An In vitro Analysis of Inflammatory Cytokine Response to *Helicobacter canadensis*

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ABSTRACT

AN IN VITRO ANALYSIS OF INFLAMMATORY CYTOKINE RESPONSE TO *HELICOBACTER CANADENSIS*

by

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Chairperson: Professor Vance J. McCracken

Helicobacter is a genus of Gram-negative helical bacteria. Colonization location divides the genus into two groups: gastric and enterohepatic. Many species of *Helicobacter* have become associated with or cause gastric, intestinal, and hepatic disease. *Helicobacter canadensis* is an enterohepatic bacterium that has become associated with enteritis and bacteremia. The first part of this study investigated the intestinal inflammatory response to *H. canadensis* in mouse explants. Colon and cecum explants taken from C57BL/6J mice were incubated with 10^8 CFU/mL *H. canadensis* for 24 hours and cytokine ELISAs were conducted on supernatants. IL-6, IL-1α, and TNF-α were not induced in either colon or cecum explants. The second part of this study involved infecting thioglycollate-elicited macrophages with 10^8 CFU/mL *H. canadensis* for 6 or 24 hours followed by cytokine ELISAs of supernatants. At 6 hours, IL-6 secretion was increased in thioglycollate-elicited macrophages infected with *H. canadensis* while IL-1α and TNF-α secretion was undetectable. At 24 hours, IL-1α secretion was significantly greater in thioglycollate-elicited macrophages treated with *H. canadensis* while IL-6 and TNF-α secretion was undetectable. Colon and cecum explant data support previous findings in our lab that *H. canadensis* does not induce inflammation, while data collected

from thioglycollate-elicited macrophage cultures indicate that *H. canadensis* stimulates inflammatory cytokines.

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CHAPTER I

LITERATURE REVIEW

Introduction

Spiral-shaped bacteria have been observed in the gastrointestinal tracts of humans and animals for over 100 years. The first documented observation of spiral bacteria is credited to Rappin, who published a thesis in 1881 describing spiral bacteria in gastric scrapings from dogs[1–3]. Rappin's results were later confirmed by Bizzozero in 1893, who observed spiral bacteria in stomach sections from dogs[2,4,5]. Three years later, Salomon conducted an extensive study investigating spiral bacteria in several animals verifying the observations made by Rappin and Bizzozero[2,5,6]. Salomon's attempts to culture the bacteria on media were unsuccessful, but his attempt to propagate the bacteria by giving gastric mucosa from infected animals to uninfected mice was successful. In 1939, Doenges became the first to observe spiral bacteria in human stomachs[7]. Of the postmortem human stomachs, 43% contained spiral bacteria while 100% of rhesus macaque stomachs contained spiral bacteria. In 1970, Lockard and Boler investigated morphological characteristics of spiral microorganisms in the gastric mucosa of dogs using electron microscopy[3]. The bacterium was named *Spirillium rapinni* and occurred in three distinct morphologies: rod (straight cylindrical shape), spiral (loose spiral with 4 to 6 turns along the length), and tight spiral (tightly coiled with 8 to 12 turns along the length). In 1975, researchers investigating the association of bacteria and ulcers observed spiral bacteria in biopsies from human patients with gastritis[8]. In that study, biopsies from 47 patients with benign gastric ulceration were

compared to biopsies from 6 normal patients. 80% of the biopsies from patients with ulcers contained Gram-negative bacteria buried deep in mucosal regions.

In 1984, Marshall and Warren observed spiral, Gram-negative, curved bacilli in 58 out of 100 patients presenting with gastritis or peptic ulcers[9]. Initially the researchers were unable to culture the bacteria using microaerophilic conditions for two days but after accidentally leaving cultures for six days, they observed growth. Curved bacilli were present in patients with chronic gastritis, duodenal ulcers, and gastric ulcers. Marshall and Warren initially named the novel bacterium *Campylobacter pyloridis*, which was later corrected to *Campylobacter pylori*.

Upon further investigation, it was determined that *C. pylori* had features not common to the *Campylobacter* genus. The cellular fatty acid composition of *C. pylori* was determined to be mainly 14:0 and 19:0 (C:D, where C is the number of carbon atoms and D is the number of double bonds) while species in the *Campylobacter* genus had 16:0, 18:1, and 16:1 as major fatty acids[10]. *C. pylori* had four sheathed flagella, something that was not common to the *Campylobacter* genus as most have a single unsheathed flagellum[10]. Comparison of 16S rRNA sequences of five *Campylobacter* species compared to *C. pylori* showed that the 16S rRNA sequence of *C. pylori* was greatly different than the reference *Campylobacters*[11]. In 1989, several tests compared *C. pylori* to the genera of *Flexispira, Spirillum,* and *Wolinella* [12]. Briefly, cellular fatty acid composition and ultra-structural features, biochemical, and growth condition tests as well as 16S rRNA sequencing were revisited. It was determined that *C. pylori* did not have characteristics seen in the *Campylobacter* genus and that it did not fit into the

Flexispira, Spirillum, or *Wolinella* genera. Thus a new genus, *Helicobacter,* was proposed and *C. pylori* was renamed *Helicobacter pylori.*

The *Helicobacter* Genus

The *Helicobacter* genus is composed of spiral, curved, or fusiform (spindle-shaped) Gram-negative bacteria. Bacteria are 0.2 to 1.2 μm in diameter and 1.5 to 10 μm long. Changes in growth conditions, such as depletion of nutrients, alteration of pH, exposure to high O_2, accumulation of metabolic products, over-passaging, and extended incubations can cause bacteria to convert to a coccoid form[13–15]. *Helicobacter* display rapid corkscrew or slow wave-like motility via unipolar or amphitrichous flagella. Members of the genus grow under microaerobic conditions (5% CO_2, 90% N_2, 5% H_2) at 37°C and grow optimally on moist agar surfaces in a thin, spreading film[16].

The *Helicobacter* genus is composed of 34 recognized species and is separated into two categories based on the location of colonization: gastric and enterohepatic[17,18]. Gastric *Helicobacters* colonize gastric mucosa and proximal duodenum. These bacteria are urease positive, breaking down urea to produce ammonia, resulting in an increase in pH that provides protection from gastric acid in the immediate area of the bacterium[2,19]. Enterohepatic *Helicobacter* species (EHS) colonize the intestines and/or liver. Some EHS have ultra-structural and physiological features similar to gastric *Helicobacters*, such as expressing urease and can sometimes be found in the stomach. When generating a phylogenetic tree of the *Helicobacter* genus (Figure 1), with the exception of *Helicobacter mustelae* and *Helicobacter typhlonius,* two distinct groups are formed which can be characterized by colonization location, gastric and enterohepatic.

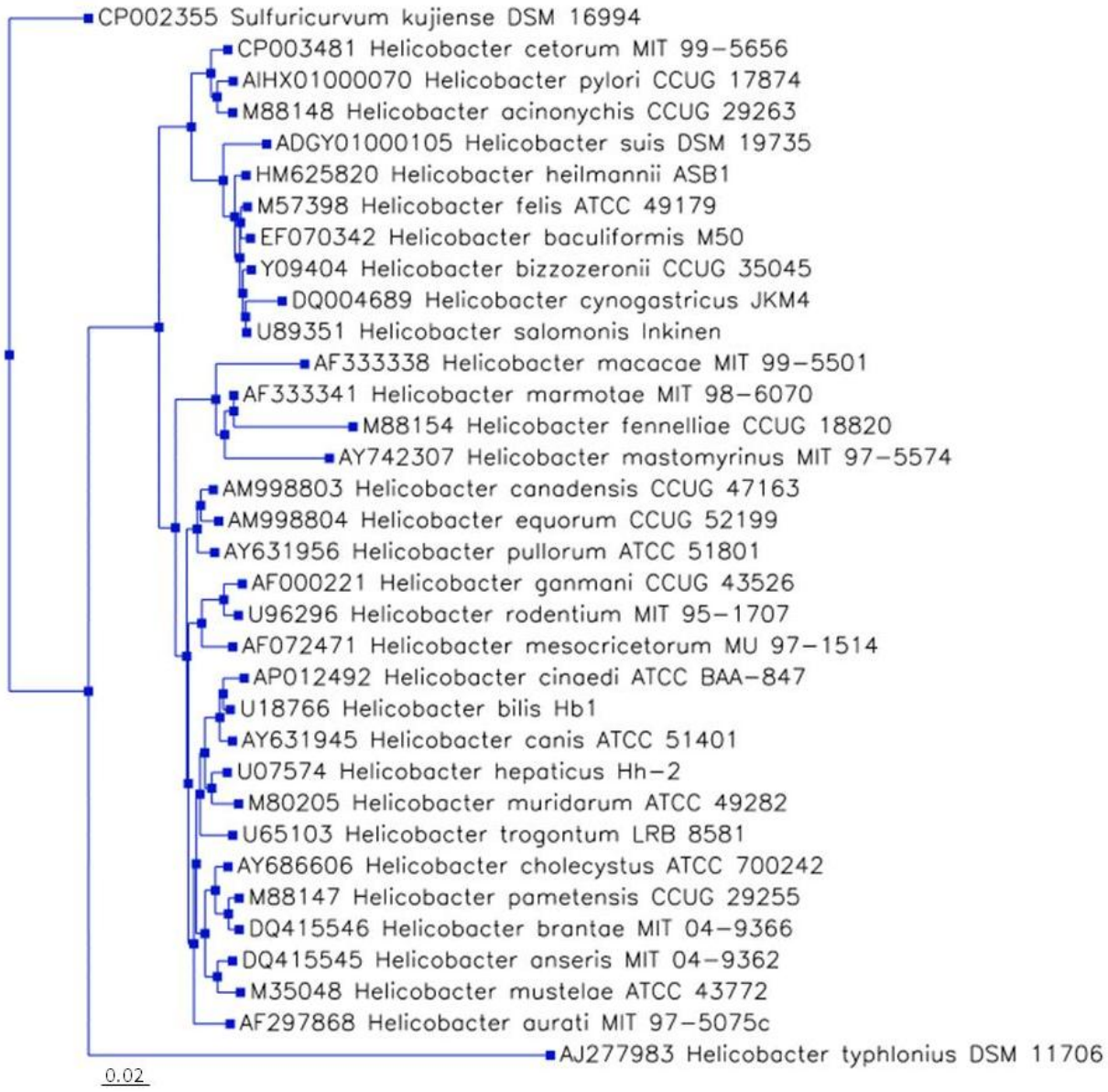

Figure 1. 16S rRNA Phylogram Tree of *Helicobacter* Genus. Phylogram tree was generated using SeqRank program found on StrainInfo website[20]. *Sulfuricurvum kujiense* (an epsilonbacteria) was outgroup selected by program. Scale bar represents degree of genetic divergence.

Helicobacter canadensis

Helicobacter canadensis has a spiral shape and nonsheathed unipolar or amphitrichous flagella[21]. It grows as a thin spreading film on tryptic soy blood agar

plates in microaerophilic conditions at 37°C. *H. canadensis* is positive for oxidase, catalase, and hydrolysis of indoxyl acetate but is negative for cytolethal distending toxin B and its cytopathic effects[21]. It is also positive for immunoglobulin A protease and two homologues of vacuolating cytotoxin[22]. Resistance to the antibiotics nalidixic acid (30mg) and cephalothin (30 mg) is also characteristic of *H. canadensis*[21].

Primarily colonizing the intestines, *H. canadensis* has been identified as a pathogenic EHS in both avian species and mammals. *H. canadensis* has been isolated from many sources: fecal samples of Canadian patients presenting with diarrhea[21], patients with bacteremia[23,24], Barnacle and Canada geese in Europe[25], Guinea fowls and pheasants in Italy[26], feces of wild rodents in China[27], and laboratory rabbits[28]. An atypical strain has been isolated from swine in The Netherlands and Denmark[29].

Intestinal Mucosa

The intestinal mucosa is composed of a mucus layer, epithelial layer, lamina propria, and a thin layer of smooth muscle. A mucus layer that varies in thickness depending on the location in the intestines protects intestinal epithelial cells from most bacteria, chemicals, and bile. The mucus layer can be divided into two layers: a loose adherent layer that is easily removable and colonized by bacteria and a firmer layer that is attached to the underlying epithelial cells[30]. The intestinal epithelium is a monolayer composed of four cells types: goblet cells, enterocytes, enteroendocrine cells and Paneth cells[31]. Enterocytes are involved in transport of macromolecules from the lumen to the lamina propria while goblet cells are mucus-secreting cells. Enteroendocrine cells secrete hormones and Paneth cells produce antimicrobial

peptides called defensins. Distributed throughout the intestinal epithelium are lymphoid follicles which contain microfold cells (M cells). M cells sample antigens and microorganisms in the lumen and present them via transcytosis to antigen-presenting cells in the lamina propria[32].

The lamina propria is a thin layer of loose connective tissue immediately beneath the epithelium and contributes to the innate immune response. The loose connective tissue of the lamina propria is rich in immune cells such as macrophages, dendritic cells, plasma cells, and T cells. Dendritic cells actively sample the lumen and travel to nearby lymph nodes to present antigens to T cells. IgA^+ plasma cells are specialized B cells that secrete IgA, which is involved in opsonization. Macrophages perform several important functions throughout the body, including the intestinal tract. M1 macrophages are pro-inflammatory while M2 macrophages are anti-inflammatory and are involved in tissue repair. M1 macrophages constantly sample the surrounding environment for invading microorganisms and stimulate other immune cells to respond to invaders[33]. To maintain tissue integrity, M2 macrophages remove dying cells and toxic material left over from cells that have undergone apoptosis. M2 macrophages produce various growth factors, such as transforming growth factor-β and platelet-derived growth factor, that promote cell proliferation and wound repair. To restore tissue homeostasis after infection or injury, M2 macrophages secrete immune regulatory proteins, such as IL-10, which is an anti-inflammatory cytokine[34].

Epithelial cells and innate immune cells, such as macrophages, recognize both commensal and pathogenic bacteria by expressing pattern recognition receptors (PRRs) on cell surfaces or intracellular compartments. PRRs recognize pathogen-associated

molecular patterns (PAMPs) such as LPS, bacterial DNA, and double-stranded DNA[31]. Once bound, PRRs can activate many inflammatory and immune functions such as phagocytosis, activation of the complement cascade, release of cytokines, and apoptosis. Macrophages express Toll-like receptors, which are cell-surface PRRs, that when bound result in the synthesis and secretion of cytokines as well as activation of other immune cells[35]. NOD-like receptors, which are intracellular PRRs, help macrophages synthesize and secrete cytokines and regulate apoptosis. Macrophage mannose receptors (MMRs) are cell-surface PRRs that are expressed in high numbers on tissue macrophages. MMRs are involved in the phagocytosis of pathogens and their delivery into lysosome compartments[36]. It is thought that MMRs serve as a link between the innate and adaptive immune systems. Another PRR involved in phagocytosis is the macrophage scavenger receptor, which recognizes a broad range of PAMPs[37]. In addition to PRRs, macrophages secrete several molecules that activate the complement cascade and enhance phagocytosis.

Cytokines are secreted chemical proteins that act locally, regionally, or systemically. Tumor necrosis factors (TNF), such as TNF-α, are cytokines produced by macrophages, T cells, and mast cells, and are involved in initiating inflammatory responses as well as apoptosis. Specifically, TNF-α contributes to vasodilatation, edema formation, recruitment of leukocytes, and induction of inflammation[38–40]. Interleukins, such as IL-1 and IL-6, are cytokines important to both the innate and adaptive immune response. IL-1 is involved in induction of fever and activation of lymphocytes and is produced by epithelial cells and mononuclear cells[41]. Fibroblasts, macrophages, T cells,

B cells, and endothelial cells produce IL-6, which induces B and T cell differentiation and cell proliferation[42–44].

Disease

It is estimated that somewhere around 10^{14} commensal bacterial cells inhabit the intestinal tract of a human adult, with each individual harboring several hundred bacterial species[45,46]. These bacteria facilitate metabolism of indigestible plant polysaccharides and produce vitamins B and K[47,48]. Competition among commensal bacteria provides protection against opportunistic pathogens through the secretion of antimicrobial peptides and restriction of nutrients and colonization sites[49]. Commensal bacteria are also involved in stimulating and regulating host immune cells, such as T cells[50]. While some commensal bacteria prevent inflammation, others promote pro-inflammatory cytokines, causing the intestinal tract to be under a constant but balanced state of mild inflammation. Dysregulation of this balanced state can contribute to inflammatory diseases such as inflammatory bowel disease (IBD), intestinal cancer, and food allergies. IBD is characterized as chronic inflammation of all or part of the digestive tract and primarily includes Crohn's disease and ulcerative colitis[51].

Several gastric Helicobacters have become associated with diseases. Since first being discovered by Marshall and Warren, *H. pylori* has been extensively studied. It is estimated that 40% of individuals in developed countries and 80% of individuals in developing countries are infected with *H. pylori;* most infected individuals are asymptomatic. Transmission of *H. pylori* is believed to be person to person via the fecal-oral, gastric-oral, or oral-oral routes[52]. *H. pylori* colonization in humans is linked to

chronic gastritis, duodenal ulcers, and gastric ulcers[9,53]. *Helicobacter suis* is a gastric *Helicobacter* which mainly colonizes the stomachs of pigs. *H. suis* is present in 60% of pigs at slaughter age[54]. The role of *H. suis* in gastric disease in pigs is poorly understood but is associated with ulcers and gastritis. *H. suis* is the most prevalent non-pylori gastric *Helicobacter* in humans with gastric complaints and is believed to be acquired through direct contact with pigs[55]. *Helicobacter felis* is another common gastric *Helicobacter* found in humans. Originally isolated from cats, *H. felis* was later also found in dogs[56]*. H. felis* infection in humans is associated with gastritis and gastric mucosal associated lymphoid tissue lymphoma[57]. Like *H. suis*, human infection with *H. felis* is believed to be zoonosis, in this case a result of transmission from cats and dogs to humans.

EHS are also associated with diseases in both animals and humans. *Helicobacter cinaedi* is a member of the normal intestinal flora of hamsters but has been found to be associated with enteritis and bacteremia in immunocompromised individuals[58,59]. The transmission route of *H. cinaedi* to humans is not known, but is thought to occur through handling of infected hamsters[60]. *Helicobacter pullorum* is frequently isolated from poultry liver, duodenum, and cecum and has been isolated from patients with gastroenteritis and diarrhea[47,48]. Since *H. pullorum* has been found on the carcasses of asymptomatic chickens, it is thought that *H. pullorum* is a foodborne illness[64].

H. canadensis has several virulence factors that could implicate its role in causing diseases in humans. It is positive for catalase which catalyzes the breakdown of hydrogen peroxide, which is toxic to bacterial cells, into water and oxygen[65]. Production of immunoglobulin A protease, which is also produced by *H. pylori*, results in the

cleavage and inactivation of IgA[66]. Vaculating cytotoxin induces vacuole formation, disrupts mitochondrial functions, and blocks T-cell proliferation[67]. Other virulence factors are resistance to antibiotics nalidixic acid (30mg) and cephalothin (30 mg).

Models

The availability of genetically different strains, short breeding cycle, short life cycle, and sometimes disease characteristics similar to humans make mice a widely used model to investigate *Helicobacter* pathogenesis. The inbred C57BL/6J strain is a widely used model to study *H. pylori* and other *Helicobacter* infections. Inbred lines are produced by conducting at least 20 brother-sister or parent-offspring matings[68]. All mice of an inbred line are virtually genetically identical. C57BL/6J mice generate a strongly polarized Th1 response, which elicits cell mediated immunity and inflammation, making C57BL/6J mice ideal for researching IBD[69]. Many genetically engineered knockout lines used to study *Helicobacter* infection are available on the C57BL/6J background. BALB/cJ is an inbred mouse line used in both cancer and immunology studies and has been used to study *Helicobacter*-induced MALT lymphoma[70,71]. BALB/cJ mice generate a Th2 response which elicits antibody production and response[69]. C3H/HeJ mice have mutated Toll-like receptor 4 gene making them insensitive to lipopolysaccharide. C3H/HeJ mice are easily colonized by *H. pylori*[72]. Mice deficient in IL-10 (IL-$10^{-/-}$) are used to investigate inflammatory bowel disease. IL-10 plays a role in suppressing inflammatory responses from cells such as macrophages and T cells and is found in high amounts during normal, non-

inflammatory conditions[33,73]. IL-10$^{-/-}$ mice develop chronic IBD and exhibit histopathological similarities to human IBD[74].

In addition to mouse lines, intestinal explants from mice, as well as humans, have been used to investigate bacteria and intestinal diseases. Intestinal explants serve as a transition to mouse studies since they are more representative of the *in vivo* environment[75,76]. Intestinal explants consist of biopsy samples that have been isolated, washed, and cut into standardized sizes[75–78]. Explants are kept in culture medium for one to two days. Mouse and human intestinal cell cultures are also used as models to investigate *Helicobacter*. Cell culture can be conducted with immortalized cells or primary cells, which are cells collected directly from animals. One such example of primary cell culture is thioglycollate-elicited macrophages. Briefly, thioglycollate is injected into the abdominal cavity causing monocytes to migrate to the cavity where they differentiate into macrophages[79]. Three to four days after injection, cells are harvested from the cavity and cultured.

Significance and Purpose

Since first being isolated from humans, further research has indicated that *H. canadensis* can be found in many wild avian species as reservoirs. Avian species, such as Canada geese, frequent and often inhabit parks, waterways, golf courses, and beaches. With their presence comes potential for fecal contamination of water, pastures, and parks that could expose humans to *H. canadensis. H. canadensis'* pathogenicity and mode of infection needs to be studied to understand its ability to cause diseases in humans.

The purpose of this study is to understand better the intestinal inflammatory response to *H. canadensis.* Previous work performed in our lab indicates that *H. canadensis* colonizes the intestines of mice but does not result in clinical signs of inflammation. We tested the hypothesis that *H. canadensis* infection has no effect on the secretion of inflammatory cytokines using mouse colon and cecum explants, as well as thioglycollate-elicited macrophages.

CHAPTER II

MATERIALS & METHODS

Animals

C56BL/6J mice originally obtained from the Jackson Laboratory were housed in the vivarium at Science Lab West building of Southern Illinois University Edwardsville. The mouse facility was maintained at 26°C ± 2°C with 12-hour light-dark cycles. Mice were kept in sterile, filter-top microisolator cages with sterile $^1/_8$" corn-cob bedding (Andersons Lab Bedding; Maumee, OH) and were housed four-to-five per cage with constant access to irradiated food (LabDiet; Brentwood, MO) and autoclaved water. Mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation. All animal experiments were performed according to the guidelines and approved protocols of the Institutional Animal Care and Use Committee of Southern Illinois University Edwardsville.

Helicobacter canadensis Culture

H. canadensis strain #700968 was purchased from American Type Culture Collection (ATCC). Cultures were initially grown on biphasic BBL Sheep Blood Agar slants (Becton, Dickinson, and Company; Sparks, MO) with trypticase soy broth (Becton, Dickinson, and Company; Sparks, MO; TSB)and later maintained on trypticase soy agar slants (Becton, Dickinson, and Company; Sparks, MO) supplemented with 5% defibrinated calf blood (Colorado Serum Company; Denver, CO). Slants were placed in a GasPak jar with a Campypak (Becton, Dickinson, and Company; Sparks, MO) to maintain

microaerophilic conditions (90% N_2, 5% H_2, and 5% CO_2) and incubated at 37°C for 48 to 72 hours. Viability of cultures was confirmed using phase-contrast microscopy.

Helicobacter canadensis Quantification: OD_{600}

Each *H. canadensis* slant culture was washed with 1 mL TSB three times and pooled. The pooled *H. canadensis* cultures were centrifuged at 5000 x g for 10 minutes at 4°C. The pellet was resuspended in 5 mL TSB and centrifuged at 5000 x g for 10 minutes at 4°C, this process was repeated three times to remove blood left from the culture medium. After final centrifugation, the pellet was resuspended in 4 mL TSB. The concentration of 1 mL of the diluted suspension was determined using an Eppendorf BioPhotometer Plus set to an optical density of 600 nm (OD_{600}). An OD_{600} of 1 corresponds to approximately 10^9 CFU/mL *H. canadensis*, as previously determined[80]. TSB was added to bring the final concentration to 10^8 CFU/mL.

Fecal DNA Extraction

Stool samples from mice were collected and stored at -20°C. Fecal DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen; Saint Louis, MO). Stool samples were placed on ice, 1.4 mL buffer ASL was added to each sample, and vortexed until homogenous. Samples were heated for 5 minutes at 70°C and centrifuged for 1 minute at full speed. One InhibitEX tablet was added to 1.2 mL supernatant and vortexed until tablet dissolved. Suspensions were incubated for 1 minute at room temperature and centrifuged at full speed for 3 minutes. Supernatants were centrifuged for 3 minutes at full speed. 200 µL of supernatant and 200 µL of Buffer AL was added to 15 µL

Proteinase K. Samples were vortexed and incubated at 70°C for 10 minutes. Lysates were centrifuged briefly and 200 µL ethanol was added. Samples were transferred to QIAamp spin columns and centrifuged at full speed for 1 minute. 500 µL Buffer AW1 was added to spin columns and centrifuged at full speed for 3 minutes. 200 µL Buffer AE was added to QIAamp spin columns and centrifuged for 1 minute at full speed. Samples were stored at -20°C until analysis.

PCR

PCR was conducted on DNA isolated from fecal samples. A universal bacterial domain primer set (341F: 5'CCTACGGGAGGCAGCAG3' and 534R: 5'ATTACCGCGGCTGCTGG3'; ATCC; Manassas, VA) was used to amplify the V3 region of the gene encoding bacterial 16S DRNA[81]. This primer set served as a control for bacterial DNA and yields an approximately 220 base-pair product. *H. canadensis* specific primers (Hcan-F1: 5'ACTAGAGATAGTGGAGTGCCTT3' and Hcan-R1:5'CGCAGTATTGCTTCTCTTTGTG3') were used to amplify the 16S rDNA gene of *H. canadensis* and yield a 262 base-pair product[29]. *Helicobacter*-specific primers (FoxC97: 5'GCTATGACGGGTATCC3' and FoxC05:5'ACTTCACCCCAGTCGCTG3') were used to amplify the *Helicobacter* genus 16S rDNA gene and yield a 1,200 base-pair product[82]. Each reaction consisted of Maxima Hot Start PCR 2X Master Mix (Thermo Scientific; Waltham, MA), PCR Grade Milli-Q water, and forward and reverse primer (at a final concentration of 0.8 µmol/µL). PCR cycling conditions were 15 minutes at 95°C followed by 30 cycles of 30 seconds at 94°C, 60 seconds at 55°C and 75 seconds at 72°C, followed by a single 10-minute extension at 72°C and hold at 4°C. DNA isolated from *H.*

canadensis was used as a positive control for *Helicobacter* and *H. canadensis*-specific primers, while DNA isolated from *H. felis* cultures served as a positive control for *Helicobacter*-specific primers. DNA isolated from *Escherichia coli* was used as a positive control for bacterial 16S rDNA primers, while PCR-grade water was used as a negative control for all primer sets. PCR samples were run on a 1% agarose gel containing ethidium bromide for 60 minutes at 90 volts and visualized using the UV light of a Biorad Universal Hood II.

Serum Collection

The area used to sacrifice mice was disinfected with 10% bleach and 70% EtOH. C57BL/6J mice were individually euthanized with CO_2 followed by cervical dislocation. Abdomens were sprayed with 70% EtOH. The pulminary cavity was opened and heart ventricle was pierced. Whole blood was collected, incubated at room temperature for 1 hour and centrifuged at 12000 x g for 10 minutes to separate serum. Serum was stored at -20°C.

Helicobacter canadensis Sonicate

Preparation

A culture of *H. canadensis* was grown at 37°C on an orbital shaker in brain heart infusion broth (Becton, Dickinson, and Company; Sparks, MO) supplemented with 5% fetal bovine serum (FBS; Biowest; Kansas City, MO). The culture was centrifuged at 5000 x g for 10 minutes and the pellet was resuspended in phosphate-buffered saline solution (PBS; GellGro; Manassas, VA) to a concentration of 10^9 CFU/mL. The

suspension was sonicated at 30% power six times (30 second bursts) on ice and cooled for 30 seconds between bursts[83]. Sonicate was centrifuged for 20 minutes at 20000 x g and filtered using a 0.22 μm filter. After quantification, sonicate was stored in aliquots at -80°C.

Quantification

A BioRad DC Protein Assay (BioRad; Hercules, CA) was conducted to determine the protein concentration of the *H. canadensis* sonicate. Undiluted sonicate and sonicate diluted in PBS (1:10, 1:50, 1:100) were added in triplicate to an Immulon ELISA plate (Thermo Scientific; Waltham, MA). A standard curve of bovine serum albumin V (1.4, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0 mg/mL; Thermo Scientific; Waltham, MA; BSA) diluted in PBS was also added to the plate in triplicate. Solution A was added to each sample and then Solution B was added. The plate was incubated in the dark for 15 minutes and read at 470nM using a BioTek PowerWave XS2 microplate reader.

Serum ELISA

Six rows of an Immulon ELISA plate were coated with 10 μg/mL *H. canadensis* sonicate diluted in PBS. The remaining two rows were coated with 10 μg/mL goat anti-mouse IgG (Southern Biotech; Birmingham, AL). Coated plates were sealed and incubated overnight at 4°C. Plates were brought to room temperature for 30 minutes and washed five times with wash buffer: 0.05% TWEEN-20 (Sigma; Saint Louis, MO) in PBS. Unbound sites were blocked with blocking buffer: filter-sterilized 5% BSA in PBS, for 1 hour at room temperature. Plates were washed 5 times with wash buffer. Samples diluted in diluent buffer (filter-sterilized 1% BSA in wash buffer), IgG standard curve

starting at 1000 ng/mL (Southern Biotech; Birmingham, AL) six two-fold dilutions and a zero, were added in triplicate. After a 2-hour incubation at room temperature plates were washed 5 times. Alkaline phosphatase-linked goat anti-mouse IgG was added as a secondary antibody. After incubating for 2 hours plates were washed 5 times and pNPP substrate solution (Sigma; Saint Louis, MO) was added to each well and incubated for 30 minutes in the dark at room temperature. The reaction was stopped with 3N NaOH and read at 405nM using a BioTek PowerWave XS2 microplate reader.

Coefficient of variation (CV) is the ratio of the standard deviation (σ) to the mean (μ), $CV = \frac{\sigma}{\mu}$, and a low value indicates low variability among replicates. After an extensive search online it was determined that assays yielding samples with CV% values greater than 10 would be rerun[84–86].

C57BL/6J Colon and Cecum Explants

The area used to sacrifice the mice was disinfected with 10% bleach and 70% EtOH. Three male, C57BL/6J mice, age 8-12 weeks were individually euthanized with CO_2 followed by cervical dislocation. Abdomens were sprayed with 70% EtOH. The abdominal cavity was opened using autoclaved dissection tools and cecum and colon were placed in a Petri dish containing R5 media [5% FBS, 1% glutamine (Thermo Scientific; Waltham, MA), 1% penicillin/streptomycin (CellGro; Manassas, VA), 0.25 μg/mL amphotericin B (CellGro; Manassas, VA), and 0.25 μg/mL gentamicin (Thermo Scientific; Waltham, MA)]. In a biological safety cabinet, colons were laterally opened, starting from the distal/rectum region moving upward to the proximal region. A small portion at the proximal region was left uncut to aid in later identification of the mucosal

layer. Ceca were cut open along the lesser curvature. Colons were pooled into a 15-mL conical tube, ceca were pooled into another 15-mL conical tube, and tissues were washed three times with PBS. After the third wash, tissues were placed in a 50-mL conical tube containing R5 and placed on a shaker at 200 rpm for 30 minutes. Two-mm punch biopsies (Robbins Instruments; Chatham, NJ) were taken from the ascending colon and cecum (Figure 2) and placed mucosal side up on 6-mm Whatman filter disks (Thermo Scientific; Waltham, MA). Punch biopsies were allowed to sit on the filter disks for 30 seconds and transferred to a 48-well plate (Corning; Corning, NY) containing R5 media. After a 6-hour incubation in a 37°C, 5% CO_2 incubator, R5 media was removed from each well and appropriate treatment diluted in RPMI 1640 + 1% L glutamine was added. Treatments consisted of 500 ng/mL TNF-α, 10^8 CFU/mL *H. canadensis*, and media. Explants from each mouse were treated in triplicate, resulting in 9 explant sections total per treatment. Plates containing treated tissue sections were placed in a 37°C, 5% CO_2 incubator for 24 hours. Culture supernatant was collected and centrifuged at 5000 x g for 10 minutes at 4°C. Resulting supernatant was stored at -20°C. The isolation of colon and cecum explants and subsequent treatments was conducted twice to obtain supernatants for cytokine ELISAs.

C57BL/6J Macrophage Collection

Three male, C57BL/6J mice, age 8-12 weeks were injected intraperitoneally with 1 mL 3% thioglycollate three days before being individually euthanized by cervical dislocation[79]. The abdomen was sprayed with 70% EtOH and the outer skin of the peritoneum was removed to expose the inner skin lining of the peritoneal cavity. Seven

mL of R10 media (10% FBS, 1% glutamine, 1% penicillin/streptomycin, 0.25 µg/mL gentamicin) was injected into the peritoneal cavity using a 25-Ga needle. The peritoneum was massaged to dislodge attached cells into the R10 media and a 21-Ga needle was used to collect the fluid.

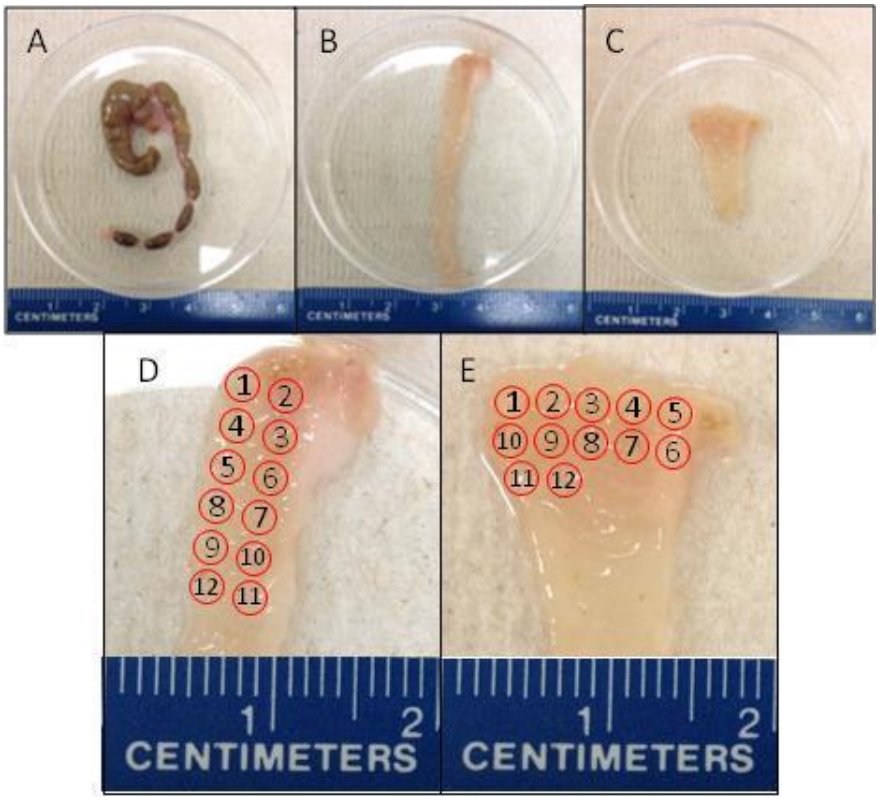

Figure 2. Colon and Cecum Isolation A: Isolated cecum and colon; **B:** Colon opened laterally from distal to proximal and washed; **C:** Cecum opened along smaller curvature and washed; **D:** Colon 2 mm punch biopsy map; **E:** Cecum 2 mm punch biopsy map.

Macrophages were centrifuged at 400 x g and resuspended in 1 M Tris base (pH: 7.5) to lyse red blood cells. Macrophages were centrifuged at 400 x g and resuspended in RPMI 1640 + 1% L glutamine to a concentration of 1 x 10^6 cells/mL. Macrophages were treated in triplicate in a 48-well tissue culture-treated plate (Corning; Corning, NY) and placed in a 37°C, 5% CO_2 incubator. Treatments consisted of 5 µg/mL *Escherichia coli* 055:B5 LPS (Sigma; Saint Louis, MO), 10^8 CFU/mL *H. canadensis*, and media. After 6 or

24 hours, culture supernatant was collected and centrifuged at 5000 x g for 10 minutes at 4°C. Supernatant was stored at -20°C. The collection of macrophages and subsequent treatments for either 6 or 24 hours was conducted twice.

Cytokine ELISA

Immulon ELISA plate were coated with the appropriate mouse capture antibody concentration; IL-6 plates were coated at 1 μg/mL (BioLegend; San Diego, CA), TNF-α plates were coated at 3 μg/mL (BioLegend; San Diego, CA), and IL-1α plates were coated at 0.5 μg/mL (BioLegend; San Diego, CA). Coated plates were incubated overnight at 4°C. Plates were brought to room temperature for 30 minutes and washed five times with wash buffer: 0.05% TWEEN-20 in PBS. Unbound sites were blocked with blocking buffer: filter-sterilized 10% FBS in PBS, for 2 hours at room temperature. Plates were washed 5 times with wash buffer. Samples diluted in blocking buffer were added in triplicate. A standard curve, six two-fold dilutions and a zero, were also added in triplicate; IL-6 standard curve started at 500 ng/mL (BioLegend; San Diego, CA), TNF-α standard curve started at 1000 ng/mL (BioLegend; San Diego, CA), and IL-1α standard curve started at 250 ng/mL (BioLegend; San Diego, CA). After a 2-hour incubation at room temperature plates were washed 5 times. Biotin-linked rabbit anti mouse IgG was added as a secondary at appropriate concentrations; IL-6 at 2 μg/mL (BioLegend; San Diego, CA), TNF-α at 1 μg/mL (BioLegend; San Diego, CA), and IL-1α at 0.15 μg/mL (BioLegend; San Diego, CA). After incubating for 1 hour plates were washed 5 times and streptavidin horseradish peroxidase (Jackson Immuno Research; West Grove, PA) at a 1:1000 dilution in blocking buffer, was added to each well and incubated

for 30 minutes at room temperature. Plates were washed 5 times and 3,3',5,5' tetramethylbenzidine (Thermo Scientific; Waltham, MA) was added to each well in the dark. Plates were incubated for 15 minutes in the dark at room temperature. The reaction was stopped with 2 M H_2SO_4 and read at 450nM with a BioTek PowerWave XS2 microplate reader. Samples with CV% values greater than 10 were rerun.

Statistical Analysis

Statistical analysis was conducted using a two-tailed t-test through the GraphPad Prism program. Statistical values were determined for data collected from two replicate experiments. All data presented in the results section are shown as the mean of two replicate experiments with SEM. Statistical significance was determined to be a P value < 0.05.

CHAPTER III

RESULTS

16S rDNA PCR

Fecal DNA was isolated from stool samples collected from 4 C57BL/6J colony mice using a QIAamp DNA Stool Mini Kit. PCR analysis was conducted using *Helicobacter* genus 16S rDNA primers (Figure 3A), *H. canadensis* 16S rDNA primers (Figure 3B), and universal bacterial 16S rDNA primers (Figure 3C). The PCR products were run on a 1% agarose gel with ethidium bromide at 90V for 1 hour. PCR confirmed that colony mice were not colonized by *H. canadensis* or other *Helicobacter* species.

Serum ELISA

ELISA was conducted to detect IgG antibodies specific for *H. canadensis* in serum collected from 3 C57BL/6J colony mice. Serum from mice infected with *H. canadensis* from a previous study were used as a positive control. IgG specific for *H. canadensis* was not detected in any colony mouse (data not shown).

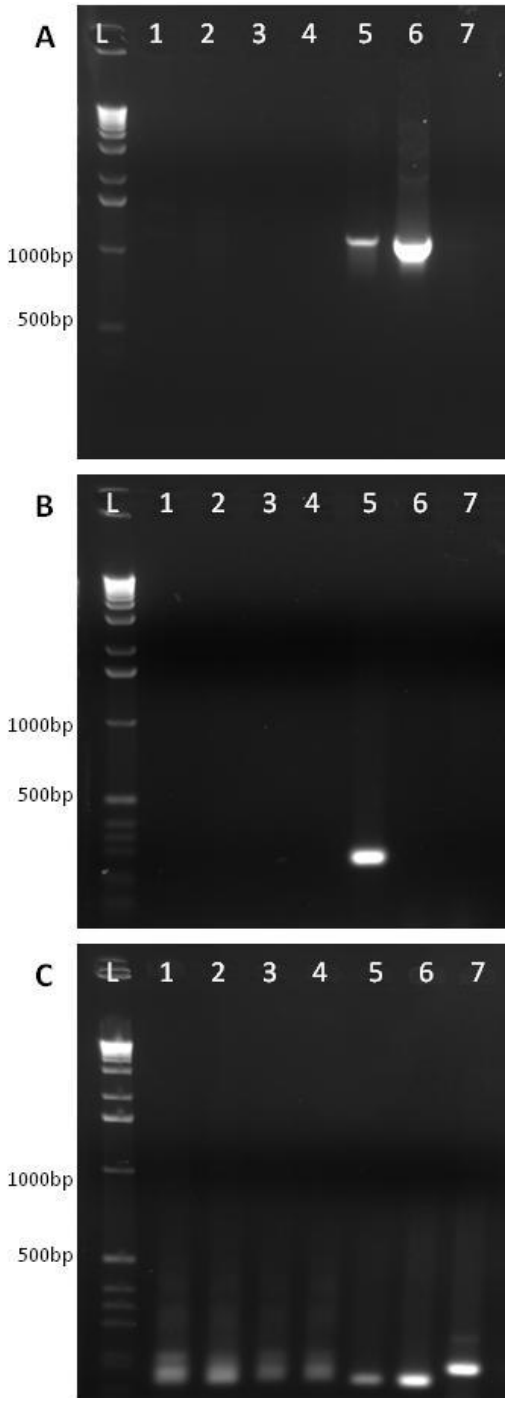

Figure 3. *Helicobacter* Genus 16S rDNA and *H. canadensis* 16S rDNA are not Present in Colony Mice. PCR analysis was conducted on stool sample DNA to amplify *Helicobacter* genus-specific, *H. canadensis*-specific, and universal bacterial 16S rDNA. **A:** *Helicobacter* genus 16S rDNA primers (1,200 bp product), **B:** *H. canadensis* 16S rDNA primers (262 bp product), **C:** universal bacterial 16S rDNA primers (approximately 220 bp product). L: 1 Kb ladder, 1-4: colony mice, 5: *H. canadensis* DNA, 6: *H. felis* DNA, 7: *E. coli* DNA, 8: water.

Colon Explants

Two-mm punch biopsies of colon explants were treated with 500 ng/mL TNF-α, 10^8 CFU/mL *H. canadensis*, or RPMI-1640 supplemented with glutamine for 24 hours. Cytokine ELISAs were conducted on culture supernatants. There was no significant difference in IL-6 secretion between untreated colon explants and explants treated with 10^8 CFU/mL *H. canadensis* (Figure 4). TNF-α and IL-1α secretion were undetectable in 10^8 CFU/mL *H. canadensis* treated and untreated colon explants (data not shown).

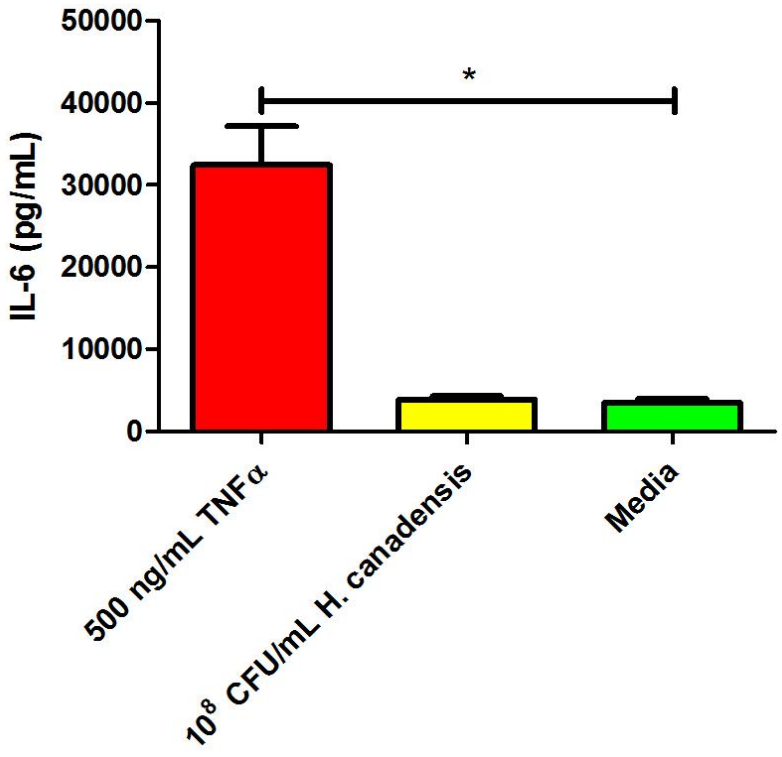

Figure 4. *H. canadensis* Infection of C57BL/6J Colon Explants for 24 Hours Does Not Alter IL-6 Secretion. 2 mm punch biopsies of colon explants were treated with 500 ng/mL TNF-α, 10^8 CFU/mL *H. canadensis*, or RPMI-1640 supplemented with glutamine for 24 hours. *indicates significance of P value <0.05

Cecum Explants

Two-mm punch biopsies of cecum explants were treated with 500 ng/mL TNF-α, 10^8 CFU/mL *H. canadensis*, or RPMI-1640 supplemented with glutamine for 24 hours. Cytokine ELISAs were conducted on culture supernatants. IL-6 secretion (Figure 5) is slightly but significantly lower in 10^8 CFU/mL *H. canadensis*-treated cecum explants (mean ± SEM: 6736 ± 778.8) than in untreated explants (mean ± SEM: 9745 ± 1218). TNF-α and IL-1α secretion were undetectable in 10^8 CFU/mL *H. canadensis*-treated and untreated cecum explants (data not shown).

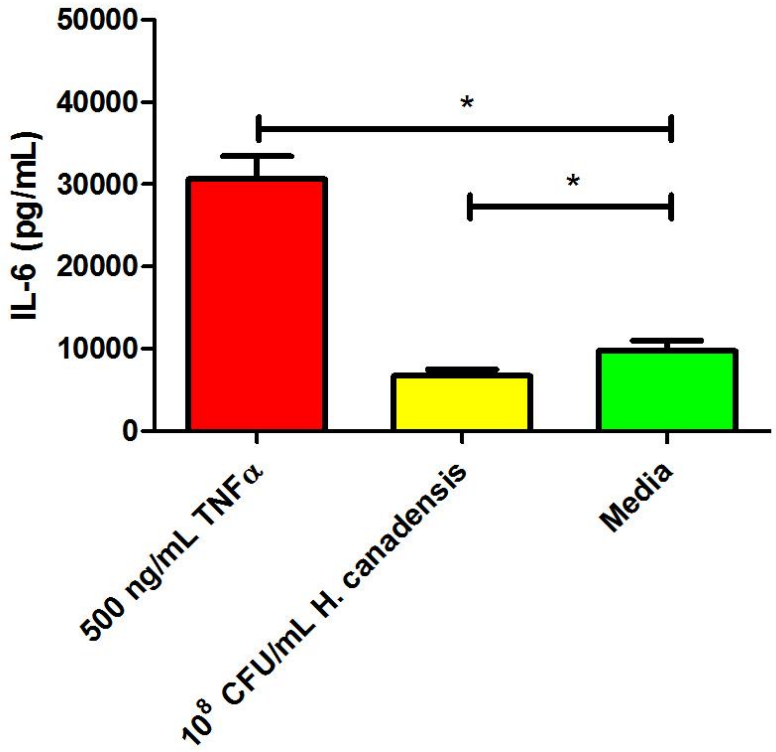

Figure 5. *H. canadensis* Infection of C57BL/6J Cecum Explants for 24 Hours Does Not Alter IL-6 Secretion. Two-mm punch biopsies of cecum explants were treated with 500 ng/mL TNF-α, 10^8 CFU/mL *H. canadensis*, or RPMI-1640 supplemented with glutamine for 24 hours. *indicates significance of P value <0.05

Thioglycollate-Elicited Macrophages

6 hour treatment

Thioglycollate-elicited macrophages were treated with 5 μg/mL LPS, 10^8 CFU/mL *H. canadensis*, or RPMI-1640 supplemented with glutamine for 6 hours. Cytokine ELISAs were conducted on culture supernatants. IL-6 secretion (Figure 6) was significantly greater in 10^8 CFU/mL *H. canadensis*-treated thioglycollate-elicited macrophages (mean ± SEM: 8217 ± 341.8) than untreated thioglycollate-elicited macrophages (mean ± SEM: 4896 ± 749.4). TNF-α and IL-1α secretion were undetectable in 10^8 CFU/mL *H. canadensis*-treated thioglycollate-elicited macrophages and untreated thioglycollate-elicited macrophages (data not shown).

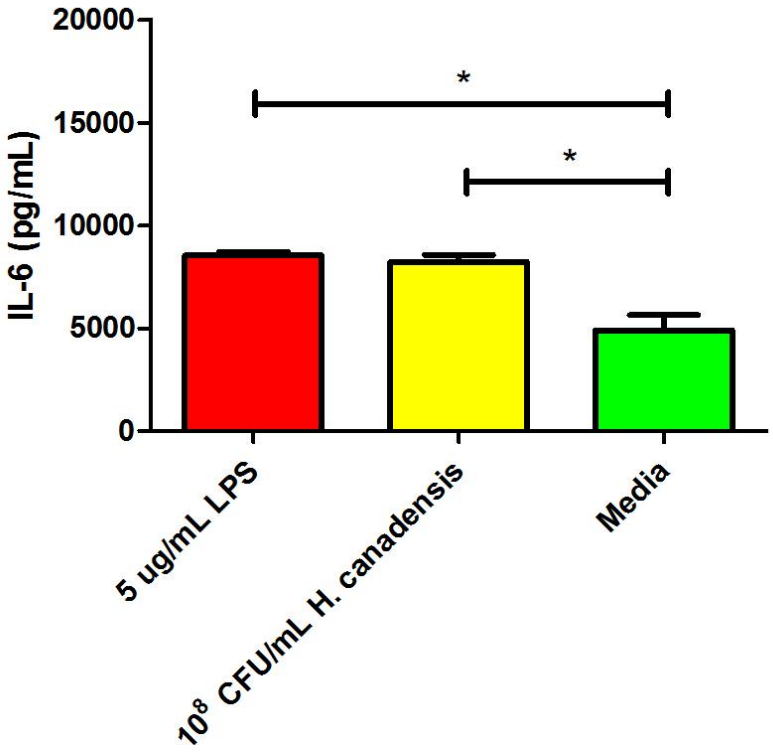

Figure 6. *H. canadensis* Infection of Thioglycollate-Elicited Macrophages for 6 Hours Induces IL-6 Secretion. Thioglycollate-elicited macrophages were treated with 5 μg/mL LPS, 10^8 CFU/mL *H. canadensis*, or RPMI-1640 supplemented with glutamine for 6 hours. *indicates significance of P value <0.05

24 hour treatment

Thioglycollate-elicited macrophages were treated with 5 μg/mL LPS, 10^8 CFU/mL *H. canadensis*, or RPMI-1640 supplemented with glutamine for 24 hours. Cytokine ELISAs were conducted on culture supernatants. IL-6 and TNF-α secretion were undetectable in thioglycollate-elicited macrophages treated with 10^8 CFU/mL *H. canadensis* and untreated thioglycollate-elicited macrophages (data not shown). IL-1α secretion (Figure 7) was significantly greater in 10^8 CFU/mL *H. canadensis* treated thioglycollate-elicited macrophages (mean ± SEM: 3362 ± 626.4) than untreated thioglycollate-elicited macrophages (mean ± SEM: 1755 ± 311.0).

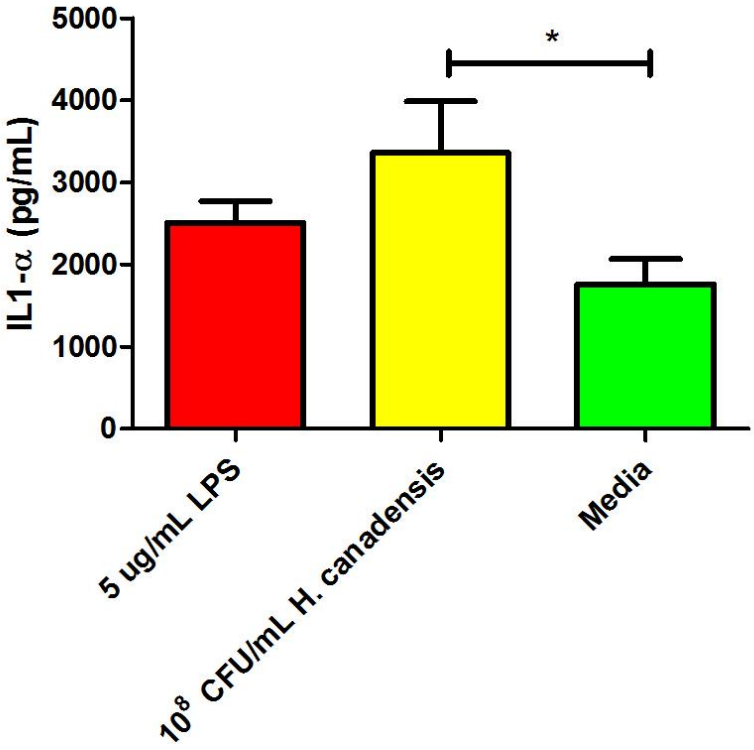

Figure 7. *H. canadensis* Infection of Thioglycollate-Elicited Macrophages for 24 Hours Induces IL-1α Secretion. Thioglycollate-elicited macrophages were treated with 5 μg/mL LPS, 10^8 CFU/mL *H. canadensis*, or RPMI-1640 supplemented with glutamine for 24 hours. *indicates significance of P value <0.05

CHAPTER IV

DISCUSSION

H. canadensis Does Not induce Inflammatory Cytokines in Colon and Cecum Explants

Colon and cecum explants were treated with *H. canadensis* to determine if infection would elicit inflammatory cytokine release. Cytokine ELISA assays showed that *H. canadensis* infection did not induce inflammatory cytokine (IL-6, TNF-α, and IL-1α) release from explants when compared to untreated explants. The results obtained support our hypothesis that *H. canadensis* does not induce inflammatory cytokine release from colon and cecum explants. This result is similar to results of previous experiments conducted in our lab involving infection of C57BL/6J mice with *H. canadensis.* These previous experiments showed that *H. canadensis* colonized the cecum but did not cause clinical signs of disease[80].

Previous publications have described laboratory mice being colonized with Helicobacters when they arrive from the vendor[87,88]. In those studies, the health certificate accompanying the mice indicated that they were *Helicobacter* free but further testing showed the presence of *Helicobacter* [89]. Any work involving our mouse colony is always conducted using biosafety level 2 (BSL2) conditions. BSL2 procedures include working with mice in a laminar flow hood, autoclaving dirty cages and water bottles, autoclaving clean cages, bedding and water bottles before use, using filter top cages and irradiated food, and routine cleaning of the mouse room. To detect if *Helicobacter* species were present in colony mice, we conducted PCR on fecal DNA and serum ELISA specific for *H. canadensis. Helicobacter* and *H. canadensis* 16S rDNA were

not present in fecal samples collected from colony mice. ELISA conducted to detect IgG specific for *H. canadensis* showed that the colony mice were sero-negative for *H. canadensis*. Our PCR and serum ELISA results show that colony mice and those used in these experiments were not infected with *Helicobacter* or *H. canadensis,* which could have otherwise skewed our results.

The genetic background of the mouse model used to investigate IBD-causing pathogens is important. Previous studies have shown that immunocompetent mice, such as those on a C57BL/6J background, infected with the enterohepatic species *H. hepaticus* do not exhibit clinical or histological signs of inflammation, while infection of immunodeficient lines, such as IL-10$^{-/-}$ mice, resulted in colitis[74,90]. It is possible that, like *H. hepaticus*, *H. canadensis* activates the immune response in immunodeficient mice while remaining clinically neutral in immunocompetent mice. Future mouse studies should utilize immunocompromised mice to better understand *H. canadensis* colonization and modulation of inflammatory responses.

Explants allow researchers to observe tissue responses while controlling culturing conditions[75,78]. Like other investigators, we chose to use explants because we wanted to investigate the inflammatory cytokine response to *H. canadensis* in as close to *in vivo* like conditions as possible. We could have included immortalized mouse colon epithelial cell lines but ATCC, a vendor, only carries two mouse colonic epithelial cell lines: CT26.WT and CT26.CL25. A PubMed search revealed that the CT26 lines are mainly used to either induce tumors or to investigate the efficacy of immunotherapies[91–94]. No literature appeared showing CT26 use in IBD or *Helicobacter* research, while many papers appeared using colon explants[75,76,95,96].

IBD is described as inflammation of the digestive tract. Ulcerative colitis is characterized by inflammation throughout the colon and can be classified into subcategories based on anatomical location[97]. Crohn's disease is most frequently characterized by inflammation in the distal small intestine and proximal colon, but can affect many parts of the gastrointestinal tract[98] . Since previous studies in our lab show that *H. canadensis* colonizes the cecum, we decided to focus on the cecum and proximal colon of C57BL/6J mice.

H. canadensis Induces Inflammatory Cytokines in Thioglycollate-Elicited Macrophages

To determine if macrophages produced inflammatory cytokines in the presence of *H. canadensis* we infected C57BL/6J thioglycollate-elicited macrophages for 6 and 24 hours. We hypothesized that *H. canadensis* would not induce the thioglycollate-elicited macrophages to secrete inflammatory cytokines. *H. canadensis* infection for 6 hours resulted in statistically greater IL-6 secretion than uninfected thioglycollate-elicited macrophages. 24 hour infections resulted in statistically significant IL-1α secretion from *H. canadensis* infected thioglycollate-elicited macrophages as compared to uninfected thioglycollate-elicited macrophages.

As with mouse strains used to study IBD, thioglycollate-elicited macrophages are ideally harvested from C57BL/6J and BALB/c mice[79]. Thioglycollate-elicited macrophages from C57BL/6J mice elicit a Th1 response while thioglycollate-elicited macrophages from BALB/c elicit a Th2 response. Since we were investigating pro-inflammatory cytokines, C57BL/6J mice were the ideal genetic background to use.

Eliciting peritoneal macrophages by using thioglycollate has been used for over 50 years. It had been believed that about 90% of the cells collected using this method were macrophages, but recent studies indicate that the percentage of macrophages is much lower[79,99]. A paper published in 2012, reported that macrophages comprised 40-45% of thioglycollate-elicited peritoneal cells, while eosinophils made up 30-40% of the cell population[99]. In addition, it was found that the presence of eosinophils reduced inflammatory cytokine response when compared to cultures that were depleted of eosinophils. This indicates that what we thought was a close to pure culture of macrophages most likely also contained eosinophils. Our results cannot be attributed to macrophages alone. It is possible that an eosinophil-depleted culture would result in stimulation of all three inflammatory cytokines that we investigated. One method to remove eosinophils from cultures is to use mouse monocyte isolation kits, which magnetically label non-monocyte cells and are retained in a column; MACS and Stem Cell Technologies are companies that sell such kits. Another method, although not currently available at SIUE, is to use a cell sorter to remove eosinophils that have been tagged by antibodies.

The production and release of inflammatory cytokines is a tightly controlled process and overproduction of these cytokines can lead to intestinal inflammation. Several studies have shown that upon stimulation, IL-6 mRNA and protein levels increase and plateau after 4-6 hours[100–102]. One mode of cytokine regulation is microRNAs (miRNA), which are small endogenous non-coding RNA molecules that act as posttranscriptional regulators of gene expression. Another mode of regulation is the half-life of cytokines; serum IL-6 has a half-life of <6 hours[102,103]. In our experiments

involving peritoneal cells, IL-6 protein expression (as observed with cytokine ELISA assays) was detectable at 6 hours but was undetectable at 24 hours. It is possible that the macrophages began producing miRNA specific for IL-6 mRNA between 6 and 24 hours as a mode of regulating the inflammatory cytokine response that *H. canadensis* was inducing and the IL-6 initially secreted degraded by the 24 hour collection time point.

Positive Controls

We tested several candidate positive controls for both explants and thioglycollate-elicited macrophages, none of which elicited secretion of all three inflammatory cytokines that we were investigating. *Salmonella typhimurium* has been used in mouse studies to induce colitis after pretreatment with the antibiotic streptomycin[104,105]. In our studies, we first pretreated explants with media that contained streptomycin, penicillin, and gentamycin and then conducted infections with several concentrations (10^9 CFU/mL, 10^8 CFU/mL, 10^6 CFU/mL) of *S. typhimurium.* We did not obtain stimulation of IL-6, TNF-α, or IL-1α for any *S. typhimurium*-treated explants. It is uncertain why this occurred and we moved on to other candidates.

We next tested mouse TNF-α protein at varying concentrations (1000, ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL. 100 ng/mL, 62.5 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL) in colon and cecum explants. We also tested TNF-α protein at varying concentrations in thioglycollate-elicited macrophage cultures (250 ng/mL, 125 ng/mL). For explants, IL-6 secretion was induced by 500ng/mL TNF-α protein but TNF-α secretion was not detected above the dosage amount while IL-1α secretion was not

induced. The macrophages did not secrete significant levels of IL-6, TNF-α, or IL-1α when treated with any concentration of TNF-α protein for 6 and 24 hours.

A23187, a calcium ionophore, and phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C, have been used to stimulate various immune cells such as leukocytes, lymphocytes and bone marrow-derived mast cells[106–108]. Unfortunately the combination of PMA and A23187 at varying concentrations (100 nM PMA + 2 μM A23187, 50 nM PMA + 1 μM A23187, 25 nM PMA + 0.5 μM A23187) were unable to stimulate IL-6, TNF-α, or IL-1α secretion from thioglycollate-elicited macrophages treated for 6 and 24 hours.

Finally, we tested lipopolysaccharide from *Escherichia coli* 055:B5 as a positive control for thioglycollate-elicited macrophages at varying concentrations (20 μg/mL, 10 μg/mL, 5 μg/mL). LPS stimulates TLR4 resulting in the release of pro-inflammatory cytokines, but unfortunately we did not see stimulation of all three cytokines at a consistent concentration. At 6 hours, 5 μg/mL LPS stimulated only IL-6 secretion while 20 μg/mL stimulated only TNF-α secretion. At 24 hours, 20 μg/mL LPS only stimulated TNF-α secretion.

Conclusion

Our results show that *H. canadensis* does not induce inflammatory cytokine secretion from colon and cecum explants. This supports previous findings in our lab and our hypothesis that *H. canadensis* does not induce an inflammatory response. The results obtained from thioglycollate-elicited peritoneal cells (originally believed to be mainly macrophages) show that *H. canadensis* infection induced IL-6 secretion at 6

hours and IL-1α secretion at 24 hours. The results from peritoneal cell cultures indicate that macrophages are responding to *H. canadensis* infection but in an unexpected manner as shown by the disappearance of IL-6 at 24 hours.

Future Studies

The first step in furthering our research with *H. canadensis* is to identify a suitable positive control for the explants and macrophages. Perhaps higher concentrations of candidate positive controls than were used in these experiments will elicit inflammatory cytokines. If not, the published literature should be further reviewed for other candidate positive controls.

After finding an appropriate positive control, the experiments outlined in this manuscript should be redone to verify the results and to verify that the ELISAs were conducted properly. A method to identify *H. canadensis* adhesion to the explants should also be used. One such method could be generating *H. canadensis*-specific fluorescent antibodies to be used for histology of explants.

Finally, other mouse lines, such as BALB/c and IL-10 $^{-/-}$, should be considered to better characterize the inflammatory cytokine response or lack thereof to *H. canadensis* infection in both explants and whole animal infections.

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