HELICOBACTER INFECTION ALTERS THE PHENOTYPE AND INFLAMMATORY RESPONSE OF MOUSE INTESTINAL MUSCLE MACROPHAGES

by

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Abstract

Helicobacter is a common intestinal pathogen of most laboratory mice from both commercial and academic sources worldwide. Not previously thought to have an effect, recent evidence indicates *Helicobacter* infection alters cytokine, chemokine, and gene expression in the stomach, intestine, and colon. Though the *in vivo* cell types responsible for these changes are currently unknown, *in vitro* results suggest macrophages are the likely source. In addition to detection and elimination of pathogens, intestinal macrophages play a role in maintaining homeostasis. By altering gene expression and cytokine production in the microenvironment, we hypothesized that *Helicobacter* infection altered the phenotype and inflammatory response of submucosal intestinal macrophages.

To test this hypothesis, we examined macrophages within whole mounts of intestinal muscle as well as isolated macrophages from *Helicobacter*-infected or uninfected mouse intestine. Macrophages from the intestinal muscle of *Helicobacter*-infected mice showed increased expression of F4/80 and CD11b, altered gene expression, and increased phagocytosis when compared to macrophages from uninfected mice. Infection also altered the macrophage response to stimuli. Macrophages from infected mice produced significantly lower concentrations of cytokines, chemokines, and PGE_2 in response to stimulation with either IFN and LPS or IL-4 and IC. These data support our hypothesis demonstrating that the intestinal muscle macrophage phenotype, function, and response to stimulation are altered by *Helicobacter* infection both *in vivo* and *in vitro*.

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Dedication

I would like to dedicate this to my mother. She is an incredible woman who taught me the true meaning of strength and determination. For better or worse, she gave me an opinionated mind and the will to use it. Without her love and encouragement I would not be where I am today.

CHAPTER 1 - Introduction

I. The Intestine

A. Mucosal Immunity

Mucosal and epithelial surfaces come into contact with environmental microorganisms immediately after birth. Due to constant direct exposure to microbes, these surfaces developed many protective mechanisms to resist invasion (reviewed in (1)). Multiple layers of epithelial cells protect the skin's surface (2), but only a single layer of epithelium covers the surfaces of the gastrointestinal (3), respiratory (4), and urogenital tracts (5). Therefore, these systems require more comprehensive protection (1). This protection comes from multiple mechanical and chemical barriers which break down and remove foreign substances (1). In addition, a very effective innate immune system and a highly specific adaptive immune system protect mucosal sites of higher organisms (1, 6, 7).

The mucosal immune system provides protection against pathogenic microorganisms. Protective mechanisms include the innate immune system and barrier function. A strong innate immune response neutralizes pathogens and prevents infections once the barrier is broken (1). Barrier function consists of preventing penetration by immunogenic components such as bacteria from mucosal surfaces (reviewed in (1, 8)). Other important roles of mucosal immunity include mucosal tolerance, which is the induction of unresponsiveness to some antigens (9), immunoregulatory functions (10), and maintaining mucosal homeostasis (1).

Mucosal immunity is dominated by innate immune processes that protect the organism within the first minutes and hours of exposure to foreign substances or infection (reviewed in (1, 6)). Innate immunity recognizes and distinguishes between the antigens of pathogenic microbes and the harmless components of microbial commensals (1, 6). Once recognized, a host defense response clears the invading pathogen (1, 6). Along with

many other molecules, this involves pattern recognition receptors (PRRs) which recognize and respond to conserved sequences present on pathogenic and commensal microorganisms (1, 11). After binding PRRs, microbial molecules, such as lipopolysaccharide (LPS) and peptidoglycan, trigger cytokine and chemokine production (1, 11). Phagocytosis by macrophages and neutrophils also removes pathogens ((12, 13) reviewed in (14)).

The primary mechanical barrier of most mucosal surfaces consists of single layer of several types of interconnected, polarized, epithelial cells (reviewed in (1)). Tight junctions reinforce the intestinal epithelial layer. These junctions connect adjacent epithelial cells and aid in forming an interconnected network (1). Tight junctions also maintain epithelial cell polarity and act as ports of entry that open and close in response to signals from the lumen, lamina propria or epithelium (1, 15). Many types of epithelial cells participate in mucosal barrier function, including enterocytes and colonocytes (1, 6). Barrier function also involves specialized epithelial cells such as goblet and Paneth cells (1, 6). Goblet cells produce mucus for protection and trefoil peptides required for epithelial growth and repair, while Paneth cells secrete antimicrobial peptides such as defensins (1, 6).

Intestinal epithelial cells play an essential part in the natural defense mechanism of mucosal surfaces. They are involved in various immune processes in addition to their absorptive, digestive and secretory functions (reviewed in (1)). The intestinal epithelium produces an array of cytokines, chemokines, and antimicrobial peptides as well as providing a physical barrier (1). Epithelial cell lines, constitutively or upon stimulation, produce a number of cytokines, chemokines and inflammatory mediators including interleukin 6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (MCP-1) (1, 16). These pro-inflammatory cytokines, also produced by macrophages and T cells, influence epithelial cell physiology and function (1). Stimulation of epithelial cells with interferon γ (IFN-γ), IL-1, or tumor necrosis factor α (TNF-α) increases expression of PRRs and other surface molecules as well as production of cytokines and chemokines (1, 17, 18).

Finally, epithelial cells present antigens which may induce anergy rather than activation (19-21).

B. Immune cells of the Intestine

The gut associated lymphatic tissue is the largest lymphatic organ of the body (22). Mucosa-associated lymphatic tissue consists of lymphatic follicles, Peyer's patches, the appendix, and freely dispersed, lamina propria lymphocytes (1, 22). These cooperate with other components of the innate immune system to initiate adaptive immune responses (1, 22). Some organized lymphatic tissues such as follicles are covered with an epithelial layer containing specialized epithelial cells called M cells (1, 23). Pinocytic M cells absorb and transport antigens from the intestinal lumen to Peyer's patches resulting in T cell activation and induction of mucosal immunity (1, 23).

Lamina propria leukocytes are a critical effector component of mucosal immunity (1). Intraepithelial lymphocytes, present in the epithelium and on the basolateral side of enterocytes, are predominately $CD8^+$ and γδ T cells which may have a limited range of specificities (1, 24-26). These T cells identify proteins not normally present in the epithelium and kill damaged or altered epithelial cells (1). Intraepithelial and lamina propria dendritic cells sample the lumen by forming transepithelial dendrites (27, 28). These cells then stimulate an immune response to detected pathogens by carrying them to the Peyer's patches and lymph nodes (29) or stimulate immune tolerance by transporting apoptotic epithelial cells to lymph nodes (30). Diffuse lymphocytes of the lamina propria are the most varied and the most active mucosal effector cells (1). They include $CD4^+$ T cells (31), B cells producing polymeric IgA (32), eosinophils (33), neutrophils (33), mast cells (34) and macrophages (35). Large numbers of macrophages are also present in the muscle layers surrounding the intestine, but their function is not well defined (36, 37).

II. Macrophages

A. General Information

Macrophages are white blood cells that display varied phenotypes and functions depending upon tissue location in the host. Many innate and adaptive immune processes involve macrophages. One important function is clearance of pathogens and apoptotic bodies via phagocytosis. Macrophages recognize and ingest fungi, bacteria, viruses, cellular debris, and immune complexes (reviewed in (38-40)). Inside vesicles called phagosomes, macrophages kill and/or break these components down by nutrient deprivation, antimicrobial peptides, or reactive oxygen and nitrogen species (reviewed in (41)).

Once a pathogen has been phagocytosed and degraded, macrophages present foreign proteins to T cells and B cells in major histocompatibility complex molecules on the cell surface (42). Though macrophages present antigen, dendritic cells are more effective (43, 44). This stimulatory signal activates T cells and B cells to produce cytokines and/or antibodies to help combat the pathogenic challenge. Additionally, macrophages produce a wide variety of cytokines, chemokines and lipid mediators in response to a range of stimuli including lipopolysaccharide, immune complexes and other cytokines (45).

Wound healing and tissue repair after injury or inflammation is another characteristic of macrophages (46). To facilitate restoration of blood flow to healing tissue, macrophages release angiogenic molecules such as vascular endothelial growth factor and fibroblast growth factor into the extracellular matrix (47). These factors, which aid in the rebuilding of blood vessels, are produced in response to hypoxia and low extracellular pH due to anaerobic cell metabolism (48). Though typically associated with tissue damage, nitric oxide (NO) produced by macrophages can promote tissue repair in the skin (49, 50). Alternatively activated macrophages also support tissue repair by production of fibronectin (51), and induction of arginase in response to Th2 cytokines

(52, 53). This leads to urea and ornithine synthesis, as well as production of polyamines that promote cell proliferation and prolines required for collagen synthesis (54).

Macrophages demonstrate a broad array of functions and phenotypes. It remains unclear whether macrophage characteristics are predetermined (45) or constantly changing (55). However, macrophages readily change their phenotype and function depending on the stimuli in the microenvironment both *in vivo* and *in vitro* (55-57). Therefore, it is likely that macrophages exist in varying activation states capable of reacting to changes in the microenvironment in response to injury or inflammation.

B. Macrophage Stimulation

The functional plasticity of macrophages allows them to respond to various stimuli in a multitude of ways. These stimuli include growth factors, cytokines, and chemokines, as well as bacteria, viruses, parasites, complement proteins, antibodies, immune complexes, damaged cells and a wide range of foreign peptides. Exposure to growth factors induces monocytes and bone marrow precursors to differentiate into macrophages (58-61). Contact with cytokines and pathogenic components either *in vivo* or *in vitro* stimulates macrophages to mature, change surface marker expression and in some cases change phenotypes (55-57, 62-65).

In vitro, macrophages differentiate from monocytes using growth factors such as monocyte-colony stimulating factor (M-CSF), also known as colony stimulating factor-1 (CSF-1) (60). M-CSF, produced by macrophages and activated T cells (66, 67), promotes proliferation and differentiation of monocytes and macrophages (68). Produced by macrophages, T cells, fibroblasts and endothelial cells, granulocyte/macrophage colony stimulating factor (GM-CSF) also stimulates the growth and differentiation of macrophages (reviewed in (61)). GM-CSF and M-CSF promote differentiation of bone marrow cells into two different phenotypes of macrophages, pro-inflammatory, type 1 (M1) macrophages and anti-inflammatory, type 2 (M2) macrophages, respectively (58,

69). These two macrophage phenotypes have different morphology, cytokine expression, and gene expression as well as slightly different surface marker expression (58, 69).

Macrophages mature and further differentiate in response to a variety of stimuli. Some stimuli induce a classically activated, M1 macrophage phenotype while others induce an alternatively activated, M2 macrophage phenotype. IFN-γ, secreted by Th1 cells in response to infection, contributes to leukocyte recruitment (70). It also induces an M1 macrophage phenotype and stimulates macrophages to kill phagocytosed bacteria (71). LPS, a gram negative bacterial surface protein, activates macrophages through Toll-like receptor 4 (TLR4) which stimulates pro-inflammatory cytokine production by M1 macrophages (72). In contrast, IL-4 is an anti-inflammatory cytokine produced by Th2 (73) and natural killer T cells (74). IL-4 induces macrophages to develop into an alternatively activated, M2 macrophage phenotype (69, 75). Immune complexes (IC), antigen-antibody aggregates, stimulate M2 macrophages to produce anti-inflammatory cytokines, but do not stimulate M1 macrophages (75).

In addition to being stimulatory, LPS activation induces production of a number of inhibitors and negative regulators of LPS signaling, thereby reducing downstream pro-inflammatory cytokine production (76, 77). ST2/T1, a member of the IL-1 receptor (IL-1R) family, is an inhibitor of LPS signaling. Mast cells (78), fibroblasts, macrophages, and Th2 T cells (77, 79) express ST2 in a membrane bound form. In response to LPS, ST2 down-regulates TLR4 expression and inhibits signaling through TLR4 and IL-1R thereby inducing tolerance (77, 80). Mice lacking ST2 do not develop tolerance to LPS (80). ST2 also exists in a soluble form (sST2) which has anti-inflammatory effects directly on macrophages (77). LPS induces both membrane-bound and soluble ST2 in alveolar macrophages (76). However, the lack of ST2 results in decreased numbers of $F4/80^+$ macrophages and increased inflammation in the lung (81). This suggests ST2 also modulates lung inflammation (76, 81). Although sST2 does not affect IL-10 or NO production, treatment of bone marrow-derived macrophages with sST2 inhibits IL-6, IL-12 and TNF-α production after LPS stimulation (77). Soluble ST2 also decreases

inflammation due to collagen-induced arthritis (82) and inhibits inflammation after intestinal ischemia reperfusion by increasing IL-10 (83).

Single immunoglobulin IL-1R-related molecule, SIGIRR (also known as Toll IL-1R 8) is a member of the TLR-IL-1R superfamily that negatively regulates immune responses (84). Macrophages and dendritic cells express SIGIRR at low levels, but epithelial cells express the molecule at high levels (85, 86). It can block both IL-1R and TLR4 signaling (85, 87-89) though these inhibitions occur through different mechanisms (90). Expression of SIGIRR on colonic epithelial cells has an important role in gut homeostasis and intestinal inflammation in response to commensal microorganisms (91). Lack of SIGIRR results in enhanced inflammation after administration of IL-1 and increased lethal susceptibility to LPS (84). In the absence of SIGIRR, PGE_2 and pro-inflammatory cytokine and chemokine concentrations increased in the intestine (92). This correlated with an increased susceptibility to intestinal cancer (92).

Interleukin-1 receptor-associated kinase of monomyeloic origin (IRAK-M) (93, 94), a molecule expressed by monocytes and macrophages (95), negatively regulates TLR signaling and plays a role in innate immune homeostasis (96). The up-regulation of IRAK-M by macrophages leads to both LPS and peptidoglycan tolerance (95, 97, 98) as well as tolerance to tumor cells (99). S-nitrosoglutathione, a NO donor, induces IRAK-M over-expression and leads to LPS tolerance (100). NO stimulates TNF-α expression by monocytes which induces IRAK-M in an autocrine manner (100). Anti-TNF-α antibodies inhibit IRAK-M expression suggesting that TNF modulates IRAK-M associated LPS tolerance (100). In a murine model of sepsis, IRAK-M mediates immunosupression and tolerance to secondary infections leading to decreased macrophage function in the lungs without altering TLR2 or TLR4 expression (101). In the absence of IRAK-M, macrophages increase inflammatory cytokine production in response to bacteria (96, 101). IRAK-M deficient mice also show significantly decreased tolerance to LPS and increased inflammation during bacterial infection (96) indicating an important role for IRAK-M in the response to LPS.

C. Types of Macrophages

Human

In vitro studies categorize activated macrophages into two types: classically or alternatively activated. Classical activation of macrophages by IFN-γ and LPS results in an M1 macrophage phenotype (70, 72). These classically activated, M1 macrophages secrete pro-inflammatory cytokines and support Th1 responses (69). Human blood-derived monocytes isolated by $CD14^+$ bead separation and cultured with GM-CSF display an M1 macrophage phenotype (69). They secrete IL-12, IL-1β, IL-6, and TNF-α upon stimulation with LPS in the presence or absence of IFN-γ (69). Alternative activation of macrophages by IL-10 or the combination of IL-4 and IC results in an M2 phenotype. Alternatively activated, M2 macrophages secrete anti-inflammatory cytokines, support Th2 responses, and play a role in regulating cellular immunity and homeostasis (69, 102). When cultured in M-CSF, $CD14^+$ human blood-derived monocytes display an M2 macrophage phenotype, secreting IL-10 upon stimulation with LPS (69).

Though having different morphologies, with M1 macrophages appearing rounded and M2 macrophages having long dendrite-like protrusions, M1 and M2 macrophages derived from human monocytes express similar levels of most surface markers (69). The only difference detected was in expression of CD163, a scavenger receptor, which was ten-fold higher on M2 macrophages (69). Both M1 and M2 macrophage phenotypes secrete IL-8, MCP-1, interferon inducible protein-10 (IP-10), and macrophage inflammatory protein 1 (MIP-1) in response to LPS stimulation (69).

Mouse

Unlike human monocytes, M-CSF induces both M1 and M2 macrophage phenotypes from mouse bone marrow (75) (Table 1). A classically activated, M1 macrophage phenotype results from LPS and IFN-γ stimulation of M-CSF cultured mouse bone marrow macrophages (75). These macrophages produce high levels of pro-inflammatory IL-12, IL-1 and TNF-α (75). In addition, M1 macrophages metabolize

arginine to NO as well as expressing inducible nitric oxide synthase (*iNOS*), one of the genes reponsible for NO synthesis (75).

Using mouse bone marrow, recent *in vitro* studies have revealed two distinct types of alternatively activated M2 macrophages (Table 1). The first M2 macrophage phenotype develops in response to M-CSF and IL-4. Unlike M1 macrophages, these cells do not express *iNOS* or produce NO (75). However they metabolize arginine to urea, which promotes tissue repair by inducing collagen production, via *Arginase-1* (*Arg-1*) (54, 75) and produce low levels of IL-10, which down-regulates Th1 responses, upon stimulation (103). The presence of *Arg-1, Fizz-1* (*found in inflammatory zone-1*), and low expression of *Sk-1* identify this type of M2 macrophage (75, 104-106). *Fizz-1* produces a cysteine-rich secreted protein that may be involved in tissue repair (105). A second M2 macrophage phenotype, developing in response to M-CSF and IC, expresses *iNOS* and produces NO similar to M1 macrophages (75). Unlike the other M2 macrophage phenotype, these macrophages produce large amounts of IL-10 and express high levels of *Sphingosine kinase-1* (*Sk-1*) (75). Other M2 macrophages, but not M1 macrophages, also express *Ym-1*, a heparin-binding lectin that may be involved in tissue repair (104, 107).

The identification of the second M2 macrophage phenotype that has characteristics of both M1 and M2 macrophages confounds the current model of macrophage classification. Although this study classified this intermediate phenotype as an M2, it suggests that the model is not sufficient to describe the observed macrophages phenotypes induced *in vitro*. In addition, this model likely does not apply to all macrophages found *in vivo* since there is high variability in the phenotypes and functions of tissue macrophages.

Table 1. Summary of Mouse Macrophage Phenotypes

	M1 MΦ-Classical	**M2 MΦ-Alternative**	
	Ca MΦ (75)	MII MΦ (75)	AA MΦ (75)
Cultured in	M-CSF	M-CSF	M-CSF
Stimulation	LPS+IFN	LPS+IFN+IC	IL-4
Cytokines	IL-12	IL-10	IL-10
NO	High	High	Very low
Urea/Arginase	Low	Low	High
Gene expression	*iNOS*, *IL-12p40*	*iNOS*, *Sk-1*, *IL-10*	*Arg-1*, *Fizz-1*, *Sk-1*^{lo}

In addition to gene expression and cytokine production, surface markers identify macrophages. CD115, a growth factor receptor also known as CSF -1 receptor or c-fms (108), and major histocompatibility complex II (MHC-II) (109, 110) are expressed on most macrophages. Most macrophages and neutrophils also display surface CD14 and TLR4, parts of the LPS co-receptor (111), and CD11b (Mac-1) (112) . However, CD83^{+} dendritic cells also express CD11b and TLR4 (113). CD36, a scavenger receptor, identifies most macrophages but endothelial cells may express CD36 and TLR4 (114). Mature macrophages specifically express F4/80 (115). Depending on tissue location and activation state, levels of these markers vary on the cell surface.

Culture with M-CSF followed by cytokine treatment has been traditionally thought to terminally differentiate macrophages. However, macrophages may reversibly change phenotypes and functions depending on stimuli present (55, 56). *In vitro*, cytokines induce an array of phenotypes in bone marrow-derived macrophages, peritoneal macrophages, and macrophages from tumor bearing mice (55, 56). Changing the cytokines present in the culture reverses or alters these phenotypes (55, 69, 75). For example, treatment of bone marrow macrophages with IFN-γ induced production of TNF-α and IL-12 upon stimulation with LPS (55). However, IFN-γ priming followed by IL-10 treatment resulted in decreased TNF-α and IL-12 along with increased IL-10 after

LPS stimulation (55). This indicates that the composition of the microenvironment prior to bacterial contact yields different immunologic responses. Additionally, mouse bone marrow macrophages activated with IFN-γ and LPS change their phenotype from M1 to M2 in response to FcγR ligation or IC stimulation after removal of IFN-γ and LPS (102). Therefore peritoneal and bone marrow-derived macrophages likely represent a malleable set of immunologically active cells rather than fixed subpopulations. However, it is unclear if further differentiated resident tissue macrophages respond to stimuli in a similar manner.

D. Intestinal Macrophages

The intestine contains the largest population of macrophages in the body (116). Recent studies investigated the phenotype and function of these macrophages in humans, rats and mice, with those from humans being better characterized (36, 37, 117-124). However, there is not a consensus on the phenotype or function of intestinal macrophages. The disparities in observed phenotypes may be due in part to isolation of macrophages from different intestinal tissues using a variety of separation and digestion methods. Nevertheless, the observed intestinal macrophage phenotypes do not coincide with a classical M1 macrophage phenotype.

Human

Analysis of several studies suggests that the isolation method may alter the expression of macrophage cell surface markers (117-120). Initial studies isolated human macrophages by treatment with EDTA to remove the epithelium (de-epithelialized) followed by enzymatic digestion of the remainder of the tissue. Then gradient sedimentation and counterflow centrifugal elutriation separated the macrophages based on size and density (117-119). These macrophages retained avid phagocytic and bactericidal activity (117, 118). Macrophages isolated from human intestinal tissue expressed MHC-II, CD13 (aminopeptidase N), CD36, TLR4, and TLR2 (118, 119), though some of these markers were expressed at very low levels. Unlike most macrophages, the enzyme digested macrophages did not express CD14, CD11b (CR3),

CD11c (CR4), or α integrin CD11a (LFA-1) as determined by flow cytometry (117-119). These macrophages had decreased responses to LPS. In response to LPS, IFN-γ, or heat-killed *Staphylococcus aureus*, they produce low levels of IL-8 and do not produce IL-1, IL-6, IL-12, TNF-α, or IL-10 (117). This decreased effect may contribute to the low level of inflammation in normal intestine despite the presence of bacteria and other pro-inflammatory molecules (117).

In a more recent study, human intestinal macrophages were isolated by enzyme digestion and sedimentation in the same manner as above or allowed to migrate out of de-epithelialized whole intestine (120). Similar to digested macrophages, human intestinal macrophages allowed to migrate out of de-epithelialized whole intestine expressed TLR2 and TLR4 (120). However, unlike the digested macrophages, migrated macrophages expressed CD14 and produced TNF-α in response to LPS and *E. coli* particles (120). It is unclear whether the observed differences in macrophage phenotype result from contact with stromal cells during migration or from removal by enzyme digestion.

Interestingly, macrophages can also be found in the muscle layers surrounding the small and large intestine (121). These macrophages are responsible for production of pro-inflammatory cytokines that contribute to postoperative ileus after surgery or transplantation in both humans (125) and rodents (126). Therefore, despite expressing abnormally low levels of most of the typical macrophage markers, intestinal macrophages appear to have normal functions.

Rat

Similar to those seen in humans, macrophages in rats form a regularly distributed network in the muscle layers surrounding the intestine. In whole muscle mounts examined by immunohistochemistry (IHC), these cells express ED2 (similar to F4/80 in mice), MHC II, CD14, and low levels of CD11a (36). $ED2^+$ macrophages in the tissue produce NO and prostaglandin E2 (PGE_2) in response to LPS treatment corresponding to increased *iNOS* and cyclooxygenase-2 (*COX-2*) expression, respectively (65). When isolated by collagenase/dispase digestion from rat intestinal muscle, these macrophages

do not proliferate in culture but do change morphology over time (36). Early after adhesion, the macrophages appear round then develop a stellate configuration with elongated processes after 2-3 days in culture (36). Initially 90% of the macrophages express ED2 and phagocytose Fitc dextran (36). If not passaged, these macrophages retain the normal resident macrophage phenotype for several weeks in culture (36). However, after three passages, ED2 staining and phagocytic activity are lost (36).

When compared to those from the peritoneum, intestinal macrophages isolated from the colon of rats by collagenase digestion have lower CD14 mRNA and surface expression than peritoneal macrophages (123). Colonic macrophages are phagocytic but, unlike those in intestinal whole muscle mounts, do not produce NO or TNF-α in response to LPS (123). Similar to those in humans, macrophages in the intestinal muscle form a network of phagocytic cells that express ED2. These cells are also observed in mice.

Mouse

$F4/80^{+}$ resident macrophages form a regularly distributed network in the muscle layers surrounding the intestines of normal and germ-free mice (122, 127, 128). Similar to human and rat, macrophages isolated from whole mouse intestine by collagenase/dispase enzyme digestion express F4/80 and MHC II (124). Macrophages isolated from only the intestinal muscle also express F4/80 and MHC II, as determined by IHC, and take up Fitc dextran (37).

Resident muscle macrophages mediate inflammation by changing the phenotype of newly recruited monocytes. For example, *in vivo*, injured skeletal muscle tissue recruits pro-inflammatory $F4/80^{lo}$ monocytes that switch to anti-inflammatory $F4/80^{hi}$ macrophages (129). *In vitro*, phagocytosis of necrotic muscle cell debris also induces pro-inflammatory macrophages to change to an anti-inflammatory phenotype (129). It is likely that intestinal muscle macrophages respond in a similar manner. This ability has important implications for both tissue damage and repair mechanisms mediated by macrophages. In addition, the absence or depletion of macrophages in the intestine decreases inflammation after surgery (126). Significantly lower production of IL-1β, IL-

6, and MIP-1α mRNA suggest that resident muscularis macrophages mediate intestinal inflammation (126). Together these data indicate resident intestinal muscle macrophages play an important role in mediating inflammation in the intestine in response to injury. This also suggests that these macrophages may be involved in the response to intestinal infections.

E. Cytokines, Chemokines, and Arachidonic Acid Metabolites by Macrophages

Macrophages are one of the primary cytokine secreting immune cells. In response to stimulation with a wide range of ligands, macrophages produce an array of pro-inflammatory, anti-inflammatory, and homeostatic cytokines (45). Macrophages also secrete other inflammatory mediators such as prostaglandins, leukotrienes, nitric oxide, and urea to regulate the immune response (45).

Cytokines and Chemokines

IL-1α and IL-1β, members of the IL-1 superfamily, are pro-inflammatory cytokines produced by macrophages and dendritic cells in response to LPS binding TLR4 (72, 130). During the inflammatory response, these cytokines increase the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes to sites of infection (131). IL-1 also causes fever by re-setting the hypothalamus (132, 133).

IL-6 is a pro-inflammatory cytokine secreted by macrophages and T cells dispersed in the lamina propria of the intestine as well as macrophages found in other tissues. These cells secrete IL-6 in response to infection, tissue damage, transplant, or graft (134, 135). IL-6, similar to IL-1, mediates fever and the acute phase response (133). In addition to being pro-inflammatory, IL-6 has anti-inflammatory effects. By inducing the expression of IL-4, IL-6 promotes Th2 differentiation and impaired Th1 differentiation (reviewed in (136)). In response to muscle contraction the muscle itself produces IL-6 (137). This may inhibit the effects of pro-inflammatory cytokines during exercise (137) and play a role in the inflammatory response in the vascular system (138). In the muscle and fatty tissue, IL-6 produced by both muscle cells and macrophages

stimulates energy mobilization which leads to increased body temperature. This can occur in response to LPS, TNF-α and IL-1β (139).

IL-10 is an anti-inflammatory cytokine produced by B cells (140), T cells (141), mast cells (142), and macrophages (143). It inhibits production of pro-inflammatory cytokines such as IFN-γ, IL-12, and TNF-α by macrophage and other cell types (144, 145). IL-10 also suppresses antigen presentation by macrophages and dendritic cells (146). Correlating with decreased pro-inflammatory responses, IL-10 stimulates Th2 cells to up-regulate anti-inflammatory cytokines in response to infection or injury (147).

IL-12 is a pro-inflammatory cytokine produced by macrophages, dendritic cells and B cells in response to bacterial challenge (148). IL-12 differentiates naive T cells into pro-inflammatory Th1 cells (149) and reduces IL-4 mediated suppression of the inflammatory response (150). In addition, macrophages produce TNF-α in response to IL-12. By controlling the levels of other cytokines, IL-12 also mediates anti-angiogenic properties (151). IL-12 controls IFN-γ production by T cells (152), which in turn increases the production of IP-10, ultimately blocking the formation of new blood vessels (151).

IP-10 (CXCL10) is a chemokine induced by IFN-γ in a variety of cell types including neutrophils (153), macrophages, endothelial cells, keratinocytes, fibroblasts (154), and activated T cells (155). LPS induced IFN-γ mediates IP-10 production (156). As a chemoattractant for monocytes and T cells (157), IP-10 promotes adhesion to endothelial cells. Also an angiostatic factor, IP-10 inhibits tumor growth and metastasis by preventing angiogenesis (151, 158).

Keratinocyte-derived chemokine (KC), the mouse ortholog of IL-8 or CXCL8, is a chemokine produced by macrophages, endothelial cells and epithelial cells that is often associated with inflammation (159). Macrophages release large amounts of KC after phagocytosis of pathogens or foreign particles (160) and in response to inflammation (161). KC then induces neutrophil infiltration and activation at the site of inflammation

(162). Also playing a role in induction of systemic inflammation and tissue damage after injury (163), MCP-1 regulates KC (164).

MCP-1, also known as CCL2, is produced by macrophages in response to LPS and IFN-γ during infection (165). Though it is not known as a potent neutrophil chemoattractant, MCP-1 mediates polymorphonuclear cell (PMN) infiltration by regulating levels of KC after injury or inflammation (164). MCP-1 also plays a role in many macrophage-associated processes as demonstrated by MCP-$1^{-/-}$ mice (reviewed in (166)). In response to myocardial ischemia, these mice exhibit reduced macrophage infiltration and activation, suppressed cytokine synthesis, and delayed phagocytic removal of dead cardiomyocytes. In addition, after myocardial infarction, myofibroblasts do not accumulate during tissue repair resulting in decreased ventricular remodeling (166).

Macrophage inflammatory proteins MIP-1α and MIP-1β, CCL3 and CCL4 respectively, are chemokines produced by macrophages, neutrophils, and dendritic cells after LPS stimulation (167-171). Both MIP-1α and MIP-1β lead to neutrophilic infiltration during inflammation followed by activation of these cells (172, 173). MIP-1α recruits macrophages to sites of inflammation. Subsequently, MIP-1α activates these cells to produce pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (174). MIP-1α may also play a role in the progression of inflammation from neutrophil-dominated to macrophage-dominated over time (169).

TNF-α is a cytokine produced primarily by macrophages in response to LPS (175, 176), but is also made by mast cells (177, 178) and endothelial cells (18). Involved in both local and systemic inflammation, TNF-α stimulates the acute phase response (179), regulates inflammation and tumorigenesis (reviewed in (180)), and induces fever (181). TNF-α also controls apoptosis, proliferation, and differentiation of immune cells (182, 183). A potent neutrophil chemoattractant (reviewed in (184)), TNF-α aids in adhesion of neutrophils to endothelial cells and transmigration to sites of infection (131, 185). In

addition, TNF-α stimulates macrophages to become more phagocytic (186) and induces the production of inflammatory molecules such as IL-1 (187).

Arachidonic Acid and Arginine Metabolites

Arachidonic acid, a precursor for several biologically active compounds, is derived from membrane phospholipids by phospholipases (reviewed in (188)). Five-lipoxygenase breaks down arachidonic acid into leukotriene B4 (LTB_4), a leukotriene involved in the inflammatory response (reviewed in (189)). LTB_4 is a potent neutrophil chemoattractant which induces the formation of reactive oxygen species and the release of lysosomal enzymes by these cells (190, 191). Produced by monocytes during inflammation (192), LTB_4 induces activation and adhesion of leukocytes to the endothelium aiding transmigration to sites of infection or damage (190, 193).

Prostaglandin E2 is also a derivitive of arachidonic acid (reviewed in (188)). PGG_2 and PGH_2 are synthesized from arachidonic acid by *COX* and converted to PGE_2 by PGE_2 isomerase ((194), reviewed in (188)). *COX-1* constitutively produces PGE_2, while in response to stimulation *COX-2* induces additional PGE_2. PGE_2 plays an important role in various biological events such as apoptosis, tumorigenesis, and inflammation (reviewed in (195)). Produced in large concentrations early in inflammation, PGE_2 promotes vasodilatation and increases vascular permeability indirectly by enhancing the release of histamine and other mediators from tissue leukocytes (reviewed in (196)). As inflammation progresses, macrophages alter PGE_2 synthesis (196). In chronic conditions, PGE_2 inhibits leukocyte activation and cytokine secretion which aids in regulating and shutting down the inflammatory response (196). The pro- or anti-inflammatory properties of PGE_2 are concentration dependent. Low constitutive levels of PGE_2 produced primarily by *COX-1* are responsible for homeostasis and physiological functions, while the induction of *COX-2* results in high levels of PGE_2 that stimulates inflammation (197).

Different types of macrophages metabolize arginine, the amino acid precursor for nitric oxide and urea, differently. In response to LPS or IFN-γ, M1 macrophages and

neutrophils use *iNOS* to metabolize L-arginine to NO (52). NO is toxic to bacteria and aids in clearance of pathogens when released into the microenviromnent (198). Besides being antimicrobial and cytotoxic, NO also plays an important role in tissue repair in the skin, with inhibition of NO production leading to decreased epithelial repair (49, 50).

In response to IL-4, IL-10, and PGE_2, M2 macrophages express *Arg-1* which metabolizes L-arginine to urea and ornithine (52, 53). Urea production can identify alternatively activated macrophages (75, 199). Ornithine production by *Arg-1* is the first step in the polyamine and proline synthesis that is neccesary for wound healing, tissue repair, smooth muscle growth, and collagen synthesis (reviewed in (200)). *Arg-1* also inhibits production of NO (200).

In conclusion, both M1 and M2 macrophages are important regulators of inflammation. They produce an array of pro- and anti- inflammatory cytokines, chemokines and other inflammatory mediators in response to stimuli (45). Since these macrophages correspond with Th1 and Th2 responses (58, 69), both M1 and M2 macrophages are critical to the immune response. Additionally, M2 macrophages may play a role in maintaining homeostasis throughout the body.

III. *Helicobacter*

A. General Information

Helicobacter, a gram negative, spiral, motile bacterium, is the causative agent of ulcers, peptic ulcer diseases, hepatic cancer, and many intestinal diseases ((201, 202) reviewed in (203, 204)). *Helicobacter* infection is difficult to detect because less than 30% of infections exhibit clinical symptoms (201). *H. pylori* is the most common human pathogenic form with more than 50% of the world's population thought to be infected (reviewed in (203, 204)). In some parts of the world, up to 80% of the population is infected by the age of 10 (202).

Helicobacter is also a common intestinal pathogen of laboratory mice. Many colonies are not routinely screened for *Helicobacter* and the infection remains undetected due to a lack of or mild symptoms. A recent study confirmed that 88% of mice from commercial and academic sources worldwide tested positive for *Helicobacter* (205). Exposure to dirty bedding transmits *Helicobacter* to mice through fecal-oral contact (reviewed in (206)). After infection with *Helicobacter,* which normally colonizes the lower intestinal tract of rodents such as mice, rats, gerbils and hamsters, most animals remain asymptomatic for long periods of time. However, a few strains of mice develop colitis that may result in rectal prolapse (206). Previously, *Helicobacter* infection was not believed to influence most experiments. However, recent evidence indicates that *Helicobacter* infection induces inflammation in mice with no disease symptoms (206).

Helicobacter is detected in animal facilities by culture methods, histopathology, and/or polymerase chain reaction (PCR) (reviewed in (206)). The conditions required for culture of the bacterium are difficult and labor intensive. Histological examination of the liver, colon and intestine is not an effective method since not all infected mice develop disease. As a result, PCR for the conserved *Helicobacter* 16s rRNA gene isolated from feces is most often used due to the sensitivity and relative ease of screening (207).

Eradication of infection in animal colonies has proven to be a challenge. Commonly used methods include restocking with *Helicobacter*-free animals, fostering, and embryo transfer ((208, 209), reviewed in (206)). Antibiotics administered in the drinking water is not effective in eliminating *Helicobacter* (210). However, amoxicillin or tetracycline in combination with metronidazole and bismuth given by oral gavage three times daily for 2 weeks was successful in treating *H. hepaticus* infected mice (210, 211). Though individual antibiotics were not effective, this combined treatment also prevented hepatitis and typhlitis disease symptoms in immunocompromised mice (212).

B. Species

H. hepaticus and *H. rodentium* are two strains commonly found in laboratory mice. *H. hepaticus,* first isolated from the livers of mice with chronic hepatitis, is a urease-positive enterohepatic bacterium (213). It induces gastrointestinal track tumors (214, 215), inflammatory bowel disease (216-218), colitis (219, 220), chronic hepatitis (214, 221), and liver necrosis (215) in susceptible strains of mice. Studied susceptible strains include IL-10 knockouts (219), A/JCr mice (reviewed in (222)), germ-free mice (223) and other immunodeficient and tumor suppressor-deficient mice (218, 220). The pathogenesis of *H. hepaticus* may be due to the presence of a cytolethal distending toxin which causes cellular distention and cell cycle arrest, actin accumulation, morphologic alterations and death of HeLa cell monolayers *in vitro* (224, 225). Though expressing active urease similar to that of *H. pylori*, the role of urease in *H. hepaticus* infection is unknown (226). Potential roles include stimulation of phagocyte chemotaxis, immune cell activation, induction of cytokines, and improving survival during passage through the stomach (226). Urease also produces ammonia which, in addition to being a source of nitrogen for protein biosynthesis, may damage host cells leading to increased pathogenesis (226).

Helicobacter rodentium was the first urease-negative *Helicobacter* species isolated from mouse intestines (227). Since it appears to be part of the normal intestinal flora, the pathogenic potential of *H. rodentium* is unknown (227). *H. rodentium* infection alone does not induce development of any clinical disease symptoms, although it can cause diarrhea in immunocompromised mice in conjunction with *H. bilus* (228). In addition, co-infection with *H. hepaticus* and *H. rodentium* is associated with exacerbated hepatitis and altered gene expression in immunocompromised mice (229).

By altering the microenvironment of the stomach, intestine and colon as well as surrounding tissues such as the pancreas, *Helicobacter* may alter the phenotype of macrophages present in these tissues. In response to *H. hepaticus* infection, thioglycollate-elicited peritoneal macrophages produce high levels of IL-6, KC and MCP-1 (230). Co-infection with *H. hepaticus* and *H. rodentium* causes a significant

increase in IP-10, MIP-1α, and IL-10 mRNA levels in the cecum (229). Though it is unclear which cells are responsible for this increase, macrophages are the likely source since *Helicobacter* induces production of cytokines by macrophages *in vitro* (230). Surprisingly, this activation occurs through TLR2, not TLR4 as expected with gram negative bacteria (230). This may occur due to the abnormal structure and composition of *Helicobacter* LPS (231).

Upon examination, Muotiala et. al. found that the unusual phosphorylation pattern and acylation of lipid A in *Helicobacter* LPS led to decreased biological activity (231). Specifically, *H. pylori* LPS has 1000 fold less mitogenic and pyrogenic activity and 500 fold lower lethal toxicity than *Salmonella typhimurium* LPS (231). The resulting minimal local inflammatory response is likely advantageous to the bacterium and may contribute to the persistence of the infection (232).

H. pylori LPS also expresses Lewis x and Lewis y blood group antigens similar to those normally found in the gastric mucosa (233). Administering *H. pylori*-induced anti-Lewis structure monoclonal antibodies resulted in gastritis in mice which suggests a pathogenic function for these antibodies (233). This may provide a mechanism for *H. pylori*-associated gastritis and other *Helicobacter*-related autoimmune diseases (233). Th1 cellular immune responses contribute to *Helicobacter*-associated hepatitis (234) and gastritis with exacerbated disease (235). A strong Th2 response results in a dramatically decreased bacterial load which may play a role in protection from disease or control of the infection (235). Since Th2 responses dominate in the intestine, this process may contribute to the persistent nature of *Helicobacter* infections and the lack of clinical symptoms in most patients.

Though it is currently unknown which cell types are responsible for elevated cytokine production in the gut during *Helicobacter* infection, macrophages may be the source. Resident macrophages play a role in maintaining the unresponsive Th2 environment of the intestine and surrounding muscle layers by producing a range of cytokines and chemokines. *Helicobacter* alters the expression of such factors by

macrophages *in vitro* (230). As a result, *Helicobacter* likely also affects gene expression. Altering gene expression may alter macrophage phenotype and function as well. Therefore, we hypothesized that *Helicobacter* infection alters the phenotype and inflammatory response of submucosal intestinal macrophages.

CHAPTER 2 - *Helicobacter* Infection Alters the Phenotype and Inflammatory Response of Mouse Intestinal Muscle Macrophages

Introduction

Helicobacter is a common intestinal pathogen of laboratory mice with 88% of mice from both commercial and academic sources worldwide testing positive for one or more *Helicobacter* species (205). *H. hepaticus* and *H. rodentium* are two species commonly found in research related rodents. In susceptible mice, *H. hepaticus* induces hepatitis and subsequent liver necrosis and tumors (reviewed in (222)) as well as intestinal inflammation and pro-inflammatory cytokine production (229). The development of disease symptoms may be due to the unusual composition and biological activity of *Helicobacter* lipopolysaccharide (LPS) (231) which likely contributes to the persistence of the infection (232). In contrast, *H. rodentium* appears to be part of the normal intestinal flora, and infection does not induce development of any clinical disease symptoms (227). However, in conjunction with other *Helicobacter* species including *H. hepaticus* and *H. bilus, H. rodentium* exacerbates hepatitis, alters gene expression (229), and induces diarrhea (228) in immunocompromised mice.

Intestinal macrophages may play a role in the response to *Helicobacter* infection. The innate immune response to other bacterial LPS is largely mediated by macrophages (230, 236), the largest population of which is found in the intestine (116). In the muscularis layers surrounding the intestine, macrophages form a regularly distributed network of cells (36, 121, 122). The function of these macrophages in response to infection has not been defined. *In vitro* studies categorize macrophage activation into two types: classically or alternatively activated. Classically activated macrophages, also known as type 1 (M1) macrophages, secrete pro-inflammatory cytokines and support Th1 responses (69). Alternatively activated macrophages, also known as type 2 (M2)

macrophages, secrete anti-inflammatory factors and play a role in regulating cellular immunity and homeostasis (69, 102). Specific genes identify M1 and M2 macrophages. M1 macrophages express inducible nitric oxide synthase (*iNOS*) , interleukin 12 (*IL-12*) and tumor necrosis factor α (*TNF-α*) (75). In contrast, M2 macrophages express Arginase-1 (*Arg-1*), *Fizz-1* (found in inflammatory zone-1), sphingosine kinase-1 (*Sk-1*), and/or *Ym-1* (54, 75, 104, 107, 237).

Recent studies investigated intestinal muscularis macrophages; however, there was not a consensus as to the phenotype or function of intestinal macrophages (36, 37, 117-124). Human intestinal macrophages isolated by enzyme digestion lack CD14 and do not produce IL-1, IL-12 or TNF-α in response to LPS (117-119). However, macrophages allowed to migrate out of the intestine do express CD14 and produce TNF-α in response to LPS (120) suggesting that isolation methods may alter phenotype. Macrophages isolated from whole mouse intestine by enzyme digestion express low levels of F4/80 and MHC II (124) as determined by flow cytometry. In contrast, macrophages isolated from only the intestinal muscle express high levels of F4/80 and MHC II as determined by immunohistochemistry (IHC) and also take up Fitc dextran (37). The disparities in observed phenotypes may be due to isolation of macrophages from different intestinal tissues using a variety of separation and digestion methods. The difference may also be associated with the method used to examine surface marker expression. Nevertheless, the observed intestinal macrophage phenotypes do not coincide with that of pro- or anti-inflammatory macrophages.

By altering gene expression and cytokine production in the microenvironment of the stomach, intestine and colon (229), *Helicobacter* may alter the phenotype of macrophages present in these and the surrounding tissues. Though it is unclear which cells are responsible for producing *Helicobacter*-induced pro-inflammatory cytokines *in vivo* (229), macrophages are the likely source since *Helicobacter* induces cytokine production by macrophages *in vitro* (230, 238, 239). Therefore, we hypothesize that *Helicobacter* infection alters the phenotype and inflammatory response of submucosal intestinal macrophages.

To test this hypothesis we examined macrophages present in whole mounts of intestinal muscle as well as isolated macrophages to determine the effects of *Helicobacter* infection. Increased expression of F4/80 in both whole mounts and isolated cells suggests *Helicobacter* infection may induce macrophage maturation. Infection also alters constitutive gene expression. Macrophages from the intestinal muscle of *Helicobacter*-infected mice express *Sk-1*, *TNF*, and significantly more *Fizz-1* than macrophages from uninfected mice. The effects of *Helicobacter* can also be seen in cultured intestinal muscle macrophages. Cultured macrophages from *Helicobacter*-infected mice significantly increase phagocytosis but constitutively produce less IP-10. In addition, *Helicobacter* infection alters the response to stimuli. Interestingly, macrophages from infected mice show a decreased response to stimulation with either LPS or IC, producing significantly lower concentrations of cytokines, chemokines, and PGE_2. These data show that the intestinal muscle macrophage phenotype, function and response to stimulation are altered by *Helicobacter* infection both *in vivo* and *in vitro*. However, the mechanism by which this occurs remains unknown.

Materials and Methods

Mice

C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and maintained in the Kansas State University Division of Biology rodent facility (Manhattan, KS). Animals were given rodent chow and water ad libitum and housed in 12h light/dark cycles. All procedures were approved by the Institutional Animal Care and Use Committee. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. Experiments were performed according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, 1996 edition).

Tissue Culture

The J774A.1 macrophage cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM, Atlanta Biologicals) containing 5% fetal bovine serum (FBS, Atlanta Biologicals), 5% NuSerum (BD Biosciences) and 1% L-Glutamine (Gibco) at 37°C with 8% CO_2.

Macrophage Isolation and Culture

Intestinal macrophages were isolated from 2-4 mo old infected or uninfected C57Bl/6J mice using a method adapted from Klaff et. al (36). Jejunum and ileum were removed from euthanized mice and placed in ice cold, Ca^{++} and Mg^{++} free Hanks balanced salt solution (HBSS, Cellgro). The intestine was flushed, cut into 1-2 inch segments, and placed in fresh Ca^{++} and Mg^{++} free HBSS on ice. Segments were slipped onto a 3.25-3.5mm knitting needle and the mesentery removed. The muscularis was gently incised along the mesenteric border and circumferentially stripped from the mucosa. Muscle tissue was minced and placed in digestion medium containing 1 mg/ml collagenase (Worthington) in Ca^{++} and Mg^{++} free HBSS supplemented with 0.4 mM $CaCl_2$ (Allied Chemical). Tissue was digested at 37°C for 30 min and pelleted by centrifugation at 1200 rpm for 10 min. The pellet was washed once with DMEM

containing 2% serum then once with serum-free OptiMEM (Gibco). The resulting pellet was resuspended in MΦ Medium (DMEM containing 5% FBS, 5% NuSerum, 10% OptiMEM, 5 ng/ml recombinant mouse M-CSF (R&D Systems), 1% L-Glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 50 μg/ml gentamicin (Gibco) then placed in 24 well plates. The plates were incubated at 37°C with 8% CO_2 overnight. Non-adherent cells were removed, fresh medium was added, and the remaining cells were cultured for up to 1 wk without changing the medium. Some sets of macrophages were cultured in MΦ Medium with the addition of 2 μl/ml of FibrOut (Chi Scientific), a reagent that prevents fibroblastic growth. Bone marrow derived macrophages were isolated from femurs of uninfected mice, red blood cells lysed using ACK lysis buffer (0.15M NH_4Cl (Fisher), 10mM $NaHCO_3$ (Fisher), 0.1mM disodium EDTA (Sigma) in dH_2O at pH 7.3), plated in MΦ Medium at $1x10^5$ cells per well in 24 well plates and grown at 37°C with 8% CO_2 for up to 7 days without changing the medium. Thioglycollate-elicited peritoneal macrophages were obtained by peritoneal lavage from mice injected with 1ml of thioglycollate 3-4 days prior. Cells were plated at $1x10^5$ cells per well in 24 well plates, allowed to adhere 1hr at 37°C, then used immediately.

Stimulation

Isolated macrophages pooled from 2-4 mice per set of stimulations were primed for 18h with fresh medium containing 30 U/ml interferon-γ (IFN-γ) (Sigma), 100 U/ml IL-4 (Antigenix), or medium alone then stimulated 18h with 10 μg/ml *Escherichia coli* LPS O55:B5 (Sigma), 150 μg/ml IgG-opsonized ovalbumin (OVA) immune complexes (IC), or medium alone. Supernatants were collected, centrifuged to remove cellular debris, and stored at -80°C until use. Bone marrow derived macrophages or thioglycollate-elicited peritoneal macrophages were used as a control for all stimulations. IgG-OVA IC was made as previously described (75, 102) by mixing a 10-fold molar excess of rabbit anti-OVA antibody (Bethyl Laboratories) to OVA (Calbiochem) for 30 min at RT.

Nitrite Determination

Nitrite concentration was determined in supernatants generated above using Griess reagent (1% sulfanilamide, 0.1% napthylethylene diamine dihydrochloride, 2%

H_3PO_4 (all from Sigma) in dH_2O). One hundred microliters of sample or standard (250-1.95 μM) was incubated with 100 μl Griess reagent for 10 min at RT. The OD550 was determined using a Model 860 microplate reader (Bio-Rad) and compared to a standard curve of $NaNO_2$ to determine concentration of nitrite in μM/ml.

Cytokine/Chemokine Production

Supernatants generated above were assayed for cytokine and chemokine production with a Lincoplex kit (Millipore) containing IFN-γ, interleukin 10 (IL-10), IL-12, IL-1α, IL-6, interferon-inducible protein 10 (IP-10), keratinocyte-derived cytokine (KC, the mouse homolog of IL-8), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and tumor necrosis factor-α (TNF-α) bead sets according to manufacturer's instructions. The plate was read on a Luminex100 machine and results analyzed with MiraiBio software (MiraiBio) subtracting fresh medium as a blank. IL-6 and IL-10 production were also determined using ELISA kits (IL-6: BioLegend, IL-10: eBioscience).

PGE_2 and LTB_4 Production

Prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4) production was measured in supernatants generated above using enzyme immunoassay (EIA) kits (Cayman Chemical) according to manufacturer's instructions. Plates were read using a Model 860 microplate reader and analyzed using software provided.

Limulus Amoebocytelysate Assay

Limulus amoebocytelysate assay (LAL, Hycult Biotechnology) was performed using supernatants generated above according to manufacturer's instructions. Plates were read using a Model 860 microplate reader and compared to a standard curve of LPS to determine concentration in ng/ml.

FITC Conjugation of Zymosan

Zymosan A particles from *Saccharomyces cerevisiae* (Sigma) were reconstituted in normal saline, activated by boiling for 1 hour, then washed with PBS. In 0.2M sodium carbonate/bicarbonate buffer (pH 9.6), 1 mg free fluorescein isothiocyanate (FITC,

Sigma) was added to10 mg zymosan and rotated in the dark for 30 min at RT. Labeled zymosan was then washed over a 0.45 um spin filter with PBS 3 times to remove any unbound free FITC. Zymosan particles were washed off the filter and resuspended in PBS. Fluorescent conjugation was verified using a Nikon eclipse 80i fluorescent microscope containing a FITC filter cube.

Phagocytosis

Isolated macrophages were removed from the plate by incubating with 0.06% EDTA for 20 min at 37°C followed by vigorous pipeting. The cells were then washed with Ca^{++} and Mg^{++} free HBSS containing 2% fetal bovine serum and 0.4 mM $CaCl_2$. In 2ml microcentrifuge tubes, macrophages were mixed with FITC-conjugated zymosan particles at a ratio of 10 particles per cell in Ca^{++} and Mg^{++} free HBSS containing 5% normal mouse sera. With $1x10^5$ cells per group, the experimental group was incubated rotating at 37°C for 20 min. To control for particles attached to the cell surface, another group was incubated rotating at 37°C for 20 min then treated with trypsin for 10 min rotating at 37°C to remove external particles. To inhibit phagocytosis, a third group was incubated on ice for 20 min and shaken every 5 min. Cells were washed with Ca^{++} and Mg^{++} free HBSS. Cytospins were prepared using $1x10^5$ cells and fixed in methanol. Fluorescence was visualized on a Nikon eclipse 80i fluorescent microscope containing a FITC filter cube, photographed with a CoolSnap CF camera (Photometrics), and analyzed using Metavue software (Molecular Devices). At least 100 cells per slide were scored and phagocytic index (total # particles ingested/100 cells scored) and % phagocytosis (# cells ingesting at least 1 particle/total # cells scored) were calculated. The macrophage cell line J774A.1 was used as a positive control in all experiments.

Immunohistochemistry

Immunohistochemistry was performed on either whole muscle mounts or cytospins. Whole muscle mounts from C57Bl/6 mouse intestine were stripped from the mucosa, placed in chamber slides, and fixed in acetone. Isolated cells were removed from the plate, prepared as cytospins, and fixed in methanol. All samples were blocked with 10% sera (Jackson ImmunoResearch) from the host of the secondary antibody in PBS for 30 min at 37°C then incubated with primary antibody overnight at 4°C (Table 2). Slides

were washed and incubated with secondary antibody as needed for 1 hour at RT or fluorescent-conjugated strepavidin for 15 min at RT. Fluorescence was visualized on a Nikon eclipse 80i fluorescent microscope, photographed with a CoolSnap CF camera, and analyzed using Metavue software. Relative fluorescence of immunohistochemical staining was quantified using Image J (NIH). The threshold was set to zero based on the isotype control and the total positive area measured.

Table 2. IHC Antibodies

Antibody	Isotype	Concentration	Fluorochrome/2°
CD11b[1]	Rat IgG2b	2.5 µg/section	Purified (Pur)/αRat IgG-FITC
F4/80[1]	Rat IgG2a	2.5 µg/section	Pur/αRat IgG-FITC
IL-6[2]	Rat IgG1	0.5 µg/section	Pur/αRat IgG-FITC, -Texas Red
αRat IgG[3]		0.375 µg/section	-FITC, -Texas Red

[1] eBioscience, [2]Biolegend, [3]Jackson ImmunoResearch

Flow Cytometry

Isolated macrophages were removed from plate by incubating with 0.06% EDTA for 20 min at 37°C followed by vigorous pipeting. The cells were washed in staining buffer (Ca^{++} and Mg^{++} free HBSS containing 2% fetal bovine serum and 0.4 mM $CaCl_2$) then incubated with FcR block (Table 3) for 15 min on ice. Primary antibody or appropriate isotype control (Table 3) was added in a volume of 100 µl for 15 min on ice. Secondary antibodies (Table 3) were used as required by incubation for 15 min on ice after washing with staining medium. Cells were washed twice and resuspended in 200 µl staining buffer for analysis. Data was collected without compensation using a FACSCalibur flow cytometer equipped with 488 nm and 635 nm lasers (BD Biosciences). Analysis was performed using WinList software (Verity Software House) with compensation set by N-Color compensation with HyperLog transformation. The macrophage cell line J774A.1 was used as a control in all experiments.

Table 3. Flow Cytometry Antibodies

Antibody	Isotype	Concentration	Fluorochrome/2°
Fc Block[1]		1 μg/10^5 cells	Purified (Pur)
CD11b[2]	Rat IgG2b	0.4 μg/10^5 cells	APC
CD14[3]	Mouse IgG1	0.2 μg/10^5 cells	Alexa 488
CD36[1]	Mouse IgA	1 μg/10^5 cells	Pur/αIgA-FITC
CD83[4]	Rat IgG1	0.4 μg/10^5 cells	PE
CD115[4]	Rat IgG2a	1 μg/10^5 cells	Biotin/SA-PE
F4/80[4]	Rat IgG2a	1 μg/10^5 cells	FITC
Pan Endo[1]	Rat IgG2a	1 μg/10^5 cells	Biotin/SA-APC
Gr-1[1]	Rat IgG2b	1 μg/10^5 cells	FITC
MHC II[1]	Mouse IgG2b	0.4 μg/10^5 cells	PE
TLR4[2]	Rat IgG2a	1 μg/10^5 cells	Biotin/SA-APC
SA-APC[4]		0.2 μg/10^5 cells	APC
SA-PE[3]		0.15 μg/10^5 cells	PE
αIgA[3]		0.2 μg/10^5 cells	FITC

[1] BDPharmingen, [2] Biolegend, [3] Caltag, [4] eBioscience

mRNA Isolation

TRIzol reagent (Invitrogen) was used to isolate total RNA from 0.5-1x10^6 macrophages. Cells were incubated at RT with TRIzol, chloroform extracted, then centrifuged 15 min at 4°C. The upper aqueous phase containing the RNA was removed to a new tube, precipitated with isopropyl alcohol at RT then pelleted. The RNA pellet was washed with 75% ethanol, air dried, resuspended in RNase free H_2O and stored at -80°C until use.

cDNA Synthesis

cDNA was synthesized from isolated mRNA using a Superscript III first strand synthesis kit (Invitrogen) or using M-MLV RT (Promega). One microgram mRNA was mixed with 1 μl random hexamers and 1 μl 10mM dNTPs in DNase and RNase free

water and incubated at 65°C for 5 min then moved to ice. Two microliters 10X reaction buffer, 4 µl 25mM $MgCl_2$, 2 µl 0.1M DTT, 1 µl RNase out, and 1 µl RT were added to the RNA and incubated in a Mastercycler (Eppendorf) 10 min at 25°C, 50 min at 50°C, 5 min at 85°C then held at 4°C. The cDNA was then incubated at 37°C with 1 µl RNase H to remove RNA, diluted with dH_2O, and stored at -20°C until use.

Helicobacter Detection

Fecal samples were collected from separately housed infected or uninfected C57Bl/6 mice and assayed immediately or stored at -80ºC until use. Total DNA was isolated according to manufacturer's instructions using a QIAmp DNA stool kit (Qiagen) and stored at -20°C until use. The species of *Helicobacter* infecting our mice were determined to be *H. hepaticus* and *H. rodentium* by an outside source (Research Animal Diagnostic Laboratory, University of Missouri).

PCR

PCR was performed using 5 µl 10X reaction buffer containing 15mM $MgCl_2$, 1 µl 50mM $MgCl_2$, 1 µl 10mM dNTPs, 80 ng each of forward and reverse primers (Integrated DNA Technologies) (Table 4), and 0.5 µl Taq polymerase (Bioline) were added to 0.5 µg DNA or cDNA in a final volume of 50 µl and incubated for 35 cycles (30 sec at 94°C, 30 sec at T_m (Table 4), 60 sec at 72°C) in a Mastercycler then stored at -20°C until used. Samples were run on a 2% agarose gel containing 0.0001% ethidium bromide and visualized using an AlphaImager (AlphaInogen). *GAPDH* sequence was obtained from Barber et.al. (240), *iNOS* primer sequence was obtained from Manthey et. al. (241), *Helicobacter* 16S rRNA primer sequence was obtained from Riley et. al. (242), all other primer sequences were designed using IDT PrimerQuest;. Relative intensity of PCR bands were quantified using Image J (NIH) and normalized to GAPDH.

Table 4. Primer Sequences

Gene	**Primer sequence**	**T_m**
Arg-1	Fwd 5' ACC TGG CCT TTG TTG ATG TCC CTA 3'	54°C
	Rev 5' AAG GTC TCT TCC ATC ACC TTG CCA 3'	

CD3	Fwd 5’ AGA GGA TGC GGT GGA ACA CTT TCT 3’	60°C
	Rev 5’ ACT CAC ACA CTC GAG CTT TCA GGT 3’	
COX-2	Fwd 5’ ATC CTG CCA GCT CCA CCG 3’	54°C
	Rev 5’ TGG TCA AAT CCT GTG CTC ATA CAT 3’	
Fizz-1	Fwd 5’ TCC AGC TGA TGG TCC CAG TGA ATA 3’	54°C
	Rev 5’ AAG CTG GGT TCT CCA CCT CTT CAT 3’	
GAPDH	Fwd 5’ CCA TGG AGA AGG CCG GGG 3’	54°C
	Rev 5’ CAA AGT TGT CAT GGA TGA CC 3’	
IL-12p40	Fwd 5’ CGT GCT CAT GGC TGG TGC AAA 3’	60°C
	Rev 5’ GAA CAC ATG CCC ACT TGC TG 3’	
iNOS	Fwd 5’ CCC TTC CGA AGT TTC TGG CAG CAG C 3’	65°C
	Rev 5’ GGC TGT CAG AGC CTC GTG GCT TTG G 3’	
IRAK-M	Fwd 5’ TAC AGT GCA CAA ATG GCA CAA CCC 3’	60°C
	Rev 5’ TTA ACT TGG CCT TCG TTG CCA CAC 3’	
SIGIRR	Fwd 5’ TGA AAG ATG GTC TGG CAT TGG 3’	54°C
	Rev 5’ TCG CCA AAG AGT GAA GGA AT 3’	
Sk-1	Fwd 5’ ATG GTC TGA TGC ATG AGG TGG TGA 3’	54°C
	Rev 5’ ACC ACA GTC CAA TGA GAA GGC ACT 3’	
ST2	Fwd 5’ ACA CTT GTC AAT TCA CAC ACG CGG 3’	60°C
	Rev 5’ TAA TCT GCC ACA GGA CAT CAG CCA 3’	
TLR4	Fwd 5’ GTA TAT GTG AAC ATC AGA AAT TCC T 3’	58°C
	Rev 5’ CAT GTT TGA GCA ATC TCA TAT TCA A 3’	
TNF	Fwd 5’ GGG ATG AGA AGT TCC CAA ATG 3’	54°C
	Rev 5’ CTC CAG CTG GAA GAC TCC TCC CAG 3’	
Ym-1	Fwd 5’ TTC CAA GGC TGC TAC TCA CTT CCA 3’	54°C
	Rev 5’ AGA CCA CGG CAC CTC CTA AAT TGT 3’	
Helicobacter	Fwd 5’ CTA TGA CGG GTA TCC GGC 3’	54°C
16S rRNA	Rev 5’ ATT CCA CCT ACC TCT CCC A 3’	

Statistics

Statistical analyses were performed using the Student's t test with significance set at a P value less than 0.05. GraphPad Prism software (GraphPad Software) was used to conduct all analyses. Numbers in the text are shown as mean ± standard error of mean (SEM).

Results

Due to the large number of research-associated mice that are infected with *Helicobacter*, approximately 88% worldwide (reviewed in (206)), the effects of this infection on the intestine need to be identified. The intestinal muscle contains a network of macrophages whose phenotype and function are still not well understood (36, 37, 117-124). This study analyzed the effects of *Helicobacter* infection on the phenotype and function of mouse intestinal muscle macrophages. Initially, we determined the phenotype of intestinal muscle macrophages by examining surface marker expression, gene expression, cytokine and chemokine production, as well as arachidonic acid and arginine metabolism. With the establishment of the intestinal muscle macrophage phenotype, we investigated the effects *Helicobacter* infection on these cells.

Helicobacter infection is detected in fecal samples by RT-PCR

To assess the infection status of the mice used in these studies, the amount of the *Helicobacter* 16S rRNA gene in fecal samples was examined by RT-PCR (Fig. 1A) and compared to a known positive control. All samples from infected mice contained similar amounts of the *Helicobacter* 16S rRNA gene when compared to the known infected control (Fig 1B). Conversely, the *Helicobacter* 16S rRNA gene was not detected in any of the separately housed, uninfected mice used in these studies (Fig 1A and 1B).

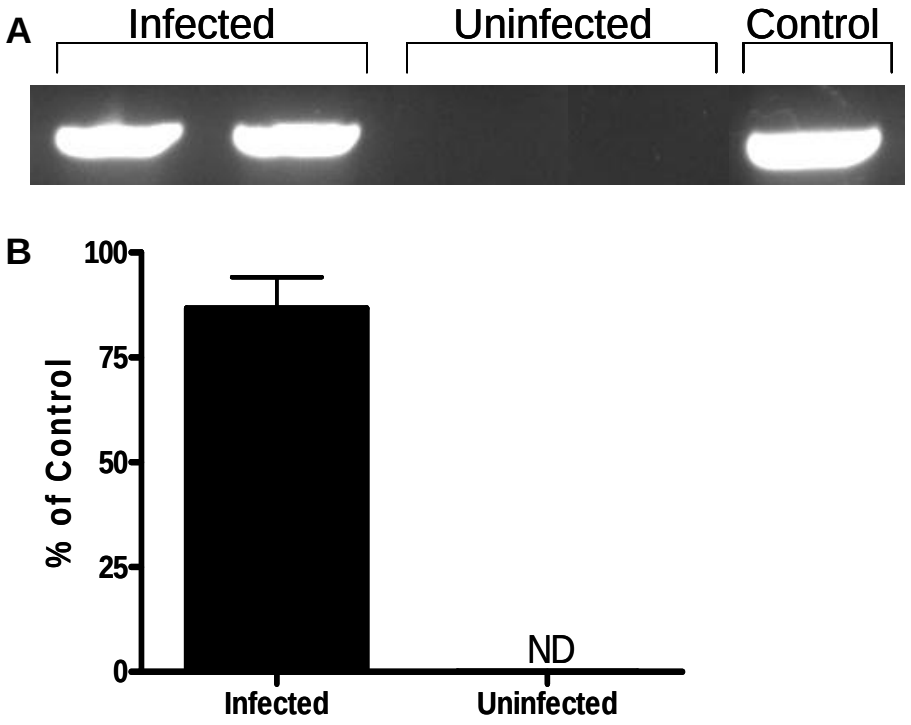

Figure 1. *Helicobacter* was detected in fecal samples from infected C57Bl/6 mice. Fecal samples were collected from *Helicobacter*-infected (■) or uninfected (□) mice, total DNA isolated and *Helicobacter* 16S rRNA gene detected by PCR as described. (A) Representative samples shown from *Helicobacter*-infected or uninfected mice. Control is a fecal sample from a known positive mouse. (B) Percent of bacterial DNA for the 16S rRNA gene compared to a known positive control set at 100, shown in arbitrary units. n=3-4 cages of mice per group, ND-none detected.

Macrophages from Helicobacter-infected mice have altered surface marker expression

Surface marker expression identifies and determines maturation state of macrophages. Whole mounts of intestinal muscle were isolated from C57Bl/6 mice and stained for F4/80, a marker of mature macrophages, and CD11b, an integrin present on most macrophages. Macrophages present in the intestinal muscle possess a stellate structure and form a network of cells as indicated by expression of F4/80 (Fig. 2A).

CD11b was also expressed (Fig. 2B), but the staining intensity was lower than F4/80. Both markers were positive when compared to isotype control stained sections (Fig. 2C). The staining was quantified as indicated and expression of F4/80 found to be significantly higher than CD11b (Fig. 2G, white bars). Therefore, the intestinal muscle contains a population of mature F4/80^{+} macrophages and a similar population that expresses CD11b.

We hypothesized that *Helicobacter* infection would alter the surface marker expression of macrophages present in the intestinal muscle. Whole mounts of intestinal muscle were isolated as described and stained for F4/80 and CD11b. Macrophages present in the intestinal muscle of infected mice (Fig. 2D-F) stained more intensely than those from uninfected mice (Fig. 2A-C) for both F4/80 (Fig. 2A and 2D) and CD11b (Fig. 1B and 1E) when compared to appropriate isotype controls (Fig. 2C and 2F). Upon quantification, F4/80 expression was almost 4 fold higher and CD11b almost 10 fold higher in whole muscle mounts from infected mice compared to those from uninfected mice (Fig. 2G). As no increase in overall cell number was observed (data not shown), these data suggest that intestinal muscle macrophages may be maturating in response to *Helicobacter* infection.

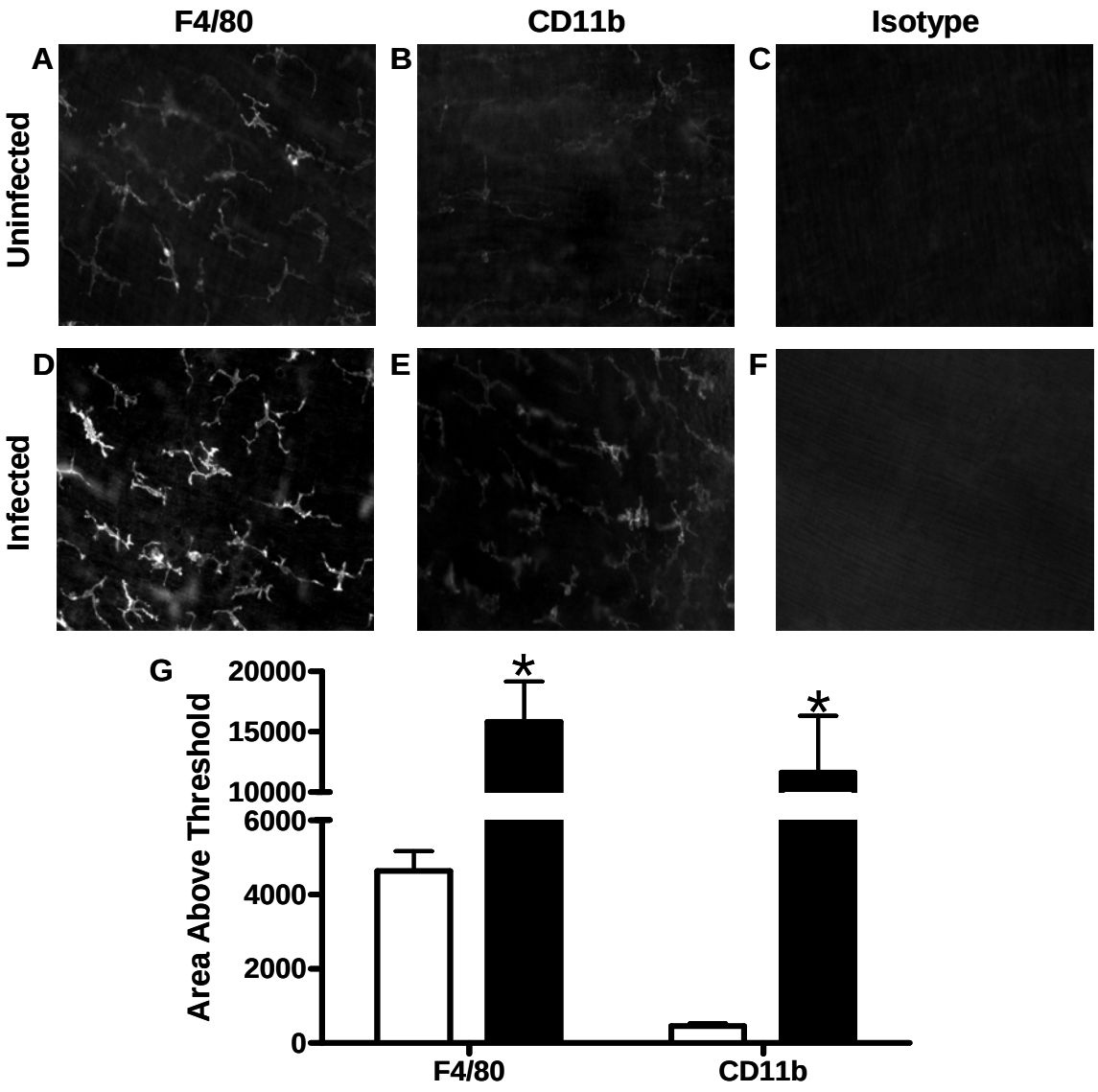

Figure 2. *Helicobacter* infection increases F4/80 and CD11b expression by intestinal muscle macrophages.

(A-F) Whole mounts of intestinal muscle were stripped from mucosa of uninfected (A-C) or infected (D-F) mice, and stained with F4/80 (A and D), CD11b (B and E), or appropriate isotype control (C and F). Photomicrographs are representative of at least 3 experiments. (G) Relative fluorescence of immunohistochemical staining from uninfected (□) or infected (■) mice was quantified using Image J. The threshold of the isotype control was set to zero and the total positive area measured. n=3-4 experiments with 4-6 representative photos analyzed per treatment, *$p<0.05$ infected compared to uninfected.

Macrophages from Helicobacter-infected mice have altered gene expression

To determine the phenotype of these macrophages, both pro-inflammatory and anti-inflammatory genes were investigated. Pro-inflammatory macrophages express *IL-12*, *TNF-α*, and *iNOS* mRNA (69, 75), while anti-inflammatory macrophages express mRNA for one or more of the genes *Fizz-1*, *Sk-1*, *Arg-1*, and *Ym-1* (54, 75, 104, 107, 237). Macrophages directly isolated from the intestinal muscle of normal mice expressed low levels of mRNA for the gene *Fizz-1* as determined by RT-PCR (Fig. 3A and C). However, macrophages produced almost no detectable amounts of *Sk-1, Ym-1* or *Arg-1* mRNA. Expression of these genes is significantly lower than anti-inflammatory, control, bone marrow-derived macrophages (BMMΦ) cultured in M-CSF and stimulated with IL-4 and IC (Fig. 3A). Intestinal muscle macrophages expressed little to no *IL-12*, *TNF-α*, or *iNOS* mRNA (Fig. 3B and C) compared to levels observed in pro-inflammatory, control BMMΦ cultured in M-CSF and stimulated with LPS and IFN-γ (Fig. 3B). Figure 3C (white bars) shows the quantification of band intensity.

Since some surface markers were altered with infection, we hypothesized that *Helicobacter* may alter gene expression as well. To test this, we analyzed macrophage gene expression. As determined by RT-PCR, macrophages directly isolated from the intestinal muscle of infected mice expressed high levels of *Fizz-1*, similar to that seen in control macrophages (Fig. 3A). Upon quantification, macrophages from infected mice expressed significantly higher levels of *Fizz-1* compared to those from uninfected mice (Fig 3C). Unlike macrophages from uninfected mice, *Sk-1* and *TNF* were expressed by macrophages from infected mice in addition to low levels of *Arg-1* (Fig. 3). Neither macrophages isolated from uninfected nor *Helicobacter*-infected mice expressed appreciable amounts of *Ym-1*, *IL-12*, or *iNOS* (Fig. 3). *Sk-1* and *TNF* expression by macrophages from infected mice is similar to that seen in control macrophages, but *Ym-1*, *Arg-1*, and *iNOS* expression is significantly lower than controls (Fig 3). Taken together these data suggest that *Helicobacter* infection may induce macrophages present in intestinal muscle to become more mature and alter gene expression *in vivo*.

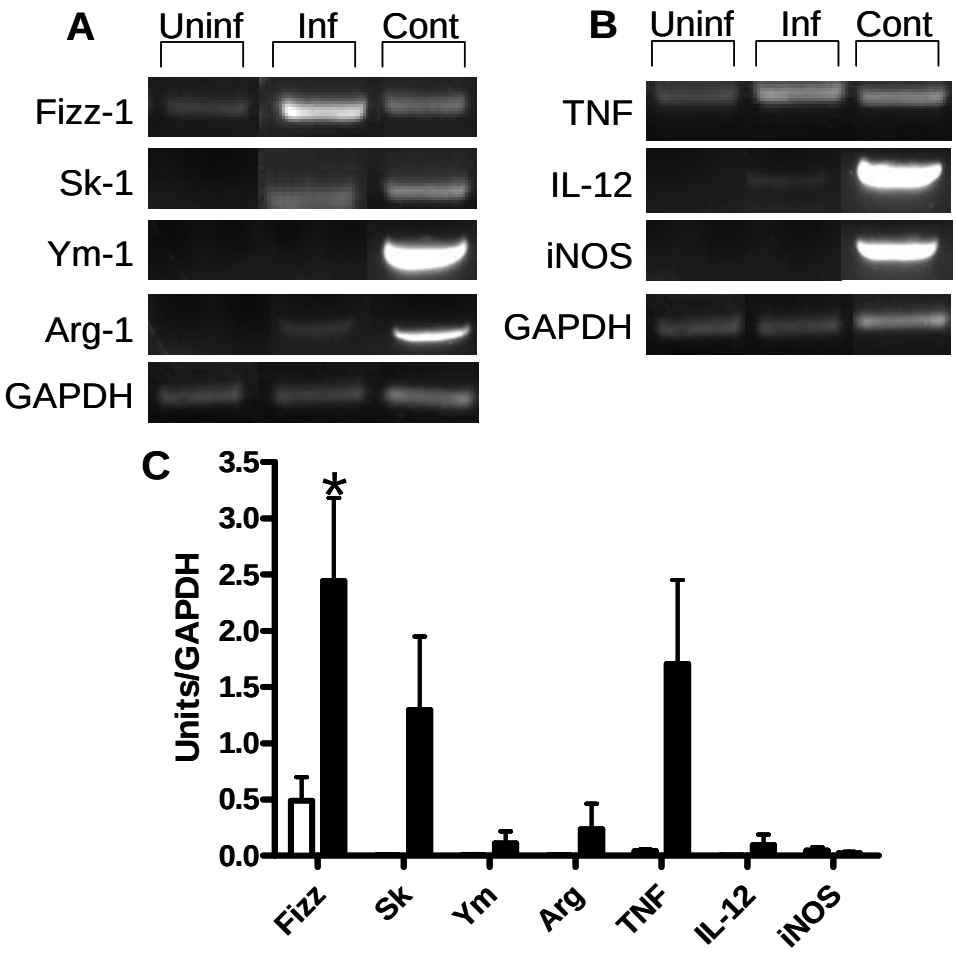

Figure 3. *Helicobacter* infection increases some gene expression by intestinal muscle macrophages.

Total RNA was isolated from intestinal muscle macrophages and (A) *Fizz-1*, *Sk-1*, *Ym-1*, *Arg-1*, (B) *IL-12, iNOS, TNF,* and *GAPDH* transcripts were determined by RT-PCR. BMMΦ stimulated with IL-4 and IC were used as anti-inflammatory controls (A) or stimulated with IFN and LPS as pro-inflammatory controls (B). (C) Relative intensity of PCR bands from uninfected (□) or *Helicobacter*-infected (■) mice were quantified using Image J and normalized to GAPDH. n=4-8, *$p<0.05$ infected compared to uninfected.

Helicobacter infection does not alter constitutive production of most cytokines or chemokines by intestinal muscle macrophages

Macrophages are involved in numerous cellular processes and produce many soluble factors. F4/80 expressing macrophages in the intestinal muscle (Fig. 4A) also express IL-6 (Fig. 4B) as indicated by co-localization in the muscle tissue (Fig. 4C) when compared to isotype control (Fig. 4D). Consequently, we measured cytokine and chemokine production by macrophages. Freshly isolated macrophages were incubated 24 hours without stimulation and the resulting supernatants used to analyze constitutive cytokine and chemokine production. We examined secreted factors associated with both pro-inflammatory and anti-inflammatory macrophages. Large amounts of IL-6 and low levels of TNF-α were detected (Fig. 5, white bars). However, when compared to pro- and anti-inflammatory BMMΦ controls, these were very low. Intestinal muscle macrophages generated significant concentrations of IP-10 as well as lower concentrations of KC (Fig. 5, white bars). Both were also significantly lower that seen in the controls. MCP-1 was the only factor produced in amounts similar to those observed by the controls (Fig. 5, white bars). Thus, many of the products secreted by these macrophages are chemokines suggesting that macrophages in the intestinal muscle may play a role in regulating leukocyte infiltration and inflammation in the intestine.

Having shown that infection alters both gene and surface marker expression, we investigated the effects of *Helicobacter* infection on the products secreted by intestinal muscle macrophages. Cells isolated from infected mice were used to analyze constitutive cytokine and chemokine production. Surprisingly, other than IP-10, none of the measured cytokines or chemokines produced constitutively by these macrophages were significantly different between those from infected or uninfected mice (Fig. 5). IP-10 production by macrophages from infected mice was almost half that produced by macrophages from uninfected mice. Although there was a trend towards increased production of TNF-α and MCP-1 by macrophages from uninfected mice, these differences were not significant (Fig. 5). These data indicate that *Helicobacter* infection alone does not alter the constitutive production of most of the cytokines or chemokines we studied.

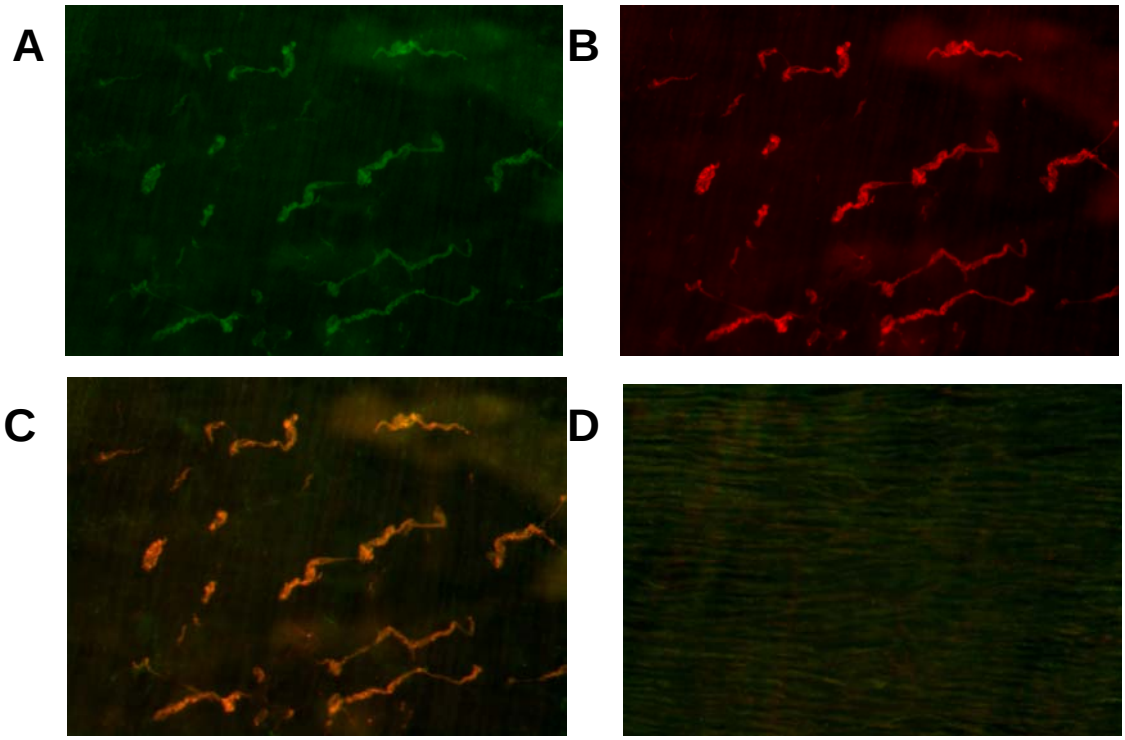

Figure 4. F4/80^{+} intestinal muscle macrophages also express IL-6.

(A-D) Whole mounts of intestinal muscle were isolated from uninfected mice and stained with (A) F4/80, (B) IL-6, (C) both F4/80 and IL-6, (D) or appropriate isotype controls. Photomicrographs are representative of at least 3 experiments.

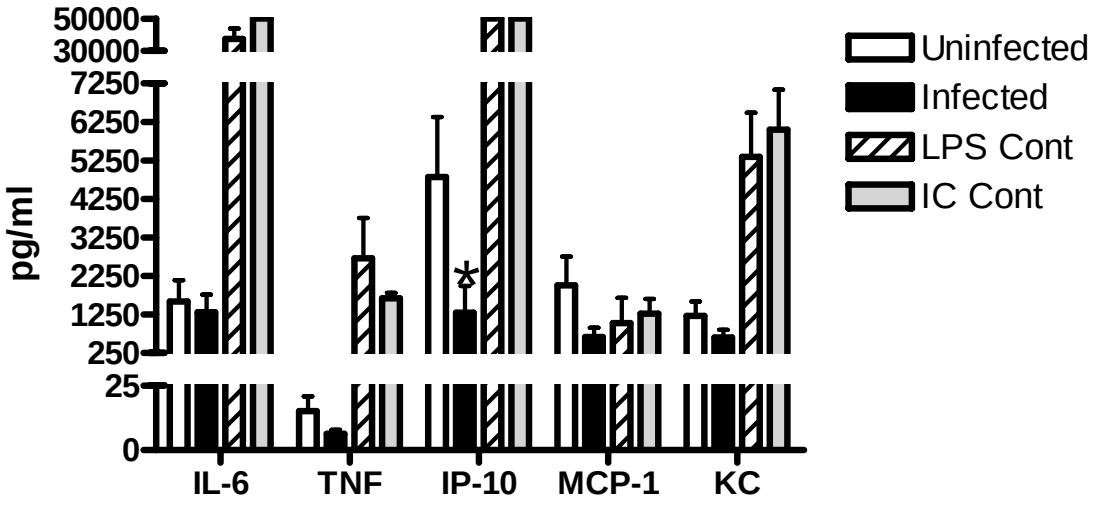

Figure 5. Intestinal muscle macrophages from infected and uninfected mice constitutively express similar levels of cytokines and chemokines.
Intestinal muscle macrophages were isolated from uninfected (□) or *Helicobacter*-infected (■) mice and cultured in medium containing M-CSF for 24 hours. Supernatants were collected and assayed for cytokine and chemokine production with a Lincoplex kit according to manufacturer's instructions (level of detection = 3–10,000 pg/ml). BMMΦ cultured in M-CSF and stimulated with IFN and LPS (▨) or IL-4 and IC (▨) were used as controls. Results were analyzed using MiraiBio software. Each point represents the average of 3-7 independent experiments measuring duplicate supernatants. *$p<0.05$ infected compared to uninfected.

Helicobacter infection does not affect constitutive arginine or arachidonic acid metabolism in intestinal muscle macrophages

The amino acid, arginine is an important precursor needed for immune function (52, 54). Pro-inflammatory macrophages metabolize arginine into nitric oxide (NO) via *iNOS* (75). Macrophages isolated from intestinal muscle do not make any detectable levels of NO compared to LPS and IFN-γ stimulated thioglycollate-elicited peritoneal macrophage controls (data not shown). Similar to macrophages from uninfected mice,

macrophages from infected mice did not produce NO (data not shown). In addition, *Helicobacter* infection did not stimulate a difference in NO production by thioglycollate-elicited peritoneal macrophages (data not shown). Therefore, infection with *Helicobacter* does not stimulate the production of NO.

As another important precursor, arachidonic acid is broken down into many inflammatory lipid mediators including LTB_4 and PGE_2. Produced in response to infection, LTB_4 is a pro-inflammatory product (reviewed in (189)). PGE_2, however, can be either pro- or anti-inflammatory depending on the concentration produced during infection. PGE_2 is pro-inflammatory during the early stages of infection when the concentration is high and anti-inflammatory late in infection when concentration is low (reviewed in (195-197)). Intestinal muscle macrophages produced LTB_4 at moderate levels (Fig. 6, white bars). Both pro- and anti-inflammatory control macrophages produced similar concentrations of LTB_4 (Fig. 6). Production of PGE_2 was significantly higher than that of LTB_4 by intestinal macrophages (Fig. 6, white bars). This trend was also seen in the controls. Unlike most cytokines and chemokines, no difference between control and intestinal muscle macrophages was observed (Fig. 6). These data show that macrophages in the intestinal muscle constitutively produce a wide range of factors with varying effects.

Investigation of arachidonic acid metabolites found that *Helicobacter* infection does not influence the constitutive amounts of LTB_4 or PGE_2 produced by intestinal muscle macrophages after 24 hours in culture (Fig. 6). However, though not different from uninfected or LPS control macrophages, cells from infected mice produced significantly more PGE_2 than IC stimulated control macrophages (Fig. 6). These data indicate that *Helicobacter* infection alone does not influence the metabolism of arginine or arachidonic acid.

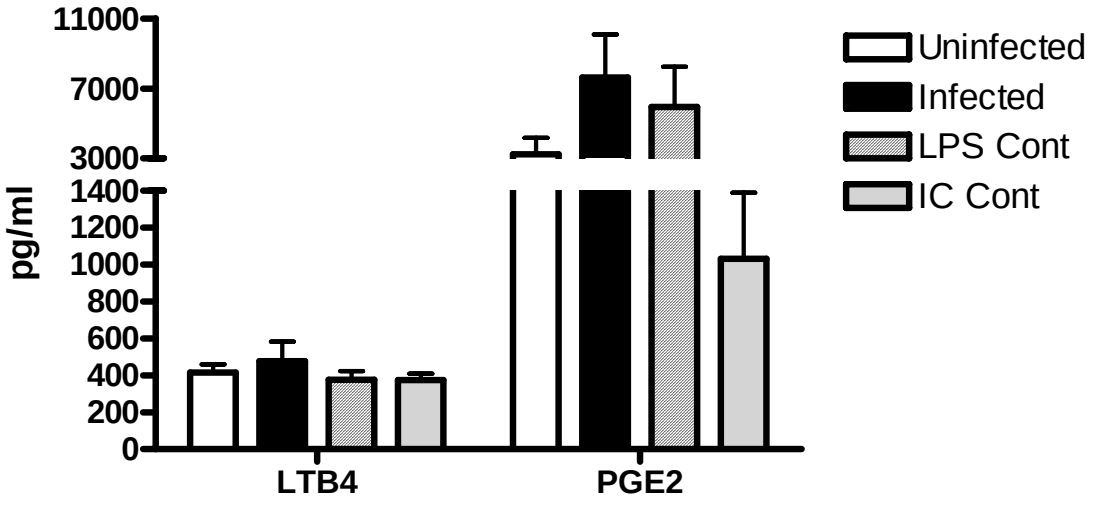

Figure 6. *Helicobacter* infection does not alter constitutive production of LTB_4 or PGE_2 by intestinal muscle macrophages.

Intestinal muscle macrophages were isolated from uninfected (□) or *Helicobacter*-infected (■) mice then cultured 24h in medium containing M-CSF. Supernatants were collected and assayed by EIA for LTB_4 or PGE_2. BMMΦ cultured in M-CSF and stimulated with IFN and LPS (▨) or IL-4 and IC (▩) were used as controls. Each bar represents the average of 5-7 independent experiments measuring duplicate supernatants

Resident muscle macrophages cultured for 7 days in M-CSF retain a phenotype similar to freshly isolated intestinal macrophages

To verify that the collected cells were macrophages and allow for more extensive studies, macrophages isolated from intestinal muscle and cultured for 7 days in medium containing M-CSF. We hypothesized that cultured macrophages would display a similar phenotype to those found in the tissue based on surface marker expression, and cytokine and chemokine production. Though somewhat heterogeneous, 70-75% of the cultured cells stained positive by IHC for F4/80 (Fig. 7A) and CD11b (Fig. 7B) when compared to appropriate isotype controls (Fig. 7C). In addition, we analyzed these cells flow cytometry. In contrast to IHC, less than 5% of the analyzed cells were positive for F4/80,

CD11b or TLR4. Flow cytometry also showed that less than 10% of the cells were positive for CD14, and less than 15% were positive for CD36 (Fig. 8, white bars). These numbers are extremely low when compared to J774A.1 macrophages (81±2.5% $CD11b^+$, 60.4±4.8% $TLR4^+$), but consistent with those previously observed in human intestinal muscle macrophages (117). To ensure that the cultures were not contaminated with fibroblasts, macrophages were cultured with FibrOut to prevent fibroblastic growth. Flow cytometry showed that surface marker expression on cells cultured with FibrOut was not significantly different when compared to those cultured under normal conditions (data not shown). In addition, flow cytometric and/or PCR analysis showed that these cultures contained few to no dendritic cells, T cells, or endothelial cells (data not shown). These data demonstrate that the cells isolated from the intestinal muscle are macrophages and that the culture is relatively free of other contaminating cell types.

Macrophages from infected mice were also analyzed for surface marker expression to investigate any changes in response to culture. Similar to muscularis tissue staining, macrophages isolated from infected mice (Fig. 7D-F) stained more intensely than those from uninfected mice (Fig. 7A-C) for both F4/80 (Fig. 7A and 7D) and CD11b (Fig. 7B and 7E) when compared to appropriate isotype controls (Fig. 7C and 7F). In contrast, macrophages from infected or uninfected mice displayed no difference in expression of F4/80, CD11b, CD14, or TLR4 when analyzed by flow cytometry (Fig. 8). CD36 expression was significantly lower in macrophages from infected mice; however, this is likely not biologically significant. Overall, these data demonstrate that the *Helicobacter*-induced increase in F4/80 and CD11b expression remains after 7 days in culture.

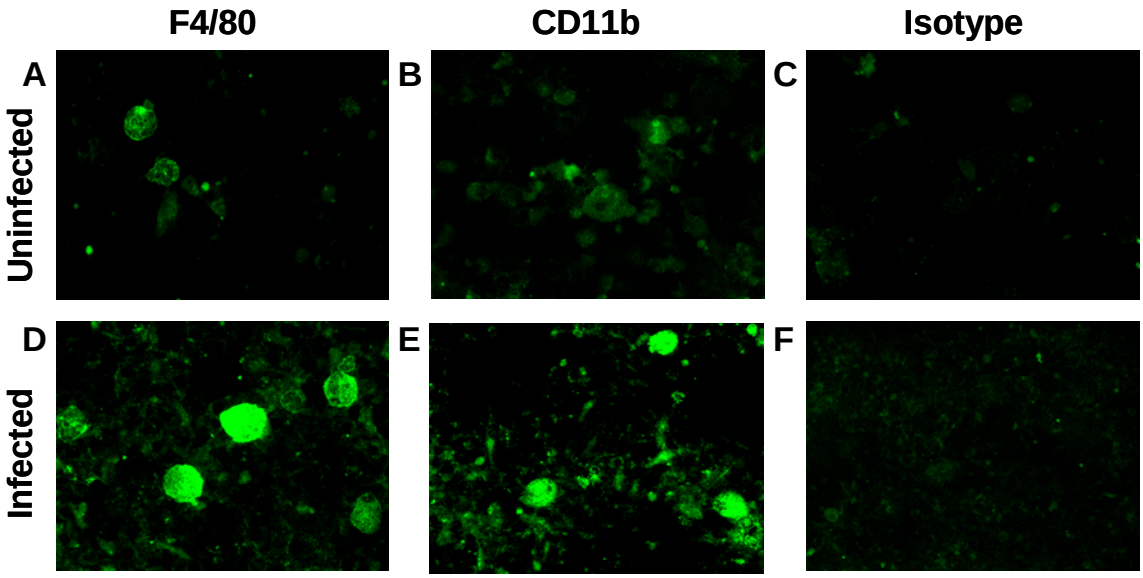

Figure 7. *Helicobacter* infection increases F4/80 and CD11b expression in cultured intestinal muscle macrophages.

Macrophages were isolated from intestinal muscle of uninfected (A-C) or infected (D-F) mice, cultured 7 days in medium containing M-CSF, then stained with F4/80 (A and D), CD11b (B and E), or appropriate isotype control (C and F). Representative photomicrographs are shown. n=3-4

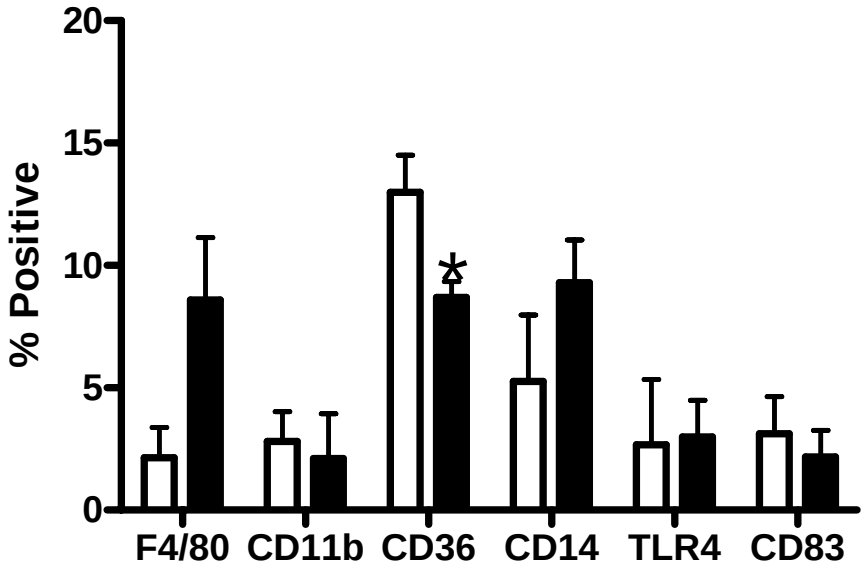

Figure 8. *Helicobacter* infection alters some surface marker expression in cultured intestinal muscle macrophages as determined by flow cytometry.
Intestinal muscle macrophages were isolated from uninfected (□) or *Helicobacter*-infected (■) mice then cultured 7 days in medium containing M-CSF. Cells were stained with the indicated antibodies or appropriate isotype control then analyzed by flow cytometry. n=3-4, *p=0.0597

To confirm that culture does not change the products secreted by intestinal muscle macrophages, we measured cytokine and chemokine production. IL-6 and KC are shown as representative cytokines or chemokines, respectively. Compared to 24 hours after isolation, constitutive secretion of these proteins did not change after 7 days in culture with M-CSF (Fig. 9). Production of cytokines and chemokines by macrophages cultured from infected mice was also examined. Concentrations of IL-6 and KC secreted by macrophages from infected mice did not significantly increase or decrease after 7 days in culture (Fig. 9). In addition, there was no significant difference between macrophages from infected or uninfected mice. Overall, when compared to freshly isolated macrophages or those found in the tissue, macrophages isolated from intestinal muscle

and cultured in M-CSF expressed similar levels of surface markers, cytokines and chemokines. Therefore, cultured intestinal muscle macrophages have a similar phenotype. All further studies used intestinal muscle macrophages cultured under these conditions.

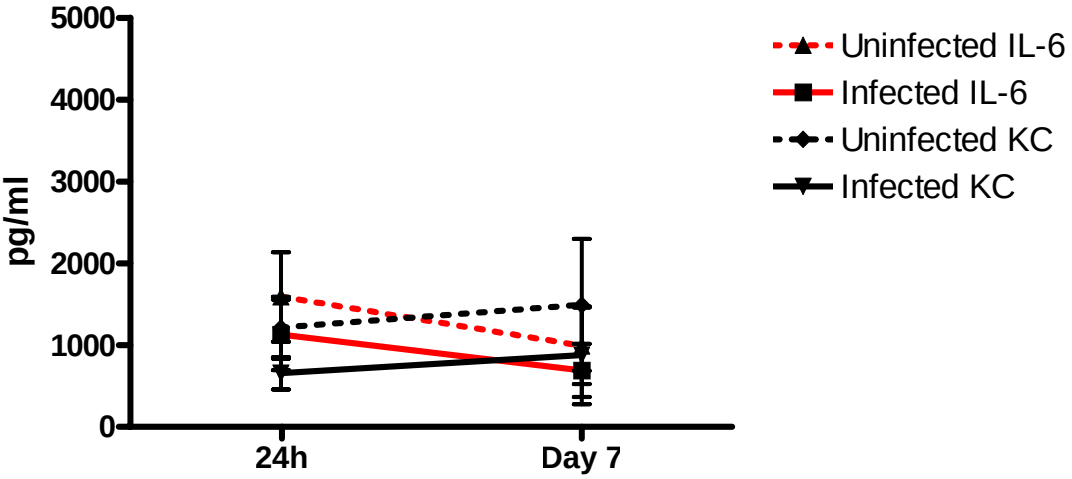

Figure 9. Isolated intestinal muscle macrophages express cytokines and chemokines at similar levels after 24 hours or 7 days of culture.
Intestinal muscle macrophages were isolated from uninfected (dashed lines) or *Helicobacter*-infected (solid lines) mice and cultured in M-CSF for 7 days. Cells were stimulated with medium alone. Supernatants were collected and assayed for IL-6 and KC production with a Lincoplex kit according to manufacturer's instructions. Results were analyzed using MiraiBio software. n=6-9

Macrophages from infected mice cultured for 7 days increased phagocytic ability

As well as production of factors that induce and regulate inflammation, a main function of macrophages is phagocytosis (reviewed in (38-40)). We hypothesized that

macrophages isolated from intestinal muscle would be phagocytic. Macrophages cultured for 7 days in M-CSF were incubated with fluorescent zymosan particles as described. To distinguish between external adherence of particles to the macrophages and internalization by phagocytosis, trypsin treatment of one of triplicate samples removed externally bound particles while ice inhibited phagocytosis in another of the triplicates. Percent phagocytosis (Fig. 10A) and phagocytic index (data not shown) were calculated from representative photomicrographs (Fig. 10C) of at least 100 cells per treatment per experiment. Approximately 25% of macrophages from the intestinal muscle phagocytosed zymosan particles (Fig 10A, white bars). Trypsin did not reduce the number of particles associated with these cells confirming phagocytosis. Treatment with ice resulted in a reduction in phagocytosis to 13.3 ± 3.7%. This indicates that intestinal macrophages are phagocytic for large particles under normal conditions.

Invading pathogens activate macrophages to phagocytose foreign particles. Therefore, we hypothesized that macrophages from infected mice would be more phagocytic than those from uninfected mice. Macrophages from infected mice were three fold more phagocytic than those from uninfected mice with approximately 80% of the cells phagocytosing zymosan (Fig. 10A). Similar to above, treatment with ice resulted in a significant reduction in phagocytosis but did not result in complete inhibition. Macrophages from infected mice also ingested five times more zymosan particles per cell, with macrophages from infected mice containing an average of 2 particles per cell while those from uninfected mice contained an average of 0.4 particles per cell. These data show that *Helicobacter* infection increases the phagocytic ability of intestinal muscle macrophages.

Due to the possibility that the presence of *Helicobacter* or LPS in these macrophage cultures could activate the cells from infected mice to have increased phagocytosis, we measured the amount of the *Helicobacter* 16S rRNA gene and total LPS present in the supernatants of cultured cells. Cultured macrophages from infected or uninfected mice did not contain the *Helicobacter* 16S rRNA gene (data not shown). Though small amounts of LPS were detected (<53.85 pg/ml), there was no difference

between supernatants from infected or uninfected mice. This confirms that the increase in phagocytosis is not due to the presence of bacteria or LPS in the macrophage cultures.

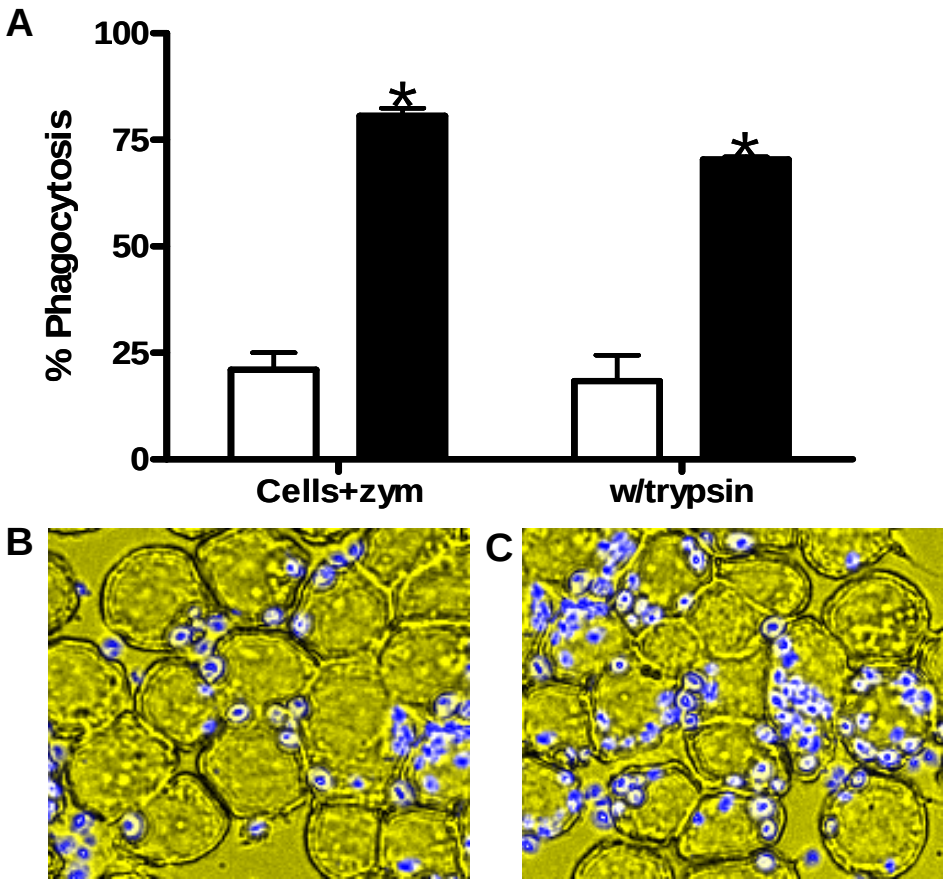

Figure 10. Intestinal muscle macrophages from *Helicobacter*-infected mice are more phagocytic.

(A) Intestinal muscle macrophages isolated from uninfected (□) or *Helicobacter*-infected (■) mice were cultured for 1 wk with M-CSF. Cells were incubated 20 min with zymosan particles at 37°C then left untreated (cells+zym) or treated with trypsin (w/trypsin) to remove externally bound particles. Cytospins were prepared, photographed (B and C), and ≥100 cells per slide were scored. Photomicrographs are representative of macrophages isolated from uninfected (B) or infected (C) mice phagocytosing fluorescent zymosan particles. n=3-4 experiments/group, *$p<0.05$ infected compared to uninfected.

Macrophages from infected mice cultured for 7 days have decreased PGE_2 production

To determine if culturing intestinal muscle macrophages for 7 days altered arachidonic acid degradation products, we examined LTB_4 and PGE_2 production. LTB_4 secreted by macrophages from uninfected mice remained unchanged in response to culture and was similar to concentrations seen in the controls (Fig. 11). In addition, infection did not alter LTB_4 (Fig. 11) and concentrations produced were similar to the controls. Surprisingly, macrophages cultured from either infected or uninfected mice produced significantly less PGE_2 after 7 days in culture (Fig. 11) when compared to day 0 (Fig. 6). Concentrations of PGE_2 produced by macrophages from infected mice were significantly lower than those from uninfected mice (Fig. 11). Though not significantly different from IC control, PGE_2 production by macrophages from infected and uninfected mice was significantly lower than the LPS control macrophages (Fig. 11). This suggested that *Helicobacter* infection may affect expression of *COX-2*, which is responsible for inducible PGE_2 synthesis. However, *COX-2* was not expressed at different levels by macrophages from infected or uninfected mice either with (Fig. 12 and 13) or without (data not shown) stimulation. Therefore, both culture and infection with *Helicobacter* decreased PGE_2 production by intestinal muscle macrophages without decreasing *COX-2* expression. Since macrophages cultured from intestinal muscle have similar patterns of surface marker expression, cytokine production and chemokine production, the decrease in PGE_2 after culture was unexpected.

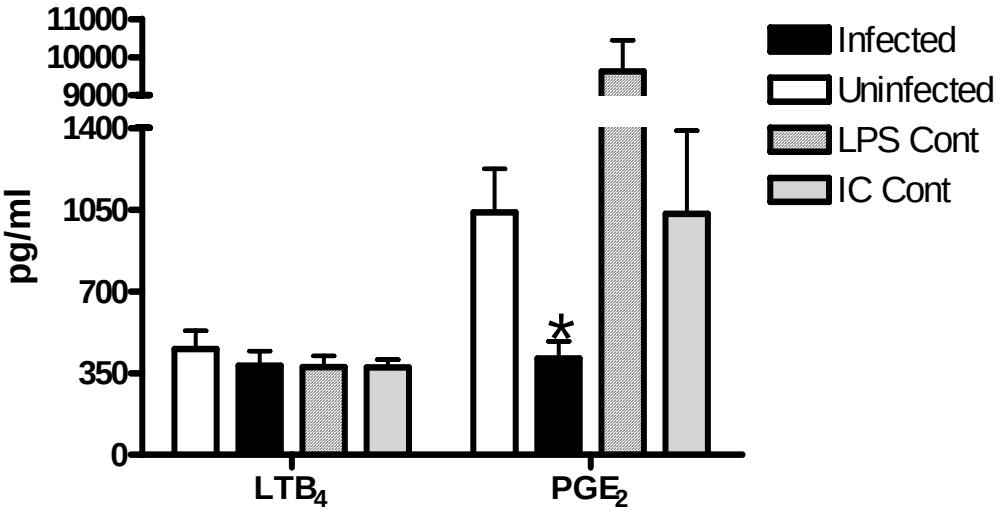

Figure 11. *Helicobacter* infection decreases constitutive PGE_2 but not LTB_4 production by cultured intestinal muscle macrophages.

Intestinal muscle macrophages isolated from uninfected (□) or *Helicobacter*-infected (■) mice were cultured 7 days in medium containing M-CSF prior to stimulation with medium alone. BMMΦ cultured in M-CSF and stimulated with IFN and LPS (▨) or IL-4 and IC (▨) were used as controls. Supernatants were collected and assayed for LTB_4 or PGE_2. n=3-9, *$p<0.05$ infected compared to uninfected.

Helicobacter infection does not alter gene expression in response to in vitro stimulation

Different types of macrophages respond to different stimulation (69, 75). IFN-γ and LPS stimulate pro-inflammatory macrophages while IL-4 and immune complexes (IC) are a known stimuli of anti-inflammatory macrophages (69, 75). Having found that infection alters the phagocytic function of isolated macrophages, we hypothesized that *Helicobacter* infection would also alter the *in vitro* response of intestinal muscle macrophages to either LPS or IC stimulation. Macrophages cultured from the intestinal muscle of infected or uninfected mice were primed then stimulated with either IFN-γ and LPS or IL-4 and IC followed by examination of gene expression. With the exception of

PGE_2, unstimulated macrophages cultured for 7 days were not significantly different when compared to freshly isolated (day 0) macrophages (data not shown). Therefore all comparisons other than arachidonic acid metabolism are made to day 0.

Similar to freshly isolated intestinal muscle macrophages, no *Ym-1*, *Arg-1*, or Sk-1 was detectable with LPS stimulation (Fig 12A and 12C, white bars) in macrophages from uninfected mice. Compared to day 0 (Fig. 3), macrophages stimulated with LPS expressed significantly decreased levels of *Fizz-1*. LPS stimulated macrophages also expressed little to no *TNF-α, IL-12* or *iNOS* (Fig 12B and 12C, white bars). In addition, LPS induced a low level of *COX-2* (Fig. 12B and 12C). All of these genes were detected in appropriately stimulated control BMMΦ (Fig. 12A and 12B). Figure 12C (white bars) shows the quantification of gene expression.

In contrast to freshly isolated and LPS stimulated macrophages, IC stimulation induced intestinal muscle macrophages from uninfected mice to express low levels of *Ym-1* and *Arg-1* (Fig. 13A and 13C). Similar to LPS stimulated macrophages, IC stimulated macrophages expressed significantly decreased *Sk-1* and *Fizz-1* (Fig. 13A and 13C) when compared to freshly isolated macrophages (Fig. 3). In addition, we observed *TNF-α, IL-12* and *iNOS* at low to undetectable levels similar to that seen with LPS stimulation. IC induced slightly higher levels of *COX-2*, but there was no significant difference between stimulations (Fig. 13B and 13C). Gene expression was quantified as described and is shown in Figure 13C (white bars). These data show that IC but not LPS stimulates the expression of the anti-inflammatory genes *Ym-1* and *Arg-1* by intestinal muscle macrophages. This further suggests that these cells have an anti-inflammatory phenotype in the absence of infection.

Macrophages isolated from the intestinal muscle of infected mice were also stimulated with LPS or IC to determine if *Helicobacter* infection alters gene expression. In response to LPS, intestinal muscle macrophages from infected mice did not express detectable levels of *Ym-1*, *Arg-1*, *IL-12*, or *iNOS* similar to that observed in the macrophages from uninfected mice (Fig. 12). LPS induced very low levels of *Fizz-1*, *Sk-*

1, and *TNF-α* in macrophages from infected mice with no observed differences between macrophages from infected or uninfected mice (Fig. 12). As indicated above, these levels were significantly decreased when compared to Day 0 (Fig. 3).

Macrophages from infected mice expressed low levels of *Ym-1* in response to IC stimulation (Fig. 13A and 13C) as well as low to undetectable levels of all other genes examined. There was no significant difference between macrophages from infected and uninfected mice for any of these products (Fig. 13). These data indicate that intestinal muscle macrophages do not respond well to LPS or IC stimulation. However, this result is not due to *Helicobacter* infection.

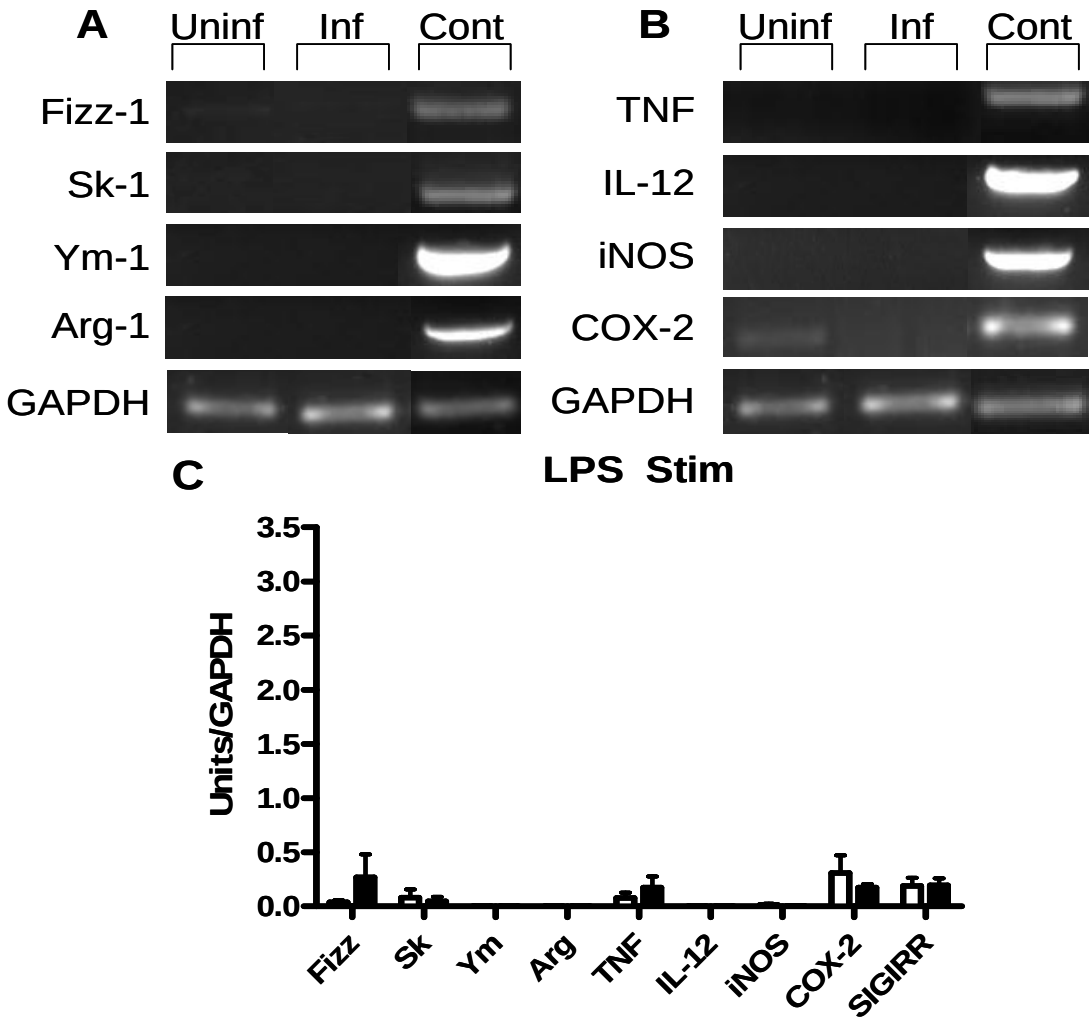

Figure 12. Stimulation with LPS does not induce gene expression by intestinal muscle macrophages.

After 7 days in culture with M-CSF, macrophages isolated from *Helicobacter*-infected or uninfected mice were stimulated with LPS and IFN-γ. Total RNA was isolated from intestinal muscle macrophages and RT-PCR used to determine levels of *Ym-1, Arg-1, Fizz-1, Sk-1* (A), *TNF, IL-12, iNOS, COX-2* (B), *SIGIRR* (C) and *GAPDH* (A and B) transcripts. BMMΦ cultured in M-CSF and stimulated with IL-4 and IC (A) or IFN-γ and LPS (B) were used as positive controls. Representative PCR bands shown. (C) Relative intensity of PCR bands from uninfected (□) or *Helicobacter*-infected (■) mice were quantified using Image J and normalized to *GAPDH*. n=4-7 experiments/group.

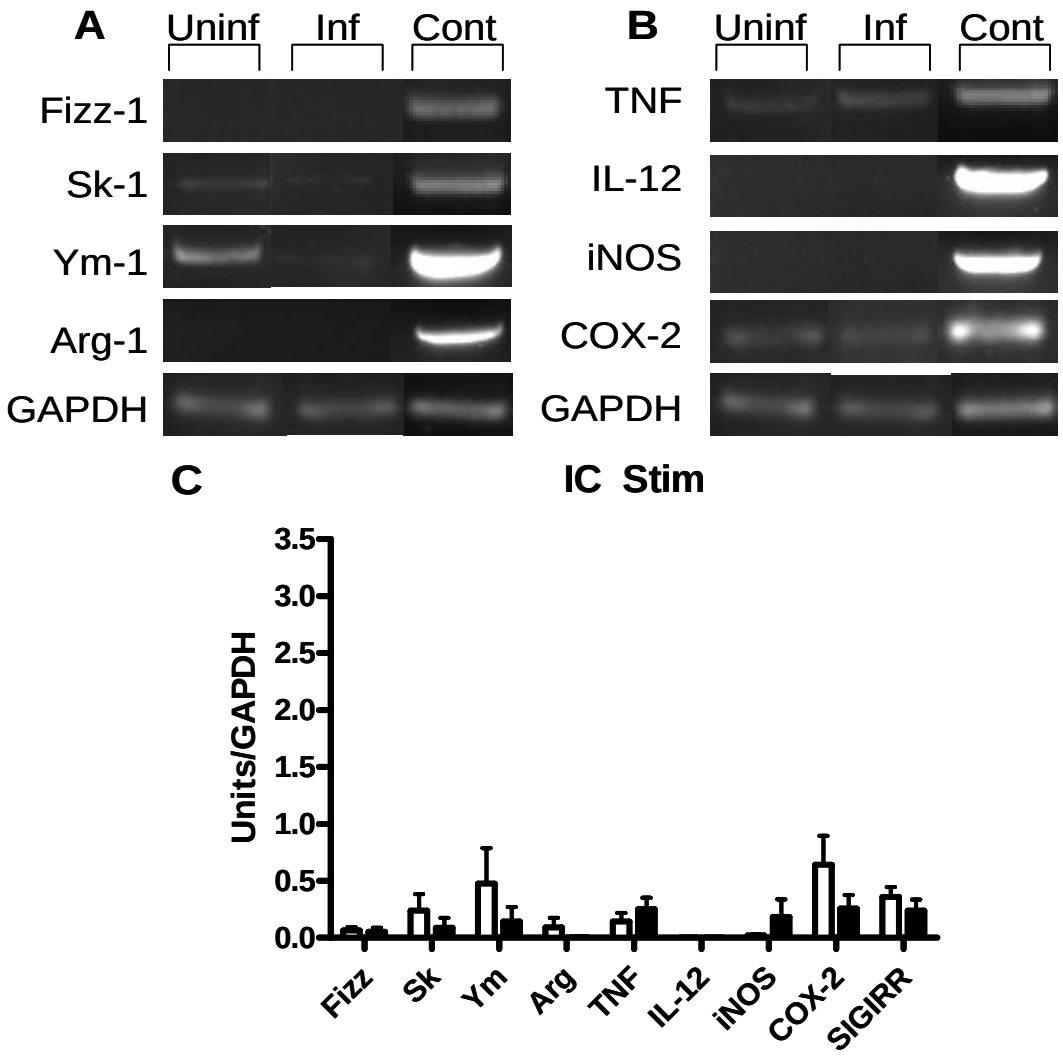

Figure 13. Stimulation with IC enhances expression of some genes by intestinal muscle macrophages.

After 7 days in culture with M-CSF, macrophages isolated from uninfected (□) or *Helicobacter*-infected (■) mice were stimulated with IL-4 and IC. Total RNA was isolated from intestinal muscle macrophages, and *Ym-1, Arg-1, Fizz-1, Sk-1* (A), *TNF, IL-12, iNOS, COX-2* (B), *SIGIRR* (C) and *GAPDH* (A and B) transcripts were determined by RT-PCR. BMMΦ cultured in M-CSF and stimulated with IL-4 and IC (A) or IFN-γ and LPS (B) were used as positive controls. Representative PCR bands shown. (C) Relative intensity of PCR bands from uninfected (□) or infected (■) mice were quantified using Image J and normalized to GAPDH. n=4-8 experiments/group.

Helicobacter infection decreases cytokine and chemokine production in response to in vitro stimulation

Macrophages produce a wide range of factors in response to both pro-inflammatory (LPS) and anti-inflammatory (IC) stimulation. To further investigate the intestinal muscle macrophages and their response to stimuli, cytokine and chemokine production as well as arachidonic acid metabolism and NO production were measured. As gene expression was not different after stimulation, we hypothesized that macrophages stimulated from infected mice would produce similar concentrations of cytokines and chemokines to those produced by uninfected macrophages.

Intestinal muscle macrophages from uninfected mice produced significantly more of the cytokines, IL-6 and TNF-α, upon stimulation with LPS (Fig. 14A, white bars) than with IC (Fig. 14B, white bars). These values were significantly lower than appropriately stimulated BMMΦ controls (Fig. 14). When compared to constitutive expression, intestinal muscle macrophages stimulated with LPS (Fig. 14A, white bars) produced significantly more IL-6 than freshly isolated macrophages (Fig. 5, white bars). However, there was no difference between constitutive cytokine levels (Fig. 5, white bars) and those produced following IC stimulation (Fig. 14B, white bars). Due to the pleiotropic effects of IL-6, these results cannot be attributed to either a pro- or anti-inflammatory phenotype.

Intestinal muscle macrophages from uninfected mice produced significantly lower concentrations of the chemokine IP-10 upon LPS stimulation (Fig. 14A, white bars) when compared to IC stimulation (Fig. 14B, white bars). Conversely, KC was significantly higher following stimulation with LPS than with IC. No difference was seen in MCP-1 production between stimulation with LPS or IC (Fig. 14, white bars). Production of IP-10 by intestinal macrophages was significantly higher than that seen in control macrophages with both stimulations, while MCP-1 was production was significantly lower (Fig. 14). After LPS stimulation, intestinal and control macrophages produced similar concentrations of KC; but with IC stimulation, BMMΦ produced significantly more KC than muscle macrophages (Fig. 14). When compared to constitutive expression (Fig. 5,

white bars), IP-10 production by intestinal macrophages was significantly higher upon stimulation with both LPS and IC (Fig. 14, white bars). Macrophages stimulated with LPS but not IC produced KC in significantly higher concentrations (Fig 14, white bars) compared to day 0 (Fig 5, white bars). MCP-1 levels were not altered in response to either stimulation (Fig. 14B and Fig 5, white bars). As expected, these data show that intestinal muscle macrophages produce cytokines and chemokines in response to both pro- and anti-inflammatory stimulation.

Macrophages cultured from infected mice were also stimulated to investigate the effects of *Helicobacter* on cytokine and chemokine production. Though macrophages from infected mice produced large amounts of IL-6, these cells produced significantly lower concentrations of IL-6 and TNF-α upon stimulation with LPS than macrophages from uninfected mice (Fig. 14A). Macrophages from infected mice also produced significantly less IL-6 and TNF-α upon stimulation with IC when compared to those from uninfected mice (Fig. 14B). Stimulation by both LPS (Fig. 14A) and IC (Fig. 14B) significantly reduced IP-10, MCP-1, and KC production by macrophages from infected mice when compared to those from uninfected mice. Therefore, these data demonstrate that *Helicobacter* infection alters the response of intestinal muscle macrophages to both LPS- and IC- mediated stimulation. The mechanism by which this occurs is unknown, but may involve production of inhibitors of macrophage activation.

Due to the decreased cytokine and chemokine responses of macrophages from infected mice, we hypothesized that *Helicobacter* infection may induce production of inhibitors that block stimulation by LPS or IC. Therefore, transcription of several negative regulators of macrophage signaling, SIGIRR, ST2 and IRAK-M, were investigated. After stimulation, intestinal macrophages expressed low levels of *SIGIRR* (Fig. 12C and 13C), *ST2* (<0.19±0.1 units/GAPDH) and *IRAK-M* (<0.07±0.03 units/GAPDH). However, macrophages from infected or uninfected mice produced similar levels of these genes. Therefore, though the mechanism remains unclear, *Helicobacter* infection alters the phenotype of intestinal muscle macrophages and their response to stimuli.

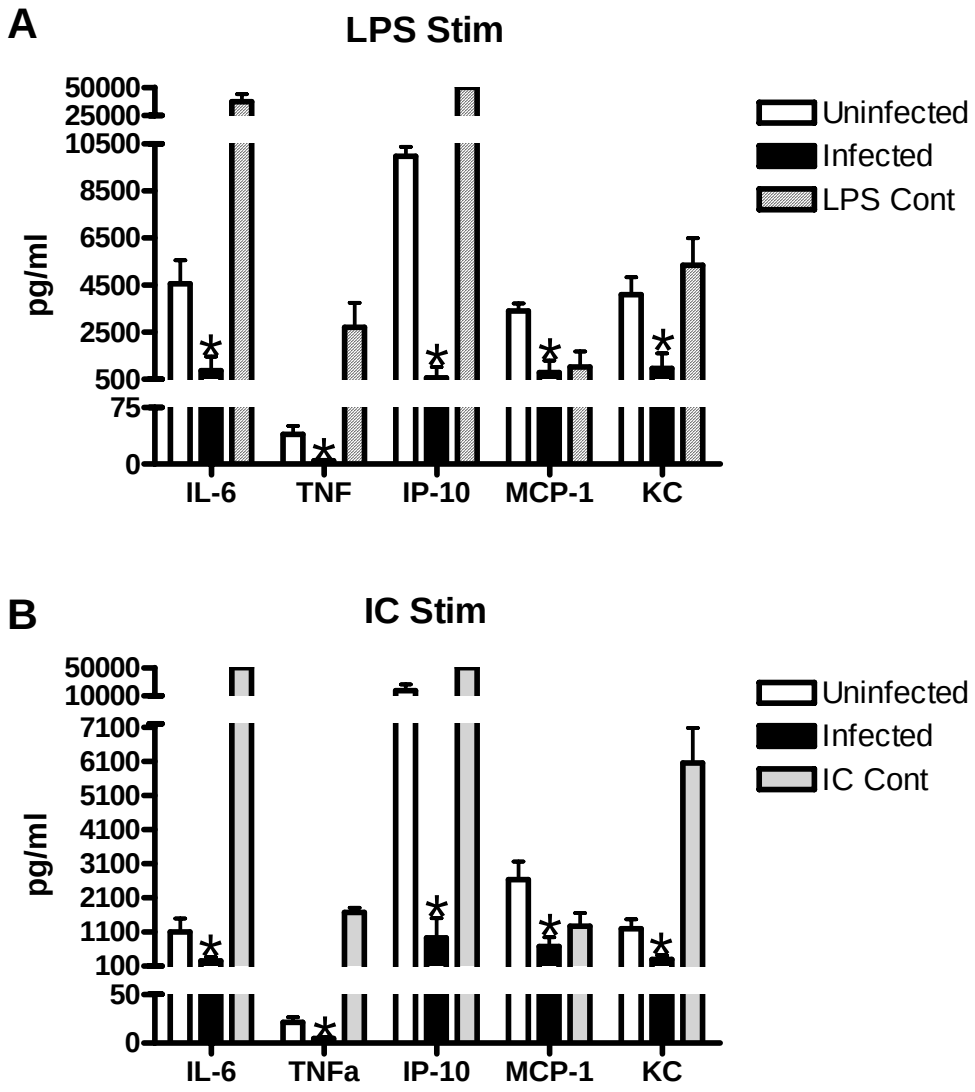

Figure 14. *Helicobacter* infection decreases production of cytokines and chemokines by intestinal muscle macrophages in response to stimulation.

Intestinal muscle macrophages were isolated from uninfected (□) or *Helicobacter*-infected (■) mice and cultured in M-CSF for 7 days prior to stimulation with (A) IFN-γ and LPS or (B) IL-4 and IC. Supernatants were collected and assayed for IL-6, TNF, IP-10, MCP-1, and KC production with a Lincoplex kit. Results were analyzed using MiraiBio software. BMMΦ cultured in M-CSF and stimulated with IFN and LPS (▨) or IL-4 and IC (▨) were used as controls. Each bar represents the average of 3-7 independent experiments measuring duplicate supernatants, *$p<0.05$ infected compared to uninfected.

Helicobacter infection decreases PGE_2 production in response to in vitro stimulation without altering COX-2 expression

Rat intestinal muscle macrophages produce PGE_2 in response to LPS stimulation (65). As a result, arachidonic acid metabolism by macrophages from infected and uninfected mice was investigated in response to stimulation. We hypothesized that, similar to cytokine production, *Helicobacter* infection would reduce the production of the arachidonic acid metabolites, LTB_4 and PGE_2, in response to stimulation.

Intestinal muscle macrophages from uninfected mice produced similar amounts of LTB_4 when stimulated with either LPS or IC (Fig. 15A, white bars). Control macrophages also released similar concentrations of LTB_4 with both stimulations (Fig. 15). When compared to constitutive LTB_4 production (Fig 6, white bar), LPS or IC stimulation did not change the concentration secreted by these macrophages (Fig. 15). These data show stimulation does not alter production of LTB_4 by intestinal muscle macrophages.

Conversely, stimulation did have an effect on PGE_2 production. LPS stimulation of intestinal muscle macrophages from uninfected mice produced significantly more PGE_2 than IC stimulation (Fig. 15B, white bars). After LPS stimulation, intestinal macrophages secreted significantly less PGE_2 than control BMMΦ macrophages. In contrast, with IC stimulation, intestinal macrophages and BMMΦ produced similar concentrations of PGE_2 (Fig. 15). Macrophages produced similar levels of PGE_2 constitutively at day 0 and upon LPS stimulation (Fig. 6 and 15B, white bars), but this was significantly higher than that seen at day 7 without stimulation (Fig. 11). Macrophages produced significantly less PGE_2 after IC stimulation (Fig. 15B, white bars) compared to day 0 (Fig. 6, white bar), but these concentrations were similar to day 7 without stimulation (Fig. 11). These data indicate that LPS induces production of PGE_2 above the constitutive levels seen at day 7 but not above day 0 concentrations. In

addition, IC stimulation does not induce intestinal muscle macrophages to produce PGE_2 above constitutive levels observed at day 7.

Investigation of arachidonic acid metabolism by macrophages from infected mice showed no difference in LTB_4 production upon stimulation with LPS or IC (Fig. 15A). There was also no difference in LTB_4 between macrophages from infected or uninfected mice (Fig 15A). However, macrophages from infected mice produced significantly less PGE_2 when stimulated with either LPS or IC compared to those from uninfected mice (Fig. 15B). Surprisingly, LPS or IC stimulation (Fig. 15B) of macrophages from infected mice did not significantly change PGE_2 production from that seen after 7 days in culture (Fig. 11). This demonstrates that without altering LTB_4 production, *Helicobacter* infection decreases PGE_2 production by intestinal muscle macrophages after stimulation when compared to those from uninfected mice.

Altered PGE_2 production again suggests that *COX-2* may be altered or inhibited. To investigate this phenomenon, we analyzed *COX-2* expression by RT-PCR. In response to stimulation macrophages from both infected and uninfected mice expressed low levels of *COX-2* (Fig. 12 and 13). Despite lower PGE_2 protein levels in macrophages from infected mice following LPS stimulation (Fig. 15B), infection did not change *COX-2* expression in intestinal muscle macrophages (Fig, 12C). *COX-2* expression was slightly but not significantly lower in macrophages from infected mice after IC stimulation (Fig. 13C). However, these results did not correlate with the observed decrease in PGE_2 production. Therefore, infection with *Helicobacter* decreases PGE_2 production without altering *COX-2* expression.

Macrophages found in the intestinal muscle of rats produce NO in response to LPS (65). Therefore, we hypothesized that mouse intestinal muscle macrophages would generate the pro-inflammatory product NO in response to stimulation with LPS but not IC and that *Helicobacter* infection would alter this response. Though produced by control thioglycollate-elicited peritoneal macrophages in response to LPS and IFN-γ, intestinal muscle macrophages from uninfected mice did not produce NO after LPS or IC

stimulation (data not shown). Similarly, no detectable amounts of NO were produced by macrophages from infected mice upon stimulation with either LPS or IC (data not shown). These results are the same as those observed with constitutive production indicating that mouse intestinal muscle macrophages do not produce NO. This further suggests that macrophages in the intestinal muscularis do not have a pro-inflammatory phenotype. Taken together with the cytokine and chemokine secretion data, this indicates that *Helicobacter* may be altering production of certain products by macrophages without altering the general phenotype of these cells.

Overall, *Helicobacter* infection alters the response of intestinal muscle macrophages to stimulation with either IC or LPS. *Helicobacter* infection results in decreased production of cytokines and chemokines (Fig. 14), as well as PGE_2 (Fig. 15B), in response to both LPS and IC stimulation.

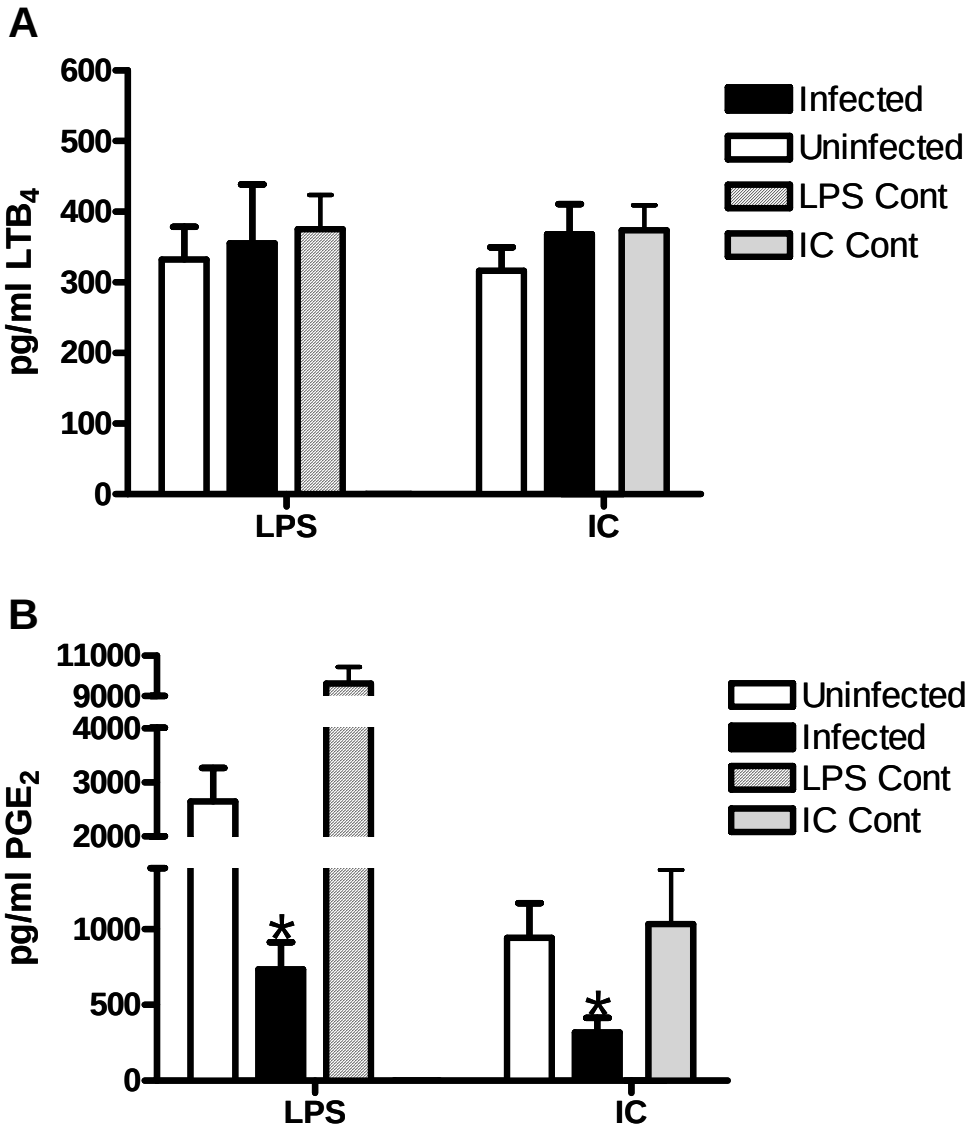

Figure 15. *Helicobacter* infection alters PGE_2 but not LTB_4 production by intestinal muscle macrophages in response to stimulation.

Intestinal muscle macrophages were isolated from uninfected (□) or *Helicobacter*-infected (■) mice were cultured 7 days with M-CSF, then stimulated with IFN and LPS or IL-4 and IC. BMMΦ cultured in M-CSF and stimulated with IFN and LPS (▨) or IL-4 and IC (▨) were used as controls. Supernatants were collected and assayed by EIA for (A) LTB_4 or (B) PGE_2. Each bar represents the average of 5-7 independent experiments measuring duplicate supernatants *$p<0.05$ infected compared to uninfected.

Discussion

Helicobacter infection alters the phenotype and function of intestinal muscle macrophages. Macrophages from infected mice express increased F4/80 and CD11b both *in vivo* (Fig. 2) and after 7 days in culture (Fig. 7). Macrophages from infected mice also demonstrate increased phagocytosis (Fig. 10). Though constitutive production is not altered (Fig. 5 and 6), macrophages from infected mice exhibit decreased cytokine (Fig. 14), chemokine (Fig. 14), and PGE_2 (Fig. 15) production in response to stimulation with both LPS and IC. In addition, *Helicobacter* alters constitutive gene expression by macrophages (Fig. 3) but does not alter gene expression after stimulation (Fig. 12 and 13). These data show that *Helicobacter* infection alters the phenotype and function of intestinal muscle macrophages as well as their response to stimulation *in vitro*.

Helicobacter infection alters cytokine and chemokine production in the intestine and colon (229, 230). Macrophages may responsible for this change, but the actual source is unknown. As a result, understanding the production of secreted factors by resident macrophages in response to infection (Fig. 5 and 6) or injury has important implications. The removal by depletion or lack of macrophages in the intestine during injury or inflammation changes the host response to the challenge (126). Therefore, altering the function of intestinal muscle macrophages likely affects the host response to injury or inflammation. *Helicobacter*, by altering macrophages in the intestine, can thereby control or modulate the inflammatory response. This may contribute to the persistence of *Helicobacter* infection. However, despite the investigation of several inducible inhibitors of LPS signaling (Fig. 12C, 13C, and data not shown), the mechanism by which this occurs remains unknown. The course of the infection may also affect macrophages. Because *Helicobacter* infection spreads slowly through a colony, the duration and level of infection may lead to some variation in the effects observed.

Despite the assessed purity of these cultures, macrophages isolated from the intestinal muscle of both infected and uninfected mice display some heterogeneity since

size and staining intensity varies (Fig 7). Due to the limitations of the method used, isolation of intestinal muscle macrophages may not exclude all other cell types and the culture may not be pure. However, flow cytometry, PCR analysis, and culture with FibrOut showed that these cultures contained few to no dendritic cells, T cells, endothelial cells, or fibroblasts. Therefore, these cultures are relatively pure indicating that the macrophage population itself may be heterogeneous.

The phenotype of intestinal muscle macrophages and their immunologic function has not been well defined. The phenotype observed in this study does not correlate with any of the known macrophage phenotypes (69, 75). Intestinal muscle macrophages from uninfected mice express more of the genes associated with an anti-inflammatory (M2) phenotype (Fig. 3 and 13) than a pro-inflammatory (M1) phenotype (Fig. 3 and 12). Macrophages from *Helicobacter*-infected mice express genes associated with both M1 and M2 phenotypes. The lack of correlation between intestinal muscle macrophages and any single macrophage phenotype may be due to the fact that these are tissue macrophages. In addition the macrophages used to determine M1 and multiple M2 phenotypes are induced *in vitro* from bone marrow and not derived directly from the mouse (58, 69, 75). The macrophages found *in vivo* are likely to be similar, but may not display exactly the same characteristics as BMMΦ artificially induced to become M1 or M2 macrophages. Although the intermediate phenotype found in the intestinal muscle in response to *Helicobacter* infection would likely be classified as an M2, it suggests that this model may not be sufficient to describe the observed phenotypes of tissue macrophages *ex vivo*. In addition, this model likely does not apply to all macrophages found *in vivo* since the phenotypes and functions of tissue macrophages varies depending on the location within the body (reviewed in (45)).

Previous studies showed that the intestine contains a network of phagocytic F4/80^{+} macrophages (36, 37, 122, 243). Most studies of intestinal muscle macrophages use Fitc-dextran (36, 37, 122, 127, 128, 243), labeled E. coli particles (120, 244), or small fluorescent microspheres (117, 118, 123). These are small particles that can be taken up by endocytosis rather than being phagocytosed. A novel aspect of this work is the use of

large zymosan particles which can only be phagocytosed (Fig. 9). Phagocytosis of large necrotic cell debris by macrophages induces an anti-inflammatory phenotype (129). Therefore the ability of intestinal muscle macrophages to ingest large particles may prove more effective in determining their role in intestinal inflammation, homeostasis, and repair. In response to *Helicobacter* infection, macrophages in the intestinal muscle were three times more phagocytic for zymosan particles (Fig. 9). Though *Helicobacter* inhibits stimulation of cytokine and chemokine production, the mechanism of inhibition of LPS and IC signaling does not affect the pathways governing phagocytosis.

The most commonly used technique for cellular identification is surface marker expression. Macrophages in this study were identified by flow cytometry and IHC using a variety of surface markers some of which were altered in response to infection. *Helicobacter* increased the expression of F4/80 or CD11b on macrophages *in vivo* (Fig. 2) or *in vitro* (Fig. 7) by IHC. The flow cytometry in this and other studies shows isolated intestinal macrophages express relatively low levels of characteristic macrophage markers such as F4/80 and CD14 (Fig. 8 and (119)). This may be due to the use of enzymes during isolation that may interfere with the staining process. However, our preliminary data using a macrophage cell line indicates that collagenase treatment does not decrease surface marker expression as analyzed by flow cytometry (data not shown). Another possibility is that the adherent nature of intestinal macrophages may cause difficulty during flow cytometric analysis. To avoid these issues, we also analyzed intestinal muscle macrophages by IHC (Fig. 7) which has been shown previously to identify macrophages similar to those we observed in the intestinal muscularis layers (36, 37, 243). As it provided better representative staining, we believe that IHC may be more accurate in determining surface marker expression for intestinal muscle macrophages.

Cytokine and chemokine production by intestinal muscle macrophages in response to stimulation has been largely undetermined in previous work. Therefore, we analyzed cytokine and chemokine production by intestinal muscle macrophages both constitutively (Fig. 5) and in response to LPS and IC stimulation (Fig. 14). Other studies showed that human intestinal macrophages produce IL-8 but not IL-6 or TNF in response

to LPS (117, 119). However, we observed production of KC, IL-6, and TNF constitutively (Fig. 5) and after stimulation with either LPS or IC (Fig. 14). One interesting observation was the constitutively high levels of IL-6 observed in macrophages in the intestinal muscle (Fig. 4 and 5). Though IL-6 is typically thought of as a pro-inflammatory cytokine, muscle contraction alone contributes to IL-6 production (137, 139). Therefore, based on the tissue location of these cells, IL-6 cannot be viewed solely as inflammatory. However our co-localization studies show that macrophages produce the majority of IL-6 in whole mounts of intestinal muscle (Fig. 4). This study also expands the range of chemokines produced by intestinal macrophages, both with and without stimulation. Previously studies showed that human monocytes cultured to have anti-inflammatory phenotype produce chemokines such as IL-8, IP-10, and MCP-1 in response to LPS (69). We obtained similar results upon stimulation of muscularis macrophages (Fig. 5 and 14). This further suggests that intestinal muscle macrophages produce a wide range of factors but do not fall into a single classification.

The production of cytokines and chemokines by intestinal macrophages in response to chronic *Helicobacter* infection has not been investigated in previous studies. *Helicobacter* infection increases MIP-1α and IP-10 mRNA in cecal tissue (229), while peripheral blood monocytes and macrophage cell lines produce IL-6, IL-8, and NO in response to *Helicobacter* stimulation *in vitro* (230, 238, 239). However, we show that *Helicobacter* infection results in decreased cytokine and chemokine production by intestinal muscle macrophages in response to stimulation with either LPS or IC *in vitro* (Fig. 14). This suggests that *in vivo* infection likely results in a different response than *in vitro* stimulation. In addition, these data show macrophages from infected mice have altered responses to *in vitro* stimulation.

Arachidonic acid metabolites are critical to inflammation and to the resolution of the response. Peripheral blood monocytes and other tissue macrophages secrete the arachidonic acid products, LTB_4 and PGE_2 in response to *in vitro* stimulation with LPS and IC (192, 245). Our data extend these findings to show that intestinal muscle macrophages constitutively produce LTB_4 (Fig. 6). Neither *Helicobacter* infection (Fig.

6) nor *in vitro* stimulation (Fig. 15) alters secretion of LTB_4. Moreover, we showed that macrophages produced PGE_2 constitutively (Fig. 6) and in response to LPS but not IC stimulation (Fig. 15). This correlates with previous studies showing that PGE_2 and *COX-2* production co-localizes with macrophage markers in the intestinal muscle after LPS stimulation (65). Chronic, *in vivo* infection with *Helicobacter* does not alter constitutive production of PGE_2. However, macrophages cultured from *Helicobacter*-infected mice secrete significantly reduced concentrations of PGE_2 (Fig. 11). *Helicobacter* infection also decreases PGE_2 in response to *in vitro* stimulation (Fig. 15). This suggests that *in vitro* stimulation is not necessarily similar to infection when determining production of arachidonic acid metabolites.

Originally considered to be a commensal bacterium, researchers did not consider *Helicobacter* infection to alter experimental studies performed on infected mice. This study and others suggest that may not be the case. Due to the large number of infected mice, alteration of the inflammatory response by *Helicobacter* has a potentially significant effect on the results of present and future animal studies as well as the analysis of previous work.

CHAPTER 3 - References

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