflammatory markers, eradication studies were conducted such that animals were inoculated with either sterile media (mock infected control animals) or *H. pylori* G27 culture (*H. pylori* cleared animals). 13 weeks after inoculation, all animals received oral eradication therapy consisting of lansoprazole (3.75 mg/kg), clarithromycin (2.25 mg/kg), and amoxicillin (4.5 mg/kg), twice daily for ten days. Sensitivity of the rat adapted strain of *H. pylori* G27 to the antibiotics was confirmed by

determination of minimum inhibitory concentration in agreement with accepted treatment guidelines[49]. Animals were then allowed to "recover" for 13 weeks prior to the collection of tissue samples. Clearance of infection was determined by the inability to culture *H. pylori* from the stomachs of animals which were inoculated with live culture. Prior infection in these animals was confirmed by serology testing for the presence of anti-*H. pylori* antibodies. Measurement of inflammatory markers from gastric tissue revealed no significant difference between mock infected and *H. pylori* cleared animals (**Figure 2.8**). These data support the inference that *H. pylori* infection was the causative agent of the observed elevation of gastric inflammatory markers.

2.3.8 *H. pylori* infection in C57BL\6 mice leads to a similar but blunted pattern of increased levels of inflammatory markers.

To determine if the observed elevation in inflammatory markers was the result of host species specific interactions, 8-week infection studies were conducted utilizing C57BL\6 mice. Animals were infected utilizing the rat-adapted strain of *H. pylori* G27 according to the same inoculation procedure utilized in rat studies. However, the animals utilized in these mouse infections were 6-week old, fully matured animals as opposed to juvenile pups in rat studies.

Following euthanasia and tissue collection, bacterial load was determined to be 8000 ± 1200 CFU/mg of gastric tissue. This bacterial load was significantly lower than that observed in Sprague-Dawley rats (*p* value: <0.01). Two possible hypotheses may explain this observation. Firstly, the gastric tissue of C57BL\6 mice may be less amenable to the growth of the rat-adapted strain of *H. pylori* G27; or, secondly, the stomachs of C57BL\6 mice may present *H. pylori* with reduced niche space as the gastric epithelium accounts for less tissue per unit mass in mice than in rats. Regardless, successful infection was observed in each inoculated animal.

Measurement of inflammatory markers from whole tissue lysate prepared from the animals' stomachs revealed a similar pattern of elevated levels of TNF-α, IL-6, and CRP in infected animals relative to mock infected animals as was seen in Sprague-Dawley rats (**Figure 2.9**). However, the magnitude of elevation was significantly reduced in infected mice in comparison to infected Sprague-Dawley rats (2-way ANOVA p value: <0.01, p values for pairwise comparison of individual inflammatory markers between infected C57BL\6 mouse vs infected Sprague-Dawley rat by Tukey's pairwise comparison: <0.01 for all comparisons). This observation of reduced inflammatory response in C57BL\6 mice has been noted in other studies utilizing gastrointestinal bacterial pathogens[39]. These data support the inference that the observed elevation in gastric inflammatory markers is not the result of species specific physiology but rather can be attributed to active infection by *H. pylori*. Additionally, these data indicate that the degree of inflammation observed during *H. pylori* infection is influenced by the host physiological response.

2.3.9 Absence of prominent *H. pylori* virulence factors has only minor influence on *H. pylori* associated gastric inflammation.

To determine the influence of prominent *H. pylori* associated virulence factors, 8-week long infections studies were conducted utilizing rat-adapted strains of transposon interruption mutants of *H. pylori* G27[47]: *H. pylori* G27 (*vacA*::Tn7), *H. pylori* G27 (*cagA*::Tn7), *H. pylori* G27 (*cagY*::Tn7), or *H. pylori* G27 (*napA*::Tn7). These mutants were selected as they represent virulence factors commonly cited as predictive in the pathological outcome of chronic *H. pylori* infections. The pore forming secreted bacterial toxin VacA has been linked to elevated risk in development of gastric disease[3,4]. VacA is has been shown to elicit an inflammatory response in cell culture based experiments[9,31]. Similarly, the *cag* pathogenicity island is associated with increased risk

for the development of both peptic ulcer disease[10,40] as well as gastric adenocarcinoma[52]. The most well studied effector protein of the *cag* pathogenicity island, CagA, interacts with SHP-2[21] to influence spreading, migration, and adhesion of epithelial cells[58]. Additionally, CagA interacts with Grb2 to activate the Ras/MEK/ERK pathway, leading to cell scattering and proliferation[37]. The CagY protein forms the sheath[12,51] of the type IV secretion system used to inject macromolecules, such as CagA, into the host cell[52]. Neutrophil-activating protein (NapA) of *H. pylori* is chemotactic for neutrophils and stimulates a high production of oxygen radicals[1,16]. It is able to activate mast cells to release TNF-α[38] as well as IL-12 and IL-23 release from neutrophils, monocytes, and dendritic cells[1].

All transposon interruption mutants resulted in significantly reduced bacterial load in comparison to wildtype *H. pylori* G27 (ANOVA p value: <0.01; all pairwise comparisons vs wildtype by Tukey's pairwise comparison test p values: <0.01; **Figure 2.10**). As comparisons of bacterial load among the transposon interruption mutants was not significant, this reduction in bacterial load is likely attributable to the influence of the transposon interruptions. However, as the fold difference in bacterial load is only approximately 1.8, it is likely that this reduction in bacterial load has only negligible impact on the course of inflammation generated by chronic infection.

Comparisons in the magnitude of elevated inflammatory markers from gastric tissue revealed that transposon interruption of these virulence factors resulted in only minor changes in gastric inflammation (**Figure 2.11**). Lack of functional *vacA*, *cagA,* or *cagY* genes was not associated with a significant change in magnitude of the measured inflammatory markers. However, lack of a functional *napA* gene was associated with a small but significant decrease in the magnitude of all three measured inflammatory markers. These data support the hypothesis that *vacA* as well as the *cag* pathogenicity island represent human specific adaptations that do not play major roles in the course of

infection in non-human animal models. As the gastric immune infiltrate is composed more predominantly of eosinophils rather than neutrophils, the true impact of the absence of a functional *napA* gene may not be accurately portrayed in rodent infections.

2.4 Discussion

Here, a Sprague-Dawley rat based model for chronic *H. pylori* infection has been developed. As *H. pylori* infection is hypothesized to begin during the earliest stages of life, protocols were established to allow for inoculation of animals shortly post-weaning. Based on histological assessment of gastric tissue, infected animals recapitulate many of the hallmark tissue adaptations that occur during chronic *H. pylori* infection prior to the development of clinical gastric disease, such as increasing immune cell infiltration and the development of glandular hyperplasia. Direct evaluation of inflammatory markers confirms that chronic *H. pylori* results in marked gastritis. Interestingly, these studies suggest that the transition from acute inflammation to chronic inflammation occurs between 2 and 8 weeks in these animals with elevated inflammatory markers remaining stable between 8 and 26 weeks of infection.

Only minor differences were observed in infections utilizing different strains of *H. pylori*, indicating that this animal model may be robust to strain specific genetic variance among pathogenic *H. pylori* strains. In comparing elevated levels of inflammatory markers between Sprague-Dawley rats and C57BL\6 mice, mice showed a similar pattern of elevated inflammatory markers; however, the magnitude of elevation was markedly reduced in mice compared to rats. This is likely due to C57BL\6 mice having a blunted inflammatory response following exposure to gastrointestinal bacterial pathogens. However, it does indicate the elevation of inflammatory markers is not unique to Sprague-Dawley rats.

In infection studies utilizing *H. pylori* G27 strains in which major virulence factors have been transposon interrupted, lack of VacA or aspects of the *cag* pathogenicity island had no significant impact on the levels of inflammation observed during infection. These findings support the hypothesis that these particular virulence factors are human specific factors that may not play a major role in *H. pylori* infection in rodents. Lack of NapA was associated with a minor but significant decrease in TNF-α and lL-6 in gastric tissue but not CRP. This indicates that NapA may serve as a minor player in the localized inflammatory response within the stomach but may not contribute to any systemic effect.

As most efforts in developing animal models for *H. pylori* infection have focused heavily on recapitulating the late-life clinical stages of disease, understanding of the early stages of chronic infection has been limited. These data lay the groundwork for an animal model which may prove beneficial for addressing questions relating to the early stages of chronic *H. pylori* infection. Furthermore, the development of a rat-based model of chronic *H. pylori* infection provides a tool amenable to evaluating many of the extra-gastric influences of *H. pylori* infection, such as cognitive and nutritional health, as rat-based animal models are well established in these fields.

2.5 Figures

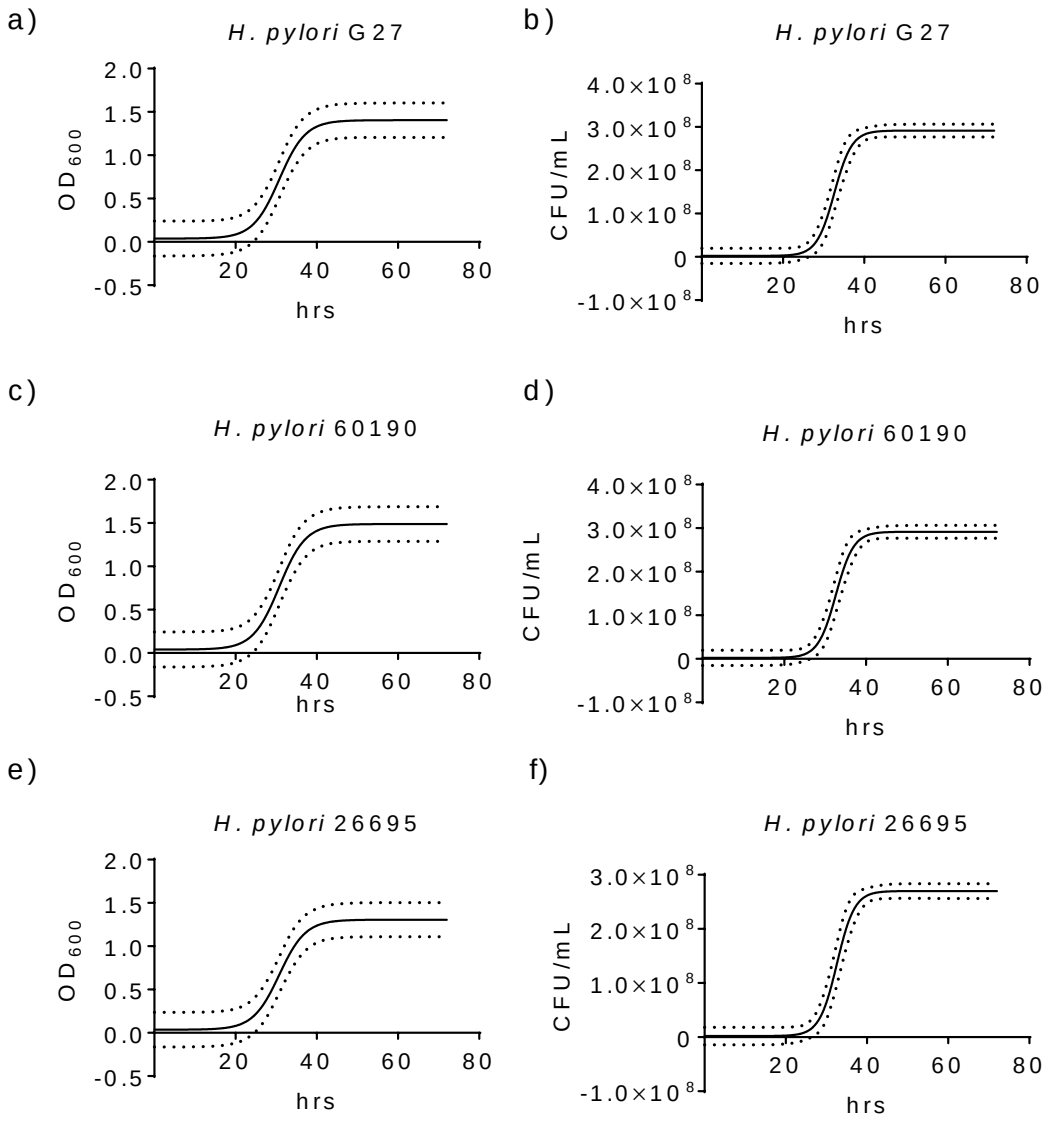

Figure 2.1. Growth curve characteristics of *H. pylori* strains utilized in infection studies. Growth curves (n=3 for each strain) for *H. pylori* strains G27 (a and b), 60190 (c and d), and 26695 (e and f) were determined using bisulfite-free *Brucella* broth under microaerophilic conditions. Samples were collected every 90 minutes and OD_{600}(a,c and e) and CFU/mL (b, d, and f) were measured. Curves were generated by non-linear regression according to the Boltzmann sigmoidal formula. Dotted lines correspond to the 95% confidence interval for each trend line. Statistical evaluation of the curves found no significant difference in the "slope" parameters, indicating that growth rate did not vary significantly among the evaluated strains. However, the "top" parameter for *H. pylori* 26695 was significantly lower (ANOVA $p < 0.01$; Tukey's pairwise test $p = 0.02$) than that observed for strain G27 and 60190, indicating that strain 26695 reaches lower cell density at stationary phase. Based on these data, inoculation cultures were grown for 36 hours prior to use in oral gavage of animals to ensure that actively growing mid-log phase culture was used.

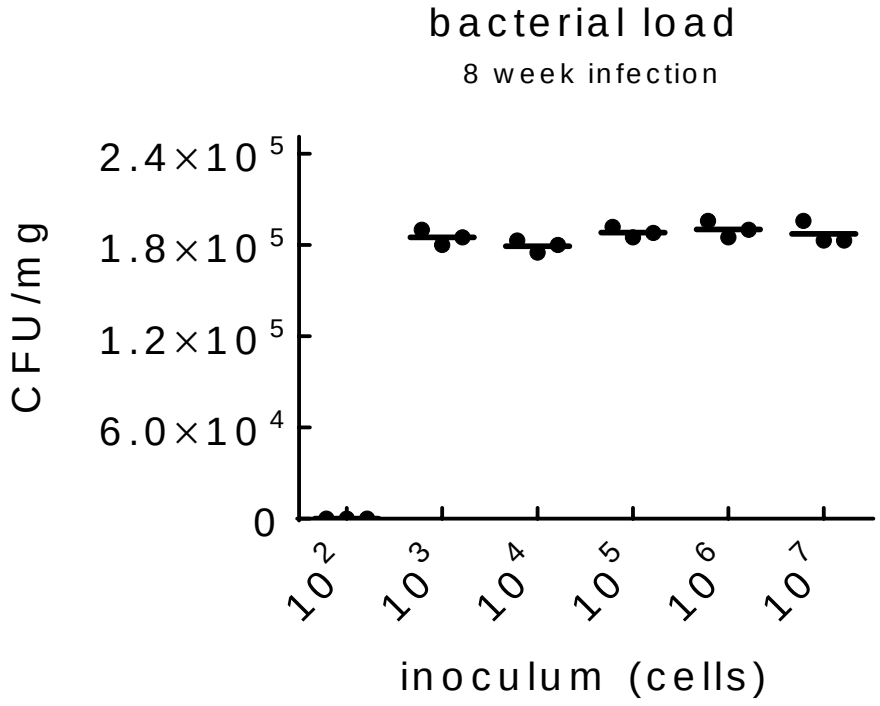

Figure 2.2. Bacterial load following 8-week long infection established by varying inoculums. To determine the influence of inoculation dosage on bacterial load, 8-week long infections (n = 1 animal per dosage with 3 technical replicates of bacterial load per animal) were established using varying inoculums of *H. pylori* G27. No infection was observed in the animal inoculated with 10^2 cells of *H. pylori*. Negligible differences were observed in bacterial load from animals that received 10^3 – 10^7 cells of *H. pylori*. These data suggest that *H. pylori* bacterial load during chronic infection is not influenced by the initial inoculum dosage. Interestingly, these data suggest that *H. pylori* exists in an equilibrium population during chronic infection.

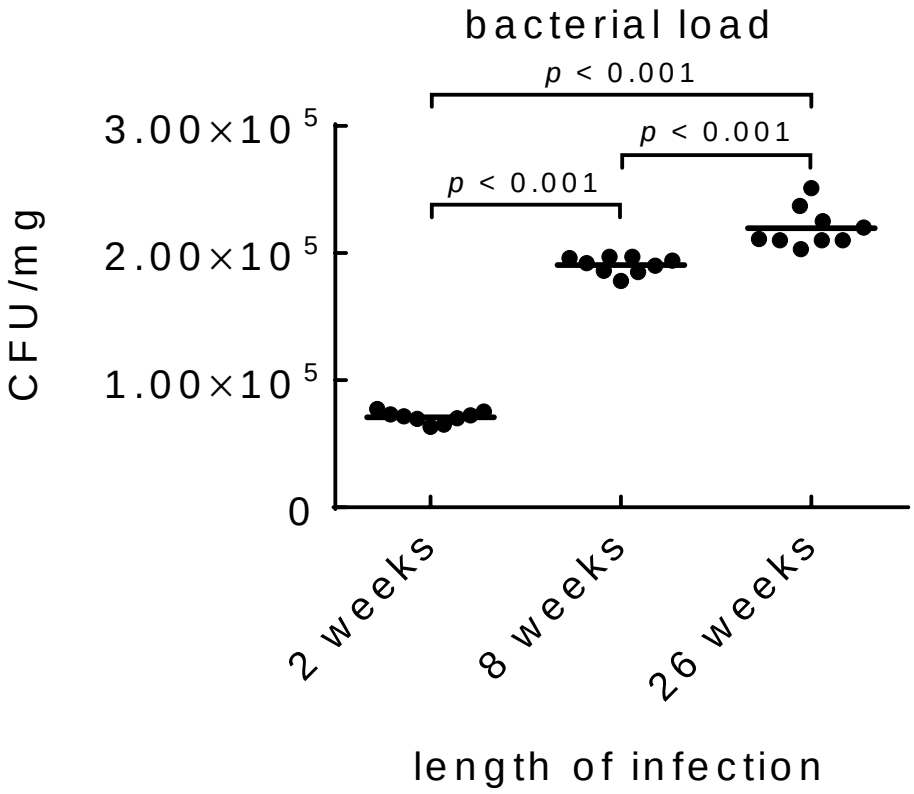

Figure 2.3. Time series of bacterial load observed following 2-, 8-, and 26-week long *H. pylori* G27 infections. Whole stomach tissue was cut into 1 cm2 pieces and washed vigorously in sterile media prior to plating on *H. pylori* selective medium (Columbia Blood Agar supplemented with vancomycin (10 µg/mL), trimethoprim (5 µg/mL), cefsulodin (5 µg/mL), and amphotericin B (5 µmg/mL)). Bacterial load was calculated as number of CFU per mg of gastric tissue. Each filled circle represents the bacterial load of an individual animal (n=9 for each time point). Data were collected across 3 independent experiments, each with 3 infected animals. Resulting data were pooled when no significant difference was detected by ANOVA (*p* values: 2 weeks = 0.6, 8 weeks = 0.3, 26 weeks = 0.4). The horizontal line corresponds to the mean bacterial load at each time point. Statistical evaluation by ANOVA ($p < 0.01$) and Tukey's multiple comparison test (*p* values reported above each comparison) supported a trend of increasing bacterial load through time.

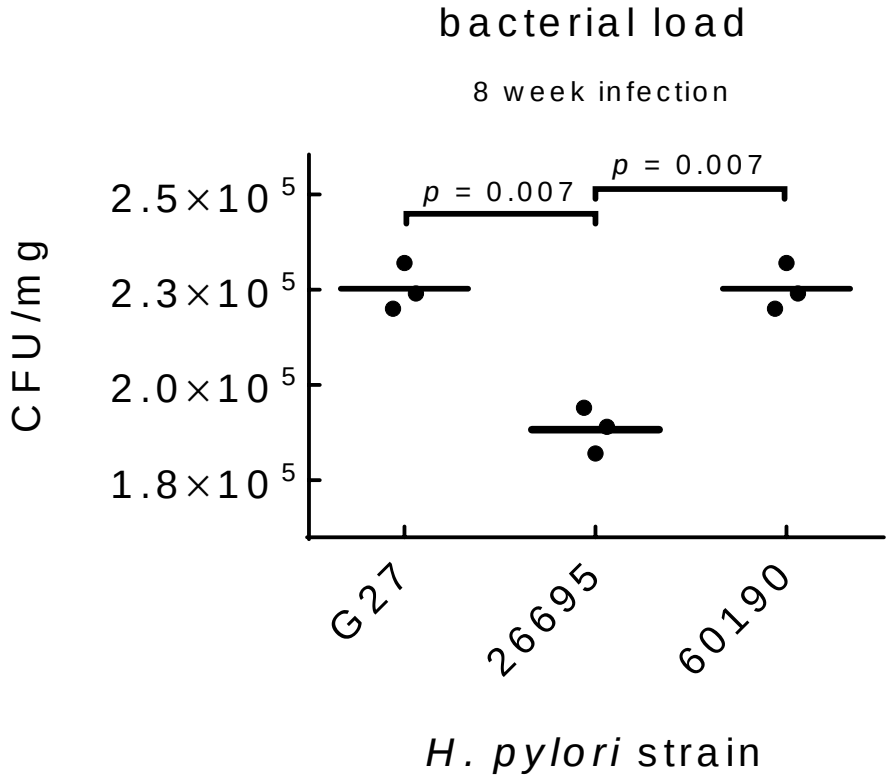

Figure 2.4. Influence of *H. pylori* strain on bacterial load following 8-week long infections. Whole stomach tissue was cut into 1 cm2 pieces and washed vigorously in sterile media prior to plating on *H. pylori* selective medium (Columbia Blood Agar supplemented with vancomycin (10 µg/mL), trimethoprim (5 µg/mL), cefsulodin (5 µg/mL), and amphotericin B (5 µg/mL)). Bacterial load was calculated as number of CFU per mg of gastric tissue. Each filled circle represents the bacterial load of an individual animal (n = 3 for each *H. pylori* strain). The horizontal line corresponds to the mean of the bacterial load. Statistical evaluation by ANOVA ($p < 0.01$) and Tukey's multiple comparison test (*p* values reported above significant comparisons) indicate that *H. pylori* 26695 infection results in significantly reduced bacterial load as compared to either G27 or 60190.

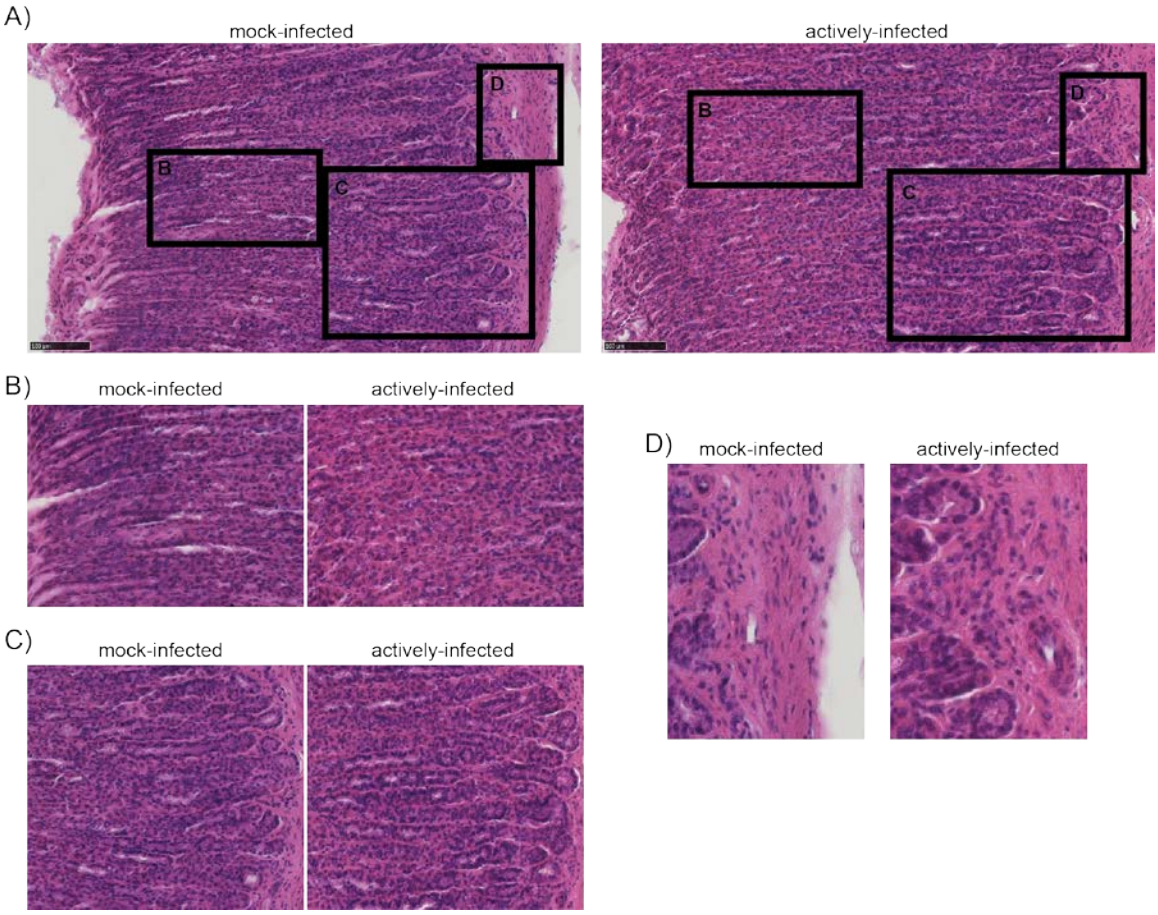

Figure 2.5. Chronic *H. pylori* G27 infection in Sprague-Dawley rats recapitulates many of the histological hallmarks associated with chronic infection in humans prior to the onset of clinical disease. Hematoxylin and eosin Y stained histological slides prepared from rat gastric tissue following 26-week long *H. pylori* G27 infections. Sections were taken along the greater curvature of the stomach at the corpus-antrum transition zone. Sections are oriented with the luminal side of the tissue on the left and serosal side to the right. Overview of the total sections (A) demonstrates the appearance of healthy, non-inflamed tissue in the mock-infected animal in comparison to actively infected animal, characterized by dysplasia of the luminal edge as well as hyperplasia of the underlying glandular cell populations. Inset images depict the presence of parietal cell hyperplasia (B) as well as chief, G, and enterochromaffin-like cell hyperplasia (C) in the actively infect animals in comparison to mock-infected. The final inset image (D) demonstrates the infiltration of immune cell populations, predominantly mononuclear cells, in the underlying submucousa in actively infected animals.

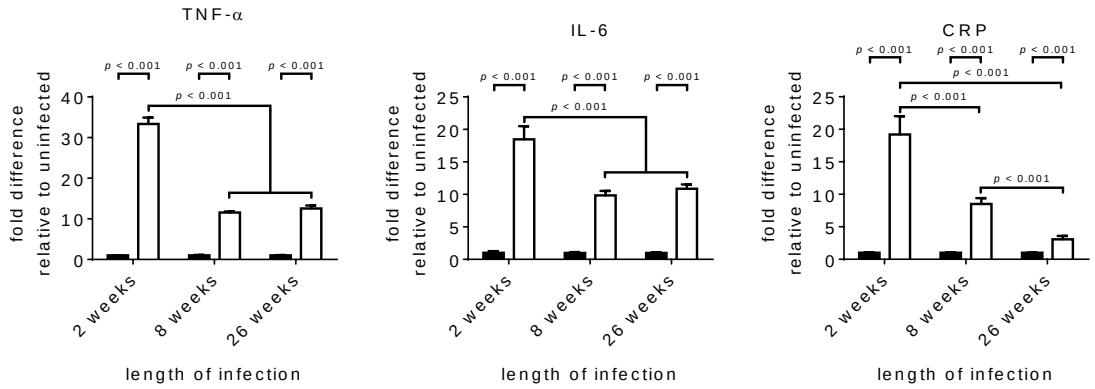

Figure 2.6. Measurement of inflamamtory markers from gastric tissue following *H. pylori* G27 infection. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* G27 infection. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by 2-way ANOVA (*p* values: *H. pylori* status: < 0.01, length of infection: < 0.01, and interaction: < 0.01 for each inflammatory marker) with comparisons between matched mock-infected and actively infected animals by Tukey's multiple comparison test (*p* values reported above each comparison).

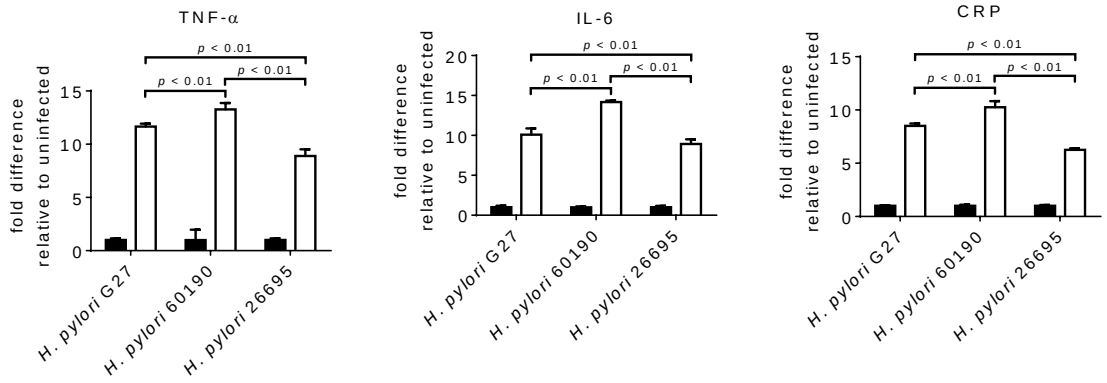

Figure 2.7. Comparison of gastric inflammatory markers following 8-week long *H. pylori* infections utilizing strains G27, 60190, and 26695. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* infection utilizing strains G27, 60190, and 26695. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by ANOVA (*p* value: < 0.01) with comparisons between *H. pylori* strains by Tukey's multiple comparison test (*p* values reported above each comparison).

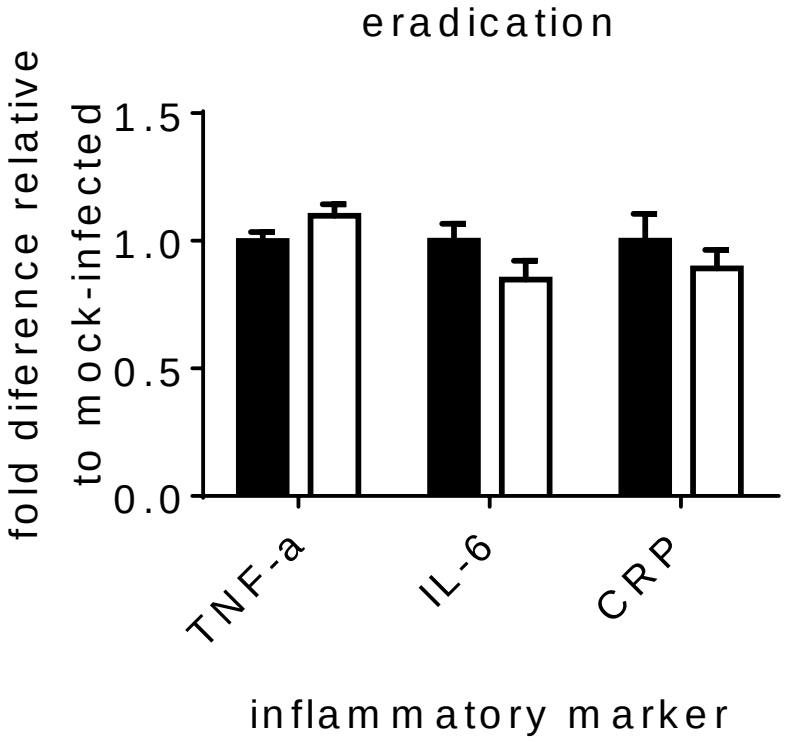

Figure 2.8. Measurement of inflamamtory markers from gastric tissue following eradication of *H. pylori* G27 infection. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP following eradication of *H. pylori* G27 infection. In eradication studies (d), 13-week infections were carried out in Sprague-Dawley rats utilizing *H. pylori* G27, followed by an eradication regimen (lansoprazole (3.75 mg/kg), clarithromycin (2.25 mg/kg), and amoxicillin (4.5 mg/kg) administered orally, twice daily for 10 days). After 13 weeks following eradication regimen, tissue samples were collected and inflammatory markers measured by quantitative ELISA. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. significant differences were not observed in levels of TNF-α, IL-6, or CRP, indicating that inflammatory marker levels returned to baseline following eradication of *H. pylori* infection.

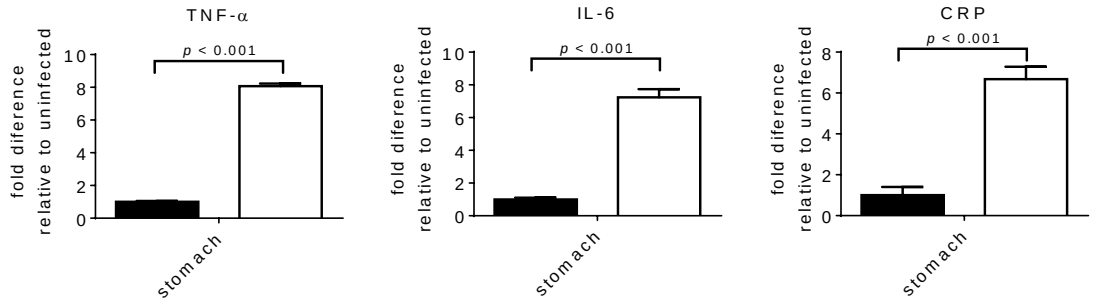

Figure 2.9. Measurement of gastric inflammatory markers in C57BL\6 mice following 8-weel long *H. pylori* G27 infections. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* infection in C57BL\6. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by student's *t*-test (*p* values reported above each comparison).

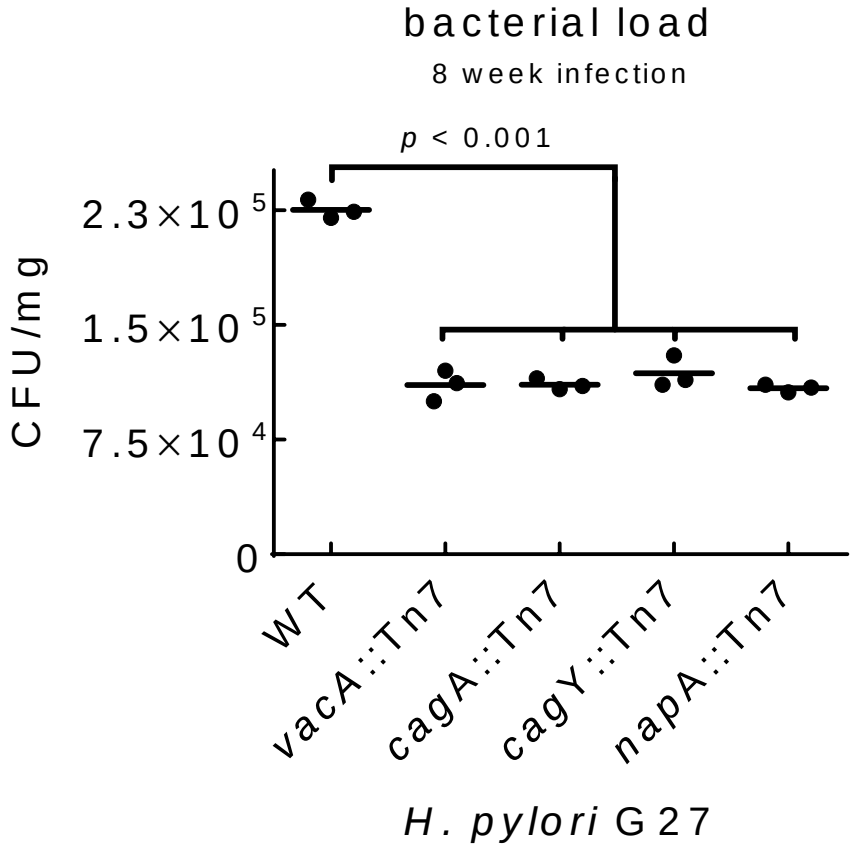

Figure 2.10. Measurement of bacterial load from 8-week long infections utilizing transposon interuption mutant of *H. pylori* G27. Whole stomach tissue was cut into 1 cm2 pieces and washed vigorously in sterile media prior to plating on *H. pylori* selective medium (Columbia Blood Agar supplemented with vancomycin (10 µg/mL), trimethoprim (5 µg/mL), cefsulodin (5 µg/mL), and amphotericin B (5 µg/mL)). Bacterial load was calculated as number of CFU per mg of gastric tissue. Each filled circle represents the bacterial load of an individual animal (n = 3 for each *H. pylori* strain). The horizontal line corresponds to the mean of the bacterial load. Statistical evaluation by ANOVA ($p < 0.01$) and Tukey's multiple comparison test (p values reported above significant comparisons) indicate that mutant strains of *H. pylori* G27 resulted in significantly lower bacterial load in comparison to wildtype. As no significant difference was observed among the transposon interruption mutants, this difference is likely attributable to the influence of the inserted Tn7 cassette.

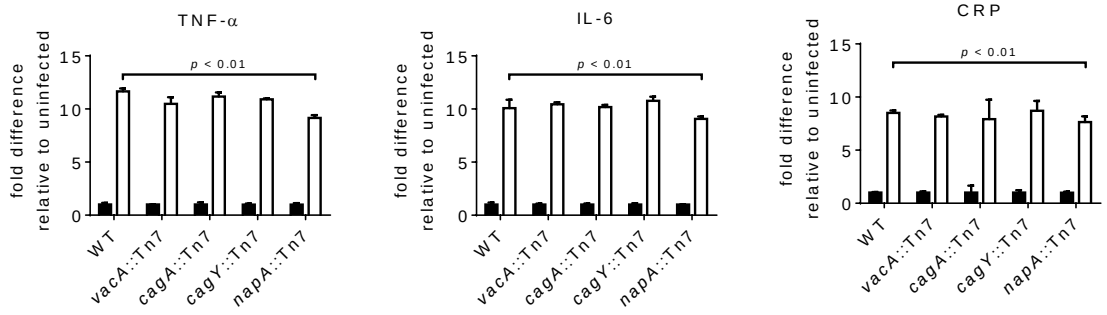

Figure 2.11. Comparison of gastric inflammatory markers following 8-week long *H. pylori* infections utilizing transposon interruption mutants of *vacA*, *cagA*, *cagY*, and *napA*. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* infection utilizing strains transposon interruption mutants of prominent virulence factors. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by 2-way ANOVA (*p* value: < 0.01) with comparisons between wildtype *H. pylori* G27 and the transposon interruption mutant strains by Tukey's multiple comparison test (*p* values reported above each comparison). Disruption of the *napA* gene was associated with a small by significant decrease in the magnitude of elevation of the measured inflammatory markers in gastric tissue.

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Chapter 3: Systemic inflammatory response to chronic *Helicobacter pylori* infection

3.1 Introduction

Chronic systemic inflammation is characterized by the elevation of circulating inflammatory markers and is associated elevated risk in the development and progression of many multi organ-system dysfunctions[1,8,10,11,28,29,31]. Two hypotheses have been put forward to explain the development of systemic inflammation as a result of localized infection. The first, termed cytokine "spill-over" describes the entrance into circulation of local produced inflammatory cytokines through inflammation mediated permeabilization of the vasculature[9,17,26,32]. The second is loss of normal immune suppressive activity by tolerogenic dendritic cells and T regulatory cells[35]. Both processes are associated with the rise in circulating pro-inflammatory markers, such as TNF-α, IL-6 and c reactive protein[34].

Epidemiological studies have suggested an association between chronic *H. pylori* infection and systemic inflammation[13,22,24,38]. Specifically, *H. pylori* seropositivity has been associated with elevated circulating of the acute phase protein, c reactive protein (CRP)[12,15,16,18,19]. These findings have led to the proposal that chronic *H. pylori* infection may increase the risk of death by myocardial infarction[3,25] as elevated circulating CRP levels are associated with increased risk of myocardial infarction[7,14,27]. Additionally, chronic *H. pylori* infection has been associated with polarization of Th1 and Th2 cell populations toward the pro-inflammatory Th1 phenotype[4,6,36].

To evaluate the role of chronic *H. pylori* infection in the development of systemic inflammation inflammatory markers were quantified from liver, spleen, and plasma samples form 2-, 8- and 26-week long *H. pylori* G27 infection studies. Eradication studies were utilized to evaluate the causal relationship between active *H. pylori* infection and systemic inflammation. Finally, the role of T cell populations in the

development of *H. pylori* associated systemic inflammation were evaluated by T cell phenotyping to determine the degree systemic immune cell population modulation that occurs during chronic *H. pylori* infection and by conducting infection studies conducted in athymic rats.

3.2 Materials and Methods

Animal care. All experiments involving the use of live vertebrate animals were conducted with the approval of the University of Illinois at Urbana-Champaign Institutional Animals Care and Use Committee (IACUC). Rat populations were composed of male Sprague-Dawley rats (Envigo, formerly Harlan Laboratories, Indianapolis, IN). Mouse populations were composed of male C57BL\6J mice (Jackson Laboratory, Bar Harbor, ME). Animals were single housed with 12 h light-dark cycle, mean temperature of 22.1°C, humidity of 50%, and allowed food and water *ad libitum*.

Active *H. pylori* infection time series. To evaluate systemic inflammation, liver, spleen and plasma tissue were collected at the time of euthanasia by necropsy from the animal populations described in Chapter 2. In brief, these animal populations consisted 2-, 8-, and 26-week infection studies, each composed of 3 independent blocks which, in turn, were each composed of 3 mock infected animals and 3 actively infected animals.

Eradication of active *H. pylori* infection studies. To evaluate systemic inflammation, liver, spleen and plasma tissue were collected at the time of euthanasia by necropsy from the animal populations described in Chapter 2. In brief, this animal population was composed of 3 independent blocks which, in turn, were each composed of 3 mock infected animals and 3 actively infected animals. Animals were infected for 13 weeks; then, both mock infected and actively infected animals underwent eradication therapy.

All animals were allowed to remain uninfected for an additional 13 weeks prior to euthanasia and collection of tissues.

Mouse infection studies. To evaluate systemic inflammation, liver, spleen and plasma tissue were collected at the time of euthanasia by necropsy from the animal population described in Chapter 2. In brief, this animal population consisted of an 8-week infection study, composed of 3 mock infected animals and 3 actively infected C57BL\6 mice.

LPS induced systemic inflammation. To provide a point of comparison to induced systemic inflammation, Sprague Dawley rats received weekly intraperitoneal injections of 500 µg/kg purified *E. coli* LPS (Sigma Aldrich, St. Louis MO) dissolved in PBS or the equivalent volume of PBS on a weekly basis for 8 weeks. Following completion of exposure experiment animals were euthanized and stomach, liver, spleen, and plasma collected for the measurement of inflammatory markers. Due to the extreme distress resulting from this exposure experiment in the experimental animal population, the animal population was limited in size to one LPS exposed animal and one PBS exposed animal.

Naproxen induced gastritis. To determine if gastritis in the absence of active *H. pylori* infection is sufficient to cause systemic inflammation, animal studies were conducted in which Sprague-Dawley rats received either naproxen (80 mg/kg; Sigma Aldrich) suspended in PBS or the equivalent amount of PBS once weekly for 8 weeks. Following completion of exposure experiment animals were euthanized and stomach, liver, spleen, and plasma collected for the measurement of inflammatory markers.

Athymic rat infection studies. To evaluate the role of T cell populations in *H. pylori* associated inflammation, 8-week long infection studies were conducted utilizing male *Foxn1*$^{rnu/rnu}$ rats (Envigo) aged 20-21 days. Animals were infected with *H. pylori* as previously described. The animal population consisted of 3 actively infected animals and 3 mock infected animals. Following completion of exposure experiment animals were euthanized and stomach, liver, spleen, and plasma collected for the measurement of inflammatory markers.

Terminal collection of tissue samples. Tissue samples were collected as described in Chapter 2. In brief, animals were placed under surgical anesthesia, and terminal blood collection was conducted by cardiac puncture. Stomach, liver, spleen and brain tissues were collected by necropsy immediately following euthanasia of the animal.

Sample processing. Collected tissue samples were processed as described in Chapter 2. In brief, plasma samples were prepared from whole blood and stored at -20°C until assayed. Remaining tissues were stored at -20°C until processed for preparation of whole tissue protein lysates.

Preparation of whole tissue protein lysates. 200 mg of gastric tissue was mechanically homogenized using mortar and pestle with the aid of liquid nitrogen. Homogenized tissue was suspended in 2 mL of RIPA buffer (Thermo Fisher) supplemented proteinase inhibitor cocktail (Thermo Fisher). Cells were lysed on ice with gentle shaking for 3 hr. Unlysed tissues and lipid fraction were removed by centrifugation at 1500 x *g* for 15 min at 4°C. Total protein concentration for tissue lysates was determined by BCA assay (Thermo Fisher). Tissue lysates were aliquoted at 200 µL and frozen at -20°C until assayed.

Cytokine quantification. Tissue lysate was diluted to 500 µg/ml to standardize the amount of protein assayed across sample. Cytokine quantification was determined by quantitative ELISA for TNF-α (Eagle Bioscience, Nashua, NH), IL-6 (Eagle Biosciences), and CRP (Sigma Aldrich) according to manufacturer's directions.

T cell population phenotyping. To evaluate changes in circulating T cell populations, 8-week long infection studies were conducted utilizing *H. pylori* G27. The experimental population consisted of 5 mock infected Sprague-Dawley rats and 5 actively infected rats. Following completion of exposure experiment animals were euthanized and whole blood collected by cardiac puncture. Buffy coat immune lymphocytes were purified from fresh whole blood differential centrifugation using Histopaque (Sigma Aldrich) and Accuspin separation tubes (Sigma Aldrich). Immune cells were cultured in RPMI 1640 (Sigma Aldrich) supplemented with 10% FBS (Sigma Aldrich). Immune cells were activated by exposure to PMA 50 ng/ml (Sigma Aldrich) and ionomycin 1 µg/ml (Sigma Aldrich) in the presence of GoligiStop (BD Biosciences, Franklin Park, NJ) for 4 hours. Following fixation and permeabilization, cells were labeled with fluorchrome-conjugated mAbs against CD4 (W/325; BioLegend, San Diego, CA), INF-γ (DB-1; BioLegend), IL-4 (OX-81; BioLegend), IL-17A (eBio17B7; Affymetrix, Santa Clara, CA), and FoxP3 (150D; BioLegend). Cells were analyzed using a BD FACS Canto II flow cytometer (BD Biosciences) and FCS Express 5 (BD Biosciences). Th1/Th2 ratio was determined as the proportion of CD4+ cells that were exclusively INF-γ+ (Th1) or IL-4+ (Th2). Th17/Treg ratio was determined as the proportion of CD4+ cells that were exclusively IL-17A+ (Th17) or FoxP3+ (Treg).

Statistical analysis. Cytokine levels from active infection time series were evaluated by two-way ANOVA of *H. pylori* infection status and length of infection with Tukey's correction for multiple comparisons. Eradication studies, mouse studies, heat killed *H. pylori* studies, *E. coli* K12 studies, naproxen induced gastritis studies, athymic rat *H. pylori* infection studies, and T cell phenotyping studies were evaluated using a 2-tailed distribution, paired *t* test. Cytokine levels from transposon insertion *H. pylori* infection studies were evaluated by two-way ANOVA of *H. pylori* infection status and *H. pylori* strain. An alpha level of 0.05 was used as a threshold for statistical significance and all error bars represent standard error of the mean (SEM). Graphpad Prism (version 6.01) was used for all statistical analyses.

3.3 Results

3.3.1 *H. pylori* infection in Sprague-Dawley rats is associated with increased levels of inflammatory markers in liver, spleen, and plasma tissue.

Utilizing tissue samples collected as part of the 2-, 8-, and 26-week long *H. pylori* G27 time series, TNF-α, IL-6, and CRP were assayed from liver, spleen, and plasma by quantitative ELISA. At 2-weeks post inoculation, liver levels of TNF-α (**Figure 3.1**) were found to be 17.5 fold elevated in *H. pylori* infected animals relative to mock-infected animals. These elevated levels decreased to 8.5-fold at 8 weeks and 7.6-fold at 26 weeks post inoculation (ANOVA *p* value: < 0.001; Pairwise comparison 2-weeks vs 8-/26-weeks by Tukey's *p* value < 0.001). Elevated levels of IL-6 in the liver (**Figure 3.1**) were on average 19.5-fold higher in *H. pylori* infected animals than in mock infected with no significant difference between time points. Elevated levels of CRP in the liver (**Figure 3.1**) were 86.5-fold elevated in *H. pylori* infected at 2-weeks post-inoculation, decreased to 34.1-fold elevated at 8-weeks, and further decreased to 13.4-fold elevation at 26-

weeks (ANOVA *p* value: < 0.001; *p* values for all pairwise comparisons by Tukey's: <0.001).

In the spleen, TNF-α levels were found to be elevated on average 8.7-fold during *H. pylori* infection across all time points with no significant difference between time points (**Figure 3.2**). Similarly, IL-6 levels were found to the elevated by 5.8-fold on average during *H. pylori* infection across all time points with no significant difference between time points (**Figure 3.2**). Spleen associated CRP levels (**Figure 3.2**) showed a trend of decreasing elevation through time with 28.4-fold elevation at 2-weeks, 8.1-fold at 8-weeks, and 5.1-fold elevation at 26-weeks post inoculation (ANOVA *p* value: < 0.001; *p* values for all pairwise comparisons by Tukey's: <0.001).

Plasma levels of TNF-α (**Figure 3.3**) were observed to be 18.8-fold elevated at 2-weeks post inoculation with a minor reduction to 16.6-fold elevated at 8-weeks and 14.5-fold elevated at 26 weeks (ANOVA *p* value: < 0.001; *p* values for all pairwise comparisons by Tukey's: <0.001). Plasma levels of IL-6 (**Figure 3.3**) were on average 3.6-fold elevated across all time points with no significant difference between time points. Similarly, plasma levels of CRP (**Figure 3.3**) were on average 4.2-fold elevated across all time points with no significant difference between time points. These data show a strong association between chronic *H. pylori* infection and stable elevation of circulating inflammatory markers with involvement of the liver, spleen, and plasma.

To assess whether *H. pylori* associated systemic inflammation was the result of strain-specific interactions, 8-week infection studies carried out utilizing *H. pylori* 60190 and 26695 likewise showed elevated levels of inflammatory markers in circulating plasma, as well as the liver and spleen (**Figure 3.4**). No significant difference was observed in the fold elevation of TNF-α (*p* value ANOVA: plasma: 0.4990, liver: 0.9997, spleen: 0.9728) or IL-6 among G27, 60190, and 26695 (*p* value ANOVA: plasma:

0.8967, liver: 0.8846, spleen: 0.7329). *H. pylori* 60190 was associated with minor but significant increase in elevation in plasma (*p* value ANOVA: < 0.001; Pairwise comparison 60190 vs G27/26695 by Tukey's *p* value = 0.003), liver (*p* value ANOVA: 0.001; Pairwise comparison 60190 vs G27/26695 by Tukey's *p* value = 0.008), and spleen (*p* value ANOVA: 0.001; Pairwise comparison 60190 vs G27/26695 by Tukey's *p* value = 0.001) CRP levels in comparison to G27 and 26695. These data indicate that strain specific variation of *H. pylori* may have little influence on *H. pylori* associated systemic inflammation.

3.3.2 Eradication of *H. pylori* infection in Sprague-Dawley rats leads to resolution of increased levels of inflammatory markers.

In eradication studies, systemic inflammatory markers were found to have returned to normal baseline levels following eradication of chronic *H. pylori* infection (**Figure 3.5**). These data are strongly indicative that not only is active *H. pylori* infection responsible for initiation of the systemic inflammatory response but is also required for maintaining the systemic inflammatory response.

3.3.3 *H. pylori* infection in C57BL\6 mice leads to a similar but blunted pattern of increased levels of inflammatory markers.

To determine if the observed systemic inflammatory response associated with *H. pylori* infection was species-specific, inflammatory markers were measured from liver, spleen, and plasma samples collected from C57BL\6 mice which had been infected with *H. pylori* G27 for 8 weeks. As was seen in gastric tissue, a similar pattern of elevated inflammatory markers was observed in mice as was seen in Sprague-Dawley rats (**Figure 3.6**). Additionally, as was seen in gastric tissue, the magnitude of elevation of

the measured cytokines was reduced in mice relative to rats by approximately 2-fold. These data support the hypothesis that the observed elevation of systemic inflammatory markers is not species-specific; however, the data do suggest that the degree of systemic inflammation associated with chronic *H. pylori* infection is potentially influenced by the host species.

3.3.4 *H. pylori* associated systemic inflammatory profile is markedly different than that associated with systemic administration of LPS.

To assess the degree of gastritis induced by chronic *H. pylori* infection to that seen during sepsis, we conducted animal studies in which animals received either mock treatment with sterile saline or once weekly intraperitoneal injection of purified *E. coli* LPS (500 µg/kg). *H. pylori* could not be recovered from the stomachs of animals at the conclusion of these studies. Measurement of gastric inflammatory markers revealed 12.4-fold higher levels of TNF-α, 13.9-fold higher levels of IL-6, and 58.3-fold higher levels of CRP in comparison to those observed following 8 weeks of *H. pylori* infection (**Figure 3.7**).

Weekly administration of LPS induced extremely elevated systemic inflammatory markers (**Figure 3.7**). LPS administration was associated with a 4.4-fold higher elevation of plasma TNF-α, 11-fold high elevation of plasma IL-6, and 9.6-fold higher elevation of plasma CRP than was observed during chronic *H. pylori* infection. In the liver, LPS administration was associated with 13.8-fold higher elevation of TNF-α, 3.5-fold higher elevation of IL-6, and 2.1-fold higher elevation of CRP than was seen during chronic *H. pylori* infection. In the spleen, LPS was associated with 17.2-fold higher elevation of TNF-α, 17.6-fold higher elevation IL-6, and 8.9-fold higher elevation of CRP than was seen during chronic *H. pylori* infection. These data help to place *H. pylori*

associated systemic inflammatory response in a broader context of severity. LPS administration more closely resembles the degree of inflammation associated with acute infection and sepsis; however, the increased levels of inflammatory markers observed during chronic *H. pylori* infection are markedly milder.

3.3.5 Administration of heat killed *H. pylori* or *E. coli* K12 is not associated with elevated levels of inflammatory markers.

To assess the necessity of active *H. pylori* infection in inducing the observed elevated inflammatory markers, infection studies were conducted utilizing heat-killed *H. pylori* G27. Bacteria were heat killed in such a way as to render them unculturable in liquid BSFB media or on Columbia blood agar plates but prevent lysis of bacterial cells as determined by phase contrast microscopy. *H. pylori* could not be recovered from the stomachs of animals 8 weeks post-inoculation. Measurement of gastric and systemic inflammatory markers found no significant elevation between mock control animals and those that received heat-killed *H. pylori* culture (**Figure 3.8**). To determine that the observed elevation of inflammatory markers was not due to perturbation of the normal gut microflora by inoculation by addition of bacterial culture, infection studies were conducted utilizing *E. coli* K12. *H. pylori* could not be recovered from the stomachs of animals 8-week post-inoculation. Measurement of gastric and systemic inflammatory markers found no significant difference between mock control animals and those that received active *E. coli* culture (**Figure 3.9**). As a whole, these data support the finding that active *H. pylori* infection is necessary for observation of elevated inflammatory markers and that a perturbation of the gut microflora by inoculation with an innocuous bacterial species is not sufficient to induce the observed elevation in inflammatory markers.

3.3.6 Naproxen-induced gastritis is not associated with the development of systemic inflammation.

To assess if gastritis in the absence of *H. pylori* infection was sufficient to induce systemic inflammation, gastritis was chemically induced by once weekly oral gavage of high dose naproxen (80 mg/kg) for 8 weeks. This approach has been previously shown to induce neutrophilic associated ulceration of the gastric epithelium[21,39]. *H. pylori* could not be recovered from the stomachs of animals at the conclusion of these studies. Measurement of inflammatory markers revealed 4-fold higher levels of TNF-α ($P < 0.0001$), 4.5-fold higher IL-6 ($P < 0.0001$), and 6.9-fold higher CRP ($P < 0.0001$) elevation in comparison to those observed following 8 weeks of *H. pylori* infection (**Figure 3.10**). These data indicate that the increased levels of inflammatory markers associated with chronic *H. pylori* infection are milder in comparison to those observed during ulceration or sepsis.

While gastritis associated with high dose oral administration of naproxen was significantly greater than that observed during chronic *H. pylori* infection, it was not associated with an elevation of systemic inflammatory markers (**Figure 3.10**). Naproxen administration was associated with a minor but significantly elevation of liver inflammatory markers. This observation is in agreement with other studies that have found the high dose naproxen administration is associated mild level of hepatotoxicity[2,5]. Ultimately, these data support the conclusion that *H. pylori* associated systemic inflammation is dependent on active *H. pylori* infection and not simply a downstream consequence of gastritis.

3.3.7 Absence of T cell populations is associated with a lack of systemic inflammation during chronic *H. pylori* infection.

As previous experiments suggested that *H. pylori* associated modulation of the immune system, especially of T cell populations, may be a key factor in determining the course of associated systemic inflammation, infection experiments were conducted utilizing athymic rats to assess the course of *H. pylori* associated inflammation in the absence of T cell populations. In congruence with athymic mouse studies[20,37], gastric inflammation was highly attenuated in athymic rats during 8 weeks of *H. pylori* G27 infection (**Figure 3.11**) showing only 4.5-fold elevation of TNF-α and 4.3-fold elevation of IL-6. CRP levels were not found to be elevated in gastric tissue, further supporting the conclusion that while CRP may be detected in other tissues, liver and adipose tissue require interaction with immune cell populations to produce and secrete this acute phase protein. Inflammatory markers measured from plasma, liver and spleen were not found to be significantly different from those observed during mock infection. These data further support the finding that *H. pylori* associated systemic inflammation is dependent on immune cells populations absent in athymic rats.

3.3.8 Chronic *H. pylori* infection is associated with modulation of circulating T cell populations.

Previous studies have suggested that chronic *H. pylori* infection manifests an immunomodulatory influence on the cell populations of the adaptive immune system[23,30,33]. To assess the degree of immune system modulation, we examined changes in the Th1:Th2 and Th17:Treg ratios following 8 weeks of chronic infection by *H. pylori* G27. The Th1:Th2 ratio increased by 1.6-fold (**Figure 3.12**), indicating a significant increase in Th1 cells relative to antagonistic Th2 cells. The Th17:Treg ratio decreased by 11.3-fold (**Figure 3.12**), indicating a significant increase in the generation

of Treg cells during *H. pylori* infection. Together, these data add a measure of nuance to the immunological response to chronic *H. pylori* infection in that while a Th1 polarization favors a pro-inflammatory state, a Treg polarization favors tolerance and suppression of inflammation. These seemingly contradictory processes suggest that *H. pylori* associated modulation of the immune system plays a key role in both inducing inflammation but also maintaining inflammation at low levels throughout chronic infection.

3.3.9 Absence of prominent *H. pylori* virulence factors has negligible influence on *H. pylori* associated systemic inflammation.

To evaluate the potential influence of major *H. pylori* virulence factors on *H. pylori* associated systemic inflammation, inflammatory markers were quantified by ELIA from liver, spleen, and plasma collected from animals follow 8-week long *H. pylori* infection studies utilizing the transposon interruption mutants described in Chapter 2 (**Figure 3.13**). In tissue collected from infected animals, no significant difference in elevated inflammatory markers was observed in each of the examined tissues. These data indicate that VacA, the *cag* pathogenicity island, and NapA have limited influence on the development of *H. pylori* associated systemic inflammation in Sprague-Dawley rats. As both VacA and the *cag* pathogenicity island have been proposed to function in a human-specific context, some caution must be exercised in direct application of these findings to natural human infection.

3.4 Discussion

Chronic systemic inflammation is characterized by the long term presence of elevated levels of circulating inflammatory markers. The presence of chronic systemic inflammation has been associated with elevated risk of multi-organ system dysfunction

and increased risk of the development of disease. Epidemiological studies have suggested a potential association between *H. pylori* seropositivity and elevated circulating inflammatory markers. Direct evaluation of markers of systemic inflammation as well as eradication studies support the hypothesis that chronic *H. pylori* infection is an etiological agent of systemic inflammation. Furthermore, the trend of decreasing inflammatory markers in the liver and spleen support the hypothesis that the immune response shifts from acute phase to chronic phase sometime between 2 and 8 weeks of chronic infection.

Similar to gastric inflammation, only negligible differences in elevated inflammatory markers were observed during infection studies utilizing different strains of *H. pylori*. Likewise, infection studies in C57BL\6 mice resulted in a similar pattern of elevated inflammatory markers as was observed in rats; however, the overall magnitude was reduced in C57BL\6 mice relative to Sprague-Dawley rats. These findings further support the hypothesis that host background plays a role in the severity of inflammation associated with chronic *H. pylori* infection.

Lack of a systemic inflammatory response to heat killed *H. pylori* or *E. coli* K12 support the hypothesis that active *H. pylori* infection is necessary for the observed patterns of elevated inflammatory markers and that administration of bacterial products or disruption of the normal gut microflora by an innocuous bacterial species in not sufficient to generate the observed changes in systemic inflammation. Additionally, the lack of systemic inflammatory response to naproxen-induced gastritis indicates that gastritis alone is not sufficient to generate the observed pattern of elevated inflammatory markers.

T cell populations appear to be key mediators in *H. pylori* associated systemic inflammation as their absence abrogates the elevation of systemic inflammatory markers

in infection studies utilizing athymic rats. Furthermore, T cell phenotyping following *H. pylori* infection indicates that chronic *H. pylori* infection is associated with modulation of both the Th1:Th2 and the Th17:Treg cell populations. Polarization of the Th1:Th2 ratio toward pro-inflammatory Th1 populations is hypothesized to be a key aspect in the development of *H. pylori* associated systemic inflammation while polarization of the Th17:Treg ratio toward tolerogenic Treg populations is hypothesized to result in suppression of *H. pylori* associated systemic inflammation to mild levels.

3.5 Figures

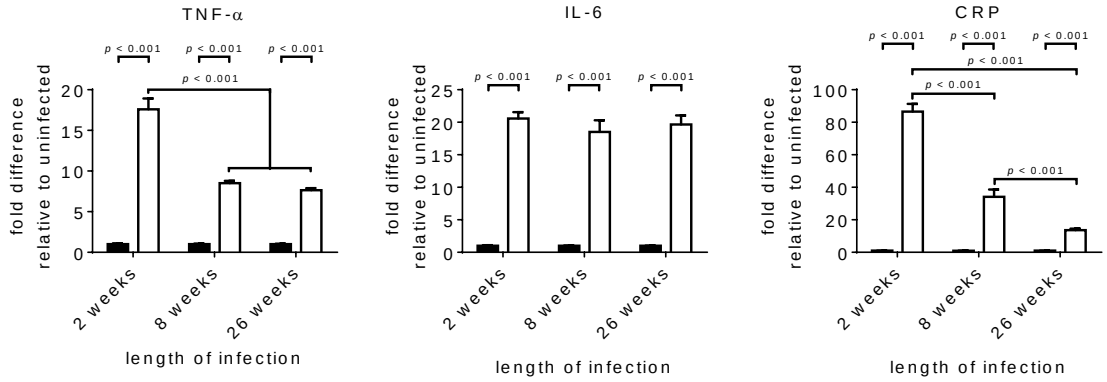

Figure 3.1. *H. pylori* G27 infection is associated with elevated inflammatory markers in liver tissue. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP from liver during *H. pylori* G27 infection. *H. pylori* associated change in inflammatory markers is reported as fold difference between actively infected animals relative to mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by 2-way ANOVA (*p* values: *H. pylori* status: < 0.01, length of infection: < 0.01, and interaction: < 0.01 for each inflammatory marker) with comparisons between mock-infected and actively infected animals by Tukey's multiple comparison test (*p* values reported above each comparison). A significant decrease in TNF-α elevation was observed between 2- and 8-week long infections as well as a significant trend in decreasing CRP through time.

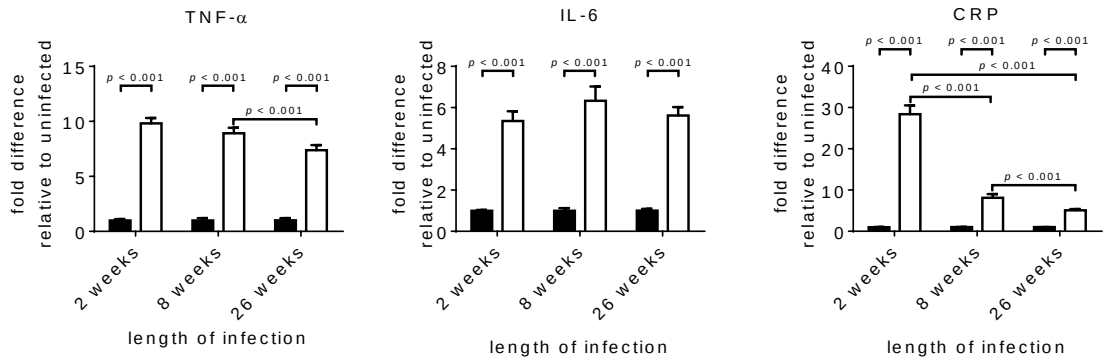

Figure 3.2. *H. pylori* G27 infection is associated with elevated inflammatory markers in spleen. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP from spleen during *H. pylori* G27 infection. *H. pylori* associated change in inflammatory markers is reported as fold difference between actively infected animals relative to mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by 2-way ANOVA (*p* values: *H. pylori* status: < 0.01, length of infection: < 0.01, and interaction: < 0.01 for each inflammatory marker) with comparisons between mock-infected and actively infected animals by Tukey's multiple comparison test (*p* values reported above each comparison). A minor but significant decrease in TNF-α elevation was observed between 8- and 26-week long infections as well as a significant trend in decreasing CRP through time.

Figure 3.3. *H. pylori* G27 infection is associated with elevated inflammatory markers in plasma. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP from plasma during *H. pylori* G27 infection. *H. pylori* associated change in inflammatory markers is reported as fold difference between actively infected animals relative to mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by 2-way ANOVA (*p* values: *H. pylori* status: < 0.01, length of infection: < 0.01, and interaction: < 0.01 for each inflammatory marker) with comparisons between mock-infected and actively infected animals by Tukey's multiple comparison test (*p* values reported above each comparison).

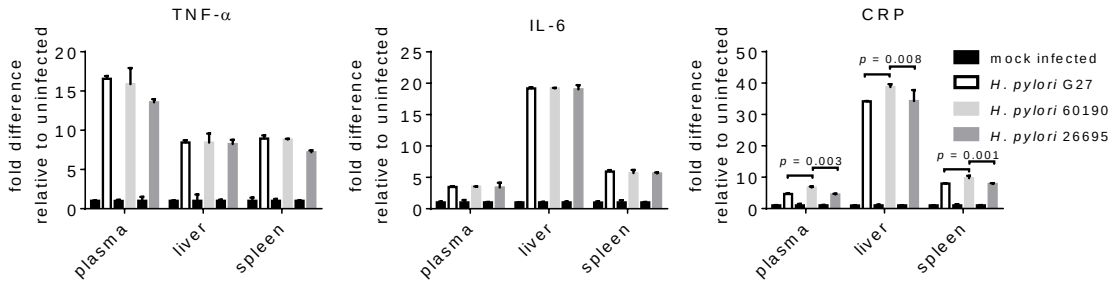

Figure 3.4. Comparison of the degree of systemic inflammation associated with various *H. pylori* strains following 8-week long infections. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* infection utilizing strains G27, 60190, and 26695. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by ANOVA (*p* value: < 0.01) with comparisons between *H. pylori* strains by Tukey's multiple comparison test (*p* values reported above each comparison).

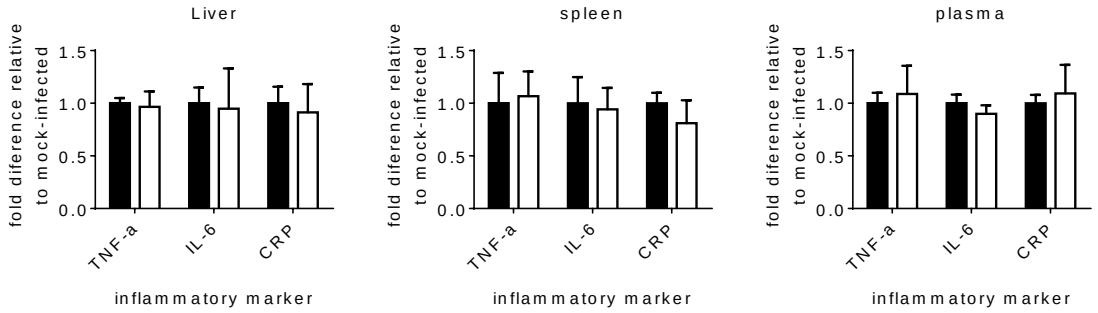

Figure 3.5. Eradication of *H. pylori* infection results in the return of elevated inflammatory markers to baseline levels. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP following eradication of *H. pylori* G27 infection. In eradication studies, 13-week infections were carried out in Sprague-Dawley rats utilizing *H. pylori* G27, followed by an eradication regimen (lansoprazole (3.75 mg/kg), clarithromycin (2.25 mg/kg), and amoxicillin (4.5 mg/kg) administered orally, twice daily for 10 days). After 13 weeks following eradication regimen, tissue samples were collected and inflammatory markers measured by quantitative ELISA. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Significant differences were not observed in levels of TNF-α, IL-6, or CRP, indicating that inflammatory marker levels returned to baseline following eradication of *H. pylori* infection.

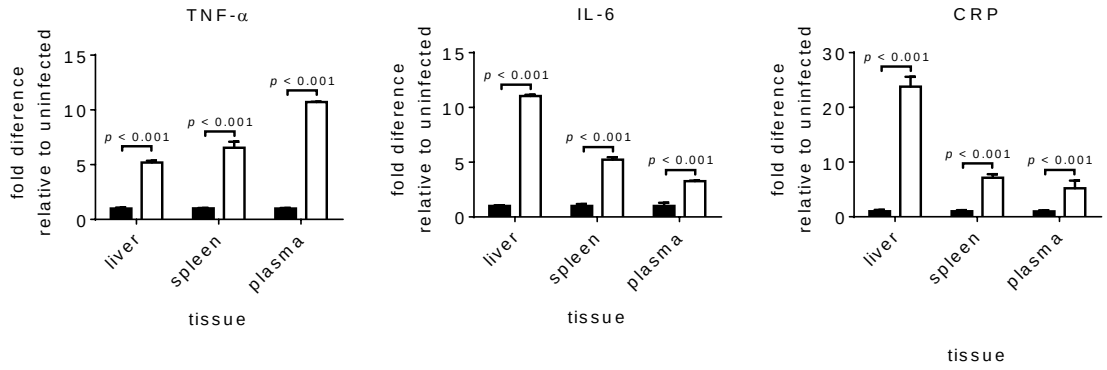

Figure 3.6. Measurement of systemic inflammatory markers in C57BL\6 mice following 8-week long *H. pylori* G27 infections. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* infection in C57BL\6. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by student's *t*-test (*p* values reported above each comparison).

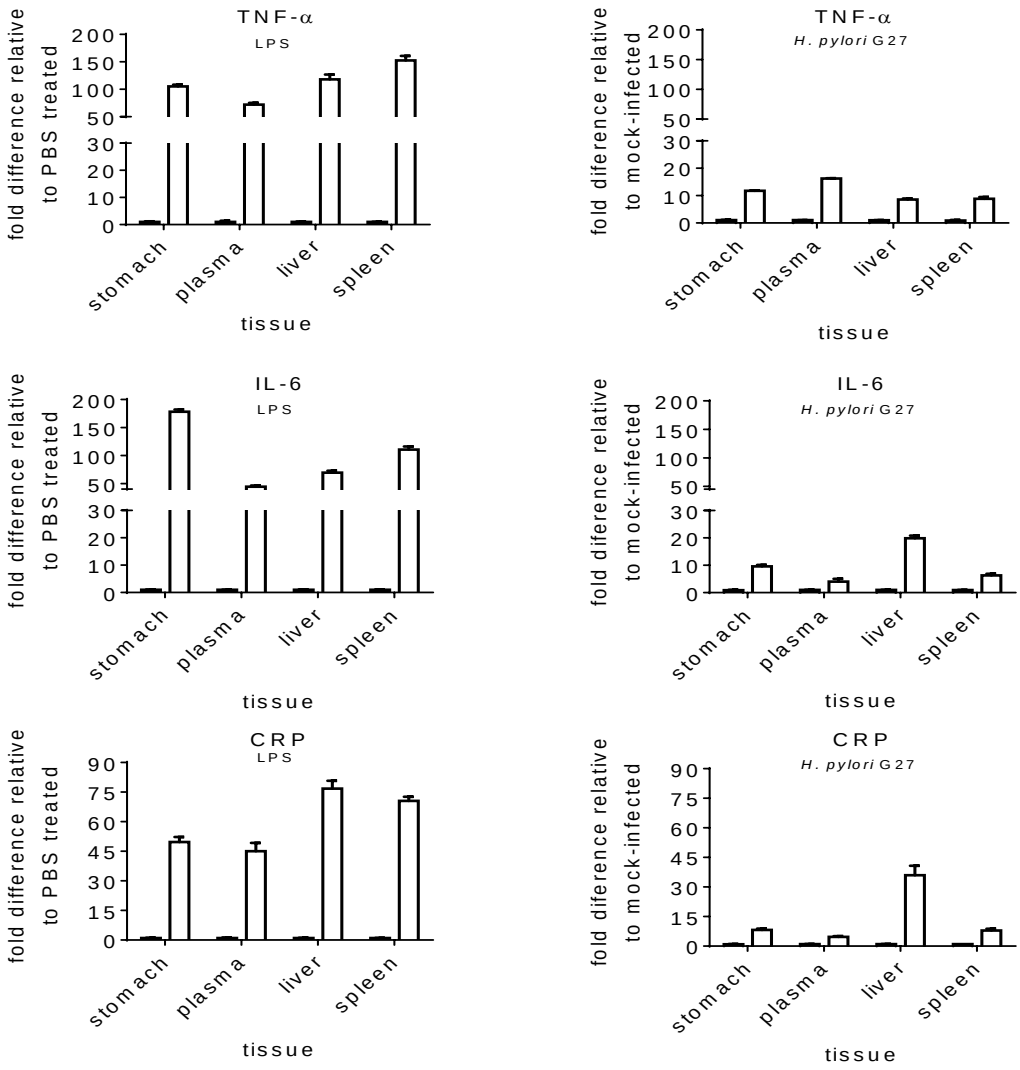

Figure 3.7. Gastric and systemic inflammatory marker profile of chronic *H. pylori* G27 infection is markedly different than that of acute systemic inflammation induced by LPS. Sprague-Dawley rats were administered LPS (500 µg/kg) by intraperitoneal injection once weekly for 8 weeks prior to quantitative ELISA based assessment of the degree of change in levels of TNF-α, IL-6, and CRP. LPS associated change in inflammatory markers is reported as fold difference between LPS-treated animals relative to mock-treated animals. The experiment was performed one time with each bar corresponding to the mean of 3 technical replicates. The error bars correspond to the standard error of the mean. Results from mock-treated animals (black bars) were averaged and normalized to 1. Results from LPS-treated animals (white bars) were averaged and rendered relative to mock-treated animals. Data gathered from 8-week *H. pylori* G27 infection studies (as reported previously) are shown to the right of LPS data as a means of comparing *H. pylori* associated changes in inflammatory markers to those seen during LPS treatment. As LPS studies were limited to treatment groups comprised of a single animal each for humane reasons, statistical testing was not conducted due to small sample size.

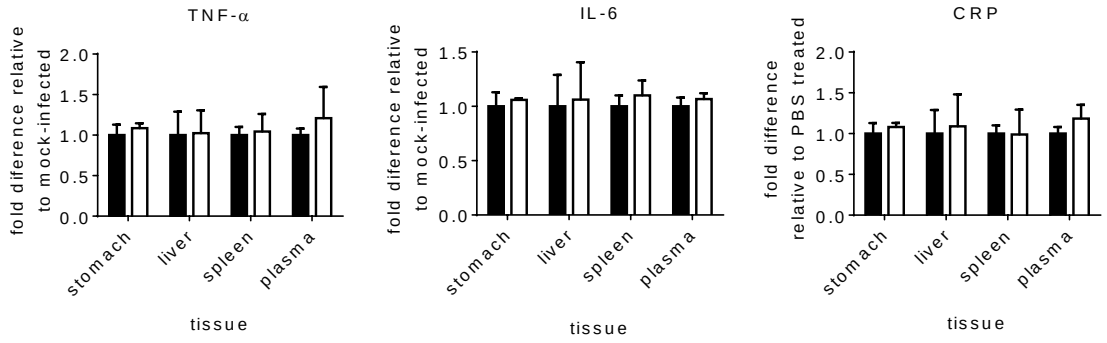

Figure 3.8. Inflammatory profile generated following exposure to heat killed *H. pylori* G27. Sprague-Dawley rats were inoculated with $1x10^7$ cells of heat killed *H. pylori* G27 in similar manner to active infection studies. 8 weeks after completion of inoculations, tissue samples were collected and inflammatory markers measured by quantitative ELISA. The experiment was performed one time with each bar corresponding to the mean of 3 technical replicates. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white and gray bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean fold difference in inflammatory markers, and the error bars correspond to the standard error of the mean. Significant differences were not observed, indicating that exposure to *H. pylori* products is not sufficient to recapitulate the inflammatory profile observed during chronic infection.

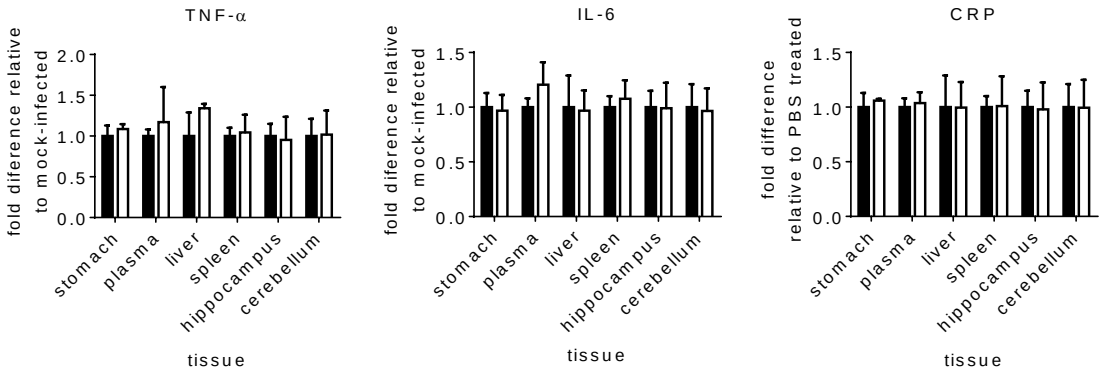

Figure 3.9. Inflammatory profile generated following exposure to *E. coli* K12. Sprague-Dawley rats were inoculated with $1x10^7$ cells of *E. coli* K12 in similar manner to active infection studies. 8 weeks after completion of inoculations, tissue samples were collected and inflammatory markers measured by quantitative ELISA. The experiment was performed one time with each bar corresponding to the mean of 3 technical replicates. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white and gray bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean fold difference in inflammatory markers, and the error bars correspond to the standard error of the mean. Significant differences were not observed, indicating that exposure to an innocuous bacterium is not sufficient to recapitulate the inflammatory profile observed during chronic infection.

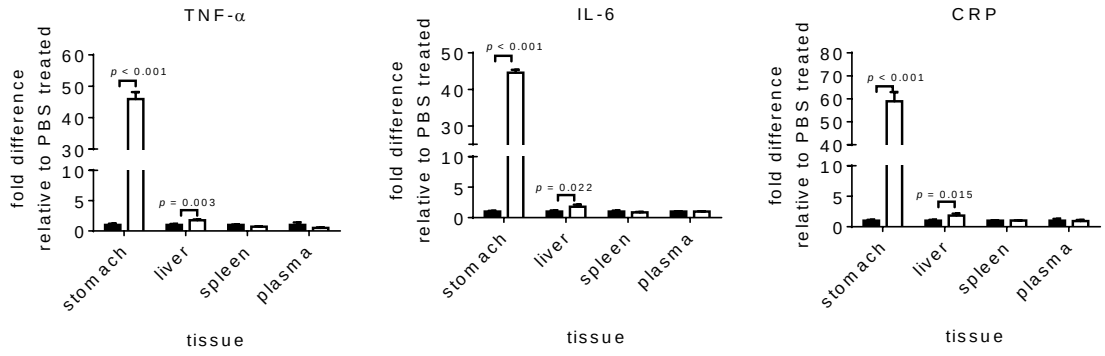

Figure 3.10. Chemically induced gastritis is not associated with the development of systemic inflammation. Gastritis was artificially induced in Sprague-Dawley rats by weekly administration of naproxen (80 mg/kg) by oral gavage. The experiment was performed one time utilizing 3 mock-treated and 3 naproxen-treated animals. Results from mock-treated animals (black bars) were averaged and normalized to 1. Results from naproxen-treated animals (white bars) were averaged and rendered relative to matched mock-treated animals. The height of each bar corresponds to the fold difference in inflammatory markers, and the error bars correspond to the standard error of the mean. Statistical evaluation by student's *t*-test indicated that naproxen treatment was associated with a robust elevation of inflammatory markers in gastric tissue (*p* values reported above relevant comparisons) as well as a significant elevation of inflammatory markers in hepatic tissue (*p* values reported above relevant comparisons). These data indicate that naproxen induced gastritis is associated with slight elevation of inflammatory markers in liver tissue but does not progress to systemic as seen during *H. pylori* infection.

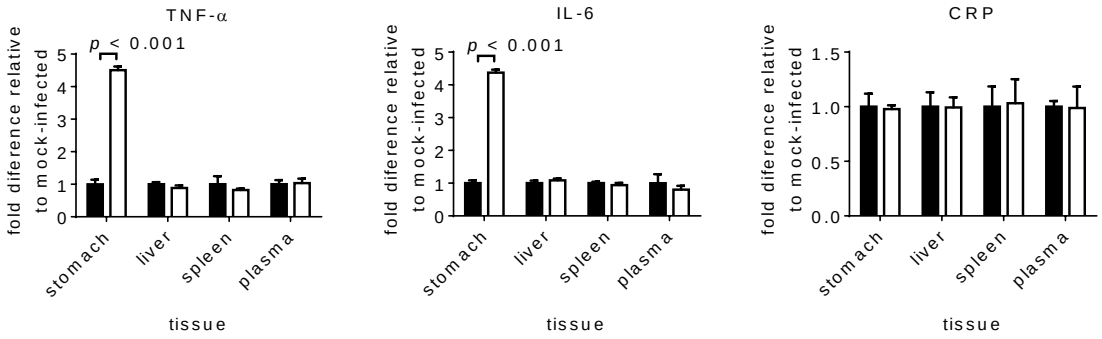

Figure 3.11. Absence of T cell populations prevents the development of systemic inflammation in response to chronic *H. pylori* infection. Quantitative ELISA based assessment of the degree of change of levels of TNF α, IL-6, and CRP following 8-week infections in athymic *FoxN1rnu*/*rnu* rats utilizing *H. pylori* G27. The experiment was performed one time utilizing 3 mock infected and 3 actively infected for each strain. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white and gray bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean fold difference in inflammatory markers, and the error bars correspond to the standard error of the mean. Statistical evaluation by student's *t*-test indicated that chronic *H. pylori* infection was associated with an increase in gastric TNF-α and IL-6. All other comparison between mock-infected and actively infected animals were not significant, indicating that T cell populations are required for the development of *H. pylori* associated systemic inflammation.

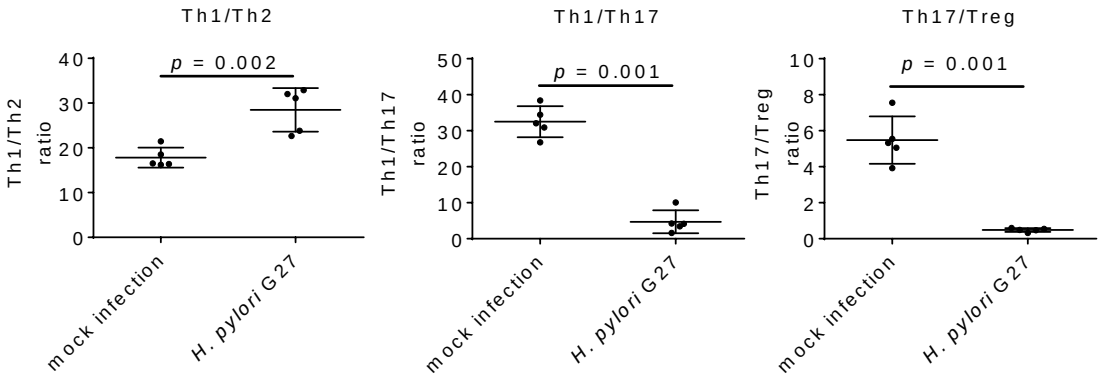

Figure 3.12. *H. pylori* infection modulates populations of circulating T cells. T cell phenotyping was conducted on Sprague-Dawley rats that had been either mock-infected or actively infected with *H. pylori* G27 after 8-weel long infections. Whole blood was collected by terminal cardiac puncture and circulating lymphocytes purified by differential centrifugation. T cell populations were quantified by flow cytometry with immune-fluorescent labeling for Th1, Th2, Th17, and Treg cell types. The experiment was performed one time utilizing 5 mock-infected and 5 *H. pylori* G27 infected animals. The Th1/Th2, Th1/Th17, and Th17/Treg ratios were calculated for each animal. Each circle corresponds to the ratio calculated for an individual animal. The longer bar corresponds to the mean, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by student's *t* test, and *p* values are reported above significant comparisons. These data suggest that *H. pylori* modulates the immune response both toward a pro-inflammatory state (elevated Th1/Th2 ratio) and simultaneously toward a tolerogenic state (depressed Th17:Treg ratio).

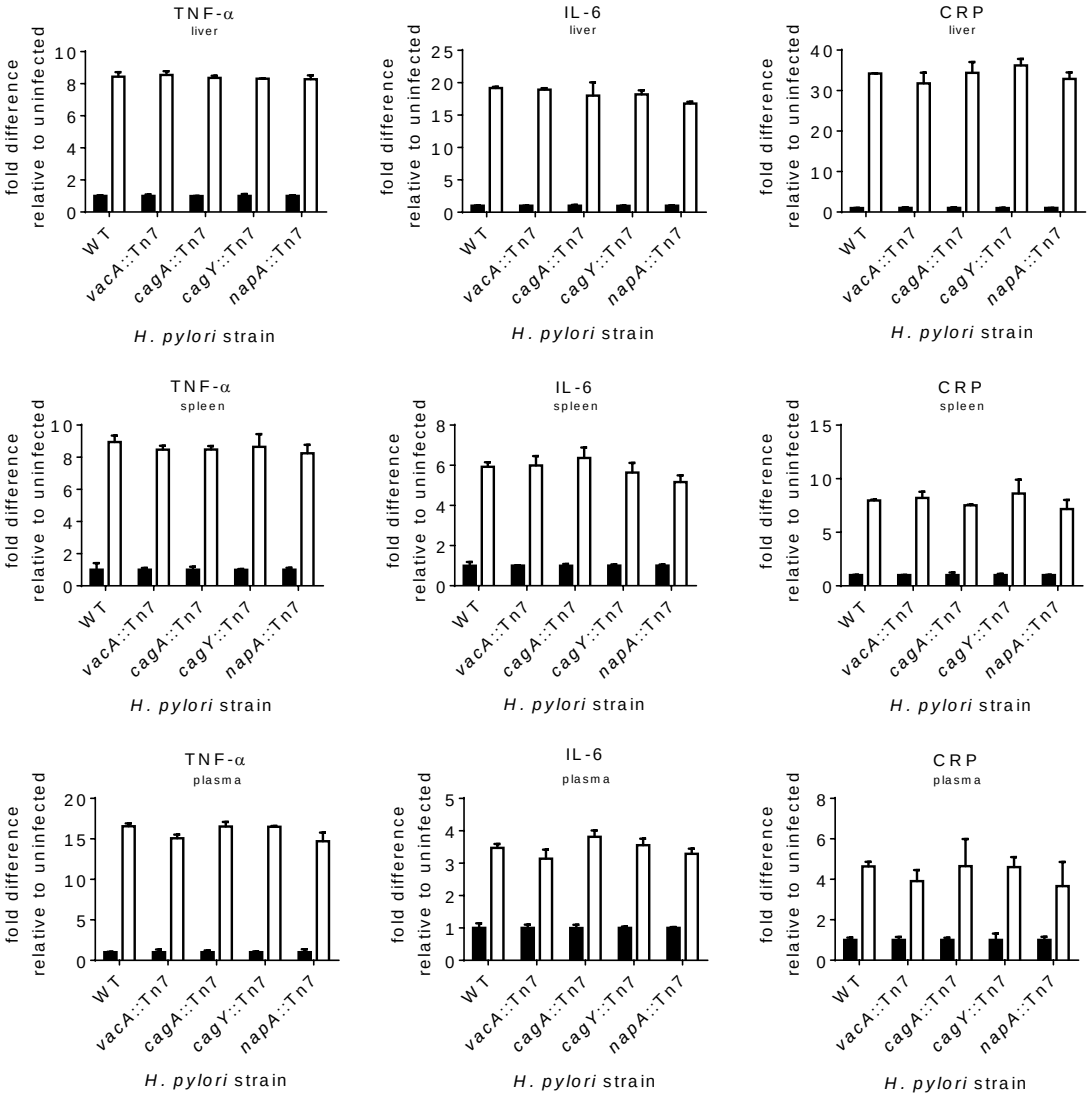

Figure 3.13. Major *H. pylori* virulence factors do not play a major role in the development of *H. pylori* associated systemic inflammation. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* infection utilizing transposon interruption mutants of prominent virulence factors. *H. pylori* associated changes in inflammatory markers are reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean fold difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by 2-way ANOVA (*p* value: 0.41 - .68) and no significant difference was observed in any marker or tissue.

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Chapter 4: Neuro-inflammatory response to chronic *Helicobacter pylori* infection

4.1 Introduction

While the central nervous system has been considered an immune privileged site with the blood-brain barrier limiting the influence of the peripheral immune system on its function, several mechanisms have been proposed to explain the development of neuro-inflammation in response to systemic inflammation. These mechanisms include neuronal recognition of inflammatory cytokines by the vagus nerve[3,12,20] as well as the neurons of the sensory circumventricular organs[4,25,28], the active transport of circulating cytokines across the blood-brain barrier[7,10,11], and the recent discovery of meningeal lymphatic vessels[1,21]. Expression of IL-1, IL-2, IL-6, and TNF-α have been shown to play various roles in disease-associated cognitive dysfuntion[8,9,23,27]. The constellation of nonspecific behavioral changes that accompany illness, such as depression, lethargy, weakness, malaise, disinterest, and inability to concentrate, are collectively referred to as "sickness behavior"[13].

The role of chronic *H. pylori* infection in the development of neuro-inflammation has only been speculated in an extremely small number of studies. These studies which noted improvements in cognition in elderly dementia[6,15,18] and Alzheimer Disease[16,17,19,26] patients following eradication of active *H. pylori* infection. In these studies, neuro-inflammation arising from elevated circulating level of homocysteine as a result of vitamin B12 deficiency was attributed to atrophic gastritis. However, no direct evaluation of neuro-inflammation was undertaken as part of these studies.

To evaluate the role of chronic *H. pylori* infection in the development of systemic inflammation inflammatory markers were quantified from hippocampal and cerebellar samples from 2-, 8- and 26-week long *H. pylori* G27 infection studies. Eradication studies were utilized to evaluate the causal relationship between active *H. pylori* infection and neuro-inflammation. Finally, the role of T cell populations in the

development of *H. pylori* associated neuro-inflammation were evaluated by conducting infection studies conducted in athymic rats.

4.2 Materials and Methods

Animal care. All experiments involving the use of live vertebrate animals were conducted with the approval of the University of Illinois at Urbana-Champaign Institutional Animals Care and Use Committee (IACUC). Rat populations were composed of male Sprague-Dawley rats (Envigo, formerly Harlan Laboratories, Indianapolis, IN). Mouse populations were composed of male C57BL\6J mice (Jackson Laboratory, Bar Harbor, ME). Animals were single housed with 12 h light-dark cycle, mean temperature of 22.1°C, humidity of 50%, and allowed food and water *ad libitum*.

Active *H. pylori* infection time series. To evaluate neuro-inflammation, hippocampal and cerebellar tissues were collected at the time of euthanasia by necropsy from the animal populations described in Chapter 2. In brief, these animal populations consisted 2-, 8-, and 26-week infection studies, each composed of 3 independent blocks which, in turn, were each composed of 3 mock infected animals and 3 actively infected animals.

Eradication of active *H. pylori* infection studies. To evaluate neuro-inflammation, hippocampal and cerebellar tissue were collected at the time of euthanasia by necropsy from the animal populations described in Chapter 2. In brief, this animal population was composed of 3 independent blocks which, in turn, were each composed of 3 mock infected animals and 3 actively infected animals. Animals were infected for 13 weeks; then, both mock infected and actively infected animals underwent eradication therapy. All animals were allowed to remain uninfected for an additional 13 weeks prior to euthanasia and collection of tissues.

Mouse infection studies. To evaluate neuro-inflammation, hippocampal and cerebellar tissue were collected at the time of euthanasia by necropsy from the animal population described in Chapter 2. In brief, this animal population consisted of an 8-week infection study, composed of 3 mock infected animals and 3 actively infected C57BL\6 mice.

LPS induced systemic inflammation. To provide a point of comparison to induced systemic inflammation, hippocampal and cerebellar tissue were collected at the time of euthanasia by necropsy from the animal population described in Chapter 3. In brief, Sprague Dawley rats received weekly intraperitoneal injections of 500 µg/kg purified *E. coli* LPS (Sigma Aldrich, St. Louis MO) dissolved in PBS or the equivalent volume of PBS on a weekly basis for 8 weeks. Due to the extreme distress resulting from this exposure experiment in the experimental animal population, the animal population was limited in size to one LPS exposed animal and one PBS exposed animal.

Naproxen induced gastritis. To determine if gastritis in the absence of active *H. pylori* infection is sufficient to cause neuro-inflammation, hippocampal and cerebellar tissue were collected at the time of euthanasia by necropsy from the animal population described in Chapter 3. In brief, this animal population consisted of 3 Sprague-Dawley rats that received naproxen (80 mg/kg; Sigma Aldrich) suspended in PBS and 3 Sprague-Dawley rats that received the equivalent amount of PBS once weekly for 8 weeks.

Athymic rat infection studies. To evaluate the role of T cell populations in *H. pylori* associated neuro-inflammation, hippocampal and cerebellar tissue were collected at the time of euthanasia by necropsy from the animal population described in Chapter 3. In

brief, 8-week long infection studies were conducted utilizing male *Foxn1*$^{rnu/rnu}$ rats (Envigo) aged 20-21 days. The animal population consisted of 3 actively infected animals and 3 mock infected animals.

Indomethacin anti-inflammatory blockade. To evaluate the role of systemic inflammation on the development of neuro-inflammation, animal infection studies were conducted utilizing a low dose indomethacin (Sigma Aldrich) anti-inflammatory blockade. All animals in the experimental population received indomethacin (2.5 mg/kg) administered via intraperitoneal injection, morning and evening each day throughout the course of the infection study beginning on the first day of lansoprazole administration. In mice this dose has been shown to be sufficient to block neuro-inflammation associated with systemic LPS administeration[22]. The experimental animal population consisted of 3 Sprague-Dawley rats that were actively infected with *H. pylori* G27 as described previously and 3 Sprague-Dawley rats that were mock-infected using sterile bisulfite free *Brucella* broth as described previously. Eight week after the competition of the inoculation protocol, animals were euthanized and stomach, liver, spleen, blood, and brain tissues collected for further analyses.

Terminal collection of tissue samples. Tissue samples were collected as described in Chapter 2. In brief, animals were placed under surgical anesthesia, and terminal blood collection was conducted by cardiac puncture. Stomach, liver, spleen and brain tissues were collected by necropsy immediately following euthanasia of the animal.

Sample processing. Collected tissue samples were processed as described in Chapter 2. In brief, plasma samples were prepared from whole blood and stored at -20°C until

assayed. Remaining tissues were stored at -20°C until processed for preparation of whole tissue protein lysates.

Preparation of whole tissue protein lysates. 200 mg of gastric tissue was mechanically homogenized using mortar and pestle with the aid of liquid nitrogen. Homogenized tissue was suspended in 2 mL of RIPA buffer (Thermo Fisher) supplemented proteinase inhibitor cocktail (Thermo Fisher). Cells were lysed on ice with gentle shaking for 3 hr. Unlysed tissues and lipid fraction were removed by centrifugation at 1500 x *g* for 15 min at 4°C. Total protein concentration for tissue lysates was determined by BCA assay (Thermo Fisher). Tissue lysates were aliquoted at 200 µL and frozen at -20°C until assayed.

Cytokine quantification. Tissue lysate was diluted to 500 µg/ml to standardize the amount of protein assayed across sample. Cytokine levels was determined by quantitative ELISA for TNF-α (Eagle Bioscience, Nashua, NH), IL-6 (Eagle Biosciences), and CRP (Sigma Aldrich) according to manufacturer's directions.

Statistical analysis. Cytokine levels from active infection time series were evaluated by two-way ANOVA of *H. pylori* infection status and length of infection with Tukey's correction for multiple comparisons. Eradication studies, mouse studies, heat killed *H. pylori* studies, E. coli K12 studies, naproxen induced gastritis studies, athymic rat *H. pylori* infection studies, and indomethacin anti-inflammatory blockade studies were evaluated using a 2-tailed distribution, paired *t* test. Cytokine levels from transposon insertion *H. pylori* infection studies were evaluated by two-way ANOVA of *H. pylori* infection status and *H. pylori* strain. An alpha level of 0.05 was used as a threshold for

statistical significance and all error bars represent standard error of the mean (SEM). Graphpad Prism (version 6.01) was used for all statistical analyses.

4.3 Results

4.3.1 *H. pylori* infection in Sprague-Dawley rats is associated with increased levels of inflammatory markers in hippocampal and cerebellar tissue.

To assess the relationship between *H. pylori* infection and neuro-inflammation, hippocampal (**Figure 4.1**) and cerebellar (**Figure 4.2**) tissues were collected as part of the *H. pylori* G27 infection time series. These tissues were selected as their functional roles have been shown to have increased sensitivity to inflammatory inhibition[2,5,14,24,29,30]. As was observed both gastrically and systemically, 2-week infection time points were associated with the highest levels of increased TNF-α and CRP. Two-way ANOVA analysis (2-way ANOVA: *H. pylori* status *p* value <0.0001, 70.9% of total variation; Length of infection *p* value <0.0001, 14.3% of total variation) with subsequent testing by Tukey's multiple pairwise test (*p* value <0.0001 for all comparisons) supported a trend of decreasing TNF-α levels in hippocampal tissue through time during chronic *H. pylori* infection. However, in cerebellar tissue, TNF-α levels remain non-significantly changed between 8- and 26-week time points (2-way ANOVA: *H. pylori* status *p* value <0.0001, 94.6% of total variation; Length of infection *p* value <0.0001, 2.5% of total variation with subsequent testing by Tukey's multiple pairwise test *p* value <0.0001 for comparisons of 2-weeks vs 8- or 26-weeks). In both, hippocampal (2-way ANOVA: *H. pylori* status *p* value <0.0001, 90.4% of total variation; Length of infection *p* value = 0.02, 1.2% of total variation) and cerebellar (2-way ANOVA: *H. pylori* status *p* value <0.0001, 94.3% of total variation; Length of infection *p* value = 0.01, 0.8% of total variation) tissue, elevated levels of IL-6 remain non-significantly different among time points. Elevated levels of

CRP showed a pattern of decreasing magnitude through time in both hippocampal (2-way ANOVA: *H. pylori* status *p* value <0.0001, 60.9% of total variation; Length of infection *p* value <0.0001, 19.1% of total variation with subsequent testing by Tukey's multiple pairwise test *p* value <0.0001 for all comparisons) and cerebellar (2-way ANOVA: *H. pylori* status *p* value <0.0001, 60.1% of total variation; Length of infection *p* value <0.0001, 19.2% of total variation with subsequent testing by Tukey's multiple pairwise test *p* value <0.0001 for all comparisons) tissue. These data indicate that chronic *H. pylori* infection is associated with elevated markers of neuro-inflammation in the hippocampus and the cerebellum. Furthermore, these data support a model in which highly potent inflammatory markers are reduced in degree of elevation through time during chronic *H. pylori* infection. However, IL-6, a key mediator of the neuro-inflammatory response remains stably elevated throughout chronic infection.

To determine if the neuro-inflammatory response was a commonality among different strains of *H. pylori*, the levels of inflammatory markers from hippocampal and cerebellar tissue were examined following 8 weeks of infection by *H. pylori* strains G27, 60190, and 26695 (**Figure 4.3**). Elevated levels of TNF-α and IL-6 were not found to vary significantly among the three strains. Hippocampal levels of CRP during infection by *H. pylori* 60190 were found to significantly elevated in comparison to those seen during 26695 infection (*p* value = 0.04) while all other comparisons regarding CRP elevation were not significant. These data indicate that specific strain of *H. pylori* is not a determinant in the degree of neuro-inflammation associated with *H. pylori* infection.

4.3.2 Eradication of *H. pylori* infection in Sprague-Dawley rats leads resolution of increased levels of inflammatory markers.

To determine the causality between chronic *H. pylori* infection and associated elevated neuro-inflammatory markers, hippocampal and cerebellar tissue was examined following eradication therapy (**Figure 4.4**). Levels of TNF-α, IL-6, and CRP associated with hippocampal and cerebellar tissue from *H. pylori* cured animals were not significantly different from those seen in mock-infected animals. These data further indicate that chronic *H. pylori* is the causative agent of the observed neuro-inflammation, and that once eradicated, elevated levels of neuro-inflammatory markers return to baseline levels.

4.3.3 *H. pylori* infection in C57BL\6 mice leads to a similar but blunted pattern of increased inflammatory markers.

To determine if the observed neuro-inflammatory response associated with *H. pylori* infection was species-specific, inflammatory markers were measured from hippocampal and cerebellar samples collected from C57BL\6 mice which had been infected with *H. pylori* G27 for 8 weeks. As was seen in gastric tissue, a similar pattern of elevated inflammatory markers was observed in mice as was seen in Sprague-Dawley rats (**Figure 4.5**). Additionally, as was seen in gastric and systemic inflammation, the magnitude of elevation of the measured cytokines was reduced in mice relative to rats by approximately 2.5- to 1.3-fold. These data support the hypothesis that the observed elevation of neuro-inflammatory markers is not species-specific; however, the data do suggest that the degree of neuro-inflammation associated with chronic *H. pylori* infection is potentially influenced by the host species.

4.3.4 *H. pylori* associated neuro-inflammatory profile is markedly different than that associated with systemic administration of LPS.

LPS administration was associated with a 7.8-fold higher elevation of hippocampal and 11-fold higher elevation of cerebellar TNF-α levels in comparison to those observed during *H. pylori* G27 infection (**Figure 4.6**). Likewise, IL-6 levels were found to be 14.4-fold higher in hippocampal tissue and 12.3-fold higher in cerebellar tissue. CRP levels were found to only be 1.9-fold higher in the hippocampus and 1.6-fold higher in the cerebellum. These data further demonstrate the reduced magnitude of *H. pylori* associated neuro-inflammation in comparison to that which would be seen during acute infection or sepsis.

4.3.5 Administration of heat killed *H. pylori* or *E. coli* K12 is not associated with elevated inflammatory markers.

To assess the necessity of active *H. pylori* infection in inducing the observed elevated inflammatory markers, infection studies were conducted utilizing heat-killed *H. pylori* G27. Measurement of hippocampal and cerebellar inflammatory markers found no significant elevation between mock control animals and those that received heat-killed *H. pylori* culture (**Figure 4.7**). To determine that the observed elevation of inflammatory markers was not due to perturbation of the normal gut microflora by inoculation by addition of bacterial culture, infection studies were conducted utilizing *E. coli* K12. Measurement of hippocampal and cerebellar inflammatory markers found no significant difference between mock control animals and those that received active E. coli culture (**Figure 4.8**). As a whole, these data support the finding that active *H. pylori* infection is necessary for observation of elevated inflammatory markers and that a perturbation of the gut microflora by inoculation with an innocuous bacterial species is not sufficient to induce the observed elevation in inflammatory markers.

4.3.6 Naproxen-induced gastritis is not associated with the development of neuro-inflammation.

Naproxen associated gastritis was not associated with a neuro-inflammatory response (**Figure 4.9**). These data support the conclusion that the neuro-inflammatory response observed during chronic *H. pylori* infection is not simply a downstream consequence of gastritis.

4.3.7 Absence of T cell populations is associated with a lack of neuro-inflammatory response during chronic *H. pylori* infection.

No significant elevation in hippocampal or cerebellar inflammatory markers was observed in tissue collected from athymic rats following 8-week long *H. pylori* G27 infections (**Figure 4.10**). These data further support the finding that *H. pylori* associated neuro-inflammation is dependent on immune cell populations absent in athymic rats.

4.3.8 Inhibition of the systemic inflammatory response by indomethacin blocks the development of *H. pylori* associated neuro-inflammation.

To determine if inhibition of the systemic inflammatory response influence the development of neuro-inflammation in response to chronic *H. pylori* infection, 8-week long infection studies were conducted in which both actively infected and mock infected animals received twice daily intraperitoneal administration of low dose indomethacin (2.5 mg/kg). Following euthanasia of animals, inflammatory markers were evaluated from stomach, liver, spleen, plasma, hippocampus, and cerebellum tissues (**Figure 4.11**). Surprisingly, low dose indomethacin administration was observed to block *H. pylori* associated inflammation at all levels. While these data are in agreement with the hypothesis that *H. pylori* associated neuro-inflammation is related to the systemic

inflammatory response, the total abrogation of *H. pylori* associated inflammation limits the conclusions that can be drawn from these data.

4.3.9 Absence of prominent *H. pylori* virulence factors has negligible influence on *H. pylori* associated neuro-inflammation.

To evaluate the potential influence of major *H. pylori* virulence factors on *H. pylori* associated systemic inflammation, inflammatory markers were quantified by ELIA from liver, spleen, and plasma collected from animals follow 8-week long *H. pylori* infection studies utilizing the transposon interruption mutants described in Chapter 2 (**Figure 4.12**). In tissue collected from infected animals, no significant difference in elevated inflammatory markers was observed in any of the examined tissues. These data indicate that VacA, the *cag* pathogenicity island, and NapA have limited influence on the development of *H. pylori* associated systemic inflammation in Sprague-Dawley rats. As both VacA and the *cag* pathogenicity island have been proposed to function in a human-specific context, some caution must be exercised in direct application of these findings to natural human infection.

4.4 Discussion

Neuro-inflammation is characterized by the presence of elevated levels of inflammatory markers within the central nervous system. The presence of neuro-inflammation has been associated with behavioral changes and the development of detrimental cognitive effects. These data support the hypothesis that chronic *H. pylori* infection is associated with the development of low grade neuro-inflammation. Data from eradication studies indicate that *H. pylori* infection is the etiological agent of the observed neuro-inflammation.

As was seen in gastric and systemic inflammation, only negligible differences in elevated inflammatory markers were observed during infection studies utilizing different strains of *H. pylori*. Likewise, infection studies in C57BL\6 mice resulted in a similar pattern of elevated inflammatory markers as was observed in Sprague-Dawley rats. However, the overall magnitude of elevation was reduced in C57BL\6 mice relative to Sprague-Dawley rats, indicating that severity of *H. pylori* associated inflammation may be modulated by the host background.

Lack of neuro-inflammatory response to either heat killed *H. pylori* of *E. coli* K12 support the hypothesis that the observed change sin neuro-inflammation are not the result of brief exposure to bacterial products or disruption of the normal gut microflora by an innocuous bacterial species, but rather result from active *H. pylori* infection of the stomach. Furthermore, the absence of a neuro-inflammatory response in athymic rats indicates the likely role of the systemic inflammatory in the development of neuro-inflammation in response to chronic *H. pylori* infection.

4.5 Figures

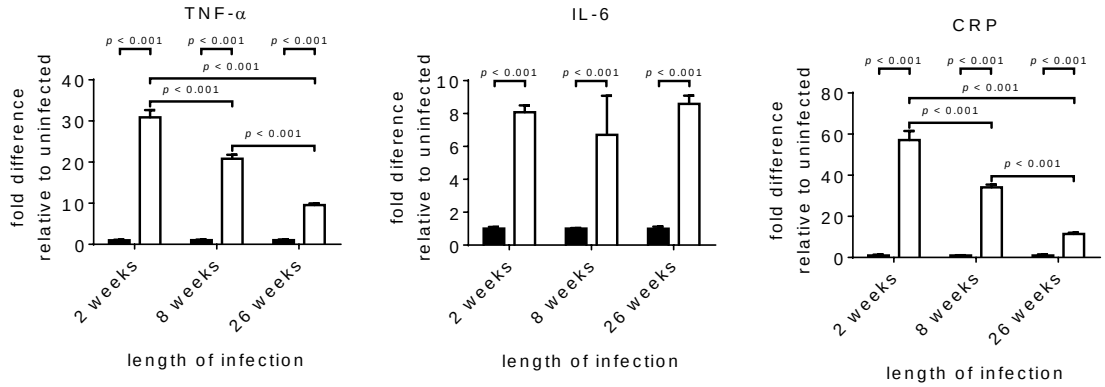

Figure 4.1. Chronic *H. pylori* infection is associated with elevated inflammatory markers in hippocampal tissue. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP from hippocampus during *H. pylori* G27 infection. *H. pylori* associated change in inflammatory markers is reported as fold difference between actively infected animals relative to mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by 2-way ANOVA (*p* values: *H. pylori* status: < 0.01, length of infection: < 0.01, and interaction: < 0.01 for each inflammatory marker) with comparisons between mock-infected and actively infected animals by Tukey's multiple comparison test (*p* values reported above each comparison). A significant trend in decreasing in TNF-α and CRP elevation was observed between 2-, 8-, and 26-week long infections.

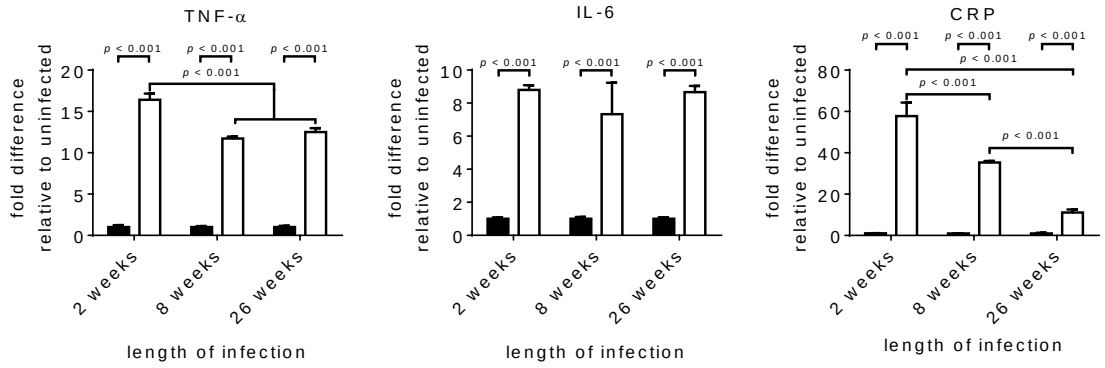

Figure 4.2. Chronic *H. pylori* infection is associated with elevated inflammatory markers in cerebellar tissue. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP from cerebellum during *H. pylori* G27 infection. *H. pylori* associated change in inflammatory markers is reported as fold difference between actively infected animals relative to mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by 2-way ANOVA (*p* values: *H. pylori* status: < 0.01, length of infection: < 0.01, and interaction: < 0.01 for each inflammatory marker) with comparisons between mock-infected and actively infected animals by Tukey's multiple comparison test (*p* values reported above each comparison). A significant trend in decreasing in CRP elevation was observed between 2-, 8-, and 26-week long infections. Additionally, a significant decrease in TNF-α was seen between 2- and 8-week infections.

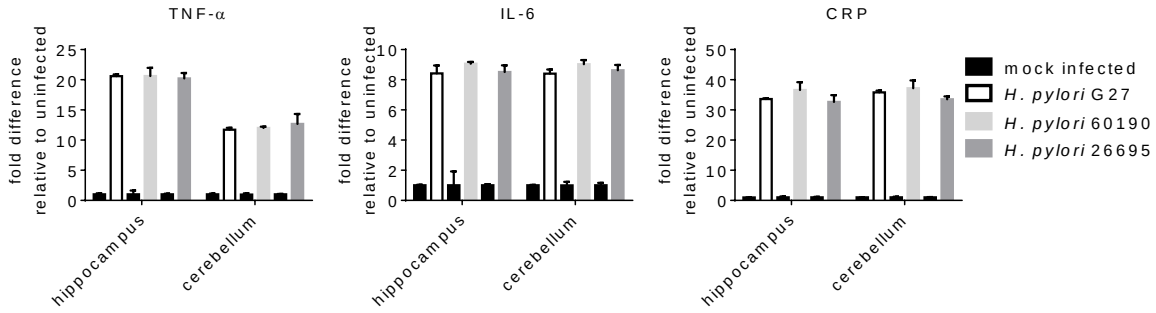

Figure 4.3. Comparison of the degree of neuro-inflammation associated with various *H. pylori* strains following 8-week long infections. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* infection utilizing strains G27, 60190, and 26695. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. No significant difference was observed in the degree of inflammation from either tissue across the three *H. pylori* strains.

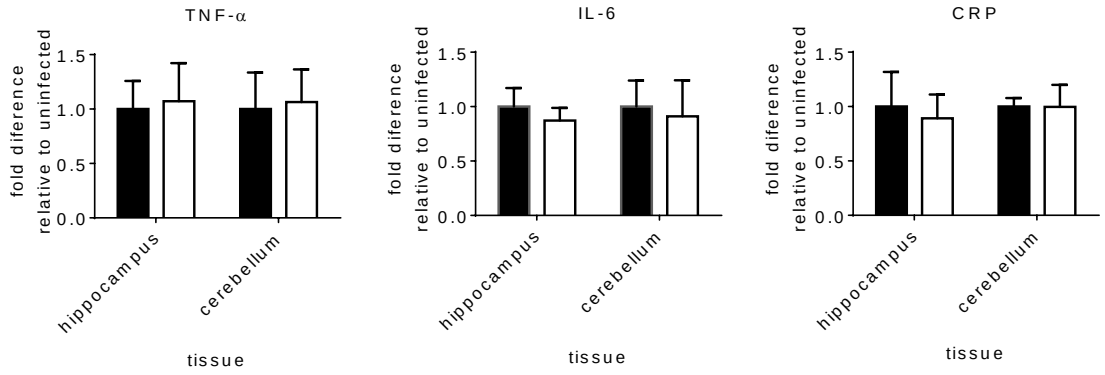

Figure 4.4. Eradication of *H. pylori* infection results in the return of elevated inflammatory markers to baseline levels. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP following eradication of *H. pylori* G27 infection. In eradication studies, 13-week infections were carried out in Sprague-Dawley rats utilizing *H. pylori* G27, followed by an eradication regimen (lansoprazole (3.75 mg/kg), clarithromycin (2.25 mg/kg), and amoxicillin (4.5 mg/kg) administered orally, twice daily for 10 days). After 13 weeks following eradication regimen, tissue samples were collected and inflammatory markers measured by quantitative ELISA. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Significant differences were not observed in levels of TNF-α, IL-6, or CRP, indicating that inflammatory marker levels returned to baseline following eradication of *H. pylori* infection.

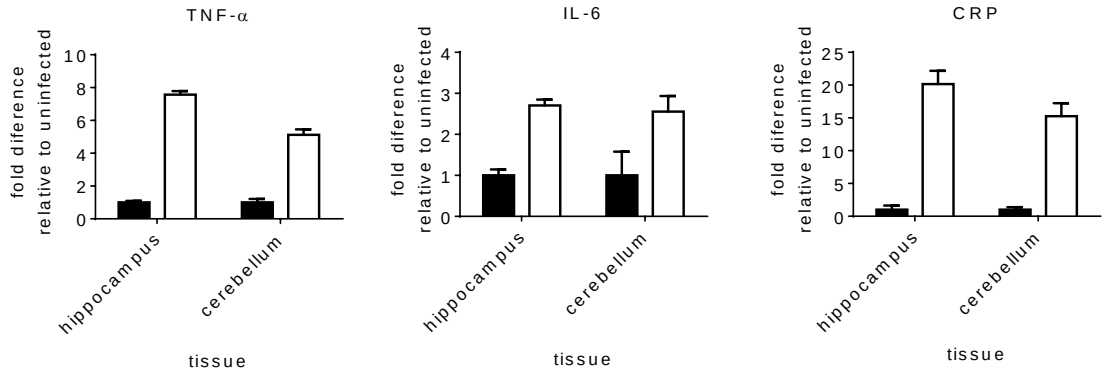

Figure 4.5. Measurement of systemic inflammatory markers in C57BL\6 mice following 8-week long *H. pylori* G27 infections. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* infection in C57BL\6. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by student's *t*-test (*p* values reported above each comparison).

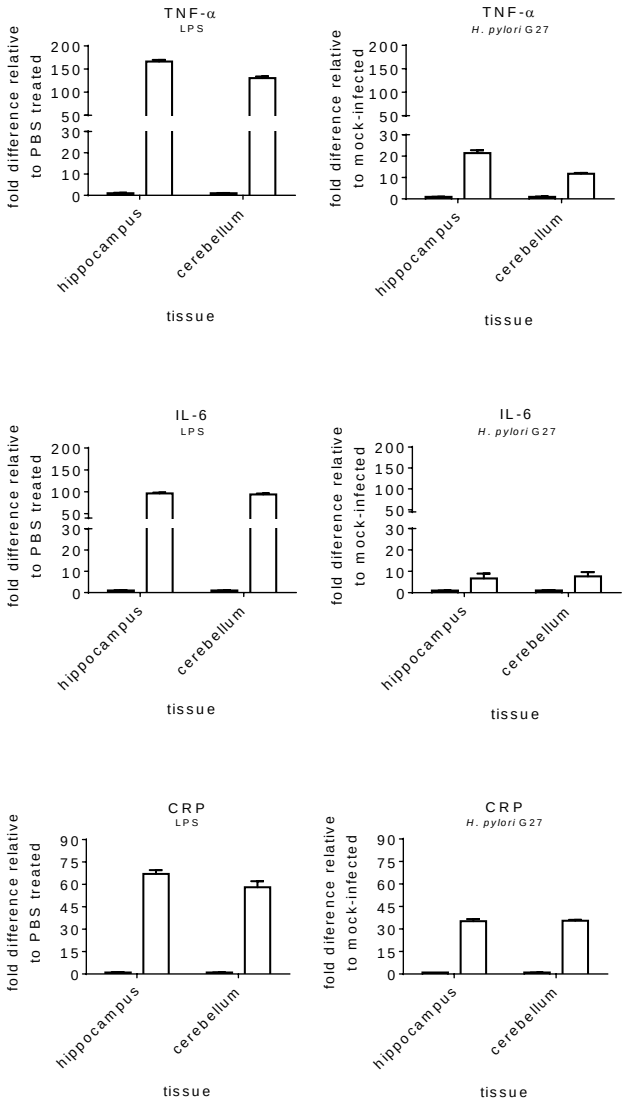

Figure 4.6. Neuro-inflammatory marker profile of chronic *H. pylori* G27 infection is markedly different than that of acute systemic inflammation induced by LPS. Sprague-Dawley rats were administered LPS (500 µg/kg) by intraperitoneal injection once weekly for 8 weeks prior to quantitative ELISA based assessment of the degree of change in levels of TNF-α, IL-6, and CRP. LPS associated change in inflammatory markers is reported as fold difference between LPS-treated animals relative to mock-treated animals. The experiment was performed one time with each bar corresponding to the mean of 3 technical replicates. The error bars correspond to the standard error of the mean. Results from mock-treated animals (black bars) were averaged and normalized to 1. Results from LPS-treated animals (white bars) were averaged and rendered relative to mock-treated animals. Data gathered from 8-week *H. pylori* G27 infection studies (as reported previously) are shown to the right of LPS data as a means of comparing *H. pylori* associated changes in inflammatory markers to those seen during LPS treatment. As LPS studies were limited to treatment groups comprised of a single animal each for humane reasons, statistical testing was not conducted due to small sample size.

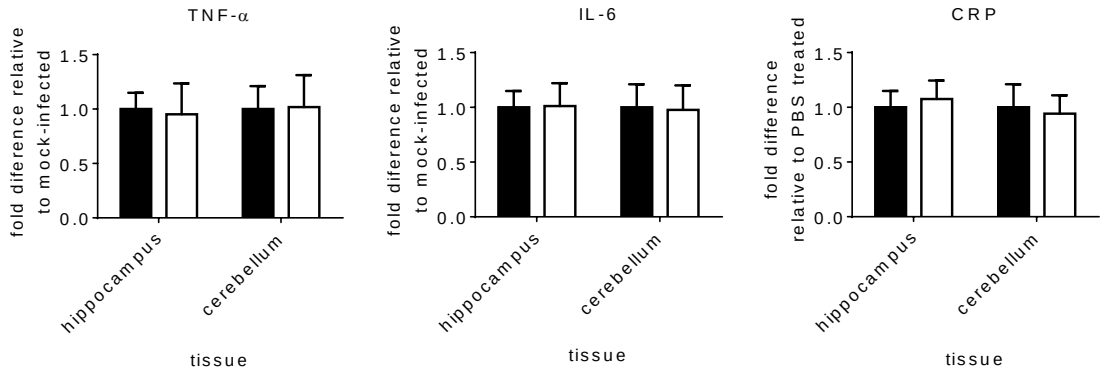

Figure 4.7. Inflammatory profile generated following exposure to heat killed *H. pylori* G27. Sprague-Dawley rats were inoculated with $1x10^7$ cells of heat killed *H. pylori* G27 in similar manner to active infection studies. 8 weeks after completion of inoculations, tissue samples were collected and inflammatory markers measured by quantitative ELISA. The experiment was performed one time with each bar corresponding to the mean of 3 technical replicates. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white and gray bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean fold difference in inflammatory markers, and the error bars correspond to the standard error of the mean. Significant differences were not observed, indicating that exposure to *H. pylori* products is not sufficient to recapitulate the inflammatory profile observed during chronic infection.

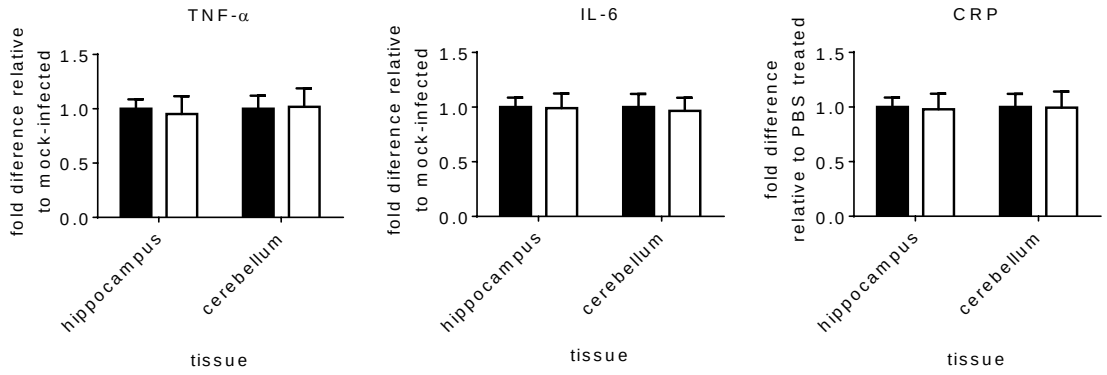

Figure 4.8. Inflammatory profile generated following exposure to *E. coli* K12. Sprague-Dawley rats were inoculated with $1x10^7$ cells of *E. coli* K12 in similar manner to active infection studies. 8 weeks after completion of inoculations, tissue samples were collected and inflammatory markers measured by quantitative ELISA. The experiment was performed one time with each bar corresponding to the mean of 3 technical replicates. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white and gray bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean fold difference in inflammatory markers, and the error bars correspond to the standard error of the mean. Significant differences were not observed, indicating that exposure to an innocuous bacterium is not sufficient to recapitulate the inflammatory profile observed during chronic infection.

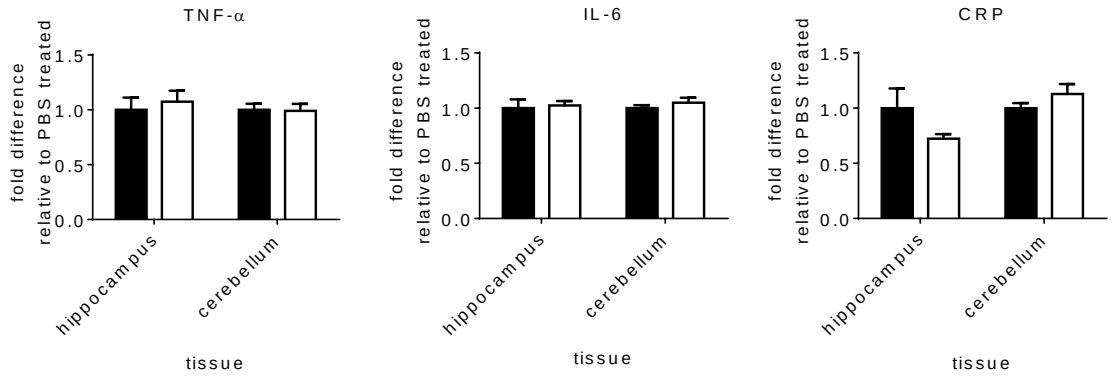

Figure 4.9. Chemically induced gastritis is not associated with the development of neuro-inflammation. Gastritis was artificially induced in Sprague-Dawley rats by weekly administration of naproxen (80 mg/kg) by oral gavage. The experiment was performed one time utilizing 3 mock-treated and 3 naproxen-treated animals. Results from mock-treated animals (black bars) were averaged and normalized to 1. Results from naproxen-treated animals (white bars) were averaged and rendered relative to matched mock-treated animals. The height of each bar corresponds to the fold difference in inflammatory markers, and the error bars correspond to the standard error of the mean. No significant difference was observed in the levels of inflammatory markers between naproxen treated and mock treated animals. These data indicate that naproxen induced gastritis does not progress to neuro-inflammation as seen during *H. pylori* infection.

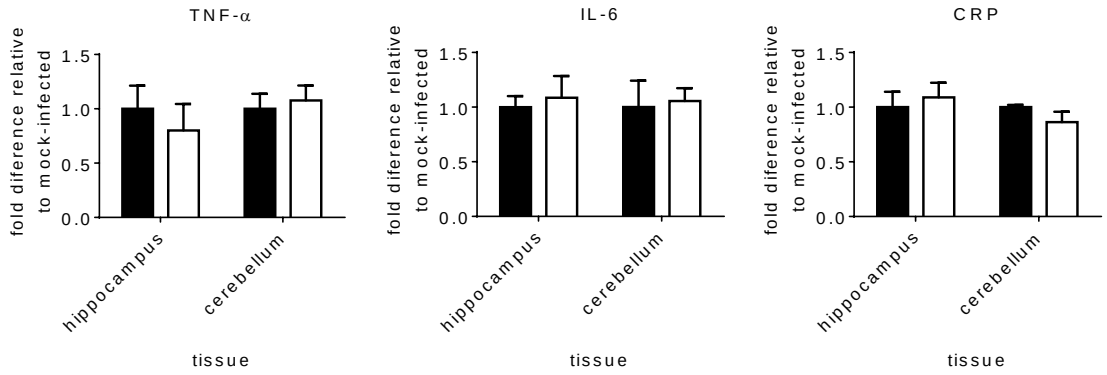

Figure 4.10. Absence of T cell populations prevents the development of systemic inflammation in response to chronic *H. pylori* infection. Quantitative ELISA based assessment of the degree of change of levels of TNF α, IL-6, and CRP following 8-week infections in athymic *FoxN1rnu*/*rnu* rats utilizing *H. pylori* G27. The experiment was performed one time utilizing 3 mock infected and 3 actively infected for each strain. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white and gray bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean fold difference in inflammatory markers, and the error bars correspond to the standard error of the mean. No significant difference was observed in the level of inflammatory markers between actively infected and mock infected animals, indicating that T cell populations are required for the development of *H. pylori* associated neuro-inflammation.

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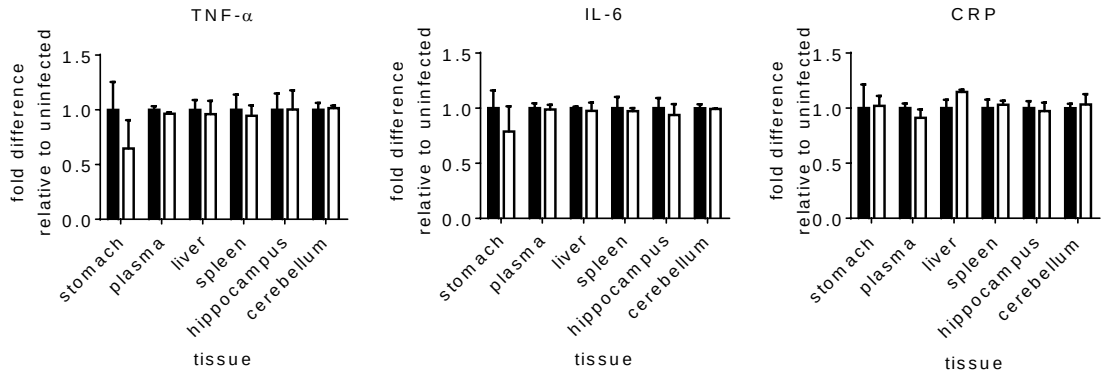

Figure 4.11. Indomethacin anti-inflammatory blockade prevent *H. pylori* associated inflammatory response. To evaluate the influence of the systemic inflammatory response on the development of the neuro-inflammatory response, 8-week infection studies were conducted in which all member of the experimental animal population received low dose indomethacin (2.5 mg/kg) twice daily by intraperitoneal injection. The experiment was performed one time utilizing 3 mock-treated and 3 actively-infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively-infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the fold difference in inflammatory markers, and the error bars correspond to the standard error of the mean. No significant difference was observed in the levels of inflammatory markers between mock-infected and actively-infected animals.

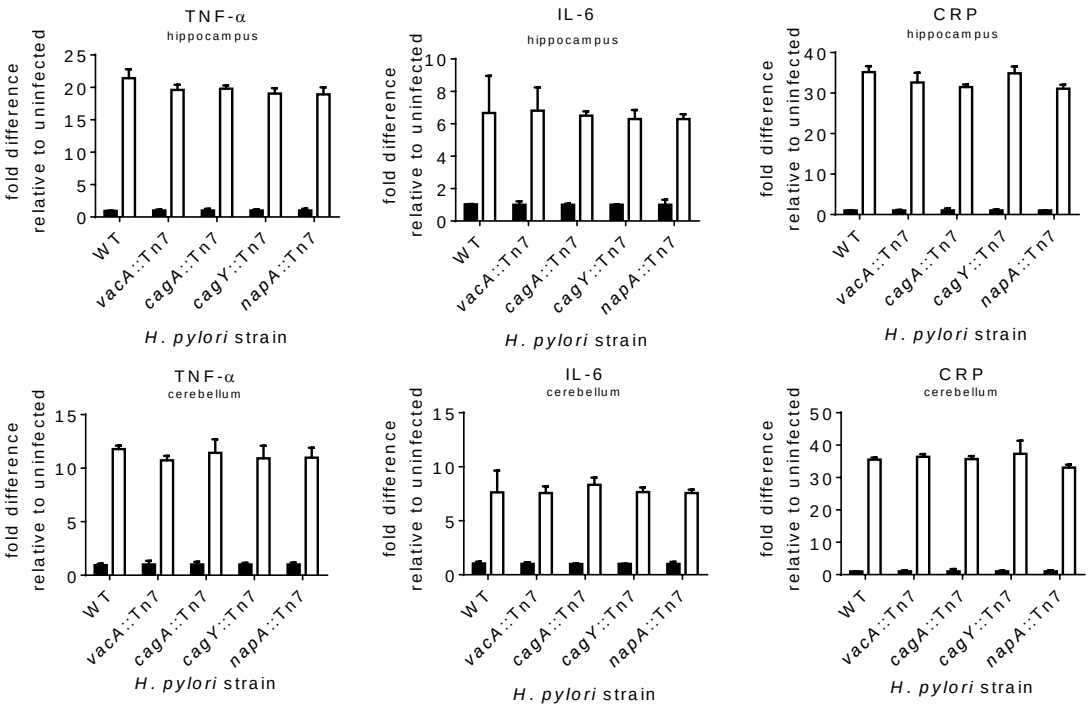

Figure 4.12. Major *H. pylori* virulence factors do not play a major role in the development of *H. pylori* associated neuro-inflammation. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP during *H. pylori* infection utilizing strains transposon interruption mutants of prominent virulence factors. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to matched mock-infected animals. Data were collected utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were statistically evaluated by 2-way ANOVA (*p* value: 0.23 - .51) and no significant difference was observed in any marker or tissue.

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Chapter 5: Influence of chronic *Helicobacter pylori* infection on cognition

5.1 Introduction

Expression of inflammatory cytokines within the central nervous system has been shown to play various roles in disease-associated cognitive dysfuntion[12,14,37,46]. The development of aspects of sickness-like behavior has been tied to IL-1, IL-6 and TNF-α levels[24,25,42]. Inflammatory cytokines have been linked to elevated risk in the development of a wide range of neuropsychiatric disorder, ranging from schizophrenia to depression to dementia[5,7,26-28,37,46,48].

In mice, chronic *H. pylori* infection was associated with abnormal changes in feeding pattern which persisted post-eradication, indicating that chronic *H. pylori* infection may result in permanent alterations to gut-brain pathways[1]. Several studies have proposed an association between Alzheimer Disease associated dementia[23] with eradication of active infection associated with improvement in survival[20] and cognitive performance[21,22]. It was hypothesized that *H. pylori* associated atrophic gastritis led to the development of vitamin B12 deficiency which allowed the elevation of circulating homocysteine levels resulting in mild neuro-inflammation[22]. A negative association was observed between *H. pylori* seropositivity and IQ score in school aged children in an Israeli cohort[36]. The observed effect was found to be independent of socioeconomic and nutritional status, eliminating the two most commonly cited variables for detrimental cognitive effects. Finally, in an epidemiological study among US adults, *H. pylori* seropositivity was associated with reduced performance during cognitive testing[2].

To evaluate the influence of chronic *H. pylori* infection on cognition, cognitive behavioral testing was conducted after 26-week long *H. pylori* G27 infection studies. Eradication studies were utilized to evaluate the reversibility of any cognitive effect associated with chronic *H. pylori* infection. Cognitive behavioral testing was conducted

in collaboration with Dr. Joshua M. Gulley's research group at the University of Illinois at Urbana-Champaign.

5.2 Materials and Methods

Animal care. All experiments involving the use of live vertebrate animals were conducted with the approval of the University of Illinois at Urbana-Champaign Institutional Animals Care and Use Committee (IACUC). Rat populations were composed of male Sprague-Dawley rats (Envigo, formerly Harlan Laboratories, Indianapolis, IN). Animals were single housed with 12 h light-dark cycle, mean temperature of 22.1°C, humidity of 50%, and allowed food and water *ad libitum*.

Establishment of *H. pylori* infections. Male Sprague-Dawley rats (Envigo), aged 19-21 days, were orally fed 3.75 mg/kg lansoprazole (Sigma Aldrich) suspended in 20% sucrose solution once daily for 7 days. On days 3 and 5 of treatment, animals received 0.5 ml of mid-log phase *H. pylori* culture diluted to $2x10^7$ cells/ml (active *H. pylori* infection) or 0.5 ml of sterile BSFB media (mock infected controls) by oral gavage using a 20 G disposable feeding needle (Thermo Fisher). Inoculum dosage was estimated by hemocytometer and verified by CFU plating. Length of infection (26-weeks) was defined as the number of weeks following competition of lansoprazole treatment prior to euthanasia and tissue collection. The experimental animal population consisted of 12 *H. pylori* G27 infected animals and 12 mock-infected animals.

Eradication of active *H. pylori* infection. Male Sprague-Dawley rats (Envigo), aged 19-21 days, were orally fed 3.75 mg/kg lansoprazole (Sigma Aldrich) suspended in 20% sucrose solution once daily for 7 days. On days 3 and 5 of treatment, animals received

0.5 ml of mid-log phase *H. pylori* culture diluted to $2x10^7$ cells/ml (active *H. pylori* infection) or 0.5 ml of sterile BSFB media (mock infected controls) by oral gavage using a 20 G disposable feeding needle (Thermo Fisher). Inoculum dosage was estimated by hemocytometer and verified by CFU plating. All animals assigned to the eradication studies underwent *H. pylori* eradication therapy 13 weeks after competition of lansoprazole treatment administered as part of the inoculation procedures. In brief, animals received eradication therapy consisting of 3.75 mg/kg lansoprazole (Sigma Aldrich), 2.25 mg/kg clarithromycin (Sigma Aldrich), and 4.5 mg/kg amoxicillin (Sigma Aldrich) suspended in 30% sucrose solution orally twice daily for 10 days. Animals were then allowed to remain cleared of *H. pylori* infection for an additional 13 weeks.

Behavioral testing. *H. pylori* infected rats and controls underwent tests for anhedonia (Saccharin preference test), exploratory activity (open-field test), and cognition (object location and T-maze tasks). These were given in succession and occurred over a period of 16 days. For the saccharin preference test, rats were given 24-hr free access in their home cage to two identical 100 mL glass sipper tubes, one containing 0.025% saccharin solution and the other containing water. On the next day, both liquids were replenished and the tubes were returned to the cage with their positions reversed; rats then had free access to the tubes for another 24 hr. The tubes were weighed before and after consumption each day. For the open-field test, rats were placed in one of four open-top circular chambers (60cm in diameter x 60cm high) located next to each other. Rats received a series of two, 10 min open-field tests where they could freely explore the open-field; tests were separated by a 60-min break in the home cage. Activity was recorded and analyzed using AnyMaze software (Stoelting; Wood Dale, IL). All chambers were wiped down with 70% ethanol solution between sessions to prevent cross-contamination. At the end of the day, each chamber was sanitized with 10%

bleach and then chambers were rinsed with 70% ethanol. For the object location test, rats were returned to the circular chambers and two identical objects (13cm x 7.5cm x 7.5cm glass mason jars with handles, filled halfway with black glass beads) were attached to the chamber floor with Velcro. Rats were allowed to interact with the jars freely during a 5-min sample phase after which they were returned to their home cage. During this time the jars were cleaned with ethanol and the position of one of the jars was changed. After 60-min elapsed, rats were returned to the chamber for a 3-min test phase where interaction with the moved and unmoved jars was assessed. After each test, all chambers and objects were thoroughly wiped down with bleach and ethanol in the same manner as in the open-field task.

For the T-maze task, rats' body weights were reduced to 85% of their free-fed weight across a 7-day period by limiting their daily food ration. Next, animals received 2 days of habituation in one of two identical T-mazes (Maze Engineers, Glenview, IL; dimensions: arms: 65cm x 10cm x 15cm, starting runway: 95cm x 10cm x 15cm). During habituation each arm of the maze was baited with a sucrose pellet (Bioserv, #F06233; Frenchtown, NJ) and rats were allowed to freely explore both of the arms and the starting runway for 5 min. Next rats received daily "choice" training sessions consisting of 20 trials where both arms were baited during 50% of trials. For each trial, rats were removed from their home cage and placed at the base of the starting runway. Trials terminated when rats either chose an arm (all 4 paws in the arm), attempted to leave the maze, or 60 sec elapsed. If rats did not make a choice, or if they failed to investigate the recessed pellet receptacle after making a choice, the trial was scored as an error. Rats were trained to a criterion of ≤ 5 errors in a single session and were required to complete at least two choice training sessions before moving on to response training. For response training, only one arm (e.g., right) was baited during each trial and the position

of the baited arm was constant during every trial in a session (but randomly assigned to each rat). Trials where rats chose the baited arm were scored as correct, while trials where the rat chose the other arm were scored as incorrect. During the first response training session, criterion was met once an animal made ten correct choices in a row (60 trials maximum/day). The day after meeting criterion, rats received the first of two reversal tests. For the first test, the previously unbaited arm (e.g., left) was baited and rats completed 60 trials, regardless of performance. On the next day, rats received an additional response training session that terminated after they met a criterion of 10 consecutive correct responses. The second reversal test was performed on the next day wherein the position of the baited arm was changed to the opposite arm and rats completed 60 trials.

Terminal collection of tissue samples. At experimental end points, whole stomach tissue, liver, spleen and brain tissues along with large volume blood samples were collected. In brief, animals were placed under surgical plane anesthesia by intraperitoneal administration of 100 mg/kg ketamine (Med Vet International, Mettawa, IL) and 10 mg/kg xylazine hydrochloride (Sigma Aldrich). Cardiovascular function was stabilized by intramuscular injection of 0.05 mg/ml atropine sulfate (Med Vet International). Animal's abdominal and thoracic cavity were opened and cardiac puncture blood draw conducted via the left ventricle using a 19 G needle. Collected blood was immediately transferred to EDTA anti-coagulated blood collection tubes (Becton, Dickinson, and Company, Franklin Lake, NJ). A 4-0 silk braided suture (MYCO Medical, Apex, NC) was used to tie a loop around the duodenum just inferior to the pyloric sphincter. The stomach was then cut free of the gastrointestinal tract just inferior to the suture and just superior to cardiac sphincter. The stomach was immediately

placed in in cold PBS prior to processing. Whole liver, spleen and brain tissue were collected by necropsy and placed into cold PBS prior to processing.

Sample processing. Blood samples were centrifuged 10 min at 1500 x *g* at room temperature to separate plasma. Plasma was centrifuged a second time for 10 min at 2000 x *g* at room temperature to remove platelets. Plasma was aliquoted at 200 µL and stored at -20°C until assayed. 200 mg pieces of liver and spleen tissue as well as the cerebellum and hippocampus from brain tissue were sterilely collected from whole tissue and frozen at -20°C. Stomachs were opened along the lesser curvature and thin strips representing the greater curvature of the stomach were collected and set aside for histology. Remaining gastric tissue was processed for determining bacterial load. After washing for bacterial load determination, 200 mg of gastric tissue was collected and frozen at -20°C.

Determination of bacterial load. Stomach tissue was roughly chopped into 1 cm^2 pieces and washed vigorously in sterile BSFB media for 10 minutes. Resulting wash serially diluted and plated onto Columbia Agar (Thermo Fisher) supplemented with 7% defibrinated sheep's blood, 10 µg/mL vancomycin (Sigma Aldrich), 5 µg/mL trimethoprim (Sigma Aldrich), 5 µg/mL cefsulodin (Sigma Aldrich), and 5 µg/mL amphotericin B (Sigma Aldrich). Plates were incubated for 5 days at 37ºC under microaerophilic conditions (5% CO_2, 10% O_2). Stomach tissue was blotted dry using sterile gauze and weighed. Bacterial load was then determined as CFU per mg of stomach tissue.

Preparation of whole tissue protein lysates. 200 mg of frozen tissue was mechanically homogenized using mortar and pestle with the aid of liquid nitrogen.

Homogenized tissue was suspended in 2 mL of RIPA buffer (Thermo Fisher) supplemented proteinase inhibitor cocktail (Thermo Fisher). Cells were lysed on ice with gentle shaking for 3 hr. Unlysed tissues and lipid fraction were removed by centrifugation at 1500 x *g* for 15 min at 4°C. Total protein concentration for tissue lysates was determined by BCA assay (Thermo Fisher). Tissue lysates were aliquoted at 200 µL and frozen at -20°C until assayed.

Cytokine quantification. Tissue lysate was diluted to 500 µg/ml to standardize the amount of protein assayed across sample. Cytokine quantification was determined by quantitative ELISA for TNF-α (Eagle Bioscience, Nashua, NH), IL-6 (Eagle Biosciences), and CRP (Sigma Aldrich) according to manufacturer's directions.

Statistical analysis. Cytokine levels from active infection studies and eradication studies were evaluated by two-way ANOVA of *H. pylori* infection status and tissue type with Tukey's correction for multiple comparisons. Saccharin preference, open field behavior, and T-maze testing were evaluated by 3-way ANOVA for *H. pylori* status, eradication therapy exposure, and testing session with Tukey's (open field behavior) or Sidak's (T-maze) multiple comparison test. Object location test was evaluated by paired student's *t* test. An alpha level of 0.05 was used as a threshold for statistical significance and all error bars represent standard error of the mean (SEM). Graphpad Prism (version 6.01) was used for all statistical analyses.

5.3 Results

5.3.1 Confirmation of *H. pylori* associated inflammation in cognitive experimental population.

Bacterial load from Sprague-Dawley rats actively infected with *H. pylori* G27 was determined following euthanasia of the animals after completion of cognitive behavioral testing (**Figure 5.1**). Bacterial load observed in the cognitive experimental population was comparable to that previously observed during inflammation studies. No *H. pylori* colonies were recovered from mock-infected animals. In the cognitive experimental population that underwent eradication therapy, no *H. pylori* colonies could be recovered from *H. pylori* cleared or mock-infected animals. However, *H. pylori* cleared animals tested positive for the presence of circulating anti-*H. pylori* antibodies while mock-infected animals tested negative, indicating the prior infection did occur in *H. pylori* cleared animals.

Inflammatory markers were measured from stomach, liver, spleen, plasma, hippocampus, and cerebellum tissue collect by necropsy. Actively infected animals were found to have a pattern of elevated inflammatory markers similar to those observed in previous 26-week long infection studies (**Figure 5.2**). In animals assigned to the eradication studies, inflammatory markers in *H. pylori* cleared animals were found to be non-significantly different in comparison to those observed in mock-infected animals (**Figure 5.3**).

5.3.2 Chronic *H. pylori* infection is not associated with anhedonia.

As cognitive behavioral testing is reliant upon reward seeking behavior, animals were evaluated for anhedonia in response to sweet taste. Performance on the saccharin

preference test was unaffected by either *H. pylori* infection or by eradication of the infection with antibiotics (**Figure 5.4**; all $p > 0.1$).

5.3.3 *H. pylori* eradication therapy in mock-infected rodents is associated with increased open field behavior.

Open field behavior was utilized to evaluate general locomotor activity levels and anxiety levels in the experimental animal populations[6,17,50]. For distance traveled in the open field chamber, there was an effect of trial ($p < 0.001$) and an interaction between infection and eradication therapy treatment ($p = 0.005$). This interaction was driven by particularly high levels of activity in mock-infected rats that received eradication therapy treatment (**Figure 5.5**). While the reason remains unclear as to why exposure to eradication therapy in mock-infected animals was associated with increased activity levels, such observations have been reported in other studies utilizing ß-lactam antibiotics in rodents[51].

5.3.4 Chronic *H. pylori* infection is not associated with changes in performance in object location memory.

Object location memory testing evaluates spatial memory in the animals[9]. Animal are introduced to two identical objects for a fixed period of time, and after a delay, exposed again to the same two objects, one of which has been moved to a new location. Animals that remember the previous exposure are expected to spend more time exploring the object in the new position. No effect of infection or eradication on the preference for the moved object (**Figure 5.6**; all $p > 0.8$). In addition, there was no evidence that the rats spent more time investigating the moved object, relative to the

unmoved object (one sample t-test vs 0.5, all $p > 0.4$). these data indicate that chronic *H. pylori* infection is not associated with direct impairment of spatial memory.

5.3.5 Chronic *H. pylori* infection is associated with lasting and irreversible impairment of learning memory and behavioral flexibility.

T maze tasks measure an integration of hippocampal driven memory and prefrontal cortex mediated decision making[10,45,47,49,52]. Performance was assessed during response training sessions and reversal tests (**Figures 5.7**). Infection affected the number of incorrect choices made across both training and test sessions ($p = 0.028$) and the number of incorrect responses also depended on session ($p < 0.001$). When incorrect response training sessions were examined alone, an effect of session ($p < 0.001$) and an interaction between *H. pylori* infection, antibiotic treatment, and session ($p = 0.048$) was found. This interaction was driven by the high level of incorrect choices during the first training session in *H. pylori* rats who had their infection eradicated with antibiotics. When reversal sessions alone were examined; however, the effect of infection failed to reach significance ($p = 0.073$) and performance improved by the second test session ($p < 0.001$).

Because the number of completed trials varied by rat, accuracy was also examined (**Figure 5.7**). Overall accuracy across the 4 days was affected by infection ($p = 0.016$) and session ($p < 0.001$). Within response training sessions, accuracy was affected by a main effect of session ($p < 0.001$) and an interaction between infection and session ($p = 0.031$). The interaction was driven by poorer performance in the *H. pylori* rats, regardless of whether the infection had been treated with antibiotics, during the second retraining session (session 3). Accuracy during the reversal tests was affected by main effects of infection ($p = 0.039$) and session ($p < 0.001$). Collectively, these data

suggest that *H. pylori* infection leads to impairment in cognition that is both lasting and potentially irreversible.

5.4 Discussion

While sickness-like behavior is tied to hippocampal IL-6 levels[42] and includes anhedonia and reduced activity levels[3,33], no effect of *H. pylori* infection was detected on either the saccharin preference test or the open-field test. This may be because chronic, rather than acute, infection was modeled. The effects of LPS on anhedonia and activity are acute and fade within 24 hours of injection[3]. A study using infection (e.g., *Calmette-Guérin* bacillus) to model chronic inflammation revealed that anhedonia fades within the first 14 days of infection, while activity levels return to normal within 7 days[19]. However, older mice in that study continued to display signs of sickness for at least 21 days, suggesting that infection may have more serious consequences on behavior in aged populations. Although *H. pylori* infection did not affect activity levels, it is worth noting that antibiotic treatment (the eradication therapy) increased activity levels among mock-infected rats. The reason for this is unclear and antibiotics did not influence saccharin preference or cognitive performance.

Rats that were infected with *H. pylori* displayed cognitive deficits during the acquisition and reversal of the T-maze task and these deficits were present even when the infection had been eradicated for 13 weeks. The cognitive deficits included increased errors during a retraining session, where rats were reinforced for choosing the arm that had been baited the day before, as well as increased errors during the two reversal test sessions. Deficits in hippocampal function can cause poorer performance on both acquisition and reversal on tasks that require spatial memory, including the T-maze task[29,53] and the water maze[43,53]. Hippocampal damage would also be expected to impair

performance on the object location task[38], although no effect of infection was found during object location performance. However, none of the groups examined displayed preference for the moved object (i.e., memory for the unmoved object); therefore, it is difficult to interpret these results. Another region that may be involved in the cognitive deficits is the prefrontal cortex, as lesions to this region can impair reversal learning in both T-maze[29] and operant[35,44] tasks. Taken together, these results suggest that *H. pylori* leads to cognitive deficits through lasting and irreversible damage to the hippocampus and/or prefrontal cortex.

These effects on cognition are enduring, as rats who had undergone the eradication regimen no longer displayed elevated inflammation within the hippocampus but they still displayed cognitive deficits. One recent study examined the long-lasting effects of early-life inflammation and reported that a single dose of LPS, when administered during the juvenile period, caused depressive-like behavior and greater sensitivity to inflammation in adult mice[8]. This is consistent with the theory that juveniles and adolescents may be especially sensitive to the effects of stress and neuro-inflammation on disrupting hippocampal and prefrontal development[13,34]. Rats in this study were infected with *H. pylori* during adolescence and maintained high levels of inflammation until their infection was eradicated, so it could be that the eradication group's deficits are caused by the early-life exposure to neuro-inflammation. This theory could be tested in a follow-up group where infection occurs during adulthood. Our results highlight the need for early intervention, as later eradication may not be enough to reverse the damage caused by early infection.

Finally, these findings raise questions regarding current established standards of care in regard to management of *H. pylori* infection in pediatric populations. Risk of the establishment of chronic *H. pylori* infection during childhood has been found to be

inversely related to socioeconomic status[11,30-32,41]. Furthermore, low socioeconomic status has been found to be a tremendous burden during cognitive development in children[4,16,18,39,40]. Current standards of care recommend testing of children for chronic *H. pylori* infection only in the cases of recurrent gastric complaint or active ulcer disease and eradication of *H. pylori* only in children with active ulcer disease[15]. The "watchful vigilance" approach espoused by the current standards of care may be placing these vulnerable populations at elevated of lasting detrimental cognitive effects. As such, these findings add to a growing body of work that suggest that the extra-gastric influence of chronic *H. pylori* infection must be considered in determination of public health policies.

5.5 Figures

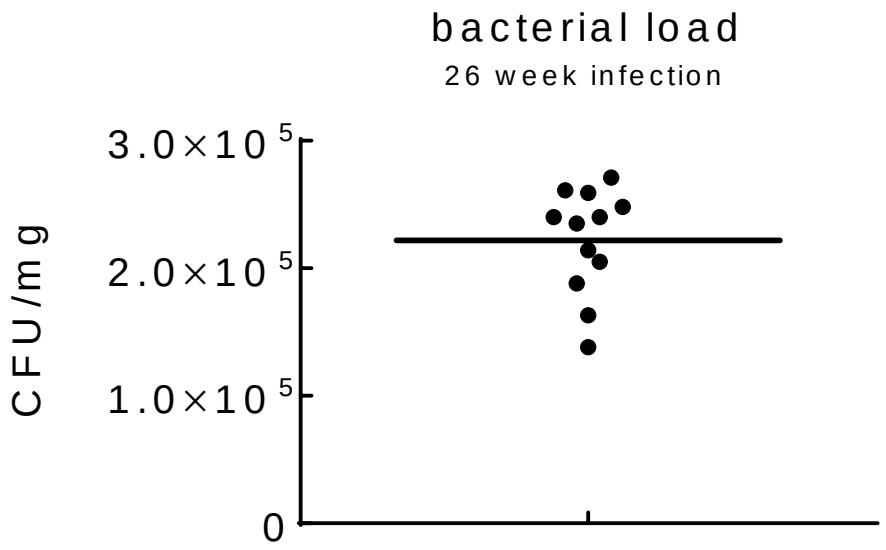

Figure 5.1. Bacterial load of *H. pylori* G27 from animals actively infected for 26 weeks that were utilized in cognitive behavioral testing. Whole stomach tissue was cut into 1 cm2 pieces and washed vigorously in sterile media prior to plating on *H. pylori* selective medium (Columbia Blood Agar supplemented with vancomycin (10 mg/mL), trimethoprim (5mg/mL), cefsulodin (5 mg/mL), and amphotericin B (5 mg/mL)). Bacterial load was calculated as number of CFU per mg of gastric tissue. Each filled circle represents the bacterial load of an individual animal (n = 12). Data were collected across 1 experiment, each with 12 infected animals.

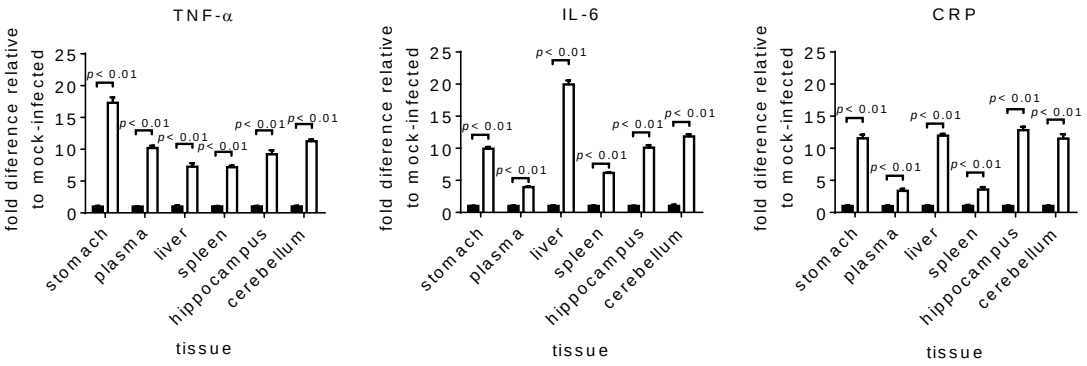

Figure 5.2. Inflammatory profile in cognitive experimental population that were utilized in active infection studies. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP following 26-week long infections utilizing *H. pylori* G27 infection. *H. pylori* associated changes in inflammatory markers are reported as fold difference between actively infected animals relative to mock-infected animals. Data were collected across 1 experiments, each utilizing 12 mock-infected and 12 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Data were analyzed by 2-way ANOVA of *H. pylori* status and tissue type ($p < 0.01$) followed by pairwise comparison's using Tukey's multiple comparison test (p values reported above significant comparisons).

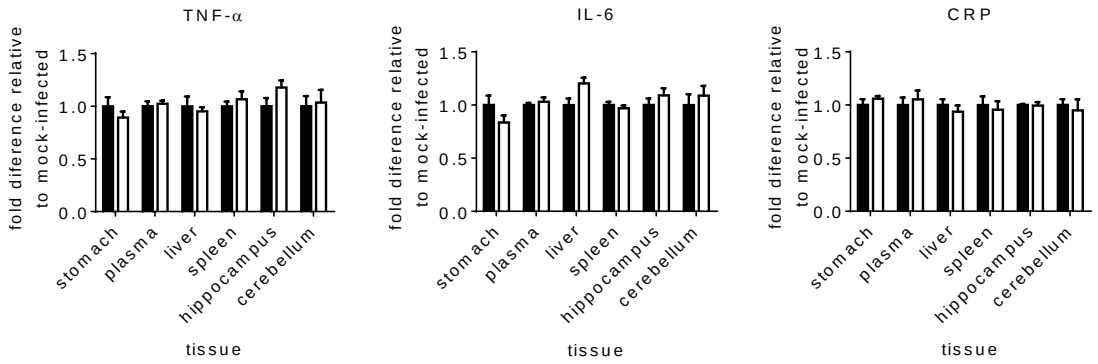

Figure 5.3. Inflammatory profile in cognitive experimental population that were utilized in eradication studies. Quantitative ELISA based assessment of the degree of change of levels of TNF-α, IL-6, and CRP following eradication of *H. pylori* G27 infection. In eradication studies, 13-week infections were carried out in Sprague-Dawley rats utilizing *H. pylori* G27, followed by an eradication regimen (lansoprazole (3.75 mg/kg), clarithromycin (2.25 mg/kg), and amoxicillin (4.5 mg/kg) administered orally, twice daily for 10 days). After 13 weeks following eradication regimen, tissue samples were collected and inflammatory markers measured by quantitative ELISA. *H. pylori* associated changes in inflammatory markers is reported as fold difference between actively infected animals relative to mock-infected animals. Data were collected across 3 independent experiments, each utilizing 3 mock-infected and 3 actively infected animals. Results from mock-infected animals (black bars) were averaged and normalized to 1. Results from actively infected animals (white bars) were averaged and rendered relative to match mock-infected animals. The height of each bar corresponds to the mean difference between inflammatory markers, and the error bars correspond to the standard error of the mean. Significant differences were not observed in levels of TNF-α, IL-6, or CRP, indicating that inflammatory marker levels returned to baseline following eradication of *H. pylori* infection.

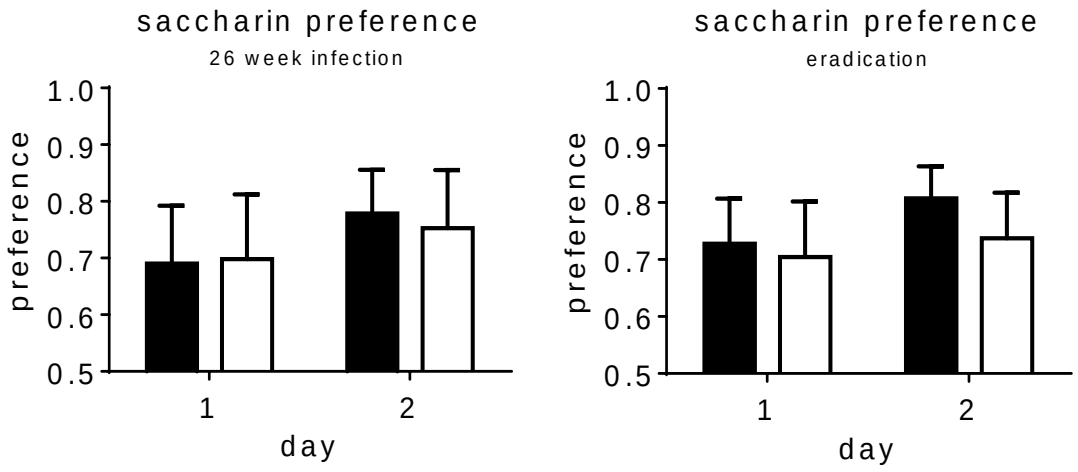

Figure 5.4. Chronic *H. pylori* infection does not induce anhedonia in infected animals. Animals were presented with two pre-measured water bottle: 1) distilled water and 2) distilled water with 0.025% saccharin. Animals were allowed to consume *ab litum* for 24 hours. At that time, the remaining water from each bottle was measured, and the process repeated for a second day. The proportion of saccharin containing water consumed by animals is reported for each treatment groups with mock-infected animals represented by black bars and *H. pylori* infected animal represented by white bars. The height of the bars corresponds to the mean for each group and the error bars represent the standard error of the mean. No significant difference was observed across any of the treatment groups (3-way ANOVA of *H. pylori* status, eradication therapy status, and day: $p > 0.3$), indicating that neither *H. pylori* status nor exposure to *H. pylori* eradication therapy induced anhedonia in the experimental animal populations.

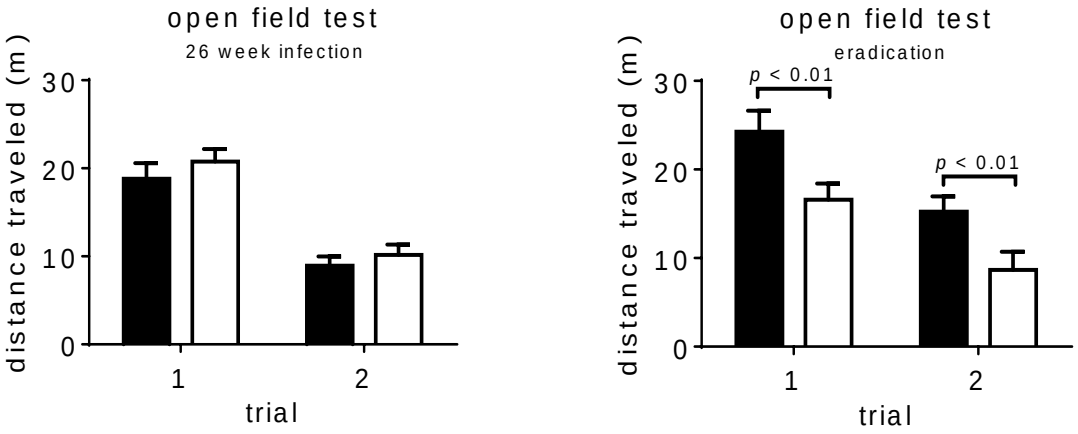

Figure 5.5. *H. pylori* status in not associated with changes in open field behavior. Animals were placed in an open field arena and allowed to freely explore for 10 minutes before being returned to their home cage for 60 minutes after which they were again allowed to explore the open field arena for an additional 10 minutes. Total distance traveled was recorded for each animal during each exposure to the open field arena. Each treatment group was composed of 12 animals with mock-infected animals represented by black bars and *H. pylori* infected animal represented by white bars. The height of each bar corresponds to the mean distance traveled and the error bars represent the standard error of the mean. Data were analyzed by 3-way ANOVA of *H. pylori* status, eradication therapy, and trial (eradication/trial $p < 0.01$) and were followed by pairwise testing by Tukey's multiple comparison test (significant p values are reported above the relevant comparisons), indicating that eradication therapy in mock-infected animals resulted in an unexplained elevation of locomotor activity.

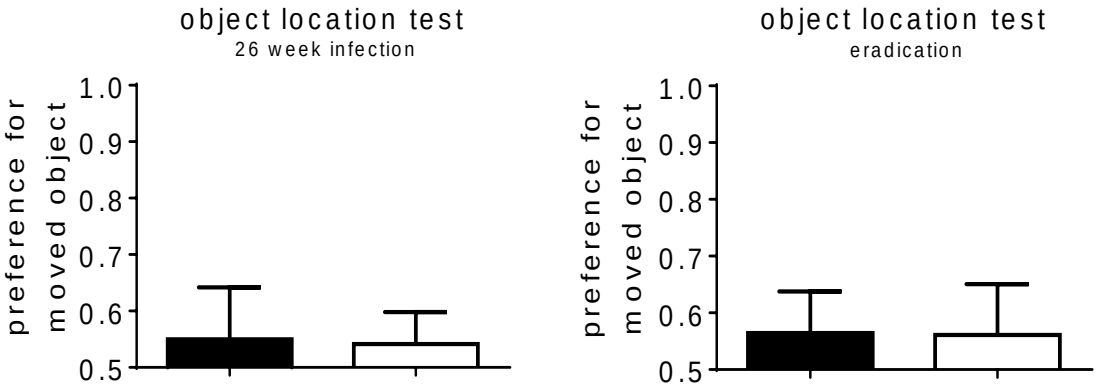

Figure 5.6. Chronic *H. pylori* infection is not associated with changes in performance during object location testing. Two identical objects were placed in the open field arena, and animals were allowed to explore each object for 5 minutes. Animals were then returned to their home cage for 60 minutes. One of the objects was then moved to a new location. The animals were then allowed to explore each object for 3 minutes. The amount of time spent exploring each object was measured and preference calculated as the proportion of time spent exploring the moved object with mock-infected animals represented by black bars and *H. pylori* infected animal represented by white bars. Each treatment group was composed of 12 animals. The height of each bar corresponds to the mean preference for the moved object and the error bars represent the standard error of the mean. Data were analyzed by one sample student's *t* test vs 0.5. No significant difference ($p > 0.4$) was found, indicating that chronic *H. pylori* did not result in a direct impairment of spatial memory (hippocampal activity).

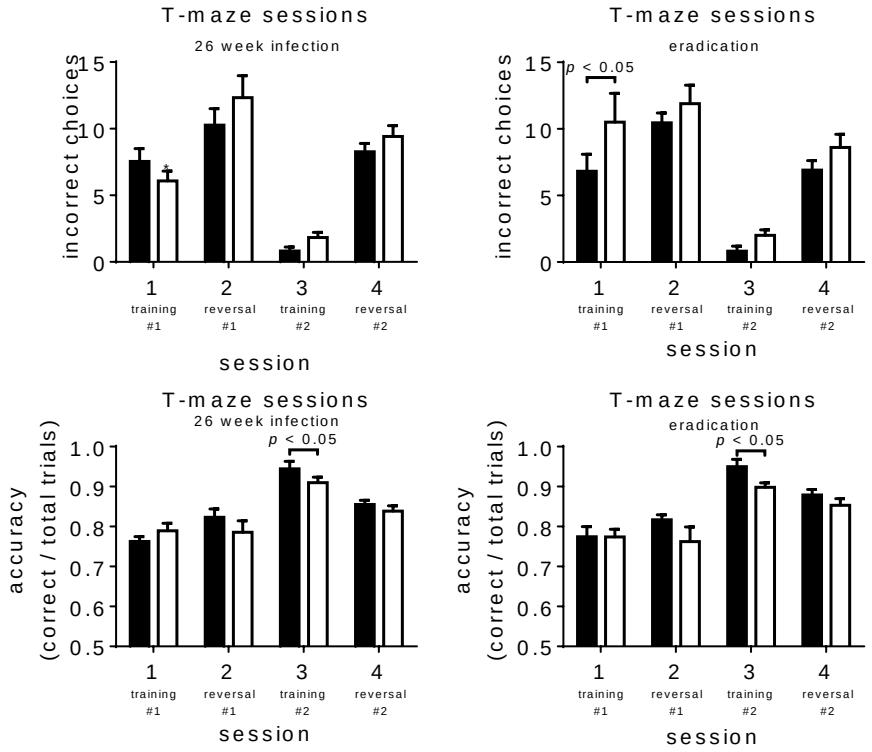

Figure 5.7. Chronic *H. pylori* infection is associated with lasting and irreversible impairment of learning memory and behavioral flexibility. Animals were trained to turn toward a baited arm (e.g., left) on the first day. For the second session, the previously un-baited arm (e.g., right) was baited to test the animal for reversibility of learned behavior. For the third session, the same arm was baited (e.g., right) to test for memory of the reversal. For the fourth session, the initially baited arm was again baited (e.g., left) to again assess behavioral flexibility. Incorrect choices were recorded when the animal chose the un-baited arm. Accuracy was recorded as the fraction of correct choices made by an animal during all trials for a given session. Data were analyzed by 3-way ANOVA of *H. pylori* status, exposure to eradication therapy, and session (session: $p < 0.001$; interaction between *H. pylori* status, exposure to eradication therapy, session: $p = 0.48$). Pairwise comparisons by Sidak's multiple comparison test were significant for accuracy during session #3 for mock-infected vs *H. pylori* infected animals. *p value < 0.01 for the comparison of animals actively infected with *H. pylori* vs *H. pylori* cleared by eradication therapy during session #1 for incorrect choices. These findings suggest that *H. pylori* status (both active infection and cleared by eradication therapy) was associated with lasting and potentially irreversible impairment of hippocampal and/or prefrontal cortex mediated cognition.

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Chapter 6: *Helicobacter pylori* associated iron deficiency

6.1 Introduction

The curious history of *H. pylori* associated iron deficiency anemia began with a single case study involving a 15 year old female patient suffering increasing fatigue and pallor with an acute onset of syncopic episodes[9]. Personal and family history were unremarkable. Patient's diet was normal concerning normal iron intake, and no gastrointestinal complaints were reported. Frank and occult blood loss was absent. Extensive laboratory tests revealed no abnormality in physiology. The patient was prescribed oral iron supplements pending follow-up upper and lower endoscopy exanimations. In the intervening two-week period, the patient's hemoglobin levels did not improve. The patient's lower endoscopy series was normal with no evidence of frank or occult bleeding was observed. Upper endoscopy revealed the presence of superficial gastritis of the gastric antrum. No evidence of frank or occult bleeding was observed. Gastric biopsy samples were positive for *H. pylori* infection. *H. pylori* infection was treated by standard eradication therapy. At a three-week follow-up, the patient's hemoglobin levels showed clear improvement, and at six-week follow-up, the patient's hemoglobin levels had returned to the mean for her age bracket. It was concluded that *H. pylori* infection resulted in a discrete micro-hemorrhagic gastritis in which microscopic bleeds resulted in iron-deficiency anemia[9].

In the decade following, a series of case studies across Europe and Asia would report similar findings of resolution of idiopathic iron deficient anemia following eradication of *H. pylori* infection[4,6,10,11,17,21,22,33,46,52]. In several epidemiology studies, *H. pylori* seropositivity was associated with reduction in iron stores[8,35,37,38,42]. An analysis of cross-sectional data from a German national health survey, *H. pylori* seropositivity was associated with a 17% decrease in serum ferritin levels after controlling for age and sex[8]. In a Danish population-based study, *H. pylori* seropositivity, independent of age, sex,

and socioeconomic status, was associated with a 40% increased risk of having a serum ferritin levels below 30 µg/L (indicative of iron-deficient anemia)[18]. In a large-scale study of Alaskan natives, *H. pylori* seropositivity was associated with an relative risk of 1.13 of low serum ferritin levels, particularly among persons less than 20 years of age[52]. In a study of Korean children aged 6-12 years, *H. pylori* seropositive was associated with a 15 ng/mL and an 11.1% increase in prevalence of iron deficiency anemia[42]. A follow-up study in Korean adolescents found that *H. pylori* associated decreases in iron stores was more common and more pronounced in females as compared to males[12].

Several hypotheses have been put forward to explain the relationship between *H. pylori* infection and iron deficiency anemia. The first proposed mechanism is that of occult blood loss secondary to chronic erosive gastritis. In a descriptive study in Alaskan natives showed high prevalence of iron-deficiency anemia associated with an atypical, grossly hemorrhagic gastritis related to *H. pylori* infection[51]. However, all subjects presented with evidence of elevated fecal hemoglobin levels prior to selection for undergoing endoscopy. Most published case studies and case series have found no evidence of bleeding lesions at time of endoscopy or reported negative fecal occult blood testing among study subjects[3,4,6,16,17,21,22,32]. The preponderance of available evidence, therefore, argues against chronic blood as an etiology for iron deficiency associated with *H. pylori* infection.

A second proposed hypothesis is that increased uptake and utilization of iron by *H. pylori* limits its availability to the host. Many *H. pylori* strains possess a 19 kDa iron-binding protein morphologically and biochemically similar to ferritin[16] which is highly expressed in the presence of iron[48]. Additionally, *H. pylori* possesses several high affinity iron transporters[49]. Another proposed mechanism is *H. pylori*'s capability to acquire iron bound by host derived lactoferrin[19] by a lactoferrin binding receptor that is expressed under iron-deplete conditions[15]. Gastric lactoferrin concentration is increased

during *H. pylori* infection, further suggesting a role for *H. pylori* lactoferrin receptor during infection[13,15]. Several membrane proteins have been described which are capable of binding heme that may also be involved in the uptake of host derived iron[50]. Finally, *H. pylori* derived neutrophil activating protein (NapA) has been shown to act as iron-binding protein[49]. Together these findings demonstrate numerous adaptations by which *H. pylori* may subvert its host's ability to access dietary iron.

A final hypothesis for the mechanism by which *H. pylori* may contribute to iron deficiency is malabsorption secondary to gastritis associated changes in gastric homeostasis. Reduction of ferric iron to ferrous iron is dependent upon the pH of the gastric juice, and ferrous iron facilitates membrane transport and uptake[26]. Absorption of ferrous iron is further mediated by gastric secreted ascorbic acid which complexes with ferrous iron and maintains solubility at the pH range encountered in the duodenum and jejunum[20], where iron absorption occurs[26]. The secretion of ascorbic acid is dramatically reduced during *H. pylori* infection[41,53]. Eradication of active *H. pylori* infection has been shown to improve gastric ascorbic acid levels[5,43].

6.2 Materials and Methods

Animal care. All experiments involving the use of live vertebrate animals were conducted with the approval of the University of Illinois at Urbana-Champaign Institutional Animals Care and Use Committee (IACUC). Animal populations were composed of male Sprague-Dawley rats (Envigo, formerly Harlan Laboratories, Indianapolis, IN). Animals were group housed (3 or 5 animals per cage) with 12 h light-dark cycle, mean temperature of 22.1°C, humidity of 50%, and allowed food and water *ad libitum*. Animals were fed standard rodent chow (Teklad F6 Rodent Diet; Envigo) containing 300 mg/kg iron.

Bacterial strains. Rat-adapted *Helicobacter pylori* G27 (*cag* PAI$^+$, *vacA* s1/m1; NCTC 13282) was cultured in media in bisulfite- and sulfite-free *Brucella* broth (BSFB) supplemented with 5% fetal bovine serum (Sigma Aldrich, St. Louis, MO) for 36 h at 37ºC under microaerophilic conditions (5% CO_2, 10% O_2). Culture was diluted to 2x10^7 cells per mL as estimated by disposable hemocytometer (inCYTO, Seonggeo-gil, South Korea). Dilution was confirmed by serial dilution and spread plating on Columbia Blood Agar (Thermo Fisher, Hanover Park, IL) supplemented with 7% defibrinated sheep's blood (Thermo Fisher), 10 µg/ml vancomycin (Sigma Aldrich), 5 µg/ml trimethoprim (Sigma Aldrich), 5 µg/ml cefsulodin (Sigma Aldrich), and 5 µg/ml amphotericin B (Sigma Aldrich). Plates were incubated for 5 days at 37ºC under microaerophilic conditions (5% CO_2, 10% O_2), prior to confirming bacterial cell density by colony counting.

Establishment of *H. pylori* infections. Male Sprague-Dawley rats (Envigo), aged 19-21 days, were orally fed 3.75 mg/kg lansoprazole (Sigma Aldrich) suspended in 20% sucrose solution once daily for 7 days. On days 3 and 5 of treatment, animals received 0.5 mL of mid-log phase *H. pylori* culture diluted to 2x10^7 cells/ml (active *H. pylori* infection) or 0.5 mL of sterile BSFB media (mock infected controls) by oral gavage using a 20 G disposable feeding needle (Thermo Fisher). Inoculum dosage was estimated by hemocytometer and verified by CFU plating. Length of infection (8-weeks) was defined as the number of weeks following competition of lansoprazole treatment prior to euthanasia and tissue collection. Active infection studies were conducted in two independent blocks each composed of 3 actively infected animals and 3 mock infected animals. Eradication studies were conducted as a single independent block composed of 5 actively infected animals and 5 mock infected animals. Nutritional intervention studies were conducted in a single independent block of actively infected animals

composed of 5 mock intervention animals, 5 iron intervention animals, 5 vitamin C intervention animals, and 5 combined iron and vitamin C intervention animals.

Eradication of active *H. pylori* infection. Animals assigned to the eradication studies underwent *H. pylori* eradication therapy 4 weeks after competition of lansoprazole treatment administered as part of the inoculation procedures. In brief, animals received eradication therapy consisting of 3.75 mg/kg lansoprazole (Sigma Aldrich), 2.25 mg/kg clarithromycin (Sigma Aldrich), and 4.5 mg/kg amoxicillin (Sigma Aldrich) suspended in 30% sucrose solution orally twice daily for 10 days. Prior infection status was confirmed by Instant-View *H. pylori* Rapid Test seropositivity assay (Alpha Scientific, Poway, CA) using whole blood samples collected by terminal cardiac puncture. Successful eradication of *H. pylori* was confirmed inability to culture *H. pylori* from stomach tissue collected during necropsy.

Nutritional intervention. Animals assigned to the nutritional intervention studies received either mock intervention (0.35 mL of 10% sucrose solution), iron supplementation intervention (0.35 mL of ferrous sulfate oral iron supplement; equivalent to 3 mg of elemental iron per animal; GeriCare, Brooklyn, NY), vitamin C supplementation intervention (250 mg/kg ascorbic acid in 10% sucrose solution; Thermo Fisher), or combined iron and vitamin C supplementation intervention (0.35 mL of ferrous sulfate oral iron supplement combined with 250 mg/kg ascorbic acid). Nutritional interventions began the day following competition of lansoprazole treatment and were administered every other day for the duration of the 8-week infection period.

Temporal measurements. Beginning 24 hours before the start of lansoprazole treatment, animals were weighed and hemoglobin and hematocrit were measured. In

brief, animals were transferred into a pre-tared clean mouse cage and weighed on portable balance (Thermo Fisher). Tail veins were dilated by gently warming by hold the animal's tail in closed hand for several minutes. The tail was cleaned and disinfected using a sterile alcohol wipe. Using a #15 scalpel blade a small nick was made transverse across the lateral tail vein. The first drop of blood was discarded. Blood was collected using 2 StateSpin SafeCrit plastic microhematocrit tubes containing heparin (Beckman Coulter, Brea, CA) and 2 Hemocue HB201 hemoglobin microcuvettes (Hemocue America, Brea, CA). Hemoglobin concentration was determined by Hemocue HB201 meter (Hemocue America). Microhematocrit tubes were centrifuge for 6 minutes at 1300 x *g* at room temperature. Hematocrit value was determined by use of a Damon IEC Microcapillary reader (Thermo Fisher).

Terminal collection of tissue samples. At experimental end points, whole stomach tissue and large volume blood samples were collected. In brief, animals were placed under surgical plane anesthesia by intraperitoneal administration of 100 mg/kg ketamine (Med Vet International, Mettawa, IL) and 10 mg/kg xylazine hydrochloride (Sigma Aldrich). Cardiovascular function was stabilized by intramuscular injection of 0.05 mg/ml atropine sulfate (Med Vet International). Animal's abdominal and thoracic cavity were opened and cardiac puncture blood draw conducted via the left ventricle using a 19 G needle. Collected blood was split between EDTA anti-coagulated blood collection tubes (2 mL; Becton, Dickinson, and Company, Franklin Lake, NJ) and serum separation tubes (remainder; Becton, Dickinson, and Company). A 4-0 silk braided suture (MYCO Medical, Apex, NC) was used to tie a loop around the duodenum just inferior to the pyloric sphincter. The stomach was then cut free of the gastrointestinal tract just inferior to the suture and just superior to cardiac sphincter. The stomach was immediately placed in in cold PBS prior to processing. Serum samples were prepared by inverting

tubes 5 times and allowing blood to clot for 30 minutes at room temperature. Samples were then centrifuged for 10 min at 1200 x *g* at room temperature to separate serum. Serum was stored at 4°C until assayed for total serum, unsaturated iron-binding capacity, total iron-binding capacity, serum ferritin, and serum soluble transferrin receptor.

Determination of bacterial load. Stomach tissue was roughly chopped into 1 cm^2 pieces and washed vigorously in sterile BSFB media for 10 minutes. Resulting wash serially diluted and plated onto Columbia Agar (Thermo Fisher) supplemented with 7% defibrinated sheep's blood, 10 µg/mL vancomycin (Sigma Aldrich), 5 µg/mL trimethoprim (Sigma Aldrich), 5 µg/mL cefsulodin (Sigma Aldrich), and 5 µg/mL amphotericin B (Sigma Aldrich). Plates were incubated for 5 days at 37ºC under microaerophilic conditions (5% CO_2, 10% O_2). Stomach tissue was blotted dry using sterile gauze and weighed. Bacterial load was then determined as CFU per mg of stomach tissue.

Determination of RBC indices. Terminal hematocrit and hemoglobin were determined immediately following collection of blood from cardiac puncture. Blood was collected using 2 StateSpin SafeCrit plastic microhematocrit tubes containing heparin (Beckman Coulter, Brea, CA) and centrifuge for 6 minutes at 1300 x *g* at room temperature. Hematocrit value was determined by use of a Damon IEC Microcapillary reader (Thermo Fisher). Blood was collected into 2 Hemocue HB201 hemoglobin microcuvettes (Hemocue America, Brea, CA) and hemoglobin concentration was determined by Hemocue HB201 meter (Hemocue America). Concentration of red blood cells was determined by disposable hemocytometer (inCYTO) using EDTA anti-coagulated blood diluted 1:200 with normal saline. Mean corpuscular volume was calculated as the hematocrit divided by the red blood cell concentration. Mean corpuscular hemoglobin

was calculated as hemoglobin divided by red blood cell concentration. Mean corpuscular hemoglobin concentration was calculated as hemoglobin divided by hematocrit.

Measurement of serum iron, unsaturated iron-binding capacity, and total iron-binding capacity. Total serum iron, unsaturated iron-binding capacity, and total iron-binding capacity were determined from serum samples within 48 hours of collection by ferrozine reaction using Iron/TIBC Reagent Set (Pointe Scientific, Ann Arbor, MI). Briefly, total serum iron was determined by releasing bound iron and reducing it to ferrous ions by use of acidic buffer prior to reaction with ferrozine reagent. Resulting violet colored complex was measured spectrophotometrically at 560 nm using a BioTek Synergy 2 plate reader (BioTek, Winooski, VT). The unsaturated iron-binding capacity was determined by additional of a known quantity of ferrous ions in alkaline buffer. Excess, unbound ferrous ions were then measured by the measured by ferrozine reaction. The difference between the known quantity of added ferrous ions and the measurement taken after binding represented the unsaturated iron-binding capacity. Total iron-binding capacity was calculated total serum iron plus the unbound iron-binding capacity.

Determination of serum ferritin and soluble transferrin receptor. Serum ferritin levels were determined by quantitative rat ferritin ELISA (Abcam, Cambridge, UK) from serum samples within 48 hours of collection. Samples were diluted 1:100 using supplied sample dilution buffer and protocol was followed according to manufactures recommendations. Measurements of absorbance at 450 nm were conducted using a BioTek Synergy 2 plate reader (BioTek). Serum soluble transferrin receptor levels were determined by quantitative rat transferrin ELISA (LifeSpan BioSciences, Seattle, WA).

Samples were diluted 1:100 using supplied sample dilution buffer and protocol was followed according to manufactures recommendations. Measurements of absorbance at 450 nm were conducted using a BioTek Synergy 2 plate reader (BioTek).

Statistical analysis. Active *H. pylori* infection experiments were conducted in two independent blocks with each block composed of 3 mock infected and 3 actively infected animals. All data was mean and variance tested prior to combining into a single data set from final analyses. Eradication studies were conducted as a single independent block composed of 5 mock infected and 5 actively infected animals. Nutritional intervention studies were conducted as a single independent block composed of 20 actively infected animals, 5 of which were assigned to mock intervention, 5 to iron supplementation intervention, 5 to vitamin C supplementation intervention, and 5 to combine iron and vitamin C supplementation intervention. Growth rates of animals were assessed by non-linear regression of weight data. Active infection and eradication studies were evaluated by two-way ANOVA (*H. pylori* status vs eradication therapy) with subsequent pairwise testing by Tukey's multiple comparison test. Nutritional intervention studies were evaluated by one-way ANOVA with subsequent pairwise testing by Tukey's multiple comparison test. Experiments were designed such that a family-wise alpha was maintained at 0.05 and overall power was maintained at 0.80 for determination of statistical significance. Graphical figures were generated in Graphpad Prism (v6.01; Graphpad Software, La Jolla, CA). All statistical analyses were conducted in SAS (v.9.3; SAS Institute, Cary, NC).

6.3 Results

6.3.1 *H. pylori* infection is associated with an iron deficient state.

Following 8 week long infections utilizing *H. pylori* G27, bacterial load was determined to be $1.83 \times 10^5 \pm 7.4 \times 10^3$ CFU/mg of gastric tissue, comparable levels to the bacterial load observed during previous studies utilizing 8 week long infections. Time series data indicate the initial hemoglobin and hematocrit response to infection by *H. pylori* is a mild increase in both markers (**Figure 6.1**). These findings are hypothesized to occur during to transient behavioral changes associated with the initial stages of *H. pylori* infection. During periods of acute distress, in this case dyspepsia associated with initial colonization of the gastric mucosa by *H. pylori*, rats will naturally reduce and/or abstain from normal feeding and drinking behaviors[7]. In turn, the development of transient dehydration results in the temporary elevation of both hemoglobin and hematocrit. However, analyses of growth rate (**Figure 6.2**) indicate that such behavioral changes are highly transient as no perturbation is observed in growth rate of actively infected animals relative to mock infected. Two weeks after completion of lansoprazole therapy, hemoglobin and hematocrit levels in actively infected animals were significantly lower than those of mock infected animals (**Figure 6.1**) and remained reduced throughout the remaining experimental period.

Following the collection of blood via terminal cardiac puncture, standard red blood cell indices were calculated for actively infected and mock infected animals (**Table 6.1**). Hemoglobin, hematocrit, red blood cell concentration, and mean corpuscular hemoglobin concentration were found to be significantly reduced in actively infected animals relative to mock infected animals. However, mean corpuscular volume, and mean corpuscular hemoglobin were not significantly affected by active *H. pylori* infection. These data indicate that active *H. pylori* infection is associated with a normocytic, normochromatic reduction in iron levels consistent with iron deficiency.

Further supporting the hypothesis that *H. pylori* infection is associated with an iron deficient state, total serum iron (the amount of iron freely present in the serum as well as that released by acid hydrolysis of serum proteins) is significantly reduced (**Table 6.2**). The unsaturated iron binding capacity and total iron binding capacity are significantly elevated while transferrin saturation is significantly reduced in actively infected animals. To verify these observations, serum ferritin and soluble transferrin receptor levels were determined by quantitative ELISA. Serum ferritin was found to be significantly reduced in actively infected animals (**Table 6.3**) to approximately 77% of the levels seen in mock infected animals. Conversely, soluble transferrin receptor levels were found to be significantly increased in actively infected animals (**Table 6.3**) to approximately 160% of the levels seen in mock infected animals.

Ultimately, these data support a model in which actively *H. pylori* infection is associated with development of an iron deficient state, persisting throughout the course of infection. This iron deficient state presents with the hallmark signs of a normocytic, normochromatic iron deficient state.

6.3.2 Eradication of *H. pylori* infection leads to rapid recovery from associated iron deficient state.

To determine if active *H. pylori* infection is causative for *H. pylori* associated iron level reduction, eradication studies were conducted. During these studies animals were either actively infected with rat-adapted *H. pylori* G27 or mock infected with sterile bacterial culture media. Four week after completing inoculation procedures, all animals underwent standard *H. pylori* eradication therapy consisting of 3.75 mg/kg lansoprazole, 2.25 mg/kg clarithromycin, and 4.5 mg/kg amoxicillin administered twice daily for 10 days. Following completion of eradication therapy, monitoring of animals continued until the completion of the 8-week study. After terminal blood collection, animals were tested

for seropositivity to *H. pylori*. All animals which were part of the actively infected group yielded a positive result (presence of anti-*H. pylori* antibodies) while those animals which were mock infected yielded a negative result. Additionally, *H. pylori* could not be recovered from the stomachs of either set of animals, indicating that *H. pylori* infection in the animals assigned to the active infection group was successfully cleared by eradication therapy.

As seen in previous active infection studies, *H. pylori* infection was associated with a transient increase in both hemoglobin and hematocrit followed by significant reduction in actively infected animals after approximately two weeks of infection (**Figure 6.1**). Again, the initial observation of an increase in hemoglobin and hematocrit is hypothesized to result from distress induced behavioral changes relating to food and water consumption which rapidly subside following the establishment of *H. pylori* infection as neither *H. pylori* status or administration of eradication therapy was associated with changes in growth rate (**Figure 6.2**). The reduction in hemoglobin and hematocrit observed during the active infection stage of these eradication studies were comparable to those seen in non-eradication studies at equivalent time points.

In *H. pylori* infected animals, hemoglobin and hematocrit levels began to show signs of recovery at approximately the mid-point of the eradication therapy regimen. Approximately 3 weeks after initiation of eradication therapy, hemoglobin and hematocrit levels of *H. pylori* infected animals were no longer significantly different than mock infected control animals (**Figure 6.1**). Evaluation of standard red blood cell indices showed no significant difference between animals in which *H. pylori* infection was cleared and mock infected animals (**Table 6.1**). Evaluation of total serum iron, unsaturated iron binding capacity, total iron binding capacity, and transferrin saturation revealed no significant difference between animals in which *H. pylori* infection had been cleared and mock infected animals (**Table 6.2**). Furthermore, serum ferritin and soluble

transferrin receptor levels were not significantly different between these two experimental groups (**Table 6.3**). Collectively, these data support the hypothesis that *H. pylori* infection is indeed the causative factor

6.3.3 *H. pylori* associated iron deficiency is malabsorptive in nature.

To evaluate whether the relationship between iron deficiency and chronic *H. pylori* infection is nutritional intervention studies were conducted over the course of 8 week long infections. Hemoglobin levels from infected animals receiving high dose oral iron supplementation (3 mg every other day) were monitored through time in comparison to those obtained from infected animals receiving placebo intervention (an equivalent volume of sterile 10% sucrose solution). Difference in bacterial load was to be insignificant with $1.79 \times 10^5 \pm 4.9 \times 10^3$ CFU/mg of gastric tissue for the placebo intervention and $1.89 \times 10^5 \pm 8.5 \times 10^3$ CFU/mg of gastric tissue for the oral iron supplementation intervention. This bacterial loads are comparable to those observed during *H. pylori* infection in the absence of any intervention. No significant difference in hemoglobin levels were observed throughout the 8-week long infection (**Figure 6.3**). No significant difference was observed in the growth rate of animals during the course of the 8-week infection. Evaluation of red blood indices following euthanasia of animals showed no significant difference between placebo intervention and oral iron supplementation intervention (**Table 6.4**). Likewise, no significant difference was observed in total serum iron, unsaturated iron binding capacity, total iron binding capacity, and transferrin saturation (**Table 6.5**) or in serum levels of ferritin and soluble transferrin receptor (**Table 6.6)**.

Together, these data indicate that the reduction in iron levels observed during *H. pylori* infection are not a direct consequence of the bacteria outcompeting its host for dietary iron. The amount of iron provided by oral supplementation far exceeds that

which could reasonably by utilized by *H. pylori*; however, no improvement in iron levels in observed in the animals receiving oral supplementation.

6.3.4 *H. pylori* associated iron deficiency results from loss of gastric mucosal associated ascorbic acid levels.

To evaluate the influence on ascorbic acid on *H. pylori* associated iron deficiency, nutritional intervention studies included infected animals which received oral ascorbic acid supplementation intervention (250 mg/kg every other day). Additionally, to determine if synergistic effects existed an additional group of infected animals received both oral iron supplementation (3 mg every other day) and oral ascorbic acid supplementation intervention (250 mg/kg every other day). Difference in bacterial load was to be significantly lower (ANOVA *p* value: < 0.01; Tukey's pairwise comparison test *p* value: < 0.01 for all comparisons) in animal receiving the ascorbic acid intervention with $1.66 \times 10^5 \pm 3.1 \times 10^3$ CFU/mg of gastric tissue in comparison to other intervention groups. However, bacterial load in animals receiving both iron and ascorbic acid supplementation intervention were $1.80 \times 10^5 \pm 7.2 \times 10^3$ CFU/mg of gastric tissue and were not significantly different in comparison to placebo intervention or iron supplementation intervention. Hemoglobin levels were found to be significantly elevated in comparison to animals receiving placebo intervention beginning approximately 1 week after inoculation with *H. pylori* and showed a trend in continual increase throughout the 8-week infection (**Figure 6.3**). No significant difference was observed in the growth rate of animals during the course of the 8-week infection. Red blood cell indices (**Table 6.4**) showed elevated levels of hemoglobin and hematocrit in both intervention group receiving ascorbic acid in comparison to placebo intervention (ANOVA *p* value: < 0.01; Tukey's pairwise comparison test *p* value: < 0.01 for all comparisons). Similarly, red blood cell count, mean corpuscular volume, mean concentration of hemoglobin, and

mean corpuscular hemoglobin concentration were found to be elevated in comparison to placebo intervention (ANOVA *p* value: < 0.01; Tukey's pairwise comparison test *p* value: < 0.01 for all comparisons). Serum iron, total iron binding capacity, and transferrin saturation were significantly elevated while unsaturated iron binding capacity was significantly reduced in comparison to placebo intervention (ANOVA *p* value: < 0.01; Tukey's pairwise comparison test *p* value: < 0.01 for all comparisons; **Table 6.5**). Likewise, serum ferritin levels were significantly elevated while serum levels of soluble transferrin receptor were significantly reduced in comparison to placebo intervention (ANOVA *p* value: < 0.01; Tukey's pairwise comparison test *p* value: < 0.01 for all comparisons; **Table 6.6**).

Together, these data indicate that high dose oral ascorbic acid supplementation is capable of rescuing *H. pylori* associated iron deficiency. Furthermore, these data are indicative that iron supplementation alongside of ascorbic acid supplementation has minimal impact on rescuing *H. pylori* associated iron deficiency. These findings support the hypothesis that gastric ascorbic acid levels play an important role in the development of iron deficiency during chronic *H. pylori* infection.

6.4 Discussion

Iron deficiency is the most common nutritional deficiency worldwide[34,36,45]. It is estimated that approximately two billion people worldwide are nutritionally deficient in iron[44]. Even with affluent nations iron deficiency remains a major public health concern. Within the United States, it is estimated that 10 million individuals are iron deficient, including 5 million who have iron deficient anemia[14]. In comparison, the prevalence of iron deficient anemia is far higher in the nations of Africa (64.6%), Asia (47.7%) and Latin America (39.5%) among children aged birth to 5 years[34].

Iron deficiency during infancy has been associated with reduction in test scores for cognitive, motor, social-emotional, and neurophysiological development in comparison to non-iron deficient infants[27]. Iron deficiency within the first few years of life is linked with impaired development of both auditory and visual centers with subtle impairments persisting for years after correction of the nutritional deficiceny[1,39]. Furthermore, iron deficiency in early childhood has been observed to have long term consequences for cognitive development with detrimental effects from a single episode of iron deficiency anemia during early childhood associated with reduced cognitive performance measured nearly 20 years later[28].

All animals infected with *H. pylori*, with the exception of those receiving oral ascorbic acid supplementation, rapidly developed iron levels below those observed in mock infected animals. Eradication of active *H. pylori* infection was followed by return of depressed iron levels to normal values. As such, these studies support not only that chronic *H. pylori* infection is associated with an iron deficient state but also that *H. pylori* infection is an etiological agent of the observed iron deficient state. While no infected animal developed clinical anemia during the course of these 8-week infection studies, infected animals did present with marginal iron deficiency which has been shown to have detrimental influence on cognitive development in rodent models[23-25]. Therefore, it is likely that *H. pylori* associated iron deficiency poses a risk factor in the potential development of detrimental cognitive effects.

Finally, it must be noted that, like *H. pylori* infection[29-31], the prevalence of iron deficiency shows clear disparity along socioeconomic standing with higher prevalence among those of lower standing[2,28,40,47]. As in the case of impairment of cognition, chronic *H. pylori* likely acts as an additional burden on these populations, placing them at elevated risk of developing iron deficiency.

6.5 Figures and Tables

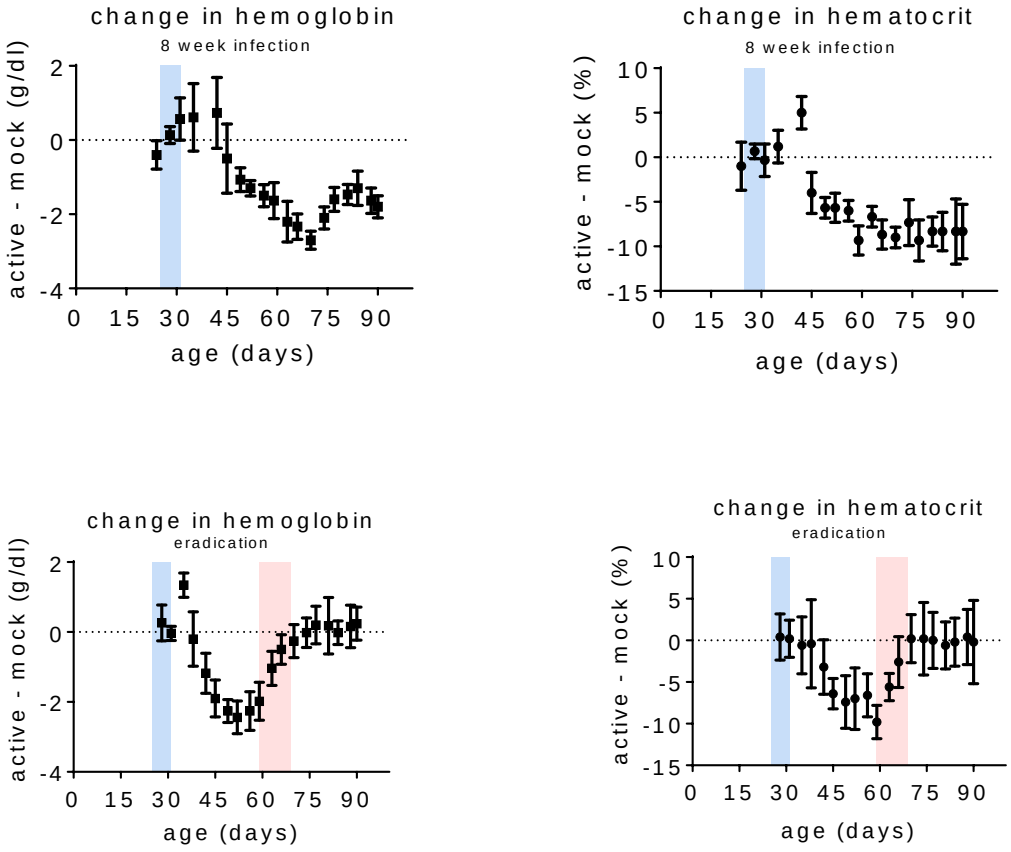

Figure 6.1. Active *H. pylori* infection is associated with a reduction in hemoglobin and hematocrit levels. Time series data for hemoglobin and hematocrit levels measured from tail vein blood during chronic *H. pylori* G27 infection. Infection experiments were carried out for 8 weeks (inoculation procedure period highlighted in blue). Animals assigned to the eradication therapy group were infected for 4 weeks prior to the administration of *H. pylori* eradication (eradication therapy highlighted in red). Eradication therapy regimen consisted of lansoprazole (3.75 mg/kg), clarithromycin (2.25 mg/kg), and amoxicillin (4.5 mg/kg) administered orally, twice daily for 10 days. Experimental populations consisted of 6 mock-infected and 6 *H. pylori* G27 animals infected for the 8-week infection group and 5 mock-infected and 5 *H. pylori* cleared animals for the eradication group. The mean difference between mock-infected and *H. pylori* infected animals is represented by a circle and the error bars correspond to the 95% confidence interval. The data indicate that active *H. pylori* infection is associated with a significant reduction in hemoglobin and hematocrit levels and that clearance of active *H. pylori* infection is associated with recovery of hemoglobin and hematocrit levels.

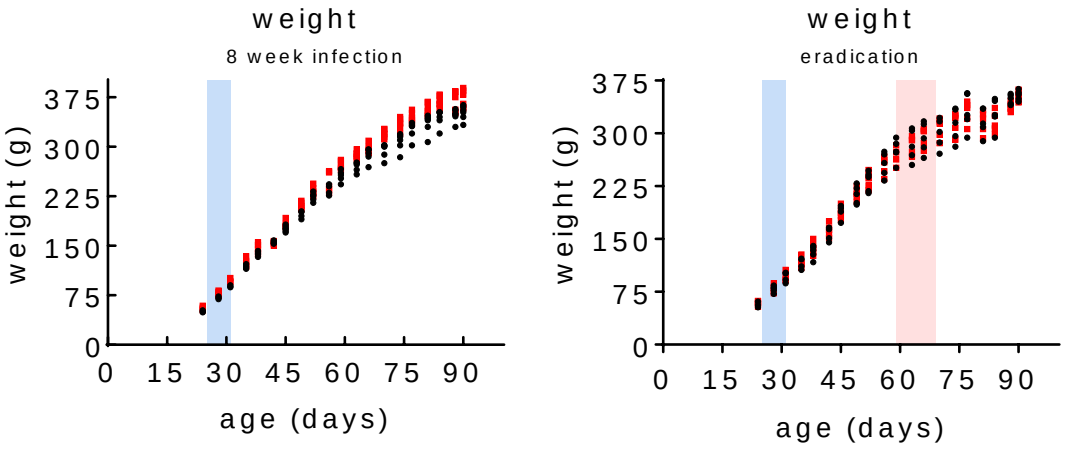

Figure 6.2. Growth rate is not influenced by *H. pylori* infection or eradication therapy. Weight measurements were conducted at each blood collection throughout the infection experiments. Infection experiments were carried out for 8 weeks (inoculation procedure period highlighted in blue). Animals assigned to the eradication therapy group were infected for 4 weeks prior to the administration of *H. pylori* eradication (eradication therapy highlighted in red). Eradication therapy regimen consisted of lansoprazole (3.75 mg/kg), clarithromycin (2.25 mg/kg), and amoxicillin (4.5 mg/kg) administered orally, twice daily for 10 days. Experimental populations consisted of 6 mock-infected and 6 *H. pylori* G27 animals infected for the 8-week infection group and 5 mock-infected and 5 *H. pylori* cleared animals for the eradication group. Each point represents the weight of an individual animal. Mock-infected animals are represented by black circles, and *H. pylori* infected animals by red squares. Data were analyzed by logistic regression analysis and no significant difference was found among the 4 experiments groups ($p > 0.4$), indicating the neither *H. pylori* infection nor eradication therapy influenced the growth of the animals.

	8 week infection		eradication	
	mock-infection n = 6	*H. pylori* G27 n = 6	mock-infection n = 5	*H. pylori* G27 n = 5
Hb (g/dL)	14.9 (0.2)	**13.1 (0.1)**	14.7 (0.2)	14.9 (0.3)
HCT (%)	53.3 (2.1)	**43.3 (0.6)**	55.4 (1.5)	54.2 (1.8)
[RBC] (M/µL)	7.1 (0.2)	**6.2 (0.1)**	7.1 (0.2)	7.0 (0.3)
MCV (fL)	75.7 (2.4)	74.0 (3.5)	78.4 (3.9)	77.3 (5.8)
MCH (gHB/M cells)	21.1 (0.8)	21.2 (0.2)	20.6 (0.6)	21.1 (1.1)
MCHC (gHb/dL)	28.0 (1.1)	**30.2 (0.5)**	26.3 (0.7)	27.3 (1.3)

Table 6.1. Chronic *H. pylori* infection is associated with changes to red blood cell indices consistent with an iron deficient state. Red blood indices for hemoglobin (Hb), hematocrit (HCT), red blood cell count ([RBC]), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured following terminal blood collection from the experimental animals. Data were by ANOVA ($p < 0.01$) followed by Tukey's pairwise multiple comparison test. Measurements significantly different ($p < 0.01$) from the mock-infected group are denoted in bold. These data indicate that *H. pylori* infection is associated with hematological changes consistent with iron deficiency in the 8-week infection group.

	8 week infection		eradication	
	mock-infection n = 6	*H. pylori* G27 n = 6	mock-infection n = 5	*H. pylori* G27 n = 5
total Fe (µg/dL)	165.7 (4.1)	**128.3 (3.7)**	164.6 (4.2)	161.2 (3.8
UIBC (µg/dL)	216.3 (9.3)	**288.3 (11.5)**	223.4 (7.4)	225.9 (10.0)
TIBC (µg/dL)	381.9 (8.6)	**416.7 (10.1)**	389.0 (10.0)	385.1 (9.5)
transferrin saturation (%)	0.43 (0.01)	**0.31 (0.02)**	0.42 (0.01)	0.41 (0.01)

Table 6.2. *H. pylori* infection is associated with reduction in total serum iron and transferrin saturation. Total serum iron (total Fe), unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), and transferrin saturation were measured following terminal blood collection from experimental animals. Data were by ANOVA ($p < 0.01$) followed by Tukey's pairwise multiple comparison test. Measurements significantly different ($p < 0.01$) from the mock-infected group are denoted in bold. These data indicated that active *H. pylori* infection is associated with changes to red blood cell indices consistent with iron deficiency.

	8 week infection		eradication	
	mock-infection n = 6	*H. pylori* G27 n = 6	mock-infection n = 5	*H. pylori* G27 n = 5
serum ferritin (pg/mL)	8126 (190)	**6270 (255)**	8260 (510)	8302 (654)
soluble transferrin receptor (pg/mL)	5721 (610)	**9180 (1123)**	5500 (243)	5517 (177)

Table 6.3. Chronic *H. pylori* infection is associated with physiological changes consistent with iron reserve depletion. Serum ferritin and soluble transferrin receptor levels were measured by quantitative ELISA following terminal blood collection from experimental animals. Data were by ANOVA ($p < 0.01$) followed by Tukey's pairwise multiple comparison test. Measurements significantly different ($p < 0.01$) from the mock-infected group are denoted in bold. These data indicated that active *H. pylori* infection is associated with physiological responses consistent with depletion of iron reserves.

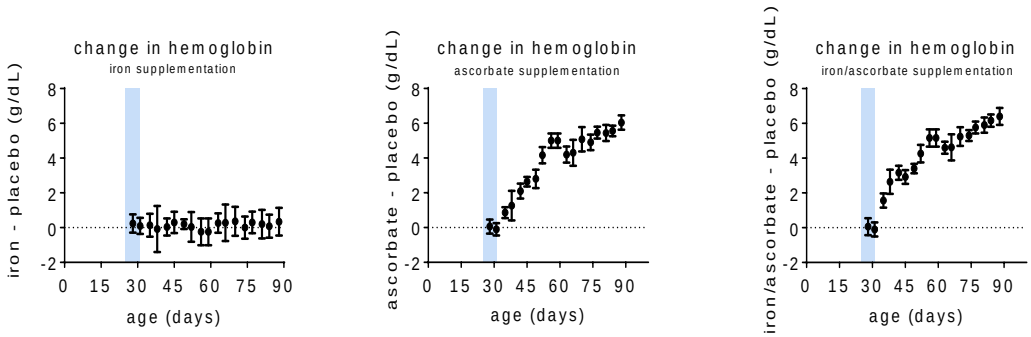

Figure 6.3. *H. pylori* associated reduction in hemoglobin levels is refractory to oral iron supplementation but rescued by oral ascorbate supplementation. Time series data for hemoglobin levels measured from tail vein blood during chronic *H. pylori* G27 infection. Infection experiments were carried out for 8 weeks (inoculation procedure period highlighted in blue). Animals assigned to the iron supplementation group received oral supplementation of 3 mg of ferrous iron every other day; animals assigned to the ascorbate supplementation group received 250 mg/kg of ascorbate every other day, and animals assigned to the iron/ascorbate supplementation group received 3 mg of ferrous iron and 250 mg/kg of ascorbate every other day. Experimental populations consisted of 5 placebo intervention (10% sucrose), 5 iron supplementation, 5 ascorbate supplementation, 5 iron/ascorbate supplementation animals. All animals were infected with *H. pylori* G27. The mean difference between placebo intervention and nutritional intervention animals is represented by a circle and the error bars correspond to the 95% confidence interval. The data indicate that *H. pylori* associated reduction in hemoglobin levels is refractory to oral iron supplementation but rescued by oral ascorbate supplementation.

	intervention			
	10% sucrose n = 5	iron n = 5	ascorbate n = 5	iron/ascorbate n = 5
Hb (g/dL)	10.4 (0.4)	10.6 (0.2)	**17.1 (0.1)**	**17.3 (0.4)**
HCT (%)	42.8 (1.9)	43.8 (0.8)	**56.4 (1.1)**	**59.6 (1.1)**
[RBC] (M/µL)	6.1 (0.2)	6.1 (0.2)	**7.8 (0.1)**	**7.9 (0.3)**
MCV (fL)	70.8 (3.2)	71.9 (3.0)	76.6 (1.6)	71.2 (3.5)
MCH (gHB/M cells)	17.1 (0.6)	17.3 (0.8)	**22.0 (0.3)**	**21.9 (0.7)**
MCHC (gHb/dL)	24.2 (0.5)	24.1 (0.3)	**28.7 (0.6)**	**30.7 (1.2)**

Table 6.4. Oral administration of ascorbate rescues *H. pylori* associated changes in red blood cell indices. Red blood indices for hemoglobin (Hb), hematocrit (HCT), red blood cell count ([RBC]), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured following terminal blood collection from the experimental animals. Data were by ANOVA ($p < 0.01$) followed by Tukey's pairwise multiple comparison test. Measurements significantly different ($p < 0.01$) from the placebo treated group (10% sucrose) group are denoted in bold. These data indicate that oral ascorbate supplementation is capable of rescuing *H. pylori* associated changes in red blood indices.

	intervention			
	10% sucrose n = 5	iron n = 5	ascorbate n = 5	iron/ascorbate n = 5
total Fe (µg/dL)	138.7 (3.3)	127.2 (9.8)	**171.6 (8.0)**	**188.8 (10.9)**
UIBC (µg/dL)	388.5 (6.5)	385.7 (7.1)	**231.1 (3.4)**	**204.9 (8.9)**
TIBC (µg/dL)	527.2 (3.0)	512.8 (9.5)	**402.7 (10.2)**	**393.7 (18.0)**
transferrin saturation (%)	0.26 (0.04)	0.25 (0.02)	**0.43 (0.01)**	**0.48 (0.01)**

Table 6.5. Oral ascorbate administration rescues *H. pylori* associated depletion of iron reserves. Total serum iron (total Fe), unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), and transferrin saturation were measured following terminal blood collection from experimental animals. Data were by ANOVA ($p < 0.01$) followed by Tukey's pairwise multiple comparison test. Measurements significantly different ($p < 0.01$) from the placebo treated group (10% sucrose) group are denoted in bold. These data indicated that oral ascorbate administration is capable of rescuing *H. pylori* associated depletion of iron reserves.

	intervention			
	10% sucrose n = 5	iron n = 5	ascorbate n = 5	iron/ascorbate n = 5
serum ferritin (pg/mL)	5760 (281)	5780 (241)	**9553 (363)**	**9781 (656)**
soluble transferrin receptor (pg/mL)	8979 (1158)	9214 (838)	**4836 (360)**	**5262 (484)**

Table 6.6. Oral ascorbate supplementation is associated with reversal of iron reserve physiological changes observed during chronic *H. pylori* infection.
Serum ferritin and soluble transferrin receptor levels were measured by quantitative ELISA following terminal blood collection from experimental animals. Data were by ANOVA ($p < 0.01$) followed by Tukey's pairwise multiple comparison test. Measurements significantly different ($p < 0.01$) from the placebo treated group (10% sucrose) group are denoted in bold. These data indicate that the physiological changes to iron reserve depletion associated with chronic *H. pylori* infection are revered by oral ascorbate supplementation.

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Chapter 7: Conclusions and future work

7.1 Introduction

The major gaps in knowledge sought to be addressed by this work include whether chronic *H. pylori* infection is associated with a systemic inflammatory response, whether chronic *H. pylori* infection is associated with the development of a neuro-inflammatory response, whether chronic *H. pylori* infection influences cognition, and whether chronic *H. pylori* infection is associated with iron deficiency. These areas of study address fundamental gaps in not only the study of *H. pylori* but also provide insight into the potential consequences of other chronic bacterial infections. While large-scale epidemiological studies have suggested links between isolated chronic bacterial infections and the development of systemic and neuro-inflammation few have been able to address aspects of causation as this study has.

7.2 Major Findings

This work addressed several major gaps of knowledge in the study of the extra-gastric influence of chronic *H. pylori* infection. From the work, new lines of research remain to fully understand and characterize the impact of bacterial associated systemic and neuro-inflammation on cognitive and nutritional health.

7.2.1 Development of a rat-based model of chronic *Helicobacter pylori* infection.

Given that *H. pylori* is a human-specific pathogen whose natural history of infection begins in early childhood and persists throughout life, the development of a rat-based model creates a powerful tool for examining the influence of chronic *H. pylori* in the absence of clinical gastric disease. In addition to the generation of strains of *H. pylori* adapted to infecting Sprague-Dawley rats, the following major findings were made:

- Chronic *H. pylori* infection in Sprague-Dawley rats results in consist bacterial load and consistent elevation of gastric inflammatory markers
- Chronic infection in Sprague-Dawley rats is largely consistent across multiple strains of *H. pylori*
- Eradication of active *H. pylori* infection in Sprague-Dawley rats results in inflammatory markers returning to baseline levels
- Chronic *H. pylori* infection in Sprague-Dawley rats recapitulates many of histological changes observed during natural infection in human prior to the onset of clinical disease
- The major *H. pylori* virulence factors VacA, *cag* pathogenicity island, and NapA are only minor players in the gastric inflammatory response to *H. pylori* infection in Sprague-Dawley rats

Altogether, these findings support the use of this animal model as a better analog to natural human infection than was previously available in the field. The identification of the tissue specific changes associated with chronic infection prior to the onset of clinical disease indicates the potential for this animal model to be utilized for addressing the early stages of *H. pylori* infection which remain poorly characterized.

7.2.2 Systemic inflammatory response to chronic *Helicobacter pylori* infection.

Given that gastric *H. pylori* infection results in prolonged activation of the inflammatory response of the immune system, it was predicted that chronic *H. pylori* infection would be associated with a systemic inflammatory response. In evaluation of the association between chronic *H. pylori* infection and systemic inflammation the following major findings were made:

- Chronic *H. pylori* infection is associated with elevated inflammatory markers in the liver, spleen, and plasma
- The elevated levels of systemic inflammatory markers are largely consistent across multiple strains of *H. pylori*
- Eradication of active *H. pylori* infection results in inflammatory markers returning to baseline levels
- *H. pylori* associated systemic inflammation is mild in comparison to systemic inflammation associated with administration of *E. coli* derived LPS
- Administration of heat killed *H. pylori* or innocuous bacteria does not result in a systemic inflammatory response consist with that observed during chronic *H. pylori* infection
- Gastritis induced chemically does not result in a systemic inflammatory response consistent with that observed during chronic *H. pylori* infection
- Absence of T cell populations abrogates *H. pylori* associated systemic inflammation
- Chronic *H. pylori* infection is associated with polarization of the Th1:Th2 ratio toward pro-inflammatory Th1 populations, but also associated with polarization of the Th17:Treg ratio toward tolerogenic Treg populations
- The major *H. pylori* virulence factors VacA, *cag* pathogenicity island, and NapA are only minor players in the systemic inflammatory response to *H. pylori* infection in Sprague-Dawley rats

Altogether, these findings support a model by which gastric infection by *H. pylori* results in development of a systemic inflammatory response. These findings support the hypotheses put forward in epidemiological studies which have found an association

between *H. pylori* seropositivity and elevated levels of circulating inflammatory markers. Furthermore, these studies indicate that *H. pylori* associated systemic inflammation resolves following eradication of active infection, indicating the *H. pylori* infection is indeed the etiological factor for the observed elevation in systemic inflammatory markers. Finally, these findings suggest that T cell populations are key players in the development of *H. pylori* associated systemic inflammation.

7.2.3 Neuro-inflammatory response to chronic *Helicobacter pylori* infection.

Given that chronic *H. pylori* infection was associated with a systemic inflammatory response, it was hypothesized that *H. pylori* may contribute to the development of neuro-inflammation. In evaluation of the neuro-inflammatory response to chronic *H. pylori* infection, the following major findings were made:

- Chronic *H. pylori* infection is associated with elevated inflammatory markers in both the hippocampus and the cerebellum
- The neuro-inflammatory response associated with *H. pylori* infection is consistent across multiple strains of *H. pylori*
- Eradication of active *H. pylori* infection results in inflammatory markers returning to baseline levels
- *H. pylori* associated neuro-inflammation is mild in comparison to that observed following systemic administration of LPS
- Gastritis that is chemically induced does not result in neuro-inflammation
- Absence of T cell populations is associated with an absence of a neuro-inflammatory response during active *H. pylori* infection

- Inhibition of the systemic inflammatory response by indomethacin anti-inflammatory blockade prevent the develop of *H. pylori* associated inflammation at all levels
- The major *H. pylori* virulence factors VacA, *cag* pathogenicity island, and NapA are only minor players in the neuro-inflammatory response to *H. pylori* infection in Sprague-Dawley rats

Altogether, these findings support a model by which the immunological response to chronic *H. pylori* generates chronic, low-grade neuro-inflammation. Furthermore, that active *H. pylori* infection is needed to both develop and maintain the observed elevation of neuro-inflammatory markers. Finally, these studies suggest that the systemic inflammatory response plays a key role in the development of *H. pylori* associated neuro-inflammation.

7.2.4 Influence of chronic Helicobacter pylori infection on cognition.

As neuro-inflammation has been linked to the development of cognitive impairment, it was hypothesized that chronic *H. pylori* infection may be associated with the development of cognitive deficits. In evaluation of the influence of chronic *H. pylori* infection the following major findings were made:

- Chronic *H. pylori* infection is not associated with anhedonia
- Chronic *H. pylori* infection is not associated with direct impairment of hippocampal mediated spatial memory
- Chronic *H. pylori* is associated with impairment of performance of learning memory and behavioral flexibility
- *H. pylori* associated impairment of learning memory and behavioral flexibility are potentially long-lasting and irreversible

Altogether, these findings suggest that chronic *H. pylori* infection is associated with detrimental cognitive effects. These effects influence learning memory and behavioral flexibility, suggesting perturbations in pre-frontal cortex mediated cognition. Furthermore, these effects persist even after eradication of active *H. pylori* infection, suggesting that these detrimental cognitive effects may be permanent.

7.2.5 *Helicobacter pylori* associated iron deficiency.

Previous epidemiological studies have suggested that *H. pylori* may be an etiological factor in the development of idiopathic iron deficiency anemia. However, systematic attempts to determine if *H. pylori* is truly a causative agent of these association and the mechanism by which iron deficiency develops during chronic *H. pylori* infection have been lacking. To address these issues, the rat-based model of chronic *H. pylori* infection developed in this work was utilized. By use of this animal model, the following major findings were made:

- Chronic *H. pylori* infection is associated with a significant decrease in hemoglobin and hematocrit
- Eradication of active *H. pylori* infection leads to a rapid normalization of hemoglobin and hematocrit
- Chronic *H. pylori* infection is associated with changes to red blood cell indices, serum iron, transferrin saturation, serum ferritin, and serum soluble transferrin receptor levels that are consistent with iron malabsorption
- *H. pylori* associated iron deficiency is refractory to oral iron supplementation
- Oral administration of high dose ascorbic acid rescues *H. pylori* associated iron deficiency

Altogether, these findings support a model by which chronic *H. pylori* infection is associated with the development of iron deficiency and that associated iron deficiency rapidly resolves following eradication of active infection, indicating that *H. pylori* infection is the likely etiological agent. Furthermore, these findings suggest that *H. pylori* associated iron deficiency is malabsorptive in nature and that the physiological cause of the malabsorption is modulation of gastric ascorbic acid levels during chronic *H. pylori* infection.

7.3 Conclusion

The completion of the aforementioned studies has added new illumination into the extra-gastric influences of chronic *H. pylori* infection. In particular, these studies support a model by which chronic gastric infection by *H. pylori* is able to induce the development of low-grade systemic and neuro-inflammation. Furthermore, these extra-gastric manifestations of inflammation have been associated with detrimental effects of pre-frontal cortex mediated cognition in the experimental animal population. Strikingly, *H. pylori* associated cognitive detriments do not resolve following eradication of active infection in the time frame evaluated in these studies, suggesting that these effects may, in fact, be permanent. Current standards of medical care in pediatric populations recommend eradication of active *H. pylori* infection only in the presence of active ulcer disease. As such, the findings of these studies indicate that such guidelines may place pediatric populations at elevated risk both due to risk of overt detrimental cognitive effects but also due to the elevated risk associated with the development of a chronic inflammatory state. The additional findings that chronic *H. pylori* infection is an etiological agent of iron deficiency further places these pediatric populations at risk as iron deficiency during childhood development is a known risk factor in many long term

pathophysiological conditions. As such, these studies lay the necessary groundwork for expanding these experiments into human populations where they hold the promise to change public health practices in meaningful ways.

7.4 Future Work

In addition to addressing several important gaps in knowledge in the study of the extra-gastric influence of chronic *H. pylori* infection, this work identified numerous future studies to be pursued.

7.4.1 Systemic inflammatory response to chronic *Helicobacter pylori* infection.

While T cell populations were shown to be key players in the development of *H. pylori* associated systemic inflammation, which specific T cells mediate and drive the systemic inflammation in response to chronic *H. pylori* infection remains unknown. For future work, isolation of the contribution of these immune cell sub-population needs to be evaluated. Furthermore, as the studies were conducted in rats, a non-natural host to *H. pylori*, validation of the findings of a systemic inflammatory response in humans is needed.

7.4.2 Neuro-inflammatory response to chronic *Helicobacter pylori* infection.

The mechanism by which chronic *H. pylori* infection induces a neuro-inflammatory response remains unknown. For future work, evaluation of the contribution of the major pathways by which systemic pro-inflammatory signaling reaches the central nervous system needs to be evaluated. Additionally, the potential role of microglial cell populations, which serve as the major immune effectors of the central nervous system, in the development and maintenance of *H. pylori* associated neuro-inflammation needs to be evaluated.

7.4.3 Influence of chronic *Helicobacter pylori* infection on cognition.

The mechanism by which chronic *H. pylori* infection negatively influences cognition remains an open question. For future work, further evaluation of the aspects of cognition impacted by chronic *H. pylori* infection is needed. Additionally, evaluation of whether establishment of chronic *H. pylori* during adulthood, after completion of cognitive development, also results in cognitive deficits is needed. Finally, the influence of chronic *H. pylori* infection on human cognition is in need of study.

7.4.4 *Helicobacter pylori* associated iron deficiency.

Ascorbic acid was found to be capable of rescuing *H. pylori* associated iron deficiency. However, the manner in which *H. pylori* infection modulates natural gastric ascorbic acid levels remains unknown. Evaluation of the pathophysiological processes that result in loss of gastric ascorbic acid needs to be evaluated. Additionally, as current standards of care in pediatric patients chronically infected with *H. pylori* recommend against eradication therapy in the absence of active ulcer disease, the application of oral ascorbic acid supplementation for management of *H. pylori* associated iron deficiency in pediatric populations is in need of study.