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THE EFFECTS OF CAMPYLOBACTER JEJUNI AND TRICHURIS SUIS ON LOCAL CYTOKINE EXPRESSION IN THE SWINE COLON

By

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ABSTRACT

THE EFFECTS OF CAMPYLOBACTER JEJUNI AND TRICHURIS SUIS ON LOCAL CYTOKINE EXPRESSION IN THE SWINE COLON

By

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Campylobacter jejuni is a significant enteric pathogen of humans, but it is a non-pathogenic resident in the gastrointestinal tract of pigs. Upon infection with the nematode *Trichuris suis, C. jejuni* opportunistically invades the mucosa in the distal colon, resulting in severe disease and pathology. The central hypothesis of this dissertation project is that natural resistance to *C. jejuni* infection in swine is due to a proinflammatory response in the intestine. A related hypothesis is that *T. suis* stimulates an anti-inflammatory response which alters the protective response to *C. jejuni*. Three experimental designs were used to test these hypotheses. In the first experiment, weaned piglets were inoculated rectally with *C. jejuni* 81-176pWM1007, then samples of the distal colon and lymphoglandular complexes were taken for quantitation of cytokine mRNA expression by real time PCR. The data showed that *C. jejuni* stimulated a proinflammatory response *in vivo*. In the second experiment, weaned piglets were orally inoculated with embryonated *T. suis* eggs, *C. jejuni* 33292, or both, then samples of the jejunum, proximal colon, and distal colon were taken for quantitation of cytokine mRNA expression by real time PCR. The data showed that *C. jejuni* stimulated a proinflammatory response in the jejunum while *T. suis* stimulated an anti-inflammatory response in the jejunum and proximal colon. When dually infected, *T. suis* down regulated proinflammatory responses to *C. jejuni* in the

jejunum and proximal colon. In the third experiment, differentiated an undifferentiated swine intestinal epithelial cells (IPEC-1) were infected with *C. jejuni* 81-176 or 33292 and IL-18 production was measured in culture supernatants by ELISA. Only differentiated cells produced significant IL-18 in response to *C. jejuni*. Taken together, these data indicate that *C. jejuni* stimulates a local proinflammatory response *in vivo*, which can be attributed in part to the interaction of *C. jejuni* with intestinal epithelial cells. *T. suis* stimulates an anti-inflammatory response and down regulates proinflammatory cytokines, abrogating the protective response to *C. jejuni in vivo*. This immunomodulation could facilitate *C. jejuni* invasion into intestinal epithelial cells and deeper tissues *in vivo*, resulting in destruction of the epithelium and severe pathology.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

*Images in this dissertation are presented in color

Chapter 1: Literature Review

Campylobacter jejuni

Basic Microbiology

Campylobacter jejuni is a gram negative, slender, curved rod measuring 0.2-0.5μm wide and 0.5-5μm long [1]. It is motile due to the presence of uni- or bipolar flagella. It grows best at 42°C in a microaerophilic atmosphere of 3-15% oxygen and 2-5% carbon dioxide [2]. *C. jejuni* is nonsaccharolytic and requires complex growth media for laboratory culture [2]. The genome is small, approximately 1.6 to 1.7Mbp, and AT rich with a GC ratio of approximately 30%. In addition, extrachromosomal elements have been discovered, including conjugative plasmids and bacteriophages [2]. *C. jejuni* is naturally transformable, able to take up DNA from the environment and incorporate it into its genome [2-4]. Several genetic tools have been used to identify variants within the species, including pulse field gel electrophoresis, restriction fragment length polymorphism typing of ribosomal and flagellar genes, and amplified fragment length polymorphism analysis [5-9]. These methods have revealed extensive genetic diversity within the species.

Clinical Disease

C. jejuni is a significant enteric pathogen of humans and is the leading cause of foodborne bacterial illness worldwide. Ingestion of as few as 500 organisms can lead to diarrheal disease in susceptible humans [1]. Symptoms develop 1 to 7 days after ingestion of bacteria, with an average 24 to 48 hour incubation period [10]. Clinically, fever,

abdominal pain, and general malaise precede diarrheal disease. Diarrhea is profuse and can range from watery to mucoid and bloody in character [11]. *C. jejuni* colonizes the mucus overlying the epithelium of the distal ileum and colon [1] and colonic biopsies from infected individuals show an acute inflammatory response with infiltration of the epithelium and lamina propria with neutrophils and mononuclear cells [10]. Histologic examination of colonic tissues from experimentally infected colostrum deprived piglets that developed diarrheal disease after *C. jejuni* inoculation showed ulcerative colitis with denuded villus tips as well as infiltration of the submucosa with neutrophils [12]. *C. jejuni* disrupts the absorptive capacity of epithelial cells by invasion, by the effect of toxins, or through induction of an inflammatory response [2]. Gastrointestinal illness due to *C. jejuni* infection usually resolves after an average of 10 days [1], but the bacteria can be excreted in the feces for up to 16 days [10]. Due to the fact that illness is usually self limiting, treatment is generally restricted to supportive care with fluid therapy. However, erythromycin is the drug of choice for severe cases or immunosuppressed individuals [11].

The most common clinical manifestation of *C. jejuni* infection is diarrheal disease, however, in rare cases extraintestinal complications arise. Local spread of the bacteria can induce cholecystitis, pancreatitis, peritonitis, and massive GI hemorrhage. Systemic spread can cause meningitis, endocarditis, septic arthritis, osteomyelitis, and neonatal sepsis [11]. One notable sequela of *C. jejuni* infection is Guillain-Barré syndrome (GBS). GBS develops in 1 in every 1000 cases of *C. jejuni* infection, developing approximately 1-3 weeks after the onset of diarrheal illness. GBS is an ascending peripheral neuropathy that is most commonly associated with Penner serotype

O:19 strains in the U.S., and type O:41 in South Africa [11, 13]. Molecular mimicry between *C. jejuni* LPS and human gangliosides results in the generation of antibodies that attack the myelin sheaths of peripheral nerves, causing nerve paralysis [11, 13]. The combination of *C. jejuni* causing significant diarrheal disease and the debilitating sequelae, though rare, warrants intense investigation of this pathogen.

Epidemiology

Epidemiologic studies have showed a high prevalence of *C. jejuni* in cases of infectious diarrhea. Diarrheal disease due to *C. jejuni* in the U. S. is more prevalent than diarrheal disease due to *Salmonella, Shigella,* and *E. coli* O157:H7 [11]. The CDC estimates 2.4 million cases of campylobacteriosis each year in the U.S [11]. Sporadic illnesses account for the majority of reported cases and are generally attributed to consumption of improperly prepared poultry products or other foods contaminated with raw poultry fluids [1]. Seasonal outbreaks occur with bimodal peaks in May and October, and are usually due to consumption of contaminated raw milk or water sources contaminated with animal feces [1]. Children and young adults, particularly young males, are most commonly infected, with most deaths occurring in infants, elderly, and immunosuppressed individuals [1]. The preferred environment for *C. jejuni* is the intestinal tract of warm blooded animals. It is a commensal in a variety of wild and domestic mammals and birds, including chickens, cattle, sheep, and swine, and animals can serve as a source of infection for humans [14, 15].

The clinical picture and epidemiology of *C. jejuni* infections is different in developing countries. Diarrheal disease due to *C. jejuni* in developing countries is usually

less severe than in developed countries and can range from watery diarrhea, without blood or inflammation, to severe inflammatory, bloody diarrhea accompanied by fever and abdominal pain [16]. Campylobacteriosis does not show seasonal peaks in developing countries and this could be attributed to climate variations, or to inadequate surveillance for epidemics [16]. In developing countries, Campylobacteriosis is generally a pediatric disease being the most frequently cause of diarrheal disease in infants less than 6 months of age [16]. In community based studies 40-60% of diarrheic children less than 5 years of age were positive for the organisms on fecal cultures [17]. Additionally, polymicrobial infection is common, with protozoa, viruses, and other enteric bacteria coinfecting individuals [18, 19]. Sources for infection are commonly contact with animals and environmental contamination from animal feces [17, 20, 21].

Virulence Factors

C. jejuni causes diarrheal disease in humans by colonizing the intestinal tract and invading the epithelium which results in decreased absorption due to loss of epithelial function [2, 10]. The events that lead to invasion of the epithelium by *C. jejuni* include colonization of the mucus layer overlying the epithelium, adherence to epithelial cells, and invasion of epithelial cells. Several virulence factors have been described that play a role in the pathogenesis of *C. jejuni* infection.

Motility:

C. jejuni are motile by means of one or two polar flagella [2]. The flagellin protein is encoded by two adjacent genes on the chromosome designated *flaA* and *flaB*

[2]. Disruption of *flaB* results in mutants that have flagella of normal length, exhibit normal motility, and invade INT-407 cells at wild type levels [22]. In contrast, disruption of the *flaA* gene results in mutants that are aflagellate or have truncated flagella and are unable to adhere to or invade epithelial cells [22, 23], or colonize the ceca of chicks [24]. Mutants with paralyzed flagella are non motile and non-invasive, but are able to adhere to cells [23]. However, non motile mutants are able to invade epithelial cells *in vitro* if centrifugation is performed to bring the bacteria into contact with epithelial cells [22]. These data suggest that motility is necessary for invasion [22, 23].

C. jejuni motility is also affected by environmental factors. *C. jejuni* display normal swarming ability on motility agar of neutral and basic pH (pH=7.3 and 8.5, respectively). However, in an acidic pH of 5.0, swarming ability is decreased despite normal morphology. *C. jejuni* displays smooth swimming patterns with longer paths and greater velocity in viscous medium that mimics the viscosity of intestinal mucous. Increased viscosity of culture medium also increases adherence to and invasion of Caco-2 cells [25]. The motility of *C. jejuni* is believed to allow penetration of the intestinal mucous layer *in vivo* and successful colonization of the host [2].

Iron acquisition:

C. jejuni requires iron for optimal growth and proper cell division. Under iron limiting conditions, *C. jejuni* produces five proteins ranging in size from 52kDa to 89kDa. Three of these proteins with molecular weights of 74kDa, 76kDa, and 82kDa proteins are surface expressed. *C.jejuni* is also capable of producing siderophores and of using the exogenous siderophores enterochelin and ferrichrome for iron acquisition [26].

C. jejuni has evolved mechanisms for obtaining iron from its environment necessary for growth. However, in the presence of oxygen iron is able to generate oxygen radicals that can cause damage to DNA, proteins, and membrane lipids [27]. Therefore *C. jejuni* has also evolved mechanisms to survive in conditions of environemental stress. Alkyl hydroperoxide reductase C (AhpC) is transcriptionally regulated by iron concentrations and has been shown to be involved in resistance to oxidative stress caused by cumene hydroperoxide (CHP) and survival in atmospheric oxygen [28]. *C. jejuni* also possesses two Fur homolog iron responsive elements, designated *fur* and *perR*. *PerR* is a repressor of *ahpC* and *katA*, and *perR* mutants show hyperresistance to CHP and hydrogen peroxide [29]. An additional protein, designated Dps, has been identified in *C. jejuni*. Dps of *C. jejuni* is structurally similar to the DNA-binding protein from starved cells family of proteins. Dps does not bind DNA, but it does bind to iron. Dps confers resistance to hydrogen peroxide [27].

Chemotaxis:

In vivo, *C. jejuni* colonizes the mucous blanket overlying the epithelium of the intestines [30, 31]. This allows them to associate closely with intestinal epithelial cells [31]. Chemotactic movement is one mechanism by which the bacteria reaches its target niche and has shown to be essential for colonization *in vivo* [32]. *In vitro* studies have identified several compounds found in the gastrointestinal environment that affect chemotaxis of *C. jejuni*. Mucin, the major component of intestinal mucous and a component of bile, attracts *C. jejuni* [33]. Amino acids and organinc acids, including L-aspartate, L-glutamate, L-serine, L-fucose, pyruvate, succinate, fumarate, citrate, L-

malate, and α-ketoglutarate also attract *C. jejuni* [32, 33]. These compounds as well as low pH and high osmolarity have been shown *in vitro* to upregulate the *flaA* promoter σ^{28}, while increased viscosity causes downregulation [34]. It is hypothesized that increased production of FlaA in response to chemoattractants would facilitate maintenance of flagellar filaments, assisting in actions necessary for survival in the host. These actions would include escaping the acid pH of the stomach and chemotaxis toward mucin or amino acids that could be used as energy sources. Decreased motility in a viscous environment simulating the mucous blanket overlying the epithelium would be advantageous because it would signal that the desired niche had been reached [34].

Adherence:

Once *C. jejuni* successfully traverse the intestinal mucus layer and locate the epithelial cells, they must adhere in order to enter the cells and translocate to deeper tissues. Several factors have been identified that mediate adherence.

C. jejuni come into close apposition with the cell surface, particularly at the intercellular spaces [35]. Fibronectin is a component of the basement membrane underlying epithelial cells and is also present in regions of cell to cell contact in the GI epithelium [36]. *C. jejuni* binds to the glycoprotein fibronectin via a 37kDa surface exposed outer membrane protein designated CadF [37]. CadF also mediates adherence to intestinal epithelial cells *in vitro*, as mutation of CadF reduces adherence by 59% [36]. CadF has also been shown to be important for *C. jejuni in vivo* as it is necessary for successful colonization of chicken ceca [38]. Jlp is a surface lipoprotein that interacts with Heat shock protein 90α, mediating adherence to HEp-2 epithelial cells. This binding

leads to activation of the transcription factors NF-kB and the signaling molecule p38 MAP kinase [39, 40]. The *pebIA* locus encodes a previously described protein, Cell Binding Factor 1 (CBF-1) that mediates adherence to HeLa cells. Disruption of this locus reduces adherence *in vitro* by 15 fold, and reduces colonization of mice intestines [41]. Flagella mediate adherence to intestinal epithelial cells *in vitro*. Aflagellated bacteria, due to mechanical removal by shearing or by mutation, have decreased adherence to INT-407 cells [42].

Invasion:

Bacterial invasion into epithelial cells is proposed to contribute to the disease and pathology caused by *C. jejuni* infection [2]. *C. jejuni* has been shown to invade intestinal epithelial cells *in vitro* and *in vivo* [12, 35, 43, 44]. Studies have indicated a role for both microfilaments and microtubules in the invasion process. Oelschleger and colleagues found that *C. jejuni* invasion into INT-407 cells is a microtubule-dependent endocytic process that results in uptake into endosomal vacuoles. Internalization requires *de novo* protein synthesis for the bacteria, but not for the host cell [35]. Hu and Kopecko also showed that microtubules played a role in invasion as depolymerization inhibited *C. jejuni* internalization into intestinal epithelial cells. Microtubule-based membrane extensions were the initial point of contact between *C. jejuni* and the host cell. Once internalized, the bacteria associated with microtubules and then migrated to the perinuclear area via association with dynein [45]. Biswas and colleagues analyzed internalization of several human and animal isolates of *C. jejuni* and found that

depolymerization of both microfilaments and microtubules inhibited invasion into INT-407 cells depending on the strain tested [46].

In addition to host factors involved in invasion, bacterial factors involved in invasion have been identified. *C. jejuni* produces proteins that are essential for internalization. Campylobacter invasion antigen (Cia) proteins are secreted by *C. jejuni* in response to physiologic stimuli, including serum, bile salts, extracellular matrix components, and contact with intestinal epithelial cells *in vitro* [47, 48]. Eight different proteins have been detected in culture supernatants. One of them, designated CiaB, is a 73kDa protein that is necessary for internalization of bacteria into INT-407 cells. In addition, CiaB itself is translocated into the host cell upon binding of the bacteria [47].

C. jejuni has been shown to encode virulence determinants on extrachromosomal elements. Four virulence genes have been detected on a 35 kb plasmid, designated pVir, found in some clinical isolates of *C. jejuni.* All four genes are homologous to type IV secretion system components. Three genes, designated *comB1*, *comB2*, and *comB3,* display significant identity to *Helicobacter pylori* genes involved in competence for natural transformation and DNA uptake. The fourth gene displays identity to a type IV secretion system component of *Agrobacterium* designated *virB11.* Mutation of *comB3* reduces adherence and invasion of INT-407 cells, and decreases frequency of natural transformation. Mutation of *virB11* also reduced adherence and invasion *in vitro* and reduced virulence in the ferret diarrheal disease model [49].

Intracellular survival:

Intracellular survival of *C. jejuni* has been demonstrated in intestinal epithelial cells and macrophages [35, 50, 51]. The bacteria can survive within macrophages for up to 7 days [50]. Two genes have been identified to be necessary for survival within cells. The gene *katA* encodes catalase, which confers resistance to hydrogen peroxide *in vitro*. Mutation of *katA* does not affect intracellular survival in HEp-2 cells, but it does eliminate recovery from peritoneal macrophages 72 hours after infection [52]. Superoxide dismutase serves to defend bacterial cells against oxidative damage by catalyzing the breakdown of superoxide radicals to hydrogen peroxide and dioxygen. In *C. jejuni*, a single gene, designated *sodB*, encodes this enzyme and has been shown to be required for survival of invasion into INT-407 cells. Mutation of *sodB* decreases intracellular survival 12 fold [51].

Lipopolysaccharide:

Lipopolysaccharide (LPS) is a constituent of the outer membrane of most gram negative bacteria that is necessary for membrane integrity and function. It is composed of 3 components: the lipid A molecule that is embedded in the membrane, the oligosaccharide core, and the O chain. The lipid A molecule contains the endotoxic activity of LPS; the core oligosaccharide is involved in immunomodulation, and the O chain is highly variable and confers antigenicity to the molecule [53]. *C. jejuni* strains produce 2 types of LPS, one that contains all the components and one that lacks the O chain and is similar to the lipooligosaccharides (LOS) of *Neisseria spp.* and *Haemophilus spp.* LOS [53, 54].

LPS and LOS function in *C. jejuni* virulence *in vitro* and *in vivo*. Carbohydrate moieties of LPS mediate adherence to INT-407 and intestinal mucous *in vitro* [42]. GalE is a UDP-glucose epimerase that catalyzes the interconversion between UDP-glucose and UDPgalactose, which is a component of *C. jejuni* LPS. Mutation of *galE* inhibits adherence and invasion of INT-407 cells and natural transformation efficiency [55]. Sialic acid moieties of *C. jejuni* LOS confer resistance to complement killing *in vitro*. Mutation of *neuC,* a gene involved in the biosynthesis of the sialic acid *N*-acetylneuraminic acid (NeuNAc), results in loss of NeuNAc and increased serum sensitivity of *C. jejuni* [56]. Carbohydrate moieties of LOS can also mimic human gangliosides and play a role in the development of autoimmune disorders. The gene *cgtA* encodes a *N*-Acetylgalactosaminyl transferase that is subject to phase variable expression due to slip-strand mispairing in a homopolymeric G tract during DNA replication. This gene plays a role in structural variation in the LOS of *C. jejuni* 81-176, which can mimic GM_2 or GM_3 gangliosides [57]. *C. jejuni* NCTC 11168 also has phase variable LOS that mimics gangliosides GM_1 or GM_2, depending on expression of the *wlaN* gene. This gene encodes a β-1,3 galactosyltransferase and is also subject to slip-strand mispairing phase variation , similar to *cgtA* [58]. *In vivo*, antibodies directed against *C. jejuni* LOS molecules that mimic gangliosides can cause autoimmune mediated neuropathy. Rabbits immunized with *C. jejuni* LOS develop nerve degeneration and paralysis which resembles the clinical signs of GBS in humans [59]. Serum IgG from these rabbits reacts with both GM_1 ganglioside and LOS *in vitro,* indicating a direct role for ganglioside mimicry by *C. jejuni* LOS in the development of autoimmune disease [59]. Taken

together, these studies have demonstrated several roles for LOS in the pathogenesis of *C. jejuni* infection.

Toxins:

Toxins have been identified from *C. jejuni* and are proposed to function in cellular destruction and clinical disease. An enterotoxin with immunologic similarity to cholera toxin and the heat labile toxin of *E. coli* has been described in *C. jejuni.* Enterotoxin causes elongation of Chinese hamster ovary (CHO) cells, increased intracellular cAMP, and fluid accumulation in a rat ligated intestinal loop model [60, 61]. However, contradictory reports of researchers unable to replicate these results have confounded this evidence [62, 63]. Cytotoxic activity has also been described by assessing rounding of Vero cells or detachment of cells from the growth plate [60, 64]. Cytotoxic activity has been detected in isolates from human patients with invasive diarrhea [64] and from animal isolates [60]. Additionally, cytotoxic activity was detected from poultry isolates tested on chicken lymphocytes using a chromium release assay [65]. Further characterization of enterotoxins and cytotoxins is necessary to elucidate their specific roles in clinical disease.

Perhaps the most characterized *C. jejuni* toxin to date is the cytolethal distending toxin. Cytolethal distending toxin (CDT) causes distension and cell disintegration of a variety of cell types *in vitro* [66-68]. In *C. jejuni*, the CDT holotoxin is composed of 3 subunits, CdtA, CdtB, and CdtC which are produced from adjacent genes. The individual subunit proteins are approximately 30kDa, 29kDa, and 21kDa, respectively. All three genes are required for toxic activity [69]. The CdtA and CdtC subunits are responsible for

binding to the host cell, while CdtB enters the host cell and exerts toxic effects [67]. CdtB has significant similarities to DNAse I enzymes and is believed to cause limited damage to DNA resulting in arrest of the cell cycle in the G2 phase [70]. The arrest is mediated by inhibition of CDC2, a kinase subunit required for entry into the M phase of the cell cycle. This arrest results in cell enlargement, nuclear disintegration, and ultimately cell death [68].

A few studies have suggested a function for CDT in induction of an immune response. Mutant *C. jejuni* lacking the *cdtB* gene are capable of colonizing NF-kB deficient mice and producing enteritis, but the disease is less severe than that seen from the wild type *C. jejuni.* In addition, serum IgG2a responses in mice infected with the mutant were lower than mice infected with the wild type strain [71]. Attenuated disease and pathology with a CdtB mutant has also been demonstrated in a piglet model of infection (Mansfield et al, unpublished results). *In vitro*, CDT stimulates production of IL-8 from intestinal epithelial cells [72]. These data support the hypothesis that CDT plays a role in development of disease and stimulating an immune response *in vivo.*

Immune responses

Humoral immunity is necessary for clearance of *C. jejuni* from the intestine and resolution of clinical disease. In human infections, *C. jejuni* stimulates secretion of IgA in feces, urine, saliva, and mammary glands [73]. Flagellin is a strong stimulant for antibody production, as evidenced by the fact that a large proportion of anti-*C. jejuni* IgG antibodies found in convalescent serum is directed against flagellin [73]. The relatively low incidence of disease in adults from endemic areas of developing countries is believed

to be due to strong humoral immunity stimulated by repeated exposure during childhood [16, 74, 75]. In a swine model of infection, lymphoglandular complexes have been discovered to be a source of IgA in *C. jejuni* infection. Twenty seven days after oral inoculation with a low dose of *C. jejuni*, B cell rich germinal centers develop in the LGCs that stain positively for IgA and *C. jejuni* [76]. These studies suggest that the adaptive immune response to *C. jejuni* is directed by Th 2 cytokines.

Several studies have also demonstrated a role for cytokines in *C. jejuni* infections. Humans with infectious diarrhea caused by *C. jejuni* have increased levels of nitric oxide gas and increased secretion of IL-1β in the stool during the acute phase of infection [77]. Intestinal colonization of *C. jejuni* infected mice is significantly reduced when they are given recombinant IL-5 or IL-6 prior to infection. In addition, recombinant IL-6 enhances specific intestinal and systemic IgA production [78]. *In vitro,* secretion of IL-1β, IL-6, IL-8, and TNFα is stimulated from intestinal epithelial cells after infection with *C. jejuni* [79] (Cunningham et al, unpublished results; Parthasarathy et al, unpublished results). In addition, intracellular levels of IL-4, IL-10, IFNγ, and TNFα are increased in *C. jejuni* infected intestinal epithelial cells [80]. Cultured human monocytes secrete IL-1β, IL-6, IL-8, and TNFα upon infection, and NF-κB is translocated to the nucleus [81]. Similar to mammalian cells, chick kidney cells and avian macrophages have increased expression of iNOS with increased release of nitric oxide, as well as increased expression of IL-1β, IL-6, IL-8 [82]. These *in vitro* studies suggest an important role for proinflammatory cytokines in host resistance to *C. jejuni* infection.

Trichuris suis

T. suis life cycle

Trichuris suis is a nematode parasite that resides in the cecum and colon of pigs and it is closely related to *T. muris*, which infects mice, and *T. trichuria* which infects humans. Morphologically it has a slender anterior end that is partially embedded in the intestinal epithelium, and a thicker posterior end that is free in the lumen. Truchuris worms are commonly referred to as whipworms due to their whip like appearance. There are 5 stages in the life cycle of *T. suis* from egg to mature adult. Non-infective barrel-shaped, tan eggs with bipolar plugs are shed from the feces of infected pigs. Infective L1 larvae develop, or embryonate, within the egg in the environment. *In vitro* studies have shown that eggs embryonate in as little as 19 days when cultured at 34°C. Embryonated eggs are ingested by the host and hatch throughout the small intestine, cecum, and colon. Hatched larvae have been detected in the distal small intestine as early as 9 hours after oral infection. The larvae burrow into the mucosa via the crypts of Lieberkühn and invade the epithelial and goblet cells lining the crypts [83]. As the larvae grow they also invade cells in the lamina propria. The first moult, or shedding of the cuticle, begins approximately 10 days after infection as the larvae enter the L2 stage. The larvae continue to develop within the crypt cells and begin to migrate through the lamina propria towards the surface mucosal epithelium [83, 84]. The second moult to the L3 stage begins 16 days after infection and the tail begins to protrude into the lumen of the colon[84]. Throughout the moults the larvae are increasing in size and development of the gastrointestinal and reproductive tracts can be observed. The third moult to the L4 stage

begins 20 days after infection. On day 32, larvae begin to undergo the last moult to the L5 or adult stage. Sexual maturation completes and fully formed eggs can be detected in the female uterus, and in the feces of the host, by 41 days, allowing the life cycle to commence again [83, 84].

Prevalence

Recent studies have found a relatively low prevalence of *T. suis* in swine herds. *T. suis* was detected in 5.6% of pigs from intensive pig farms in Guangdong Province, China that do not use a strategic parasite control [85]. In Munsterland, Germany 8% of sows sampled from a 144 breeding farms were found to have *T. suis* ova in their feces. These farms routinely used anthelminthics in sows [86]. A study of Danish organic swine herds detected *T. suis* from 4% of weaners, 13% of fatteners, and <1% of sows [87]. Examination of a feral swine population in the US found that 10% of the pigs harbored *T. suis* [88]. Despite the apparently low prevalence of *T. suis* in swine herds, economic losses due to poor growth and death warrant further study of disease caused by these nematodes [89].

Mucohemorrhagic diarrhea

Mucohemorrhagic diarrhea is a significant health problem in the swine industry that can result from infections with a variety of bacteria and parasitic worms. *Treponema hyodysenteriae, Salmonella typhimurium, Salmonella typhisuis, Trichuris suis,* and *Campylobacter spp.* have all been found as causative agents. However, in many cases two or more agents act synergistically, causing significant enteric pathology [90, 91].

Trichuris suis infection causes a disease commonly known as "21 day scours" due to the fact that diarrheal disease commonly occurs approximately 3 weeks after pigs are placed in an area contaminated with infective *T. suis* eggs [90]. Diarrheal disease associated with severe infection is profuse, reddish-brown, mucoid, and bloody. In addition to diarrhea, pigs become anorexic, anemic, and can have significant weight loss. Penetration of the nematode into the mucosa of the cecum and proximal colon causes inflammation, hemorrhage, and in severe cases necrosis [44, 89-91]. *T. suis* was once believed to be innocuous, but further study of these nematodes has revealed that they are an important pathogen in the swine industry.

Histological lesions caused by *T. suis* infection are quite profound. Infection induces thickening of all layers of the intestine, sloughing of degenerate epithelial cells, and dilation of crypts with mucous. The lamina propria and submucosa become infiltrated by inflammatory cells, including neutrophils, lymphocytes, plasma cells and eosinophils [44, 89, 91]. In the distal colon, lymphoglandular complexes become enlarged by increased numbers of lymphocytes and macrophages, and several species of bacteria can be isolated from abscessed LGCs [44, 76, 89, 91]. Microscopic studies have shown that *T. suis* causes significant pathology both in the proximal colon where the worms reside and in the distal colon far from sites of worm attachment.

Disease and pathology caused by *T. suis* infection is exacerbated by interaction with the microbial component of the swine colon. In 1975 Rutter and Beer demonstrated, through infections of conventionally reared, specific pathogen free and gnotobiotic pigs, that some component of the normal gut flora of pigs acted synergistically with *T. suis* to cause severe mucohemorrhagic enteritis [92]. Further studies by Mansfield and Urban

confirmed Rutter and Beer's findings, also showing that severe pathology caused by secondary bacterial infection was ameliorated by antibiotic treatment [91]. Additionally, fenbendazole treatment of both naturally and experimentally infected pigs removes all adult *T. suis* and reverses gross pathology in 7 days (Mansfield et al., unpublished results). These studies demonstrate the complex nature of the polymicrobial infection involving *T. suis* that results in severe disease.

T. suis excretory/secretory product (ESP)

The pathologic effects of *T. suis* infection are mediated both by direct interaction of the worm with host tissues and through the actions of secreted worm products. Adult *T. suis* produce an excretory/secretory product (ESP) composed of secreted products that function in invasion and feeding *in vivo*, and excreted products that are byproducts of its metabolism. Crude ESP can be collected from culture media of adult *T. suis* maintained *in vitro* after removal from infected pigs [93] and several components have been isolated and partially characterized. A 45kDa zinc metalloprotease was localized by immunocytochemical staining to the stichosome. The protease displayed proteolytic activity against fibrinogen and elastin and is proposed to have a role in feeding and tissue penetration [93]. Phenol oxidase was localized to the reproductive tract of the female worms and caused brown pigmentation of the worms in an oxygenated environment. Phenol oxidase is proposed to function in tanning of the eggs [94, 95]. Thiol protease activity, localized to the gut of the worm, is hypothesized to function in digestion and absorption of nutrients [96]. Serine protease inhibitors were isolated from culture fluids and soluble worm extracts. These protease inhibitors showed activity against trypsin,

chymotrypsin, and elastase, and may function in down-regulating host mast cell, and other immune cell, effector mechanisms [97, 98]. Recently, antimicrobial activity which inhibits the growth of *C. jejuni, E. coli,* and *S. aureus in vitro* in a dose-dependent manner has been described and may play a role in defending the worm against bacteria in the intestinal environment [99]. A 20kDa glycoprotein isolated from culture media was detected in a Western blot assay using serum from *T. suis* infected pigs. Serum from uninfected pigs, or pigs infected with the parasites *Oesophagostomum dentatum, Trichuris spiralis, Ascaris suum*, or *Toxoplasma gondii* did not cross react, indicating that the 20kDa protein was specific for *T. suis* and could be used in diagnostic tests for infection [100].

In addition to identifying components of ESP, two recent studies have described its effects on host cells. Treatment of intestinal epithelial cells with crude ESP causes a dose dependent cytotoxic effect, and decreased transepithelial electrical resistance of differentiated cells. *T. suis* ESP treatment of swine intestinal epithelial cells prior to infection with *C. jejuni* decreases invasion, most likely due in part to its antibacterial activity [101]. Also, ESP has been shown to induce secretion of IL-6 and IL-10 from both differentiated and undifferentiated swine intestinal epithelial cells (Parthasarathy et al, unpublished results). These studies suggest a potential role for ESP in immunomodulation and the development of pathology *in vivo*.

Immunity

Preliminary studies have demonstrated that *T. suis* infection induces a strong immune response in conventionally reared pigs. Humoral immunity develops during

primary infection with the development of antibodies directed against a 20kDa glycoprotein isolated from *in vitro* culture fluids of adult worms. These *T. suis* specific antibodies were detected in serum from pigs 21 days after natural or experimental infection [100]. In addition, expression of IL-10 mRNA is increased 10 fold over uninfected controls in the mesenteric lymph nodes of pigs experimentally infected with *T. suis*. Pigs infected on a contaminated dirt lot have evidence of secondary bacterial infection in the distal colon and expression of both IL-10 and IL-12p40 are significantly increased in draining mesenteric lymph nodes compared to uninfected controls [102]. Pigs exposed to low level trickle infections with *T. suis* eggs over 4 weeks have decreased worm survival and worm fecundity during secondary challenge with 4000 infective eggs [103]. Taken together, these data suggest that *T. suis* stimulates a Th 2 directed immune response and that protective immunity can be achieved through low level exposure. Additional research is necessary to further characterize the *T. suis* stimulated immune response.

Studies in humans have also described a Th 2 directed immune response to the human whipworm, *T. trichuria*. People that live in areas endemic for whipworm infection have antigen specific serum antibodies that are predominantly of the IgG1 and IgG4 isotypes, with lower levels of IgG2 and IgG3. Antigen specific IgA and IgE are also present, with higher levels of IgE in less heavily infected individuals [104-106]. Whole blood cultures stimulated with *T. trichuris* ESP antigens or whole worm extracts secrete IL-4, IL-9, IL-10, IL-13, TNFα, and to a lesser extent IFNγ [104, 106]. Additionally, whole blood cultures stimulated with whole worm extracts display a significant proliferative response [106]. Similar to the swine whipworm, only a few studies have

been conducted to characterized specific components of the immune response to this nematode and further research is necessary.

In contrast to studies in swine and humans, extensive research in murine models has characterized the roles of specific mediators in the immune response to *Trichuris* infection. Inbred mouse strains have been developed that are either resistant or susceptible to chronic infection with *T. muris* [107-110]. Resistant strains like BALB/K, BALB/c and C57BL/6 mount a Th 2-directed immune response with increased production of IL-4, IL-5, IL-9, IL-10, IL-13, *Trichuris* specific IgG1, and increased serum levels of IgE [107, 109, 111, 112]. These strains develop significantly greater tissue eosinophilia upon infection than susceptible mice and are able to expel the larval stages of *T. muris* between 14 and 21 days after infection [107, 112]. Susceptible mouse strains like AKR and B10.BR mount a Th 1 directed immune response with increased production of IFNγ and high levels of *Trichuris* specific IgG2a as well as IgG1. These strains are unable to expel the larval stages of *T. muris* and become chronically infected [107, 111, 112]. These dichotomous strains have allowed researchers to define the roles of specific immune mediators in the disease process.

Alteration of the polarized immune responses of inbred mouse strains to *T. muris* infection through administration of stimulatory antigens or recombinant cytokines have elucidated the roles of specific cytokines in the immune response. Inoculation of susceptible AKR mice with *Schistosoma mansoni* egg antigens prior to infection with *T. muris* results in production of IL-4, IL-5, IL-10, IgG1 and IgE and subsequent worm expulsion [113]. Administration of recombinant IL-4 to AKR mice facilitates parasite expulsion while blocking IL-4 activity in BALB/K mice with antibodies inhibits parasite

expulsion [114]. Exogenous recombinant IL-9 causes increased intestinal mastocytosis, increased serum IgE, and decreased worm burden in *T. suis* infected AKR mice [109]. In addition, neutralization of IL-9 with antibodies decreases colonic muscle hypercontractility and inhibits worm expulsion in normally resistant C57BL/6 mice [110]. Exogenous recombinant IL-12 increases production of parasite specific IgG2a and impaired worm expulsion in BALB/K mice [115]. Antibody depletion of IFNγ activity in normally susceptible AKR mice infected with *T. muris* results in parasite expulsion [114].

In addition to using exogenous agents, genetic manipulation of inbred mice to abrogate production of certain cytokines has also proved to be a useful tool for studying the effects of Th 2 cytokines in *T. muris* infection. IL-10 knockout mice are susceptible to chronic infection with *T. muris* and have decreased IL-5 and IL-13 production, decreased peripheral eosinophilia, and decreased intestinal mucous production [112]. IL-4 knockout mice infected with *T. muris* have decreased production of IL-5, IL-9, and IL-13 compared to wild type mice and are unable to generate parasite specific IgG1. IL-13 knockout mice also have impaired cytokine production initially, but by 21 days after infection, IL-4, IL-5, and IL-9 production is at or above the levels produced by infected wild type mice. This demonstrates an early role for IL-13 in promoting the development of Th 2 responses stimulated by *T. muris* infection [116]. In addition to the roles of specific cytokines and antigen specific antibody in *T. muris* infection, cell mediated immunity is also important. SCID mice are normally susceptible to chronic *T. muris* infection, but reconstitution with purified CD4+ T cells from *T. muris* infected BALB/c mice resulted in expulsion of the parasite in the absence of antigen specific serum antibodies [108]. These studies have demonstrated the critical roles of the Th 2 cytokines

IL-4, IL-5, IL-9, IL-10, and IL-13 in resolution of *T. muris* infection and helped elucidate their specific effects *in vivo*.

Rationale for study

Campylobacter jejuni is a significant enteric pathogen in humans causing severe diarrheal disease and in rare cases debilitating autoimmune disorders [2, 10, 11, 13]. Severe diarrheal disease and colonic pathology in humans is due to invasion of the intestine by *C. jejuni* with subsequent inflammation [2, 10]. Because pigs can become colonized with *C. jejuni* within 24 hours after birth and can carry the bacteria without clinical signs of disease, they serve as a potential source of infection for humans [15, 44]. *T. suis* infection of pigs harboring *C. jejuni*, either by natural or experimental infection, results in mucohemorrhagic diarrhea and severe pathology that mimics the clinical disease of Campylobacteriosis in humans [44, 91]. When *T. suis* is present concurrently, *C. jejuni* invade epithelial cells, significant inflammation develops, and mucohemorrhagic diarrhea is evident clinically [44]. This suggests that in both human and swine infections, bacterial invasion is the key event in the development of clinical disease and pathology. In the case of dual infection in swine, it is believed that immunomodulation induced by *T. suis* abrogates the natural resistance to *C. jejuni*, allowing invasion of intestinal tissues.

Studies performed to date to elucidate the *in vivo* immune responses associated with *C. jejuni* infection in humans and swine have focused mainly on immunoglobulin production in serum and in lymphoid tissues [73, 76]. *In vitro* studies have focused mainly on cytokine responses from cultured cells ([72, 79]; Parthasarathy et al, unpublished results; Cunningham et al, unpublished results). The results indicate that intestinal epithelial cells are an important source of proinflammatory cytokines which may function in host resistance *in vivo.* Indeed, studies have shown a critical role for

epithelial cell derived IL-8 in control of experimental *Shigella flexneri* infection in rabbits [117].

Characterization of the swine immune response to *T. suis* is severely limited and has focused on production of a relatively limited panel of cytokines in draining lymph nodes and secreted in the feces ([102]; Parthasarathy et al, unpublished results; Cunningham et al, unpublished results). Comparison of cytokine expression between intestinal tissue at the site of infection and the draining lymph nodes in animal models of nematode infection have shown that the cytokine profiles are not identical [112, 118]. *T. suis* infection results in significant pathology in the proximal colon due to direct interaction of the worms with the intestinal mucosa, and in the distal colon due to secreted worm products or immunomodulation [44, 91, 92]. Therefore, it is important to study local cytokine responses in the region where the worms reside and also in distant areas where worms are not present to determine the nature of the local immune response and ascertain what effect those cytokines may have on the development of pathology. In this dissertation, we have begun to characterize the swine local immune responses to *C. jejuni* and *T. suis* in an effort to expand our knowledge of the immune response to *T. suis* and to determine the nature of the immunomodulation that occurs during dual infection.

Hypothesis

The central hypothesis for this dissertation project is that a proinflammatory response plays a role in swine natural resistance to *C. jejuni.* A related hypothesis is that a *T. suis* stimulated anti-inflammatory response alters the protective proinflammatory

response. Thus *T. suis* infection would facilitate *C. jejuni* invasion in the colon and severe pathology would develop.

Short term goals

The short term goals are to address the following questions:

1. Does *C. jejuni* that expresses Gfp colonize the distal colons of pigs when inoculated rectally?
2. Does rectally inoculated *C. jejuni* that expresses Gfp stimulate an immune response in the distal colon?
3. What is the nature of the cytokine response in the distal colon stimulated by rectally inoculated *C. jejuni*?
4. What is the nature of the local cytokine response in the small and large intestines of pigs infected orally with *C. jejuni, T. suis*, or both?
5. Is there evidence of immunomodulation in the colons of dually infected pigs?
6. Do epithelial cell derived cytokines play a role in swine resistance to *C. jejuni* infection as determined by *in vitro* infection of swine intestinal epithelial cells?

In summary, the goal of this study was to determine the nature of the local cytokine response to *C. jejuni* and *T. suis* and to determine whether cytokine dysregulation could play a role in the pathology and disease caused by dual infection.

Chapter 2 summarizes the studies on the effects of rectal inoculation with *C. jejuni* on colonization, localization of the bacteria in the distal colon, and on local cytokine mRNA expression. Proinflammatory cytokines are upregulated in the intestines

of animals experimentally or naturally infected with enteric bacteria and they have been shown to be critical for elimination of the bacteria and resolution of disease [117, 119-121]. We measured mRNA expression of both proinflammatory and anti-inflammatory in the distal colons and LGCs of pigs rectally inoculated with *C. jejuni*. The nature of the cytokine response to *C. jejuni* in lymphoid and nonlymphoid tissue in the distal colon is discussed.

Chapter 3 summarizes the study on the effect of oral infection with *C. jejuni, T. suis*, or both on local cytokine expression in the intestines of pigs. Cytokine responses in resistant mice infected with *T. muris* are predominantly Th 2 directed with increased production of IL-4, IL-5, IL-10, and IL-13 [107, 109, 111, 112]. These cytokines can also be characterized as anti-inflammatory as they downregulate proinflammatory cytokines [122, 123]. We measured mRNA expression of both proinflammatory and anti-inflammatory cytokines in the jejunum, proximal colon, and distal colons of pigs orally inoculated with *C. jejuni, T. suis*, or both. The nature of the cytokine responses and the differences in responses between the intestinal segments are discussed.

Chapter 4 summarizes the *in vitro* studies on proinflammatory cytokine production from intestinal epithelial cells infected with *C. jejuni*. *C. jejuni* stimulates production of IL-1β, IL-6, IL-8, and TNFα from both swine and human intestinal epithelial cells (Cunningham et al, unpublished results, Parthasarathy et al, unpublished results; [72, 79, 80]. Intestinal epithelial cell derived IL-8 is critical for control of infection in a rabbit ligated ileal loop model of *Shigella flexneri* infection [117]. We measured secreted IL-18 protein and IL-18 mRNA expression from *C. jejuni* infected IPEC-1 swine intestinal epithelial cells. The nature of the responses from differentiated

and undifferentiated IPEC-1 cells and the role of transcriptional regulation of IL-18 production are discussed. A proposed model for epithelial cell derived IL-18 in resistance to *C. jejuni* infection *in vivo* is also discussed.

Chapter 5 summarizes the previous chapters and discusses a proposed model for disease and pathology during dual infection with *C. jejuni* and *T. suis* due to cytokine dysregulation.

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Chapter 2: "*Campylobacter jejuni* invades distal colonic lymphoglandular complexes causing upregulation of proinflammatory cytokines in a swine rectal challenge model"

Abstract

Campylobacter jejuni is the leading bacterial cause of foodborne illness worldwide. Orally inoculated pigs have been used to study diarrheal disease, intestinal pathology, and humoral immune responses due to *C. jejuni*. These studies have shown that *C. jejuni* mainly targets the distal colon. We used a rectal inoculation technique to target the distal colon in order to assess pathogenesis of *C. jejuni* infection at specific time points. Weaned piglets were inoculated rectally with 10^9 cfu of *C. jejuni* expressing green fluorescent protein (Gfp), and were euthanized 1 or 24 hours after inoculation. Samples of the distal colon were taken for *C. jejuni* detection and localization, and cytokine analysis. *C. jejuni* was detected in 93.75% of inoculated pigs. Immunostaining detected organisms associated with surface epithelium, within crypts, and within crypts of lymphoglandular complexes in the distal colon. Several proinflammatory cytokines, including IL-8, IL-18, IL-1β, TNF-α, GM-CSF, iNOS, and MCP-1, were significantly upregulated in the distal colon and lymphoglandular complexes 1 hour after inoculation. These results show that rectal inoculation of *C. jejuni* is a useful technique for targeting the distal colon and eliciting cytokine responses. Proinflammatory cytokines are likely important in the pathogenesis of *C. jejuni* infections in pigs.

Introduction

Campylobacter jejuni is the most frequently reported bacterial cause of foodborne illness worldwide. The CDC estimates 2.4 million cases of campylobacteriosis occur each year in the U.S. [1]. Children and young adults are most commonly affected, developing profuse, watery diarrhea with blood, mucus, and leukocytes 1 to 7 days after ingestion of the organism [2, 3] . In most cases symptoms persist for 5 to 8 days and include abdominal cramping, fever, and malaise [3]. In rare cases death occurs, most commonly in infants, elderly, and immunosuppressed individuals [2]. Most *Campylobacter* infections in humans result in self-limiting gastrointestinal disease. However, extra intestinal infections can result in local complications including cholecystitis, pancreatitis, peritonitis, and massive GI hemorrhage. Further spread can result in meningitis, endocarditis, septic arthritis, osteomyelitis, and neonatal sepsis [1]. Notable sequelae of *C. jejuni* infection include Reiters syndrome and Guillain-Barré syndrome (GBS) [1-4] . Reiters syndrome occurs in 1% of people affected with campylobacteriosis. Symptoms commonly arise 7 to 10 days after onset of diarrheal illness and include a sterile, reactive arthritis, urethritis, and conjunctivitis [2]. Guillain-Barré syndrome, an ascending peripheral neuropathy, is an autoimmune disorder that develops in 1 in every 1000 cases of *C. jejuni* infection and is most commonly associated with Penner serotype O:19 strains [1-4]. Food sources that have been associated with infection include raw milk, poultry products, shellfish, and fresh produce [2]. Live animals are a significant reservoir for *C. jejuni* and a source of infection for humans as *C. jejuni* is a commensal in the gastrointestinal tract of a variety of wild and domestic animal species including poultry, cattle, sheep, and swine [5-8]. Campylobacteriosis has

incited an abundance of research aimed at elucidating virulence mechanisms and immune responses in an effort to develop therapies to treat and prevent infection with this pathogen.

Immune responses to *C. jejuni* have been characterized in both human infections and animal models. *C. jejuni* stimulates a humoral immune response in humans with production of specific IgA in feces, urine, saliva, and mammary glands [9]. Flagellin is an important antigen for stimulating immune responses, as serum taken from patients up to 2 months post infection contained a large proportion of antibodies directed against flagellin [9]. Humoral immunity affords protection against reinfection with homologous strains [10], which is believed to account for the relatively low incidence of diarrheal disease in adults in developing countries compared to developed countries [11, 12]. Recently, Mansfield and Gauthier demonstrated that lymphoglandular complexes (LGCs) in the distal colons of pigs are targeted by *C. jejuni*. The LGCs expand significantly after infection due to development of B cell rich germinal centers that produce IgA [13].

Cytokines also play an important role in *C. jejuni* infections. Mice orally administered recombinant IL-5 or IL-6 have reduced intestinal colonization after infection. In mice given IL-6, specific intestinal and systemic IgA responses are also enhanced [14]. In vitro studies on human cells show that *C. jejuni* induces IL-8 secretion and NF-κB translocation in intestinal epithelial cells [15-17]. *C. jejuni* infected monocytes secrete IL-1β, IL-6, IL-8, and TNF-α, and NF-κB is translocated to the nucleus [18].

Several animal models have emerged to aid in the study of various aspects of *C. jejuni* infection. Inbred mice have been used to study leukocyte responses, cytokine

responses, immunoglobulin responses, and colonization [19-22]. Ferrets have been used to study diarrheal disease, abortion, colonization, and antibody responses to *C. jejuni* infection [23]. Pigs have been used to study diarrheal disease, pathology, and immune responses [13, 24, 25].

Rectal immunization protocols to study immune responses and vaccine efficacy have shown that direct inoculation of infectious agents into the distal colon is an effective, non-invasive method for eliciting and studying local immune responses [26, 27]. In this study we used rectal inoculation of *C. jejuni* to study the early immunological response to initial invasion events in the distal colon. Our results indicate that rectal inoculation of *C. jejuni* elicits measurable changes in cytokine expression in the distal colon and lymphoglandular complexes (LGCs). Expression of several proinflammatory cytokines was significantly increased in the LGCs, which have previously been shown to function as antigen sampling structures that are specifically targeted by *C. jejuni* [13, 25].

Materials and Methods

Animals

Four week old male and female outbred Landrace-Yorkshire cross pigs were obtained from the Michigan State University (MSU) swine farm. Animals were housed in the MSU Research containment facility in groups of 4 for social support. Individual infection groups were housed in separate rooms to eliminate cross contamination. All animal housing and handling complied with MSU animal use guidelines and National Institutes of Health guidelines for humane use of laboratory animals.

Bacteria

C. jejuni strain 81-176 which had been electroporated with the plasmid pWM1007 [28] was used for all experiments. The plasmid pWM1007 contains the *gfp* gene under the influence of a constitutively active consensus *Campylobacter* promoter. Bacteria were grown, from frozen stocks, on Bolton (Oxoid Inc., Basingstoke, Hampshire, England) agar plates without antibiotics at 37°C, 5% CO_2 in humidified air for 48 to 72 hours. Isolated colonies were subcultured onto Bolton agar with 50 μg/mL of kanamycin to select for gfp-expressing clones. Four milliliter (4 mL) aliquots of Bolton broth without antibiotics, in a 6-well polystyrene tissue culture plate (Costar®, Corning, Corning, NY), were inoculated from isolated colonies from agar plates with kanamycin. The broth was incubated at 37°C, 5% CO_2 in humidified air at 200 RPM until the early log phase of growth, as determined by optical density (OD). The culture was diluted 1:100 in 75 mL fresh Bolton broth without antibiotics and incubated under the previously described

conditions until an OD_{600} of approximately 0.1 was reached. Serial dilutions and plate counts from previous experiments have shown that this OD corresponds to the early log phase of growth and a concentration of approximately $1x10^8$ cfu/mL. The bacteria were collected and pelleted by centrifugation and resuspended in serum free medium (SFM) (Sigma Inc., St. Louis, MO) for rectal inoculation of pigs. A subsample of the inoculum were examined microscopically using a Nikon Eclipse E600 with an Epi-fluorescence system (Mager Scientific, Dexter, MI) for darting motility and fluorescence prior to infecting pigs. Also, a sample from the prepared inocula was serially diluted and plated to determine actual concentration. Each pig was inoculated with approximately $2x10^9$ cfu *C. jejuni* in a total volume of 10 mL SFM.

Experimental design and inoculation procedure

Pigs were divided into 4 groups as follows: Group 1: Control, euthanized after 1 hour; Group 2: Infected, euthanized after 1 hour; Group 3: Control, euthanized after 24 hours; Group 4: Infected, euthanized after 24 hours. All infected pigs received *C. jejuni* resuspended in 10 mL SFM. All control pigs received 10 mL SFM without bacteria. All pigs were tested for the presence of *C. jejuni* both before and after inoculation by culture of rectal swabs or fecal samples. Rectal swabs or fecal samples were placed in Cary Blair (Difco Laboratories, Detroit, MI) transport medium for later culture in the laboratory. After samples were taken for *C. jejuni* culture, the pigs were anesthetized with intramuscular injections of 4.4 mg/kg Telazol (Fort Dodge, Overland Park, KS) and 2.2 mg/kg xylazine (Butler, Columbus, OH). Once the pigs were fully anesthetized, they were placed in lateral recumbency on heating pads and covered with towels to maintain

body temperature throughout the procedure. For each pig, the perineum was cleaned with three scrubs, alternating betadine and isopropyl alcohol for each scrub, and a purse string suture pattern was placed around the anus using 0 silk suture and left untied. A sterile 14 French feeding tube was inserted into the anus and advanced 10 to 15 cm into the distal colon. The appropriate inoculum was slowly injected through the tube, which was immediately removed. The purse string was tightened, and the hindquarters were elevated slightly. All pigs remained anesthetized for at least 1 hour, after which they were either euthanized or allowed to recover from anesthesia, depending on group assignment. Pigs that were allowed to recover from anesthesia were reanesthetized, as previously described, 24 hours after inoculation. All pigs were euthanized with an intravenous injection of 86 mg/kg sodium pentobarbital (Fatal Plus®, Vortech Pharmaceuticals, Dearborn, MI).

Sample collection

The colons of euthanized pigs were removed from the abdomen and opened. A separate set of sterile instruments was used for each group to eliminate cross contamination between infection groups. Full thickness samples of the distal colon and individual LGCs were collected. Samples were fixed in 3.7% formaldehyde for histology and immunohistochemistry, embedded in OCT compound for laser capture microdissection, or placed in cryovials and snap frozen in liquid nitrogen for nucleic acid isolation.

C. jejuni isolation and identification

Rectal swabs or fecal samples were streaked onto Preston selective agar (Oxoid Inc., Basingstoke, Hampshire, England) and incubated at 42°C, 5% CO_2 in humidified air for 48 hours. Colonies isolated from rectal swabs or fecal samples of experimentally infected pigs were scored according to 5 criteria: 1) the bacteria grew on *Campylobacter* selective media under microaerophilic conditions (5% CO_2 at 42°C); 2) colonies were round, raised, had pink pigmentation, and were 1-2mm in diameter-morphology that was consistent with *Campylobacter spp.*; 3) microscopic examination revealed curved motile rods; 4) bacteria were positive for PCR amplification of the quinolone resistance determining region (QRDR) of the *gyrA* gene of *C. jejuni* [29]; and 5) bacteria fluoresced green when examined at 488 nm wavelength on a Nikon Eclipse E600 with an Epi-fluorescence system (Mager Scientific, Dexter, MI). Pigs were considered positive for the strain of *C. jejuni* used for infections if all 5 criteria were met.

We found culture alone to be inadequate for detection of *C. jejuni* from experimentally inoculated animals, therefore we used 3 different PCR techniques to detect *C. jejuni* from tissue DNA. Total DNA was isolated from approximately 25 mg of frozen tissue using a Qiagen DNEasy tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The DNA was quantified spectrophotometrically (Beckman, Fullerton, CA). To detect *C. jejuni* from experimental animals, the *C. jejuni* QRDR target was amplified by PCR from 500ng of total DNA isolated from full thickness tissue samples using the previously designed primers JL238 (5' TGG GTG CTG TTA TAG GTC GT 3') and JL239 (5' GCT CAT GAG AAA GTT TAC TC 3') [29]. Amplicons were electrophoresed on a 1.8% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer with

0.2 μg/mL ethidium bromide. The fragments were visualized by UV illumination on an Alpha Imager Gel documentation system (Alpha Innotech Corporation, San Leandro, CA).

A Restriction Fragment Length Polymorphism (RFLP) assay to detect and distinguish *C. jejuni, C. coli, C. lari*, and *C. upsaliensis* by analysis of a portion of the 23S rRNA gene was used to detect thermophilic campylobacters in experimental animals [30]. The target was amplified from 500ng of total pig tissue DNA using primers THERM1 (5'-TAT TCC AAT ACC AAC ATT AGT-3') and THERM4 (5'-CTT CGC TAA TGC TAA CCC-3'). The amplicons were digested overnight with either *AluI* or *Tsp5091* following the manufacturers protocols (New England Biolabs, Beverly, MD), and the resultant fragments were separated electrophoretically on a 2% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer with 0.2 μg/mL ethidium bromide. The fragments were visualized by UV illumination on an Alpha Imager Gel documentation system (Alpha Innotech Corporation, San Leandro, CA).

Primers were designed to amplify a 543 bp fragment from the *gfp* gene (Genbank Number AF292556) using SeqWeb software (Genetics Computer Group, Madison, WI). The forward primer sequence is 5'AGT GGA GAG GGT GAA GGT GAT G 3'; the reverse primer sequence is 5' AAG GGC AGA TTG TGT GGA CAG G 3'. A 25 μL PCR reaction with 1X PCR buffer (Tris-HCL, pH 8.3, 50 mM KCl), 1.0 mM $MgCl_2$, 0.05 mM each dATP, dTTP, dCTP, dGTP, 37.5 ng of each primer, 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 500ng total tissue DNA was used to amplify the target. Amplification parameters were initial denaturation at 94°C for 10 minutes, followed by denaturation for 1 minute at 94°C, annealing for 1 minute at

62°C, extension for 1 minute at 72°C for 39 cycles, and a final extension at 72°C for 10 minutes. The amplicons were electrophoresed on a 1.8% agarose gel, stained, and visualized as previously described.

Histology and Immunohistochemistry

Fixed tissue samples were embedded in paraffin and 5 μm sections were cut and adhered to charged slides. Slides were routinely dehydrated in progressively higher concentrations of ethanol, deparaffinized and rehydrated. For histological evaluation, slides were stained with Mayer's hematoxylin and eosin and cover slipped. For immunostaining, sections of selected tissues were incubated in preheated antigen retrieval buffer (Dako, Carpinteria, CA) for 20 minutes at 100° C. Slides were removed from the steamer and left in the retrieval solution at room temperature to cool down for 20 minutes, rinsed in distilled water and transferred to Tris-HCL buffer. Endogenous peroxidase was blocked for 15 minutes with 3% hydrogen peroxide. Non-specific immunoglobulin binding blocking was done by a 10 minute incubation with a protein-blocking agent (Dako, Carpinteria, CA) prior to application of the primary antibody. A rabbit polyclonal anti-*C. jejuni* antibody (Biogenesis, New Fields, UK) was used as the primary antibody at a concentration of 1:100. Sections were stained with a Dako autostainer using a labeled streptavidin-immunoperoxidase staining procedure (Dako Corp., Carpinteria, CA). The immunoreaction was visualized with AEC (Dako Corp., Carpinteria, CA). Sections were counterstained with Mayer's hematoxylin, then dehydrated, cleared and mounted. Sections of distal colons from pigs in which infection with *C. jejuni* had been confirmed by PCR served as positive controls.

Determination of antibody specificity

Healthy, conventionally reared pigs have been shown to have more than one species of *Campylobacter* residing as commensals in their intestines [7]. Due to the likelihood that the pigs used in this study also harbored other Campylobacter species and the possibility of cross reactivity with those species, we tested the specificity of the polyclonal antibody for *C. jejuni*.

IPEC-1 cells are a non-immortalized swine intestinal epithelial cell line derived from neonatal pigs. All cell culture reagents were obtained from Invitrogen unless otherwise indicated. IPEC-1 cells were grown in DMEM/F12 media supplemented with 5% fetal bovine serum (FBS) and 1% Insulin-Transferrin-Selenium(ITS) in Costar culture flasks (Costar®, Corning, Corning, NY). Cells were washed in versene and detached with 0.05% Trypsin-EDTA, then seeded at a density of $5x10^5$ cells per well onto glass chamber slides coated with 20μg/mL fibronectin (Sigma, St. Louis, MO). The cells were incubated at 37°C, 5% CO_2 until confluent (2-3 days). *C. jejuni* 81-176 expressing gfp, *C. coli* 43134, *C. lari* 43675, and *C. upsaliensis* 43954 were streaked for isolation onto Bolton agar plates (Oxoid Inc., Basingstoke, Hampshire, England) and incubated for 72 hours at 37°C, 5% CO_2. Isolated colonies were streaked for lawn growth and incubated for 20 to 24 hours. Lawns were harvested from plates with sterile swabs, resuspended in DMEM/F12 supplemented with 5%FBS and 1%ITS, and adjusted to an OD_{600} of 0.1. IPEC-1 cells were inoculated with an MOI 100:1 of each *Campylobacter* species and incubated for 3 hours at 37°C, 5% CO_2. The supernatants were removed and

the cells were fixed in 10% formaldehyde, then stained as previously described with the anti-*C. jejuni* antibody.

Laser Capture Microdissection

Five micron sections were cut from tissue samples embedded in OCT compound on a Reichert-Jung cryotome (Reichert-Jung , Bensheim, Germany) and adhered to poly-L-lysine (Sigma, St. Louis, MO) coated slides. Slides were rinsed in ethanol, stained with Mayer's Haemotoxylin, dehydrated in progressively higher concentrations of ethanol, then rinsed in xylene. Crypt units and submucosal samples, avoiding the surface mucosa, were microdissected using a Pixcell II laser capture Microdissection system (Arcturus, Mounrainview CA). Samples were adhered to CapSure HS LCM Caps (Arcturus, Mounrainview CA) and stored at -20°C for later DNA isolation. For DNA extraction, caps were incubated in Proteinase K digestion buffer (1mg/ml Proteinase K (Qiagen, Valencia, CA) /1% Tween 20 (Sigma) in TE Buffer pH.8.0) overnight at 37°C. Samples were heated to 95°C for 10 minutes to inactivate the Proteinase K and stored at -20°C. To determine if the inoculating strain was present in the crypts and submucosa, 10μL of DNA were used as a template in a PCR reaction with primers designed in our laboratory to detect the *gfp* gene. RFLP analysis of the *Campylobacter* 23S rRNA gene was also performed to determine if other *Campylobacter* strains were present in the crypts and submucosa[30].

Cytokine Analysis

To determine the effect of rectal inoculation of *C. jejuni* on local immune responses in the distal colon and LGCs, mRNA expression of 14 cytokines, chemokines and iNOS was measured using a real time PCR assay. Total RNA was isolated from frozen tissue samples from each pig using Trizol reagent (GibcoBRL Life Technologies). Briefly, $3mm^3$ tissue samples were homogenized in Trizol using a Polytron® tissue homogenizer (Kinematica, Cincinnati, OH). RNA was extracted with chloroform, precipitated with isopropanol, and washed with ethanol. Pellets were air dried, then resuspended in DEPC treated H_20 (GibcoBRL Life Technologies). DNA contamination was removed by treatment with approximately 27 Kunitz units of DNase I for 15 minutes. Samples were repurified on a Qiagen RNEasy column (Qiagen, Valencia, CA). RNA concentration and integrity were determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). For each sample, 10 μg of total RNA was reverse transcribed with random primers using a Stratagene First Strand RT-PCR kit (Stratagene, La Jolla, CA). First strand cDNA was used as the template in real time PCR reactions. Real time PCR primers and probes designed using the Primer Express (Applied Biosystems, Foster City, CA) software package were used to analyze 14 cytokines, chemokines, and iNOS (Table 2). The 18S rRNA probe was obtained from Applied Biosystems and was used as a constitutively active gene for normalization. PCR was performed using a commercially available kit (Brilliant kit, Stratagene, La Jolla, CA) on an ABI PRISM 7900HT (Applied Biosystems). Amplification conditions were as follows 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C 15 seconds and 60°C for 1 minute. Fluorescence signals measured during amplification were processed post

amplification. Threshold cycle (C_t) values were determined using values of 0.2 for TET, 0.1 for VIC, and 0.05 for FAM reporter dyes.

Statistical Analysis

The cytokine mRNA expression data for each tissue was analyzed separately using Statview 5.0 for Macintosh (Abacus Concepts, Berkeley, Calif.). The C_t value for the 18S ribosomal subunit was subtracted from the C_t value for each cytokine message to normalize for RNA content prior to statistical analysis. One-way analysis of variance (ANOVA) was performed to determine the statistically significant differences between treatments. A Fisher's least squares difference test was performed to determine differences between infected and control animals at each time point. P values of 0.05 or less were considered statistically significant.

Results

Campylobacter isolation and identification

C. jejuni was not isolated from any fecal samples taken prior to infection (data not shown). One hour after infection, *C. jejuni* was not recovered from any infected or control pigs (Table 1). Twenty four hours after inoculation, *C. jejuni* was recovered from 1 of 4 infected pigs and 0 of 4 control pigs. All *C. jejuni* isolates recovered from pigs were confirmed to be the inoculating strain by examination using fluorescence microscopy.

PCR analysis of total DNA isolated from pig tissues proved to be a more sensitive assay for the detection of *C. jejuni* from experimentally inoculated pigs. One hour after inoculation, *C. jejuni* was detected in all 4 infected pigs and none of the control pigs using primers specific for the QRDR of the *gyrA* gene of *C. jejuni.* Twenty four hours after inoculation, *C. jejuni* was detected in 1 of 4 infected pigs and 1 of 4 control pigs (Figure 1). The infected pig that was positive on culture 24 hours after inoculation was the same pig that was positive by amplification of the QRDR target.

Analysis of the *Campylobacter* 23S rRNA RFLP demonstrated that both *C. jejuni* and *C. coli* were present in our experimental animals (Figure 2). One hour after inoculation, 3 of 4 infected pigs had patterns indicative of *C. jejuni* and the remaining pig had a pattern indicative of *C. coli.* All control pigs had patterns indicative of *C. coli.* Additionally, 24 hours after inoculation, all infected and control animals had patterns indicative of *C. coli* only. Neither *C. lari* nor *C. upsaliensis* were detected from any pig. These data strongly suggest that all of the pigs were colonized by *C. coli* prior to

infection and at one hour after experimental infection *C. jejuni* is the predominant species, but by 24 hours *C. coli* is predominant.

Detection of the *gfp* gene proved to be the most sensitive assay for detection of *C. jejuni* from experimentally inoculated pigs. One hour after inoculation, the *gfp* target was detected from all infected pigs and none of the controls (Figure 3). Twenty four hours after inoculation, the target was detected from 2 of 4 infected pigs and none of the controls.

Histology and Immunohistochemistry

Distal colonic tissue samples were examined microscopically to determine if lesions were induced by rectal inoculation with *C. jejuni.* Additionally, the location of *Campylobacter* organisms in the samples was determined by immunohistochemistry using a rabbit polyclonal anti-*C. jejuni* antibody. Control pigs showed no degenerative, proliferative, or inflammatory lesions. Most experimentally infected pigs also showed no degenerative, proliferative, or inflammatory lesions (data not shown).

Tests to determine the specificity of the polyclonal antibody used for immunocytochemical staining showed strong cross reactivity with *C. lari* as well as *C. jejuni*, but no cross reactivity with *C. coli, C. hyointestinalis,* or *C. upsaliensis* (Figure 4). Microscopic examination of tissues stained with the polyclonal antibody showed positively stained rods along the surface epithelium, within the mucus of the crypts and the crypts of the LGCs, and associated with goblet cells (Figure 5A through D). Staining patterns were similar at both 1 and 24 hours and were detected in both infected and control animals.

Interestingly, one pig had more severe lesions at 1 hour post inoculation characterized by multifocal distention of crypts with mucus, neutrophils, and small curved rods that stained positively on IHC (Figure 5E). Crypts within the LGCs were also distended with mucus, neutrophils, and positively staining curved rods. Lymphocytes, neutrophils, and plasma cells had infiltrated the epithelium and superficial propria, and there was evidence of epithelial degeneration.

Immunocytochemical staining of rod shaped bacteria in the distal colonic crypts suggested that *Campylobacters* were located in the crypts. However, cross reactivity of the antibody with *C. lari* as well as *C. jejuni* necessitated a more specific technique to determine if the bacteria in the crypts were the same strain used for inoculation. We used laser capture microdissection to specifically isolate bacteria and enterocytes from the crypts and submucosa to test by PCR for *C. jejuni* specific targets.

Laser Capture Microdissection

Laser capture microdissection was performed to isolate crypt units and submucosa, avoiding the surface epithelium, to determine if the *C. jejuni* strain used for inoculation was present. A PCR using primers specific for the *gfp* gene amplified the target only from the infected pig that was tested, while the control animal was negative (Figure 6). Additionally, RFLP analysis of the *Campylobacter* 23s rRNA gene was performed to determine if other *Campylobacter* species were present in crypts and submucosa. The infected animal had banding patterns consistent with *C. jejuni* and *C. coli*. A faint pattern that could be consistent with *C. upsaliensis* was also seen. The control animal had banding patterns that were consistent with *C. coli*, and an additional

band at ~150bp that did not fit the patterns of *C. jejuni, C. coli, C. lari*, or *C. upsaliensis* (Figure 7).

Cytokine mRNA expression

We measured mRNA expression of 14 cytokines, chemokines, and iNOS in both the distal colon and LGCs to determine if *C. jejuni* had a significant impact on cytokine expression (Table 2). One hour after infection, expression of IL-18, TNFα, and IL-8 was significantly upregulated 2 to 3 fold over controls in the distal colon and IL-8 remained upregulated 24 hours after infection. At the same time in the LGCs expression of IL-18, TNFα, IL-8, GM-CSF, IL-1β, iNOS, MCP-1, IL-6, and IL-10 was upregulated 3 to 20 fold over controls (Figure 8). Downregulation of mRNA expression was not seen for any target measured. These data indicates that a proinflammatory response was initiated by *C. jejuni* infection, and the response was more robust in LGCs compared to the distal colon.

Discussion

Rectal inoculation of *C. jejuni* stimulates a proinflammatory cytokine response in the colon characterized by increased expression of IL-1β, IL-6, IL-8, IL-18, GM-CSF, iNOS, MCP-1, and TNFα. In samples of the distal colon excluding LGCs, expression of IL-18, TNFα, and IL-8 was significantly upregulated 1 hour after inoculation, and IL-8 remained upregulated 24 hours after inoculation. These data correlate with cytokine production from *in vitro* infections of intestinal epithelial cells and monocytes with *C. jejuni*. Swine intestinal epithelial cells, IPEC-1, secrete IL-1β, TNFα, IL-6, IL-8 and IL-18 upon infection with *C. jejuni* (Parthasarathy and Mansfield, unpublished data; Jones et al, unpublished data; Cunningham and Mansfield, unpublished data). Human intestinal epithelial cells, INT-407, have been shown to secrete IL-8 in response to *C. jejuni* [15, 16]. In addition, intracellular production of IFNγ, TNFα, IL-4, and IL-10 is increased with *C. jejuni* infection [31]. In our model immunohistochemistry detected *Campylobacter* organisms associated with surface epithelial cells, goblet cells, and within the mucus of distal colon crypts. Histologically, no inflammatory changes were seen in infected pigs, therefore the significantly increased cytokines are likely due to the interaction of *C. jejuni* with intestinal epithelial cells. *C. jejuni* targets lymphoglandular complexes in the distal colons of pigs after oral inoculation, stimulating an immunoglobulin response [13]. LGCs from *C. jejuni* infected pigs are composed of the follicle associated epithelium, B cell rich germinal centers, T cells, and cells with macrophage morphology [13, 25]. *In vitro* human monocytes secrete IL-1β, IL-6, IL-8, and TNF-α upon infection with *C. jejuni* [18]. In our rectal challenge model, IHC

demonstrated *Campylobacter* organisms within the entrapped crypts of the LGCs and within the follicle associated with leukocytes. In the LGCs IL-1β, IL-6, IL-8, IL-18, GM-CSF, iNOS, MCP-1, and TNFα were significantly upregulated. In addition, the response was more robust than in the non-lymphoid portions of the distal colon with 3 to 20 fold increases in cytokine expression compared to 2 to 3.5 fold increases in the distal colon. In contrast to the distal colon, the increased cytokines in the LGC are likely due primarily to the interaction of *C. jejuni* with leukocytes, which are capable of producing an expanded cytokine profile and greater levels of cytokines [15, 18]. Ultimately, both tissues showed induction of a proinflammatory response. The enhanced response in LGCs supports other data showing that *C. jejuni* specifically targets the lymphoid tissue [13, 25].

Proinflammatory cytokines are induced by a variety of enteric pathogens. *Salmonella, Shigella*, and *Campylobacter* increase rectal nitric oxide gas and stool IL-1β levels in humans during the acute phase of infection [32]. *Shigella* stimulates production of IL-1β, IL-8, and IL-18 in animal models of infection. These cytokines are critical for initiating inflammation that limits invasion of the bacteria into the lamina propria and ultimately eliminates the bacteria from the host [33, 34]. *Salmonella* stimulates expression of IL-1, IL-6, IL-8, IL-18, and TNFα from intestinal tissues of pigs [35, 36]. In mice, wild type *Salmonella* decreases expression of IL-18 in Peyer's patches and mesenteric lymph nodes, which is believed to be a mechanism for evading the immune response and establishing an infection [37]. *Helicobacter pylori* stimulates increased expression of IL-1β, IL-6, IL-18 and TNFα in the gastric mucosa of humans and nonhuman primates [38-40]. Given that several enteric bacterial pathogens induce

proinflammatory cytokines *in vivo*, and *C. jejuni* induces proinflammatory cytokines *in vitro*, it is not surprising that expression of several proinflammatory cytokines was increased in the distal colons and LGCs of rectally infected pigs as these cytokines likely play a role in resistance to *C. jejuni* infection.

In our model, rectally inoculated *C. jejuni* behaved in a similar manner to orally inoculated bacteria. Oral inoculation studies have demonstrated *C. jejuni* within entrapped crypts and in the follicle associated epithelium of distal colonic LGCs of germ free piglets [25], and within superficial mucosal epithelial cells of the colons of colostrums deprived piglets [24]. These bacteria have traversed the gut and encountered the acidic pH of the stomach and bile in the small intestine, two environmental conditions that can affect the virulence of the organism [41]. The *C. jejuni flaA* promoter can be upregulated by acidic pH, bovine bile, and deoxycholate which suggests that these stimuli enhance production of flagella, an important virulence determinant [41, 42]. Additionally, bile stimulates the synthesis of Cia proteins, which are essential for *C. jejuni* invasion into intestinal epithelial cells [41]. However, these environmental stimuli are not absolutely necessary for *C. jejuni* virulence. *In vitro*, routinely cultured bacteria invade intestinal epithelial cells [15, 28, 43] and elicit cytokine responses [15-17]. *In vivo*, ferrets rectally infected with *C. jejuni* become colonized and develop diarrhea similar to orally infected animals [23]. Our data correlates with these findings, demonstrating *Campylobacter* organisms associated with epithelial cells and goblet cells, within distal colonic crypts, and within crypts of the LGCs at both 1 hour and 24 hours after inoculation. PCR analysis of microdissected crypts confirmed that *C. jejuni* 81-176 expressing gfp was present in infected pigs and not controls. Thus it was not entirely

unexpected that cytokine expression was significantly increased in the distal colon and LGCs.

Positive staining with immunohistochemistry on the mucosal surface, in the crypts, and in the entrapped crypts of the LGCs of rectally infected pigs suggests that *C. jejuni* is present in these areas. PCR analysis of DNA from full thickness tissue samples and microdissected crypts and submocosa confirm that the inoculating strain of *C. jejuni* had invaded the crypts and submucosa of infected pigs. Positive staining was also seen in control pigs but PCR analysis of DNA samples did not corroborate the presence of *C. jejuni* specifically. Cross reaction of the polyclonal antibody with closely related *Campylobacter* species could account for positive staining in control pigs. All control pigs harbored *C. coli,* as determined by RFLP analysis, but our tests to determine antibody specificity showed cross reaction only with *C. lari* (Figure 5). *C. lari* has been detected by culture in the feces of healthy pigs [7] as well as from abscessed LGCs from pigs with mucohemorrhagic diarrhea due to *Trichuris suis* infection [44]. All control and infected animals from our rectal inoculation study were negative by PCR for the *C. lari lpxA* gene (data not shown) [45]. Interestingly, Mansfield and Urban isolated an unidentified *Campylobacter* species from abscessed LGCs as well as *C. jejuni*, *C. coli* and *C. lari* [44]. These bacteria were all part of the resident flora of conventionally reared pigs prior to *T. suis* infection, and could also have been present in the pigs used in our experiment. In light of this, the changes in cytokine expression in the distal colon and LGCs could also be due in part to perturbation of the resident bacterial population upon introduction of exogenous *Campylobacter*.

Due to the low incidence of *C. jejuni* cultured from pigs after infection, we used three different PCR methods to detect *C. jejuni* in DNA isolated from pig tissue samples: amplification of the *C. jejuni* QRDR, RFLP of *Campylobacter* 23S rRNA gene, and amplification of the *gfp* gene. The RFLP analysis gave valuable information about the species of *Campylobacter* present in the distal colons of the experimentally infected piglets, but it was not as sensitive for detection of the inoculation strain as was expected. Therefore, we developed a PCR assay to detect the *gfp* gene that would only be present in the samples if the inoculating strain was present in the tissue. The PCR protocols for amplification of *C. jejuni* QRDR and *Campylobacter* 23S rRNA gene were originally designed for use on bacterial cultures, but we were able to adapt the protocols and successfully amplify *Campylobacter* targets from mixed DNA samples containing both pig and bacterial DNA. We found these assays to be more rapid and sensitive for *Campylobacter* detection and identification in infection studies than traditional culture methods. Overall, we believe that culture alone is insufficient to detect and distinguish *Campylobacters* that are present in the gut of conventionally reared pigs. PCR analyses were more sensitive and specific for detection and identification and did not rely on the ability to recover viable bacteria, as would be needed for traditional speciation by biochemical means.

The single pig that was infected for only one hour but had significant microscopic lesions was an unusual finding. Clearly there was an inflammatory response occurring in this animal as was evident by infiltration of inflammatory cells into the lamina propria and the existence of multifocal crypt abscesses (Figure 5E). It is unlikely that the abscessation was caused by the *C. jejuni* inoculum as this pig was only infected for one

hour. However, it is interesting to note that there were *Campylobacter* organisms associated with the abscessed crypts. Whether the organisms were from the inoculum or from the resident population the pathology supports the hypothesis that *Campylobacter spp.* are opportunistic pathogens in the distal colons of pigs. *Campylobacter* organisms invade abscessed LGCs in the distal colons of both immunocompetent pigs infected with the whipworm *T suis* [44] and germ free piglets infected with *T. suis* and *C. jejuni* [25]. In this dual infection model, it is hypothesized that the whipworm infection facilitates secondary infection by resident gut flora through dysregulation of the immune response, or by direct effects on enterocytes that permit bacterial invasion [44] (Parthasarthy, unpublished results). It is possible that in the rectally inoculated animal from our study, some undetermined agent facilitated *Campylobacter* invasion similar to *T. suis* facilitation of *C. jejuni* invasion.

Rectal inoculation proved to be a useful tool for studying the pathogenesis of *C. jejuni* infection in a swine model. Rectal inoculation allowed targeted delivery of a specific amount of bacteria to the distal colon and a specific time frame to study the effects on cytokine expression in the distal colon. The fact that significant changes in cytokine expression were measured after rectal inoculation with *C. jejuni* confirms that the bacteria are capable of eliciting a response when delivered by a non conventional route. This model allows for delivery of the agent of interest to a discrete area of the intestine and assessment of responses at more accurate time points. We believe that this model will continue to be useful for studying the complex interaction between pathogen and host *in vivo.*

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Inoculation	Culture	*C. jejuni* QRDR	*gfp* PCR	23S rRNA RFLP	
				C. jejuni	*C. coli*
***C. jejuni* 1hr**	0 of 4	3 of 4	4 of 4	3 of 4	1 of 4
Control 1hr	0 of 4	0 of 4	0 of 4	0 of 4	4 of 4
***C. jejuni* 24 hr**	1 of 4	1 of 4	2 of 4	0 of 4	4 of 4
Control 24hr	0 of 4	1 of 4	0 of 4	0 of 4	4 of 4

Table 1: Summary of *Campylobacter* detection and identification. *Campylobacters* were detected by culture of feces, or PCR for *C. jejuni*, *gfp*, or *Campylobacter* 23S rRNA targets using total DNA isolated from distal colonic tissue samples as the template.

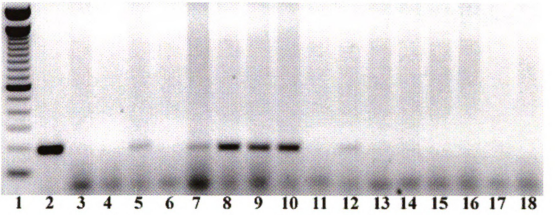

Figure 1: Amplification of the QRDR of *C. jejuni gyrA* from distal colon DNA from experimentally infected pigs. Lane 1: 100bp DNA ladder; Lane 2: DNA from *C. jejuni* expressing gfp; Lanes 3-6: 24 hour infected pigs; Lanes 7-10: 1 hour infected pigs; Lanes 11-18: uninfected controls.

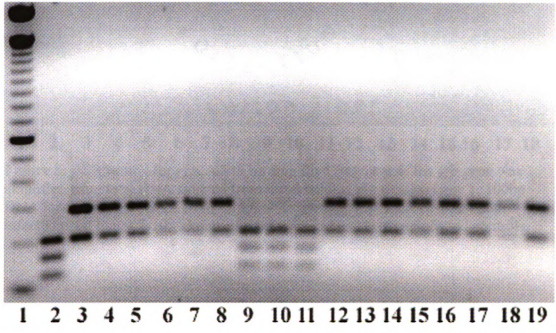

Figure 2: *Alu I* digest of *Campylobacter* 23s rRNA gene amplified from pig tissues by PCR. Lane 1: 100bp DNA ladder; Lane 2: *C. jejuni*; Lane 3: *C. coli* ; Lanes 4-7 : 24 hour infected pigs ; Lanes 8-11 : 1 hour infected pigs ; Lanes 12-19 : uninfected controls.

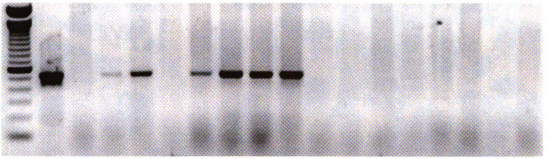

Figure 3: PCR testing for gfp. A 543 bp amplified fragment of the *gfp* gene was amplified from total DNA isolated from distal colonic pig tissues. Lane 1: 100bp DNA ladder; Lane 2: DNA from *C. jejuni* expressing gfp; Lanes 3-6: 24 hour infected pigs; Lanes 7-10: 1 hour infected pigs; Lanes 11-18: uninfected controls.

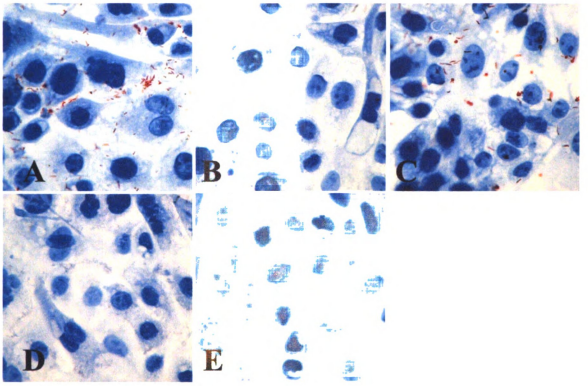

Figure 4: Immunohistochemistry of *Campylobacter* infected IPEC-1 cells. Immunohistochemistry was performed on IPEC-1 cells infected with one of five *Campylobacter* species to determine antibody specificity. A: *C. jejuni* 81-176 expressing gfp; B: *C. coli* 43134; C: *C. lari* 43675; D: *C. upsaliensis* 43954 ; E : *C. hyointestinalis* 35217. (100X magnification)

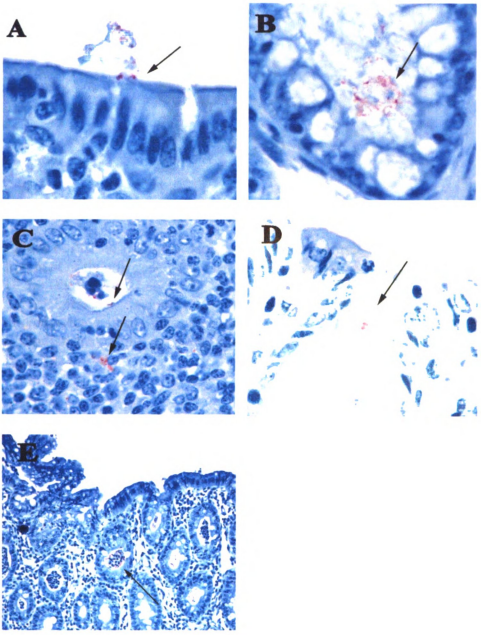

Figure 6: Immunohistochemistry of distal colon samples using the polyclonal antibody detected *Campylobacter spp.* organisms associated with the brush border and in the lumen (A), within the crypts (B), within the crypts of the LGCs and associated with inflammatory cells (C), and associated with goblet cells, possibly within the cytoplasm (D) (100X magnification). One pig had organisms within crypt abscesses (E) (20X magnification).

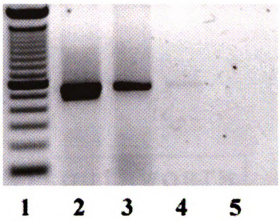

Figure 6: PCR testing DNA isolated from LCM samples. Primers designed to amplify a fragment of the gfp gene were used to test DNA isolated from LCM samples. Lane 1: 100bp DNA ladder; Lane 2: DNA from *C. jejuni* expressing gfp; Lane 3: DNA from a full thickness tissue sample taken from an infected animal; Lane 4: DNA from microdissected crypts and submucosa from an infected animal; Lane 5: DNA from microdissected crypts and submucosa from a control animal.

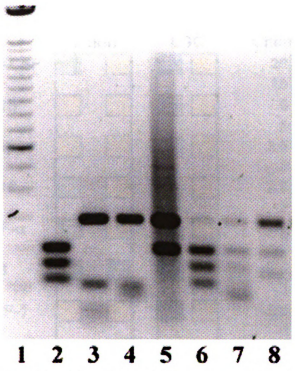

Figure 7: *Alu I* digest of *Campylobacter* 23s rRNA gene PCR product amplified from microdissected crypts and submucosa. Lane 1: 100bp DNA ladder; Lane 2: *C. jejuni* 81-176 expressing gfp; Lane 3: *C. lari* 43675; Lane 4: *C. upsaliensis* 43954; Lane 5: *C. coli* 43134; Lane 6: DNA from a full thickness tissue sample taken from an infected animal; Lane 7: DNA from microdissected crypts and submucosa from an infected animal; Lane 8: DNA from microdissected crypts and submucosa from a control animal.

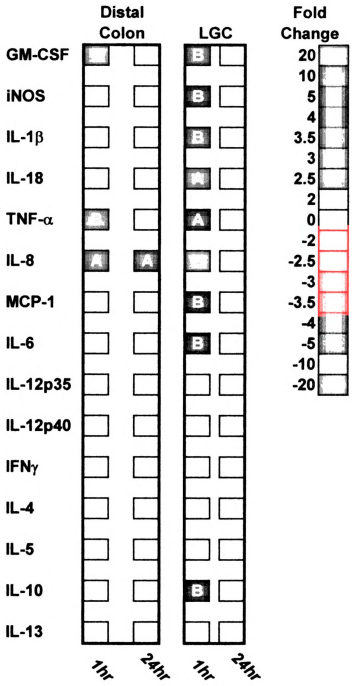

Figure 8: Cytokine expression in distal colonic tissues. Expression of mRNA from a panel of cytokines was measured by Real Time PCR. The data represents the average fold change in expression from infected animals when compared to control animals. Letters denote statistically significant changes. A: $p \leq 0.05$; B: $p \leq 0.01$.

Gene	Genbank Accession	Primer	Sequence 5'-3'	Concentration
IL-6	M86722	Forward	AATGTCGAGGCTGTGCAGATT	300 nM
		Reverse	TGGTGGCTTTGTCTGGATTCT	900 nM
		Probe	TET-AGCACTGATCCAGACCCTGAGGCAAA-BHQ1	200 nM
IL-12p35	L35765	Forward	GCCCAGGAATGTTCAAATGC	300 nM
		Reverse	GGGTTTGTTTGGCCTTCTGA	300 nM
		Probe	TET-CAACCACTCCCAAAATCTGCTGAAGGC-BHQ1	200 nM
IL-12 p40	U08317	Forward	AAGCTGTTCACAAGCTCAAGTATGA	300 nM
		Reverse	TCTTGGGAGGGTCTGGTTTG	300 nM
		Probe	TET-ACCAGCAGCTTCTTCATCAGGGACATCA-BHQ1	200 nM
IL-4	X68330	Forward	GCCGGGCCTCGACTGT	300 nM
		Reverse	TCCGCTCAGGAGGCTCTTC	300 nM
		Probe	6FAM-CTTCGGCACATCTACAGACACCACACG-TAMRA	200 nM
IL-5	AJ010088	Forward	GACTGGTGGCAGAGACCTTGAC	300 nM
		Reverse	CTTCAATGCATAGTTGGTGATTTGT	300 nM
		Probe	6FAM-CTGCTCTCCATTCATCGAACTCTGCTGAT-TAMRA	200 nM
IL-10	L20001	Forward	TGAGAACAGCTGCATCCACTTC	300 nM
		Reverse	TCTGGTCCTTCGTTTGAAAGAAA	300 nM
		Probe	6FAM-CAACCAGCCTGCCCCACATGC-TAMRA	150 nM
IL-13	AF385626	Forward	CTGACCACCAGCATGCAGTACT	50 nM
		Reverse	GCTGCAGTCGGAGATGTTGA	900 nM
		Probe	6FAM-TGCCGCCCTGGAATCCCTCA-TAMRA	150 nM

Table 2 (cont'd)

Gene	Genbank Accession	Primer	Sequence 5'-3'	Concentration
IL-6	M86722	Forward	AATGTCGAGGCTGTGCAGATT	300 nM
		Reverse	TGGTGGCTTTGTCTGGATTCT	900 nM
		Probe	TET-AGCACTGATCCAGACCCTGAGGCAAA-BHQ1	200 nM
IL-12p35	L35765	Forward	GCCCAGGAATGTTCAAATGC	300 nM
		Reverse	GGGTTTGTTTGGCCTTCTGA	300 nM
		Probe	TET-CAACCACTCCCAAAATCTGCTGAAGGC-BHQ1	200 nM
IL-12 p40	U08317	Forward	AAGCTGTTCACAAGCTCAAGTATGA	300 nM
		Reverse	TCTTGGGAGGGTCTGGTTTG	300 nM
		Probe	TET-ACCAGCAGCTTCTTCATCAGGGACATCA-BHQ1	200 nM
IL-4	X68330	Forward	GCCGGGCCTCGACTGT	300 nM
		Reverse	TCCGCTCAGGAGGCTCTTC	300 nM
		Probe	6FAM-CTTCGGCACATCTACAGACACCACACG-TAMRA	200 nM
IL-5	AJ010088	Forward	GACTGGTGGCAGAGACCTTGAC	300 nM
		Reverse	CTTCAATGCATAGTTGGTGATTTGT	300 nM
		Probe	6FAM-CTGCTCTCCATTCATCGAACTCTGCTGAT-TAMRA	200 nM
IL-10	L20001	Forward	TGAGAACAGCTGCATCCACTTC	300 nM
		Reverse	TCTGGTCCTTCGTTTGAAAGAAA	300 nM
		Probe	6FAM-CAACCAGCCTGCCCCACATGC-TAMRA	150 nM
IL-13	AF385626	Forward	CTGACCACCAGCATGCAGTACT	50 nM
		Reverse	GCTGCAGTCGGAGATGTTGA	900 nM
		Probe	6FAM-TGCCGCCCTGGAATCCCTCA-TAMRA	150 nM

Table 2 (cont'd)

Chapter 3: "*Trichuris suis* down regulates proinflammatory cytokines in the proximal colon of pigs concurrently infected with *C. jejuni*"

Abstract

The swine whipworm *Trichuris suis* and the bacterial pathogen *Campylobacter jejuni* synergize in the colons of pigs to cause mucohemorrhagic diarrhea and severe pathology. We hypothesize that dysregulation of cytokine immune responses play a role in pathogenesis of this disease. We analyzed cytokine expression in the jejunum, proximal and distal colons of pigs infected with either or both of these pathogens to test this hypothesis in the areas where the pathogens reside. Weaned piglets were orally inoculated with 2500 embryonated *T. suis* eggs. Twenty one days after inoculation with *T. suis* eggs pigs were orally inoculated with $2x10^8$ cfu of *C. jejuni* or sterile milk. All pigs were euthanized and tissue samples were taken for analysis of cytokine expression 23 days after initial inoculation. *C. jejuni* was detected by PCR in proximal colon samples from pigs dually infected with *C. jejuni* and *T. suis*. *T. suis* stimulated increased expression of IL-13 in the proximal colon, while expression of IL-8, MCP-1, and IL-12 were decreased in pigs infected with *T. suis* alone, or concurrently with *T. suis* and *C. jejuni*. In the distal colon, increased expression of IL-1β, IL-6, IFNγ and IL-13 was measured in response to *T. suis* alone. In the jejunum, *C. jejuni* infection stimulated increased expression of MCP-1, TNFα, IL-12p40, IFNγ, IL-4, and IL-10, while infection with *C. jejuni* and *T. suis* concurrently caused decreased expression of GM-CSF. These data indicate that *C. jejuni* stimulates a proinflammatory response and *T. suis* down

regulates proinflammatory responses, both in single infection and concurrent infection with *C. jejuni*.

Introduction

Trichuris suis is a worm parasite that invades the cecum and proximal colon of swine causing inflammation, hemorrhage, and in severe cases necrosis [1]. The severity of colonic pathology is increased by interaction with large intestinal flora. Rutter and Beer demonstrated this phenomenon when they showed that specific pathogen free and gnotobiotic pigs developed significantly milder lesions upon *T. suis* infection than conventionally reared pigs with a normal population of intestinal flora [2]. The role of resident bacteria in Trichuriasis was confirmed by Mansfield and Urban, who showed amelioration of disease and pathology by antibiotic treatment of *T. suis* infected pigs. Additionally, several species of bacteria, including *Campylobacter jejuni,* were isolated from abscessed lymphoglandular complexes in the distal colons of pigs that did not receive antibiotics concurrently [3]. Further studies with germ free piglets showed that *T. suis* and *C. jejuni* synergize to produce severe disease and colonic pathology, while infection with either agent alone resulted in mild disease and pathology [4]. Severe disease in this dual infection model is believed to result in part because of inappropriate cytokine responses which ultimately cause dysregulation of the immune response necessary to clear the bacteria in the short term.

Cytokines serve to initiate, direct, and sustain immune responses to infectious agents. Helminths typically stimulate IL-4, IL-5, IL-9, IL-10, and IL-13 production [5-8]. Together these cytokines clear helminth infections by recruiting eosinophils, enhancing IgE production, stimulating mucus production, and promoting intestinal smooth muscle contractions that help to expel worms [8-11]. Enteric bacterial pathogens, like *Salmonella* and *Shigella*, stimulate the production of IL-8, IL-1β, IL-18, and TNFα [12-

16]. These cytokines initiate inflammation in the gut, control the spread of invading bacteria, and facilitate elimination of the bacteria from the intestine [12, 13, 16]. Inappropriate cytokine responses hinder clearance of pathogens and resolution of disease. Mice normally resistant to the intestinal nematodes *Nippostrongylus brasiliensis* and *T. muris* develop chronic disease with suppression of Th 2 cytokines when given exogenous IL-12 [17, 18]. Additionally, concurrent infections with the intracellular protozoan parasite *Eimeria ferrisi* and the nematode *N. brasiliensis* increases worm fecundity and exacerbates disease in inbred mice normally resistant to this chronic nematode infection [8].

T. suis infected pigs reared in confinement have increased expression of IL-10 in mesenteric lymph nodes and mild evidence of secondary bacterial infection in the colon. *T. suis* infected pigs reared on dirt lots have increased expression of both IL-10 and IL-12 in mesenteric lymph nodes and develop severe disease with evidence of extensive secondary bacterial infection in the colon [5]. It is believed that IL-12 is stimulated by invasive bacteria, and it exacerbates disease and pathology. We examined the local cytokine responses in the jejunum, proximal colon, and distal colon of pigs singly or dually infected with *T. suis* and *C. jejuni*. Our data show that IL-13 is significantly increased in the proximal colon of *T. suis* infected pigs while IL-12 and IL-8 are significantly decreased. The cytokine profile in the proximal colons of *T. suis* infected pigs did not appear to be significantly altered by the presence of *C. jejuni*. In contrast, IL-1β, IL-6, and IFNγ are up-regulated in the distal colon of pigs infected with *T. suis* only which may indicate an inflammatory reaction to secondary infection from some component of the resident microbial population.

Materials and Methods

Animals

Four week old male and female outbred Landrace-Yorkshire cross pigs were obtained from the Michigan State University (MSU) swine farm. Animals were housed in the MSU Research containment facility in groups of 6 for social support. Individual infection groups were housed in separate rooms to minimize cross contamination. All animal housing and handling complied with MSU animal use guidelines and National Institutes of Health guidelines for humane use of laboratory animals. All animals were tested for the presence of *T. suis* prior to inoculation by microscopic examination of fecal floats for ova.

Bacteria

C. jejuni strain ATCC 33292 that had been rederived by passage through a pig and stored after the second passage was used for experimental infections. The bacteria were grown on Bolton Agar supplemented with 5% sheep's blood for 48 hours at 37°C, 5% CO_2. Isolated colonies were subcultured for lawn growth for 18-20 hours. The lawns were harvested with sterile swabs and resuspended in sterile milk to a final concentration of $1x10^8$ cfu/mL.

T. suis embryonated eggs

T. suis eggs were collected from adult worms using previously described methods [19]. Eggs were centrifuged 2-3 times in deionized distilled water at 10,000 rpm for 5 minutes to remove debris. The eggs were resuspended in deionized distilled water and incubated at 34°C, 5% CO_2 for 19 days to stimulate embryonation. After 19 days porcine bile extract (Sigma Chemical Co., St. Louis, MO) was added to the eggs at a final concentration of 10mg/mL. The eggs were incubated for an additional 3-5 days, then examined microscopically for L1 larval development and movement. Embryonated eggs were collected, centrifuged at 10,000rpm for 10 minutes, and then resuspended in sterile water. A subsample was loaded onto a hemocytometer and examined microscopically to enumerate embryonated eggs, diluted to 1,250 eggs per mL, and stored at 4°C until inoculation.

Experimental design and inoculation procedure

All pigs were tested for the presence of *C. jejuni* by culture of rectal swabs prior to initial inoculation, and immediately prior to euthanasia. Rectal swabs were transported to the laboratory in Cary Blair (Difco Laboratories, Detroit, MI) transport medium for Campylobacter isolation and identification.

For experimental inoculations (Figure 1), weaned piglets were divided into 4 groups: Group 1- Uninfected control; Group 2- *C. jejuni*; Group 3- *T. suis*; Group 4- *C. jejuni* and *T. suis*. On day 0, groups 3 and 4 were inoculated by oral gavage using a sterile

12 gauge steel ball feeding needle with 2500 embryonated *T. suis* eggs resuspended in 2 mLs sterile milk. Groups 1 and 2 were given 2 mLs of sterile milk only. Twenty one days after inoculation with *T. suis* eggs, groups 2 and 4 were inoculated with $2x10^8$ cfu of *C. jejuni* by oral gavage. Groups 1 and 3 were given 2 mLs of sterile milk. Twenty three days after initial inoculations, all pigs were euthanized with an intravenous injection of 86 mg/kg sodium pentobarbital (Fatal Plus®, Vortech Pharmaceuticals, Dearborn, MI).

Sample collection

To eliminate cross contamination, a separate set of sterile dissection instruments was used for each group. For sample collection, the abdomen was opened by a midline incision and the small and large intestines were exteriorized. Full thickness samples of the jejunum, proximal colon, and distal colon were collected using sterile scissors and forceps. One set of samples was fixed in 3.7% formaldehyde for histology and immunohistochemistry. A second set was placed in cryovials and snap frozen in liquid nitrogen for nucleic acid isolation.

***C. jejuni* isolation and identification**

All pigs were tested for the presence of *C. jejuni* by culture of rectal swabs prior to inoculations, and immediately prior to euthanasia. Swabs were placed in Cary Blair transport media (Difco Laboratories, Detroit, MI) and stored on ice for transport to the laboratory where they were processed. Rectal swabs were streaked onto Preston selective agar (Oxoid Inc., Basingstoke, Hampshire, England) and incubated at 42°C, 5% CO_2 in humidified air for 48 hours. Cells from isolated colonies were tested by PCR using the

previously designed primers JL238 (5' TGG GTG CTG TTA TAG GTC GT 3') and JL239 (5' GCT CAT GAG AAA GTT TAC TC 3') which are specific for the quinolone resistance-determining region (QRDR) of the *gyrA* gene of *C. jejuni* [20].

In addition to culture of rectal swabs, tissue samples from each segment of intestine were tested for the presence of *Campylobacter* organisms from each pig. Total DNA was isolated from approximately 25 mg of frozen tissue using a Qiagen DNEasy tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The DNA was quantified spectrophotometrically (Beckman, Fullerton, CA). The *C. jejuni* QRDR target was amplified by PCR from 500ng of total DNA isolated from full thickness tissue samples using the previously designed primers JL238 and JL239 [20]. Amplicons were electrophoresed on a 1.8% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer with 0.2 μg/mL ethidium bromide. The fragments were visualized by UV illumination on an Alpha Imager Gel documentation system (Alpha Innotech Corporation, San Leandro, CA).

A Restriction Fragment Length Polymorphism (RFLP) assay to detect and distinguish *C. jejuni, C. coli, C. lari,* and *C. upsaliensis* by analysis of a portion of the 23S rRNA gene was used to detect thermophilic *Campylobacters* in experimental animals [21]. The target was amplified from 500ng of total pig tissue DNA using primers THERM1 (5'-TAT TCC AAT ACC AAC ATT AGT-3') and THERM4 (5'-CTT CGC TAA TGC TAA CCC-3'). The amplicons were digested overnight with either *AluI* or *Tsp5091* following the manufacturers protocols (New England Biolabs, Beverly, MD), and the resultant fragments were separated electrophoretically on a 2% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer with 0.2 μg/mL ethidium bromide. The fragments were

visualized by UV illumination on an Alpha Imager Gel documentation system (Alpha Innotech Corporation, San Leandro, CA).

Histology and Immunohistochemistry

Tissues were examined microscopically to detect *T. suis* larvae, to evaluate histological changes, and to localize specifically stained *Campylobacter* organisms. Fixed tissue samples were embedded in paraffin and 5 μm sections were cut and adhered to charged slides. Slides were routinely dehydrated in progressively higher concentrations of ethanol, deparaffinized and rehydrated. For histological evaluation, slides were stained with Mayer's hematoxylin and eosin and cover slipped. For immunostaining, sections of selected tissues were incubated in preheated antigen retrieval buffer (Dako, Carpinteria, CA) for 20 minutes at 100° C. Slides were removed from the steamer and left in the retrieval solution at room temperature to cool down for 20 minutes, rinsed in distilled water and transferred to Tris-HCL buffer. Endogenous peroxidase was blocked for 15 minutes with 3% hydrogen peroxide. Non-specific immunoglobulin binding blocking was done by a 10 minute incubation with a protein-blocking agent (Dako, Carpinteria, CA) prior to application of the primary antibody. A rabbit polyclonal anti-*C. jejuni* antibody (Biogenesis, New Fields, UK) was used as the primary antibody at a concentration of 1:100. Sections were stained with a Dako autostainer using a labeled streptavidin-immunoperoxidase staining procedure (Dako Corp., Carpinteria, CA). The immunoreaction was visualized with AEC (Dako Corp., Carpinteria, CA). Sections were counterstained with Mayer's hematoxylin, then dehydrated, cleared and mounted.

Cytokine Analysis

T. suis resides in the cecum and proximal colons of pigs [2], while *C. jejuni* can colonize throughout the small and large intestine of pigs (Mansfield et al, unpublished results; [22]. To determine the effect of infection with *T. suis*, *C. jejuni*, or both on local immune responses in areas where these pathogens are present, we took samples from the jejunum, proximal colon, and distal colon. mRNA expression of 15 cytokines, chemokines, and iNOS was measured from each tissue using a real time PCR assay. Total RNA was isolated from frozen tissue samples from each pig using Trizol reagent (GibcoBRL Life Technologies). Briefly, $3mm^3$ tissue samples were homogenized in Trizol using a Polytron® tissue homogenizer (Kinematica, Cincinnati, OH). RNA was extracted with chloroform, precipitated with isopropanol, and washed with ethanol. Pellets were air dried, then resuspended in DEPC treated H_20 (GibcoBRL Life Technologies). DNA contamination was removed by treatment with approximately 27 Kunitz units of DNase I for 15 minutes. Samples were repurified on a Qiagen RNEasy column following the manufacturer's protocol (Qiagen, Valencia, CA). RNA concentration and integrity were determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). For each sample, 10 μg of total RNA was reverse transcribed with random primers using a Stratagene First Strand RT-PCR kit (Stratagene, La Jolla, CA). First strand cDNA was used as the template in real time PCR reactions. Real time PCR primers and probes designed using the Primer Express (Applied Biosystems, Foster City, CA) software package were used to analyze 14 cytokines (Chapter 2, Table 1). The 18S rRNA probe was obtained from Applied Biosystems and was used as a constitutively active gene for normalization. PCR was performed using a

commercially available kit (Brilliant kit, Stratagene, La Jolla, CA) on an ABI PRISM 7900HT (Applied Biosystems). Amplification conditions were as follows: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C 15 seconds and 60°C for 1 minute. Fluorescence signals measured during amplification were processed post-amplification. Threshold cycle (C_t) values were determined using values of 0.2 for TET, 0.1 for VIC, and 0.05 for FAM reporter dyes.

Statistical Analysis

The cytokine mRNA expression data for each tissue was analyzed separately using Statview 5.0 for Macintosh (Abacus Concepts, Berkeley, Calif.). The C_t value for the 18S ribosomal subunit was subtracted from the C_t value for each cytokine message to normalize for RNA content prior to statistical analysis. One-way analysis of variance (ANOVA) was performed to determine the statistically significant differences between treatments. A Fisher's least squares difference test was performed to determine differences between infected and control animals at each time point. P values of 0.05 or less were considered statistically significant.

Results

C. jejuni isolation and identification

C. jejuni was not isolated from fecal cultures of any pigs before or after infection (Table 1). We previously found culture alone to be inadequate for detection and identification of *C. jejuni* from experimentally infected pigs. In addition, to correlate our results from analysis of cytokine expression we needed to determine if *C. jejuni* was present in all intestinal segments sampled. Therefore we used 2 PCR assays to detect *C. jejuni* from the jejunum, proximal colon, and distal colon separately. *C. jejuni* was not detected from any tissue samples from any group using primers specific for the QRDR of the *gyrA* gene of *C. jejuni.* Analysis of the *Campylobacter* 23S rRNA RFLP demonstrated that *C. jejuni, C. coli,* and a Campylobacter species that gave a unique pattern were present in our experimental animals (Figure 2).

We had previously determined that the polyclonal antibody raised against *C. jejuni* used for immunohistochemistry cross reacted with *C. lari.* We used primers specific for the *lpxA* gene of *C. lari* to detect *C. lari* from tissue samples [23]. The target was not amplified from any tissues of any pigs (data not shown).

Histopathology

Haemotoxylin and Eosin (H&E) stained sections of jejunum, proximal colon, and distal colon of each pig were examined microscopically for the presence of *T. suis* larvae

and histological changes due to infection. *T. suis* larvae were seen in proximal colon sections from 5 of 6 pigs in group 3, and 5 of 6 pigs in group 4. No larvae were seen in groups 1 or 2 (Figure 3).

Control animals (Group 1) had no proliferative, inflammatory, or degenerative lesions in any of the tissues examined and were therefore used as a normal comparison for all infection groups. All lesions were confined to the colon. Colonic lesions due to *C. jejuni* or *T. suis* infection were similar to those described in germ free piglets [4]. Pigs in group 2 (*C. jejuni* only) developed focal mild crypt distension with sloughed epithelial cells, mucus and bacteria in the crypts in the distal colon (Figure 4A) and LGCs (Figure 5 A). There was no significant inflammatory infiltrate into the lamina propria on day 23 after inoculation. Pigs infected with *T. suis* only developed mild to moderate colitis with goblet cell hyperplasia and crypt distension with mucus in superficial lamina propria. Lesions were found only in the areas where *T. suis* larvae were located. A mixed inflammatory infiltrate was present in the lamina propria and included lymphocytes, plasma cells, neutrophils, and eosinophils (Figure 4B). Where present crypts of LGCs were not distended (Figure 5B). Pigs infected with both *C. jejuni* and *T. suis* developed mild colitis with goblet cell hyperplasia and crypt distension with mucus in superficial lamina propria in the areas where *T. suis* larvae were located. A mixed inflammatory infiltrate in the lamina propria included lymphocytes, plasma cells, neutrophils, and eosinophils. Entrapped crypts of the LGCs were distended with mucus and few sloughed epithelial cells (Figure 5C).

Immunohistochemistry

Sections from jejunum, proximal colon, and distal colon were stained with a polyclonal antibody directed against *C. jejuni* to determine the location of the bacteria. We had previously determined that the antibody cross reacted with *C. lari* (Chapter 2, figure 4) and therefore concluded that positive staining in tissue sections could be due to either *C. jejuni* or *C. lari*. Positively stained organisms were not detected in jejunal sections of any groups (data not shown). Positively stained organisms were detected in the colonic crypts and on the mucosal surface from all groups, regardless of infection status (Figure 6). Staining patterns were focal to multifocal in uninfected controls and pigs infected singly with either *C. jejuni* or *T. suis*. Pigs dually infected with *T. suis* and *C. jejuni* exhibited a diffuse staining pattern with more intense staining (Figure 6).

Cytokine expression

We measured expression of mRNA from 15 cytokines, chemokines, and iNOS in each segment of intestine sampled in an effort to determine if *T. suis* or *C. jejuni* had a significant effect on cytokine expression, and to determine if the affected cytokines were indicative of a specific response.

Cytokine expression patterns were distinct in each segment of the intestine tested (Figure 7). In the proximal colon of the *T. suis* only and dual infection groups, IL-13 expression was increased 3 to 4 fold while IL-12 and IL-8 expression was decreased 2 to 4 fold when compared to uninfected controls. In the dual infection group, expression of

IL-5 and MCP-1 were each decreased 2 fold compared to controls. These data indicate that *T. suis* stimulates IL-13 in its local environment while decreasing expression of proinflammatory cytokines. In the dual infection group this occurred despite the presence of *C. jejuni* in the tissue as confirmed by PCR analysis.

In the distal colon of pigs infected with *T. suis* only, expression of IL-1β, IL-6, IFNγ, and IL-13 was increased 3 to 4 fold over controls. Infection with *C. jejuni* only or dual infection with *C. jejuni* and *T. suis* did not significantly affect expression of any cytokines, chemokines, or iNOS in the distal colon. These data indicate that a predominately proinflammatory response has been initiated in the distal colons of pigs infected with *T. suis* only.

In the jejunum, expression of MCP-1, TNFα, IL-12p40, IFNγ, IL-4, and IL-10 were increased 3 to 5 fold in the *C. jejuni* only group when compared to controls. Expression of GM-CSF was decreased 5 fold in the dual infection group when compared to controls. These data indicate that *C. jejuni* stimulates a predominately proinflammatory response in the jejunum, while *T. suis* may be down regulating proinflammatory cytokines.

Discussion

Oral infection of weaned piglets with *T. suis* and *C. jejuni* elicited significant changes in cytokine expression in both the small and large intestine. The effects of *T. suis* were predominantly in the proximal colon, with some effects in the distal colon. *C. jejuni* affected cytokine expression mainly in the jejunum, with few changes in the colon. *T. suis* stimulated 3 to 4 fold increases in IL-13 expression in the proximal colon in groups 3 and 4, which is typical of a helminth infection. Concurrently, IL-8 was significantly decreased in both groups, and in the case of dual infection, MCP-1 was also decreased. Increased expression of IL-8 in the distal colon at both 1 and 24 hours after inoculation with *C. jejuni* suggested an important role for this cytokine in host resistance to *C. jejuni*. Additionally, IL-8 has been shown to be critical in controlling the spread of *Shigella flexneri* in a rabbit ligated intestinal loop model of infection [12]. Histological studies of *T. suis* infection have shown that bacteria invade and proliferate in the lamina propria and submucosa surrounding the site of worm attachment [2, 3], and *C. jejuni* was detected in 4 of 6 pigs (66%) in group 4 by RFLP analysis. Taken together, these data suggest that decreased expression of IL-8 due to the presence of *T. suis* may facilitate *C. jejuni* invasion and proliferation in the lamina propria and submucosa, similar to the *Shigella* model. Decreased expression of IL-8 can be attributed at least in part to IL-13, as IL-13 and IL-4 have been shown to inhibit IL-8 production from intestinal epithelial cells [24].

T. suis also significantly decreased expression of both subunits of IL-12 in the proximal colon. IL-12 is produced mainly by antigen presenting cells, including monocytes, macrophages, and dendritic cells, and drives a Th 1 directed response by

inducing IFNγ production from T cells [25, 26]. However, IL-13, which is increased in resistant mouse strains infected with *T. muris* [9], has been shown to downregulate production of IL-12 [27, 28]. Thus the decreased expression of both subunits of IL-12 in the proximal colons of pigs infected with *T. suis* can be attributed to the immunomodulatory effects of IL-13. Our data is in contrast to the data of Mansfield and colleagues who measured increased expression of IL-12 expression in the mesenteric lymph nodes of *T. suis* infected pigs with evidence of secondary bacterial infection [5]. However, Mansfield and colleagues used competitive RT-PCR, which is not as accurate for quantitative analysis of cytokine expression as real time PCR. Another explanation for this discrepancy is that they measured expression in a draining mesenteric lymph node while we measured expression in the intestinal tissue where worms reside. Comparison of cytokine expression in draining lymph nodes and intestinal tissues directly in contact with the pathogen shows that the cytokine responses are not identical [6, 29]. Detailed analysis of cell types and cytokine expression comparing intestinal tissue to draining lymph nodes would enhance our knowledge of the swine immune response to *T. suis*.

In addition to decreased expression of proinflammatory cytokines in the proximal colon, IL-5 was decreased in the dual infection group. This was an unexpected finding. IL-5 functions in eosinophil recruitment and it has been previously shown to be up regulated in helminth infections along with other classical Th 2 cytokines [8]. However, while treatment of helminth infected mice with anti-IL-5 antibody abolishes peripheral and tissue eosinophilia, this does not enhance survival of *Heligmosomoides* worms [10]. In experimental *C. jejuni* infections, oral IL-5 pretreatment of mice reduces intestinal

colonization by *C. jejuni*, suggesting a protective effect of this cytokine for the host [30]. It follows that IL-5 may be down regulated by *C. jejuni* as a mechanism to evade the immune response and establish infection. IL-5 expression was decreased in the proximal colon of pigs infected with only *C. jejuni*, however the decrease was not statistically significant. Nevertheless, decreased expression in both groups that received *C. jejuni* suggests that IL-5 plays an important role in pathogenesis of Campylobacteriosis *in vivo*.

In contrast to the proximal colon, IL-1β, IL-6, IFNγ and IL-13 were up regulated in the distal colon of pigs infected with *T. suis* only. These effects can be attributed in part to the effects of ESP in the distal colon. Up regulation of IL-1β, IL-6, IFNγ is indicative of an inflammatory reaction. *In vitro*, ESP treatment of differentiated IPEC-1 cells causes a dose dependent cytotoxic response and significantly decreased TER [31]. ESP also stimulates secretion of IL-6 from both differentiated and undifferentiated IPEC-1 cells *in vitro* (Parthasarathry et al, unpublished results). The destructive effects of ESP on intestinal epithelial cells could stimulate an inflammatory reaction in the distal colon. Another possibility for the inflammatory response seen is *T. suis* stimulated secondary bacterial infection in the distal colon. Several resident bacterial species have been cultured from abscessed lymphoglandular complexes in the distal colons of *T. suis* infected pigs, including *C. jejuni*, *C. coli,* an unidentified *Campylobacter* species, and *E. coli* [3]. *C. jejuni* and *C. coli* have been shown to induce IL-8 secretion from cultured human INT-407 intestinal epithelial cells [32]. *C. coli* was detected in the distal colons of 2 of 6 pigs infected with *T. suis* only and an unidentified *Campylobacter spp.* was detected in 3 of 6 pigs from this group by RFLP analysis of the Campylobacter 23S rRNA gene. Thus it is possible that either of these species invaded tissues in the distal

colon and initiated an inflammatory response. Increased expression of IL-13 could also be attributed to the effects of excretory/secretory product (ESP) produced by the 4th stage larvae in the proximal colon that have traveled to the distal colon. A 20kDa glycoprotein isolated from ESP collected from adult worms in vitro can be recognized by serum antibodies from *T. suis* infected pigs as early as 21 days after inoculation [33]. This indicates that the larval stages present in pigs 21 days after infection are stimulating an immune response. Taken together, these data indicate that direct effects of ESP on intestinal epithelial cells could account for increased cytokine production *in vivo* at sites distal to worm attachment.

In the jejunum of pigs infected with *C. jejuni* only, expression of MCP-1, TNFα, IL-12p40, IFNγ, IL-4, and IL-10 was increased. Although PCR testing and IHC did not confirm the presence of *Campylobacters* in this tissue, it is still likely that the response is due to *C. jejuni* stimulation. *C. jejuni* stimulates intracellular production of MCP-1, TNFα, IFNγ, IL-4, and IL-10 from INT-407 cells [34, 35], thus *C. jejuni* interaction with intestinal epithelial cells in the jejunum could account for the cytokine changes seen. In the jejunum of pigs dually infected with *C. jejuni* and *T. suis*, expression of GM-CSF is decreased 5 fold compared to uninfected controls, suggesting that similar to the proximal colon *T. suis* infection down regulates expression of proinflammatory cytokines. These effects could be due to systemic IL-13 and IL-10 produced by lymphocytes stimulated by worm antigens in draining lymph nodes.

In a separate study, secreted levels of IL-1β, IL-8, TNFα, IL-4, IL-6, and IL-10 were measured from the feces of these experimentally infected pigs (Cunningham et al, unpublished results; Parthasarathy et al, unpublished results). On day 23, which

corresponds to the day tissues were taken for analysis of cytokine expression, IL-1 β secretion was increased in the feces of pigs infected with *C. jejuni* only or dually infected with *T. suis* and *C. jejuni* when compared to controls. No other cytokines were increased in the feces on day 23. Increased secretion of IL-1 β in the feces on day 23 correlates with increased expression in the distal colon of the dual infected group and in the jejunum of the *C. jejuni* only infected group. Interestingly, IL-1 β expression was also significantly increased in the distal colon in the *T. suis* only group, but there was not a corresponding increase in fecal secretion. Taken together, these data suggest that *C. jejuni* stimulated increases in IL-1 β, even in the presence of *T. suis*. It also suggests that IL-1β production may be at least in part transcriptionally regulated, but a direct correlation is not evident from these studies. On day 22, which corresponds to 24 hours after inoculation with *C. jejuni*, secretion of IL-1 β, TNFα, and IL-10 were increased in all infection groups compared to controls, and IL-4 was increased in the dually infected group. This suggests that the maximal effects of *T. suis* and *C. jejuni* infection on production of these cytokines occurs at a time earlier that tissues were taken for expression analysis. Additional experiments measuring both secreted protein in feces and mRNA from tissues over time are necessary to clarify this issue.

We found that culture alone is not sensitive enough for detection of *C. jejuni* from conventionally reared pigs. This correlates with infection studies of orally inoculated day old chicks. *C. jejuni* was only recovered by culture of cecal contents from 64% to 72% of infected animals [36, 37]. We found that to increase the sensitivity of detection in our study, we needed to use PCR assays. RFLP analysis of the *Campylobacter* 23S rRNA gene was able to detect *C. jejuni* in proximal colon samples from 66% (4 of 6) of pigs

dually infected with *C. jejuni* and *T. suis*, and 16% (1 of 6) from each of the control, *T. suis* only, and *C. jejuni* only groups. Interestingly, RFLP analysis showed a distinct banding pattern that was not consistent with *C. jejuni*, *C. lari*, *C. coli*, or *C. upsaliensis* in some pigs (Figure 1, panel A, lanes 7 and 15). Fermer and Engvall noted that PCR product of the correct size was amplified from one strain of *C. mucosalis*. This amplicon was digested with the restriction enzymes and had a unique RFLP pattern, distinguishable from *C. jejuni*, *C. lari*, *C. coli*, or *C. upsaliensis* [21]. Unfortunately the authors failed to show the RFLP pattern for *C. mucosalis* and we were unable to compare the unique pattern generated in this study to the pattern for *C. mucosalis*. In addition, positive staining on immunohistochemistry was seen in colonic samples from all groups. Although antibody validation tests showed that the antibody cross reacts with *C. lari*, all pigs were negative when tested for the presence of *C. lari* by PCR (data not shown). Taken together, these data indicate that multiple Campylobacter species were present in the colons of our experimental animals and likely impacted the cytokine responses elicited by experimental infections. Further investigation of *Campylobacter* species that are present in our experimental groups is currently being pursued by cloning and sequencing *Campylobacter* 16S rRNA genes amplified from tissue samples.

Histologically, the lesions observed in pigs infected singly or dually with *T. suis* and *C. jejuni* were consistent with what has been demonstrated previously. Colostrum deprived piglets orally inoculated with a large dose ($4x10^8$ cfu) of *C. jejuni* have normal small intestines. In contrast, lesions in the colon include diffuse mild to moderate erosive colitis and typhlitis with infiltration of the lamina propria with small numbers of neutrophils [22]. Germ free piglets develop only mild colonic lesions in response to a low

dose (10^6 cfu) of *C. jejuni* with low level infiltration of the lamina propria and submucosa with lymphocytes, macrophages, neutrophils, and eosinophils [4]. *T. suis* infection causes goblet cell hyperplasia and a mixed inflammatory infiltrate in the proximal colons of both germ free and conventionally reared pigs [3, 4]. The mild to moderate colitis with goblet cell hyperplasia and mixed inflammatory infiltrate in the lamina propria and submucosa elicited in our study confirmed that pigs inoculated with *T. suis* eggs did become infected, and allowed for correlation between the presence of the worm and cytokine changes seen in the colon.

The balance between inflammatory cytokines and anti-inflammatory cytokines allows for defense against invading pathogens, while preventing fatal outcomes by an overwhelming inflammatory response. Inflammatory cytokines are produced in the early stages of infections to combat bacteria and in some cases eliminate the threat before a specific humoral or cell-mediated response is generated. In normal immunocompetent individuals, anti-inflammatory cytokines are naturally produced after induction of inflammatory cytokines to temper the response and prevent serious tissue destruction. Based on time course studies in murine disease models, anti-inflammatory cytokines are generally produced after the inflammatory cytokines have eliminated the pathogen. Helminths generally stimulate a Th2 directed response with increased production of IL-4, IL-5, IL-10, and IL-13 in gut associated lymphoid tissues [8, 38, 39]. These cytokines are essential for modifying the enteric environment to be inhospitable for helminths, ultimately resulting in expulsion of the pathogen and thus resolution of disease [8, 10, 11, 29]. A byproduct of these cytokines is downregulation of proinflammatory cytokines [24, 27]. In the case of dual infection, the anti-inflammatory response to the worm may

actually have a detrimental effect on the protective proinflammatory response toward bacteria. Indeed, there has been one reported case of dual *C. jejuni* and *T. suis* infection in a human with severe colitis, toxic megacolon, and acute renal failure [40]. The lack of an appropriate level of proinflammatory cytokines could facilitate invasion of tissues by resident bacterial populations, causing cellular destruction. The data presented in this study support this hypothesis.

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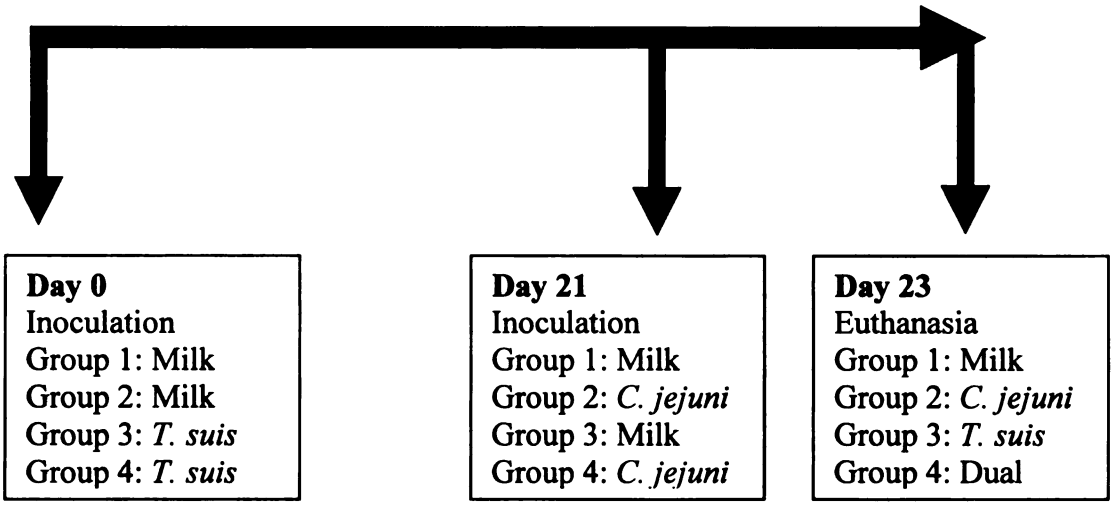

Figure 1: Inoculation timeline: Weaned piglets were divided into 4 groups of 6 pigs each and inoculated with 2,500 embryonated *T. suis* eggs, 10^8 cfu *C. jejuni* 33292, or both as shown. All inoculations were by oral gavage in 2mL of sterile milk.

			QRDR		Campylobacter 23S rRNA RFLP		
	Infection	Culture	Feces	Tissue DNA	Cj	Cc	Campy spp.
Jejunum	Control	0/6	0/6	0/6	0/6	5/6	2/6
	C. jejuni	0/6	0/6	0/6	0/6	2/6	5/6
	T. suis	0/6	0/6	0/6	0/6	3/6	2/6
	Dual	0/6	0/6	0/6	1/6	4/6	1/6
Proximal colon	Control	0/6	0/6	0/6	1/6	4/6	5/6
	C. jejuni	0/6	0/6	0/6	1/6	2/6	4/6
	T. suis	0/6	0/6	0/6	1/6	4/6	1/6
	Dual	0/6	0/6	0/6	4/6	4/6	2/6
Distal colon	Control	0/6	0/6	0/6	0/6	0/6	6/6
	C. jejuni	0/6	0/6	0/6	0/6	0/6	6/6
	T. suis	0/6	0/6	1/6	0/6	2/6	3/6
	Dual	0/6	0/6	0/6	0/6	2/6	6/6

Table 1: Summary of *Campylobacter* detection and identification. *Campylobacters* were detected and identified from pigs by fecal culture, PCR for the *C. jejuni* QRDR on both fecal and tissue DNA, and RFLP analysis of the thermophilic *Campylobacter* 23S rRNA gene using tissue DNA.

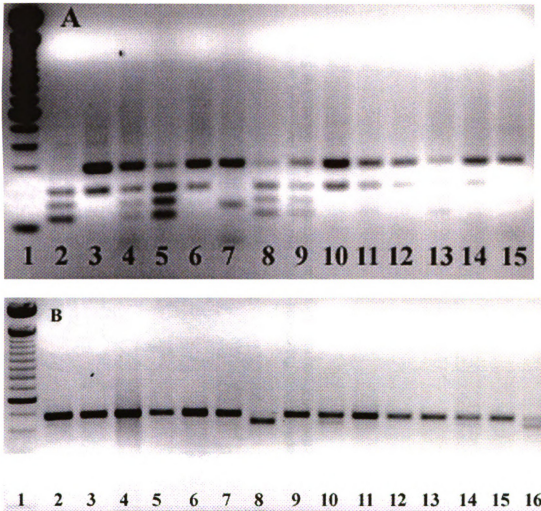

Figure 2: RFLP analysis of the *Campylobacter* 23S rRNA gene. Panel A: *AluI* digest of proximal colon samples from infected pigs . Lane 1: 100bp DNA ladder; Lane 2: *C. jejuni*; Lane 3: *C. coli*; Lanes 4 through 15: DNA from infected pigs. Panel B: *Tsp5091* digest of proximal colon samples from infected pigs. Lane 1: 100bp DNA ladder; Lane 2: *C. jejuni*; Lane 3: *C. coli*; Lane 4: *C. hyoilei*; Lanes 5 through 16: DNA from infected pigs.

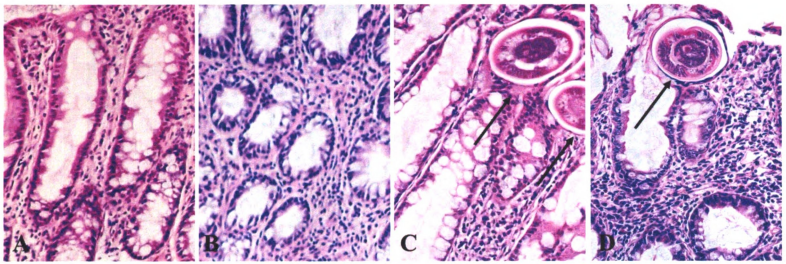

Figure 3: H&E stained sections of colonic tissue. H&E stained sections from the colons of pigs were examined for *T. suis* larvae. Panel **A**: Control; **B**: *C. jejuni* only; **C**: *T. suis* only; **D**: Dual infection, 10X magnification. Larvae (arrows) were detected in 5 of 6 pigs from each of the *T. suis* only and dual infection groups.

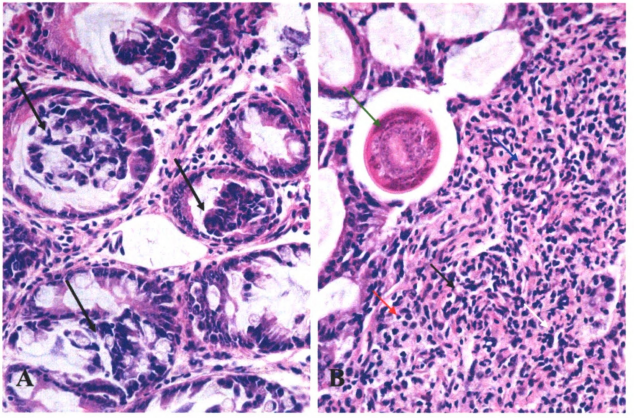

Figure 4 : H&E stained sections of colonic tissue. H&E section from a *C. jejuni* infected pig (panel A) showing sloughed epithelial cells in the crypts (black arrow), and a *T. suis* infected pig (panel B) showing the mixed inflammatory infiltrate in the lamina propria associated with the larvae (green arrow). Inflammatory cells included eosinophils (black arrow), plasma cells (blue arrow), lymphocytes (red arrow), and neutrophils (white arrow) (40X magnification).

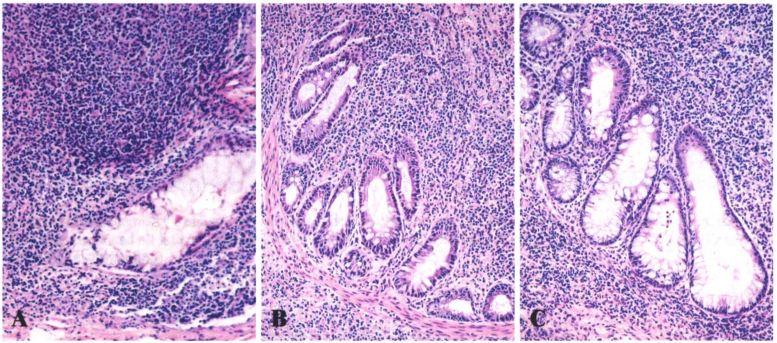

Figure 5: H&E sections of LGCs from infected pigs. Crypt distension was present in groups 2 (panel A) and 4 (panel C), but crypts from group 3 (Panel B) were not distended. (10X magnification)

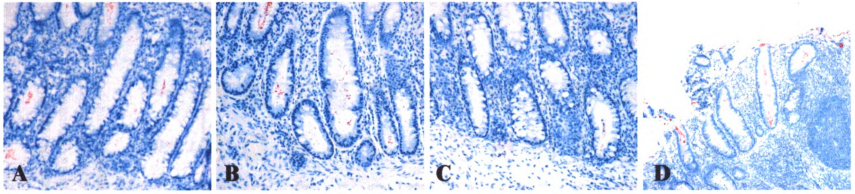

Figure 6: Immunohistochemistry from colonic sections. The polyclonal antibody raised against *C. jejuni* positively stained organisms in the crypts of controls and all infection groups. Panel A: Group 1; Panel B: Group 2; Panel C: Group 3 (20X magnification) ; Panel D: Group 4 (10X magnification)

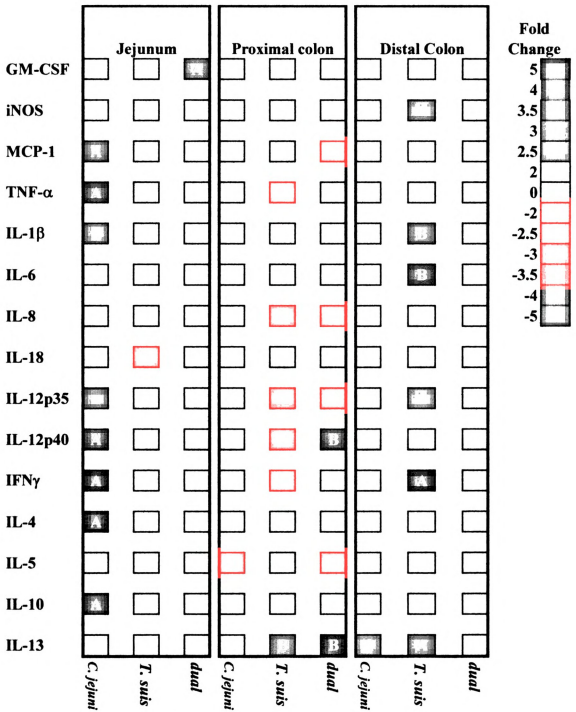

Figure 7: Cytokine expression in intestinal tissues. Expression of mRNA from a panel of cytokines was measured by Real Time PCR. The data represents the average fold change in expression from infected animals when compared to control animals. Letters indicate statistically significant changes: A: $p \leq 0.05$; B: $p \leq 0.01$

Chapter 4: "*Campylobacter jejuni* stimulates IL-18 secretion from differentiated intestinal epithelial cells"

Abstract

Campylobacter jejuni is the leading bacterial cause of foodborne illness worldwide. Clinical signs of campylobacteriosis mimicking human disease have been reproduced in piglets. Upon infection, the bacteria penetrate the intestinal mucus layer, and adhere to and invade intestinal epithelial cells, resulting in inflammation. We have previously shown that *C. jejuni* induces proinflammatory cytokines in the distal colon and lymphoglandular complexes of rectally inoculated piglets one hour after infection. Additionally, *C. jejuni* has been shown to stimulate secretion of the proinflammatory cytokines IL-1β, TNFα, IL-6, and IL-8 from intestinal epithelial cells and monocytes. Interleukin 18 is a pleiotrophic cytokine that is constitutively expressed in intestinal tissues and plays a critical role in initiating an immune response to pathogenic bacteria. Because intestinal epithelial cells play an important role in the pathogenesis of *C. jejuni* infection *in vivo*, we hypothesized that *C. jejuni* induces IL-18 from cultured swine intestinal epithelial cells. Differentiated and undifferentiated IPEC-1 cells were infected apically with *C. jejuni* strain ATCC 33292 or 81-176. Supernatants were collected at 0, 1, 3, 6, 8, and 24 hours after infection and analyzed for IL-18 protein by ELISA. RNA was isolated from cells 8 hours after infection to assay for IL-18 mRNA expression by RT-PCR. Differentiated cells secreted significantly higher levels of IL-18 protein as early as 3 hours after infection, compared to uninfected controls. Undifferentiated cells did not

secrete significant levels of IL-18 at any time point. Expression of IL-18 mRNA was not significantly increased by *C. jejuni* 8 hours after infection from either differentiated or undifferentiated cells. These results indicate that differentiated intestinal epithelial cells are a significant source of IL-18. Epithelial cell derived IL-18 may play an important role in the pathogenesis of *C. jejuni* infection *in vivo.*

Introduction

Campylobacter jejuni (C. jejuni) is the most frequent bacterial cause of foodborne illness worldwide, with the CDC estimating 2.4 million cases of campylobacteriosis each year in the U.S. [1]. Clinical signs include a prodrome of fever, abdominal pain, and malaise followed by the onset of profuse, watery diarrhea with blood, mucus, and leukocytes [2]. Microscopic examination of colonic tissues from humans and experimentally infected animals demonstrates that *C. jejuni* infection stimulates an acute inflammatory response with infiltration of the epithelium and lamina propria with neutrophils and mononuclear cells [3-5]. Immunohistochemical staining of colonic tissues shows *C. jejuni* associated with epithelial cells and goblet cells, within deep crypts of the colon, and within lymphoglandular complexes (LGCs) in entrapped crypts and in the follicle associated epithelium [3, 4]. The pathogenesis of *C. jejuni* infection involves colonization of the intestinal mucous overlying the epithelium, adherence to intestinal epithelial cells, and invasion of epithelial cells [2, 5]. *C. jejuni* is chemoattracted to mucin, a major component of intestinal mucous, and polar flagella combined with its spiral morphology facilitate efficient movement through the mucous to come into contact with the epithelium [2, 6, 7]. *C. jejuni* binds to epithelial cells, intercellular junctions, and the basement membrane via adhesins, including CadF, Jlp, CBF-1, and flagella [8-13]. Bound bacteria are internalized by microtubule- and microfilament-dependent processes [14-16]. Epithelial cell destruction can occur due to the effects of invasion and toxins produced by *C. jejuni,* including enterotoxin, cytotoxins, and cytolethal distending toxin

[17-21]. Cellular destruction and loss of epithelial function leads to decreased absorption which manifests clinically as diarrhea [2, 5].

Elimination of the bacteria and resolution of disease is dependent upon induction of a humoral immune response and cytokine production. *C. jejuni* stimulates specific serum IgG as well as secretory IgA in feces, urine, saliva, and mammary glands [22]. In a swine model of infection, anti-*C. jejuni* IgA has been localized to B cell rich germinal centers of colonic lymphoglandular complexes [23]. Oral pretreatment of mice with recombinant IL-5 or IL-6 prior to oral challenge with *C. jejuni* significantly reduces intestinal colonization. In addition, recombinant IL-6 enhances specific intestinal and systemic IgA production [24]. *In vitro*, *C. jejuni* stimulates secretion of IL-1β, TNFα, IL-6, and IL-8 from intestinal epithelial cells and monocytes [25-27] (Parthasarathy and Mansfield, unpublished data; Cunningham and Mansfield, unpublished data). These studies indicate that a humoral immune response is necessary for resolution of infection and that proinflammatory cytokines may be important for host resistance to *C. jejuni* infection *in vivo*.

IL-18 is a pleiotrophic cytokine capable of initiating and regulating both innate and adaptive immune responses *in vivo* [28-31]. Mouse peritoneal macrophages and human peripheral blood mononuclear cells produce TNFα, IL-1α, IL-1β, IL-6, IL-8, and MCP-1 upon stimulation with IL-18 [32, 33]. Naïve T cells proliferate and produce IFNγ when stimulated with IL-12 and IL-18 [29, 34, 35]. NK cell cytotoxicity and Th 1 cell differentiation are enhanced by stimulation with IL-12 and IL-18 [29, 34, 35]. In the presence of IL-2, IL-18 stimulates IL-13 production from NK cells and T cells [35]. IL-18 is produced by a variety of cell types and is widely distributed through tissues *in vivo*

[35, 36]. IL-18 is both transcriptionally and post translationally regulated. The IL-18 promoter region is responsive to lipopolysaccharide (LPS) and increased mRNA expression can be induced in LPS stimulated swine macrophages and splenocytes *in vitro* [36, 37]. Immature IL-18 protein requires cleavage by IL-1β converting enzyme (ICE), also known as Caspase-1, for activation and secretion from cells [38]. In addition, IL-18 binding protein regulates IL-18 activity *in vivo* [39]. IL-18 mRNA and protein are constitutively expressed in a variety of cell types, including intestinal cells, suggesting a role for IL-18 as a first line of host defense against pathogens [35].

IL-18 is constitutively expressed in intestinal tissues and plays a critical role in initiating an immune response to pathogenic bacteria [34, 35, 40, 41]. Also, as discussed in Chapter 2, *C. jejuni* stimulates significant expression of IL-18 and other proinflammatory cytokines in the distal colon and lymphoglandular complexes 1 hour after rectal inoculation. Because enterocytes are the first line of defense against enteric bacteria, we hypothesized that intestinal epithelial cells are a source of IL-18 during *C. jejuni* infections. To test this hypothesis, differentiated and undifferentiated cultured Intestinal Pig Epithelial Cells (IPEC-1) were infected with *C. jejuni* for 0, 1, 3, 6, 8, and 24 hours. IL-18 production was analyzed using two methods. IL-18 secretion in culture supernatants was measured by ELISA. IL-18 mRNA expression in cells was measured by RT-PCR. Differentiated cells secreted significant levels of IL-18 protein as early as 3 hours after infection. Undifferentiated cells did not secrete significant levels of IL-18 at any time point. Expression of IL-18 mRNA was not significantly increased by *C. jejuni* at any time from either differentiated or undifferentiated cells. These results indicate that

differentiated intestinal epithelial cells are a significant source of IL-18. Epithelial cell-derived IL-18 may be important for host resistance to *C. jejuni* infection *in vivo.*

Materials and Methods

Cells

IPEC-1 is a non-immortalized undifferentiated cell line derived from the small intestine of neonatal pigs. These cells will differentiate, developing tight junctions and distinct apical and basolateral surfaces, when grown on porous supports for 10 to 14 days [42]. The transepithelial electrical resistance (TER) can be measured across the monolayer to indicate whether the cells have achieved a differentiated state. Differentiated IPEC-1 cells represent the mature enterocytes located on the villus tips in the intestines.

When IPEC-1 cells are seeded at the appropriate density onto flat bottom polystyrene plates and grown for only 2-3 days, the cells form confluent monolayers that remain undifferentiated. Undifferentiated cells represent the rapidly dividing cells found in the base of the crypts in the intestine. We used IPEC-1 cells in both differentiated and undifferentiated states to determine if IL-18 production was stimulated upon *C. jejuni* infection.

All culture media and growth supplements were obtained from Invitrogen unless otherwise stated (Invitrogen, Frederick, MD). Growth flasks and transwells were obtained from Corning (Costar, Corning, NY). Cells were routinely cultured at 37°C, 5% CO_2 in DMEM/F12 supplemented with 5% Fetal Bovine Serum (FBS) and 1% Insulin-Transferrin-Selenium (ITS) in T75 flasks. To seed cells for infection studies, the cells were gently washed with versene and released from the flask with 0.05% Trypsin-EDTA. Detached cells were collected and pelleted by centrifugation at 1200rpm for 5 minutes.

The supernatant was removed and the cells were resuspended in RPMI supplemented with 5%FBS. A subsample of the cells was mixed with a 1:1 volume of trypan blue (Sigma, St. Louis, MO), then loaded onto a hemocytometer (Hausser Scientific, Horsham, PA) and viable cells were counted on a Nikon inverted microscope (Mager Scientific, Dexter, MI).

Cells to be tested in a differentiated state were seeded at a density of $3x10^5$ cells onto 6.5mm diameter, 3.0μm pore size transwell inserts which had been previously coated with 20μg/mL fibronectin (Sigma, St. Louis, MO). Cells were incubated for 12-14 days to allow differentiation. Prior to infection TER was measured from each well using an electrode to confirm confluency and tight junction formation (EVOMX, World Precision Instruments, Sarasota, FL). TER values greater than $600\Omega/cm^2$ indicated that the cells were differentiated.

Cells to be infected in the undifferentiated state were seeded at a density of $3x10^5$ cells per well onto 24 well plates that had previously been coated with 20μg/mL of fibronectin (Sigma, St. Louis, MO). The cells were incubated for 2-3 days to allow formation of a confluent monolayer.

Scanning Electron Microscopy

Differentiated IPEC-1 cells grown on transwells for 12 days were examined by scanning electron microscopy to verify their state of differentiation. The cells were seeded onto transwells and incubated for 12 days as previously described. The medium was removed, the membrane was excised, and the cells were fixed in 4% glutaraldehyde in 0.1M sodium phosphate buffer. The cells were further processed and examined at the

Center for Microscopy at Michigan State University (East Lansing, MI). The cells were fixed in 1% osmium tetroxide, dehydrated through and ethanol gradient, then dried in a critical point dryer (Balzers, Lichenstein). Dehydrated membranes were mounted on aluminum stubs and sputter coated with 7nm of gold (Emscope SC500 sputter coater, UK). The cells were visualized on a scanning electron microscope (JEOL 6400V, Japan).

Bacteria

Campylobacter jejuni strains ATCC 33292 and 81-176 were used for infections of IPEC-1 cells. Both strains were originally isolated from humans with enteritis. The *C. jejuni* strain ATCC 33292 used in these infections had been rederived by passage through a pig in our laboratory and stored as second passage stocks. *C. jejuni* strain 81-176 was kindly donated by Carol Pickett. Bacterial cultures were streaked for isolation onto Bolton Agar plates and incubated for 48 to 72 hours at 37°C, 5% CO_2. Isolated colonies were subcultured for lawn growth and grown for approximately 20 hours. Lawns were harvested with sterile swabs and resuspended in RPMI without phenol red or FBS. The OD_{560} was measured on a Beckman DU 530 UV/Vis spectrophotometer (Beckman Coulter, Inc, Fullerton, CA) and subsequently adjusted to an OD_{560} of 0.1 in RPMI. Previous studies in our lab have shown that this measurement corresponds to approximately $5x10^8$ cfu/mL. A subsample of each inoculum was examined microscopically to assess morphology and darting motility of the bacteria.

Experimental Design

To determine the effect of *C. jejuni* on IL-18 secretion, IPEC-1 cells were exposed to each strain of bacteria apically at a multiplicity of infection (MOI) of approximately 40:1. Previous studies in our laboratory have shown this to be the optimal concentration of *C. jejuni* for stimulation of IL-6 and IL-10 secretion from IPEC-1 cells. RPMI without phenol red or FBS was used as the negative control and Concanavalin A (Con A) was used at a concentration of 100μg/mL as the positive control. Supernatants were removed at 0, 1, 3, 6, 8, or 24 hours after infection then centrifuged for 10 minutes at 6000rpm and 4°C to pellet bacteria. The supernatants were transferred to polypropylene tubes (DOT Scientific, Burton, MI) and stored at -80°C until ELISA was performed to measure the concentration of IL-18 protein. For differentiated IPEC-1 cells, supernatants from the upper and lower chamber were stored and analyzed separately to determine if IL-18 was secreted from the apical or basolateral surface. Expression of IL-18 mRNA was measured from IPEC-1 cells in both differentiated and undifferentiated states by RT-PCR of total RNA extracted from cells infected for 8 hours.

ELISA

Secreted IL-18 protein was measured from culture supernatants by an indirect ELISA developed in our laboratory. Recombinant porcine IL-18 and anti-swine IL-18 primary antibody were obtained from R and D systems (Minneapolis, MN). Biotin conjugated anti-goat IgG secondary antibody, Extravidin®, bovine serum albumin (BSA), and the ready to use peroxide containing tetramethyl benzidine (TMB) substrate were obtained from Sigma (Sigma, St. Louis, MO). ELISA plates were washed 3 times

with Tris-buffered saline with 0.5% Tween-20 (TTBS) between incubations and all incubations were for 1 hour at room temperature unless otherwise indicated. Prior to measuring IL-18 from experimentally infected IPEC-1 cells, the optimal concentrations for the primary antibody, secondary antibody, and Extravidin were determined experimentally (data not shown). For preparation of the standards, recombinant porcine IL-18 was diluted in RPMI 1640 without phenol red to 100ng/mL, 50ng/mL, 25ng/mL, 12.5ng/mL, 6.25ng/mL, 3.125ng/mL, and 1.56ng/mL final concentrations. Supernatants and standards were coated in duplicate onto Nunc Immuno Maxisorp 96 well plates (VWR, West Chester, PA) overnight at 4°C. Plates were blocked for 90 minutes with 3% BSA at 37°C, then bound IL-18 was detected by 2 hour incubation with anti-swine IL-18 antibody diluted to 750ng/mL in 1%BSA in TTBS. Primary antibody was detected by biotin conjugated secondary antibody diluted 1:10,000 in 1%BSA in TTBS. The biotin conjugate was reacted with Extravidin® diluted to 1μg/mL in 1%BSA in TTBS, followed by color development for approximately 15 minutes with the peroxide containing TMB substrate. Color development was stopped by addition of 6N H_2SO_4 after approximately 15 minutes. Absorbance was measured at 450nm on an EL800 Universal Microplate Reader and the concentration of IL-18 protein in each sample was calculated by KCjunior® software (Bio-Tek instruments, Winooski, Vermont).

Cytokine expression

Total RNA was isolated from IPEC-1 cells using the Trizol® method. Briefly, cells were lysed in Trizol® reagent (Invitrogen, Frederick, MD), RNA was extracted with chloroform, precipitated with isopropanol, and washed with ethanol. Pellets were air

dried, then resuspended in DEPC-treated H_20 (Invitrogen, Frederick, MD). Concentration of RNA was determined spectrophotometrically (Beckman). Samples were checked for degradation by electrophoresis on a 1.8% agarose gel in 1X TAE buffer with 0.2μg/mL ethidium bromide. Gels were visualized by UV illumination on an Alpha Imager 2000 Documentation and Analysis system (Alpha Innotech Corporation, San Leandro, CA). RNA samples were stored at -80°C. For each sample, 5ug of total RNA was reverse transcribed with Oligo dT primers using a Stratagene (Stratagene, La Jolla, CA) First Strand RT-PCR kit. First strand cDNA was used as the template in further PCR reactions

Swine IL-18 and β_2-microglobulin expression was measured from IPEC-1 cDNA samples using previously described primers [35, 43]. The primer sequences for IL-18 were forward 5′ TAT GCC TGA TTC TGA CTG TT 3′ and reverse 5′ ATG AAG ACT CAA ACT GTA TCT 3′ [35]. The primer sequences for β_2-microglobulin were forward 5′ CTG CTC TCA CTG TCT GG 3′, and reverse 5′ ATC GAG AGT CAC GTG CT 3′ [43]. For each target, a 25 μL PCR reaction consisted of 1X Perkin Elmer PCR buffer (Tris-HCL, pH 8.3, 50 mM KCl), 1.0mM $MgCl_2$, 0.05mM each dNTP, 37.5ng of each primer, 0.625U Perkin Elmer AmpliTaq Gold, and 1μL of first strand cDNA in DEPC treated water. Each sample was amplified in triplicate.

Prior to measuring expression in our sample set, the optimal number of amplification cycles for each target was determined for measurement of expression during the exponential phase of amplification. We measured expression from positive and negative controls every 2 cycles from 20 to 40 cycles and plotted the values on a graph (Figures 5 and 6). All samples were amplified with a 10 minute hot start at 94°C followed

by 39 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, with a final extension time of 10 minutes at 72°C.

The PCR product was electrophoresed on 1.8% agarose gels in 1X TAE with 0.2μg/mL ethidium bromide. The gels were visualized by UV illumination on an Alpha Imager 2000 Documentation and Analysis system, and density of individual bands was measured using the Line Densitometry function. β_2-microglobulin was used as the constitutively expressed gene for normalization of cytokine expression. Ratios of specific cytokine to housekeeping gene were calculated by dividing the density values obtained from the IL-18 target by those obtained from the β_2-microglobulin target [35].

Statistical Analysis

The data were analyzed in Excel (Microsoft, Redmond, WA). An F test for sample variance was performed to compare means of infected cells to uninfected cells. The appropriate T test was then used to determine the effect of treatment on IL-18 production at each time point. P values of 0.05 or less were considered statistically significant.

Results

Scanning Electron Microscopy (SEM)

A representative SEM image of IPEC-1 cells grown on transwells for 12 days is shown in figure 1. TER values measured from 12 day old IPEC-1 cells ranged from 600 to 800 Ω/cm^2. The cell monolayers were confluent and cells had developed microvilli on their apical surfaces, indicating that they had achieved a differentiated state.

IL-18 secretion

IPEC-1 cells in a differentiated state secreted significant amounts of IL-18 into the culture supernatant by 3 hours after *C. jejuni* infection (Figure 2). In the upper chamber, secretion was virtually undetectable immediately after infection (0 hr) and 1 hour after infection. Secretion peaked at approximately 12 ng/mL 3 hours after infection, and remained elevated at both 6 and 8 hours after infection. By 24 hours, IL-18 was still detectable, but the levels had decreased significantly. In the lower chamber, IL-18 secretion was very low and was not significantly induced by *C. jejuni* infection at any time point (Figure 3). IL-18 secretion in response to *C. jejuni* infection was not significantly different between strains.

In contrast, IPEC-1 cells in an undifferentiated state did not secrete significant amounts of IL-18 in response to *C. jejuni* at any time (Figure 4).

IL-18 mRNA expression

IL-18 expression was measured by RT-PCR from IPEC-1 cells in both the differentiated and undifferentiated state 8 hours after infection with *C. jejuni*. Prior to measuring expression from experimental samples, the optimal cycle number to use was determined by measuring the density of the IL-18 and β2-microglobulin targets amplified from stimulated (100ng each of Calcium ionophore and phorbol myristate acetate) and unstimulated cells (DMEM or RPMI medium only) over several cycles (Figure 5). The optimal cycle number for IL-18 measurement from experimental samples was selected from the linear portion of the amplification curve for each target (Figure 6). The optimal cycle numbers selected for measurement of target expression from experimental samples were 31 for IL-18 and 27 for β2-microglobulin.

IL-18 and β2-microglobulin targets were amplified from IPEC-1 cells in both differentiation states with the exception of IPEC-1 cells in the differentiated state treated with Con A (data not shown). IL-18 expression was not significantly impacted by *C. jejuni* infection at any time point in IPEC-1 cells in either differentiation state (Figure 7).

Discussion

We hypothesized that intestinal epithelial cells infected with *C. jejuni* are a source of IL-18. Furthermore, early release of IL-18 from epithelial cells assaulted by *C. jejuni* may serve to initiate a protective inflammatory response *in vivo*. Our experiments show that *C. jejuni* stimulates IL-18 secretion from IPEC-1 cells that have achieved a differentiated state. Secretion peaked at 3 hours and remained elevated at 6 and 8 hours after infection before decreasing significantly by 24 hours after infection. *In vitro*, *C. jejuni* stimulates secretion of a panel of proinflammatory cytokines from intestinal epithelial cells and monocytes, including IL-1β, TNFα, IL-6, and IL-8 [25-27] (Parthasarathy and Mansfield, unpublished data; Cunningham and Mansfield, unpublished data). Epithelial cell-derived cytokines play an important role in controlling the spread of pathogenic bacteria *in vivo* [44]. IL-18 facilitates neutrophil accumulation in tissues of mice during lethal endotoxemia, and it has also been shown to activate neutrophils, inducing granule release and enhancing the respiratory burst [45, 46]. Neutrophil infiltration of the lamina propria and submucosa is a consistent finding in animal models of *C. jejuni* infection [3, 4, 47]. The effects of IL-18 on neutrophil functions likely serve to kill invading *C. jejuni* and contain the infection. IL-18 also stimulates production of IL-8, TNFα, and IL-1β, which would sustain the inflammatory response and activate other mechanisms for eliminating bacterial pathogens [32, 33, 45, 46].

IL-18 secretion from differentiated IPEC-1 cells was apparently not transcriptionally regulated. IL-18 mRNA was constitutively expressed in these cells, but

expression was not significantly increased by infection with *C. jejuni.* The IL-18 promoter sequence contains LPS response elements and IL-18 expression is induced in LPS stimulated swine alveolar macrophages, adherent splenocytes, and non adherent splenocytes [36, 37]. Differentiated IPEC-1 cells stimulated with 200μg/mL of LPS from *E. coli* O26:B6 secrete significant amounts of IL-6 (Parthasarathy et al, unpublished results), but not IL-18 (data not shown). This concurs with data from Okazawa and colleagues who found that LPS stimulation of the human colonic cell line HT-29 did not result in increased intracellular accumulation of IL-18 or increased secretion [48]. These data indicate that LPS-induced transcriptional regulation does not increase production of IL-18 from intestinal epithelial cells. Constitutive expression of IL-18 protein in the intestine suggests that rapid production of IL-18 is important for early responses to pathogens [35, 36]. Therefore, it is likely that post translational modification of IL-18, via cleavage with Caspase-1, rather than increased expression of mRNA, is the main mechanism for IL-18 secretion in the intestine. The data from differentiated IPEC-1 cells support that hypothesis.

In contrast to differentiated IPEC-1 cells, *C. jejuni* infection did not stimulate increased production of IL-18 protein or mRNA from undifferentiated IPEC-1 cells at any time assayed over 24 hours. Similar to differentiated cells, undifferentiated cells express IL-18 mRNA constitutively (data not shown). Undifferentiated cells are capable of secreting 7ng/mL of IL-18 after 8 hours of stimulation with Con A (Figure 4). It has been demonstrated that undifferentiated IPEC-1 cells secrete IL-10 in response to Con A, but not in response to *C. jejuni,* while differentiated IPEC-1 cells do secrete IL-10 in response to *C. jejuni* infection (Parthasarathy et al, unpublished results). IL-1β stimulated

differentiated HT-29 human intestinal epithelial cells have impaired production of IL-8 when compared to undifferentiated cells. This effect is due to decreased c-Jun N-terminal kinase (JNK) and IκB kinase (IKK) activity with subsequently reduced NF-κB DNA binding after stimulation of the IL-1β receptor [49]. Impaired secretion of IL-18 (and IL-10) from undifferentiated IPEC-1 cells may be due to altered intracellular signaling pathways, but further study is necessary to elucidate this.

IL-18 mRNA and immature protein are constitutively expressed in swine intestinal epithelial cells and gut associated lymphoid tissues throughout the intestine [35, 36]. *Salmonella, Shigella, Yersenia*, and *Helicobacter* infections induce increased expression of IL-18 mRNA and maturation of IL-18 protein to the active form, which are critical for resolution of infection [34, 36, 41, 50-52]. We have also shown, as discussed in Chapter 2, that *C. jejuni* induces IL-18 and other proinflammatory cytokines in the distal colon and lymphoglandular complexes of rectally inoculated pigs. We propose that in our swine model of *C. jejuni* infection, IL-18 released from *C. jejuni* infected epithelial cells stimulates production of IL-1β, TNFα, IL-6, IL-8, and MCP-1 from epithelial cells and leukocytes locally, inducing an inflammatory response. Also, neutrophil infiltration of the epithelium and lamina propria with subsequent activation of their bacterial killing mechanisms by IL-18 may be critical for reducing pathogen numbers.

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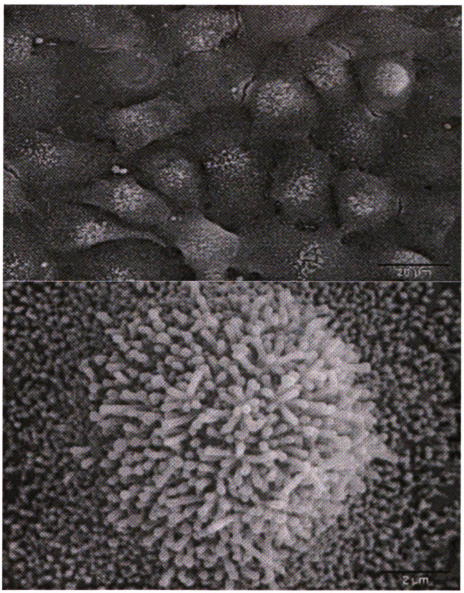
Figure 1: Scanning electron micrograph of polarized IPEC-1 cells (12 days) on transwell membranes. Top panel is overview of cells; bar represents 20 μm. The apical finger-like microvilli are seen in the bottom panel; bar represents 2 μm. Micrographs are representative of three samples each.



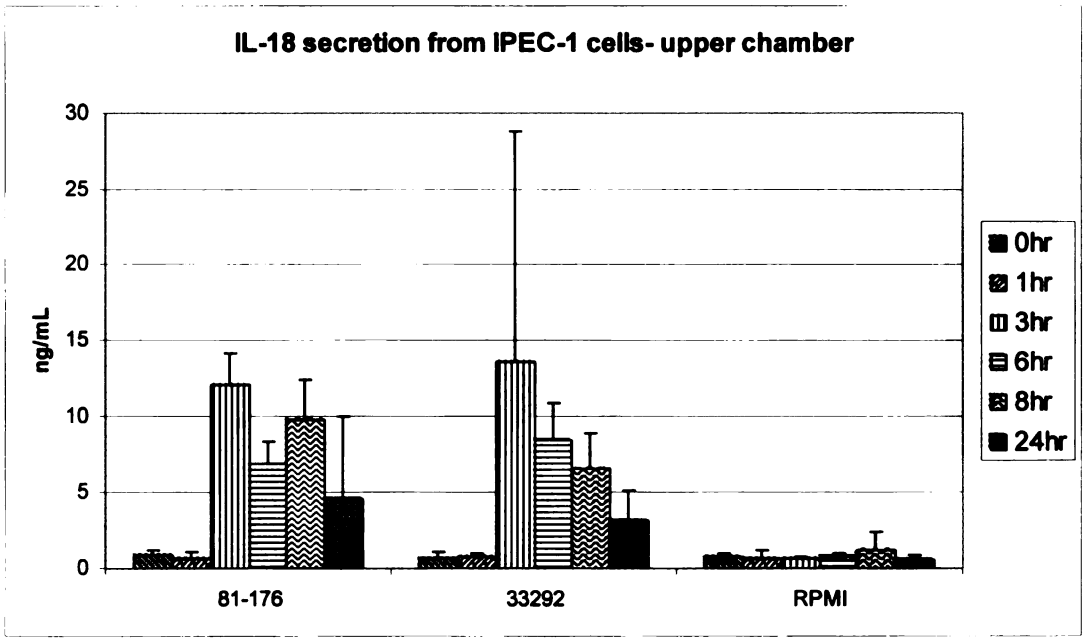


Figure 2: IL-18 secretion from differentiated IPEC-1 cells. Apical secretion of IL-18 in response to apical infection with *C. jejuni* was measured by ELISA from the upper chamber of differentiated cells. Secretion from *C. jejuni* infected cells was significantly greater than medium only controls at 3, 6, 8, and 24 hours after infection. ($p \leq 0.05$).

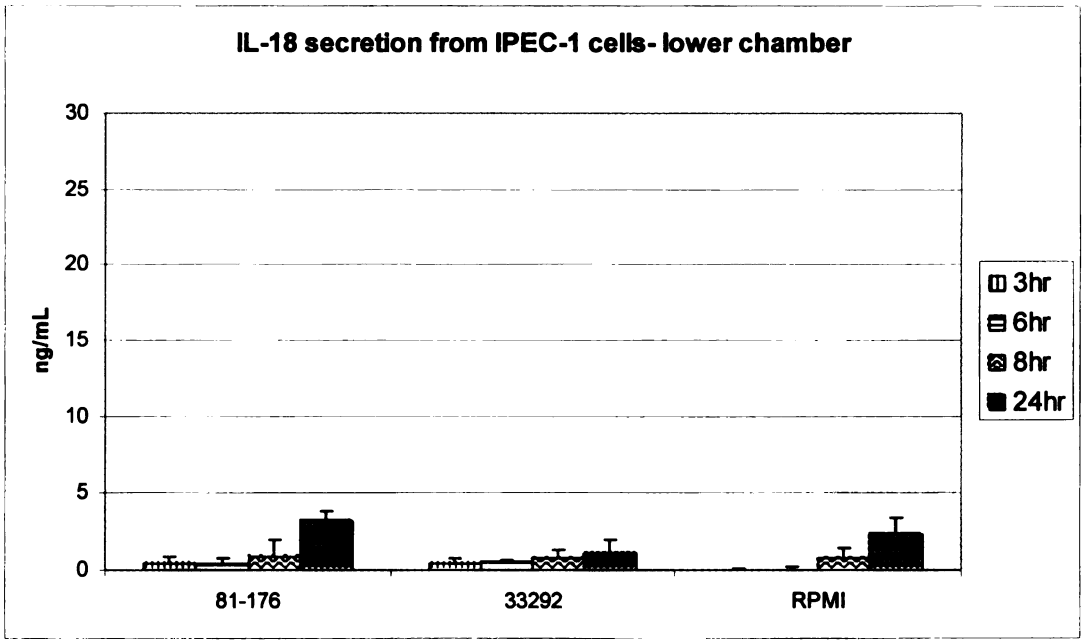


Figure 3: IL-18 secretion from differentiated IPEC-1 cells. Basolateral secretion of IL-18 in response to apical infection with *C. jejuni* was measured by ELISA from the lower chamber of differentiated cells. Secretion from *C. jejuni* infected cells was not significantly greater than medium only controls at any time after infection. ($p \leq 0.05$).

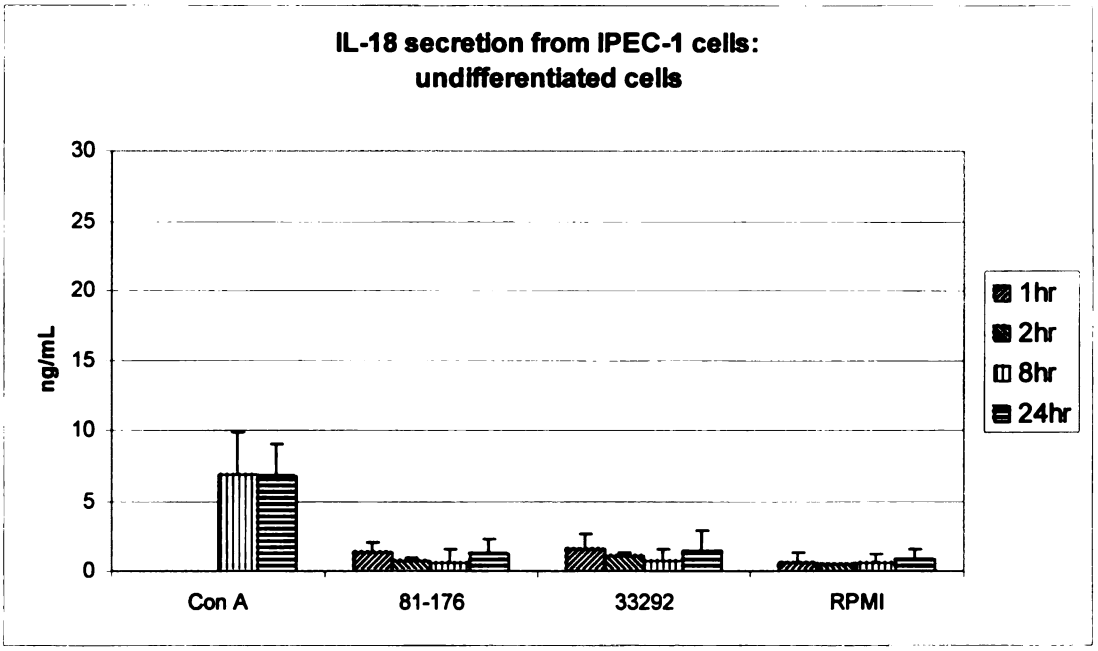


Figure 4: IL-18 secretion from undifferentiated IPEC-1 cells. IL-18 secretion in response to *C. jejuni* infection of undifferentiated IPEC-1 cells was measured by ELISA. Undifferentiated IPEC-1 cells secreted an average of 7ng/mL of IL-18 in response to Concanavalin A, however IL-18 secretion from *C. jejuni* infected cells was not significantly greater than medium only controls at any time after infection. ($p \leq 0.05$).

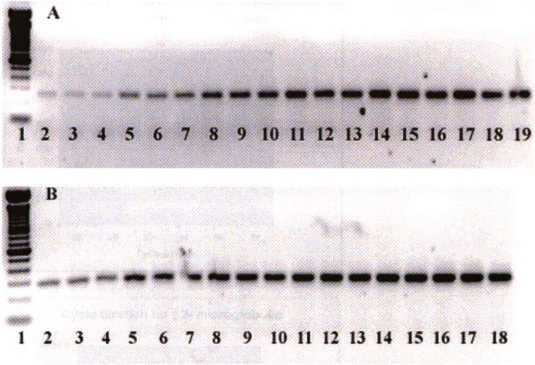
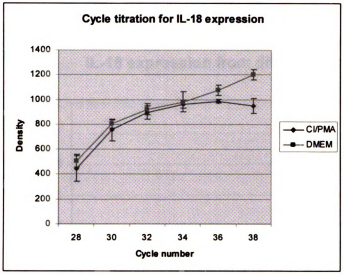


Figure 5: Optimization of amplification parameters. We titrated the cycle number needed to measure IL-18 and β2-microglobulin expression within the linear portion of the amplification curve. Samples were amplified in triplicate, electrophoresed on an agarose gel, and density of the bands was measured. **Panel A:** Cycle titration for IL-18 expression. Lane 1: 100bp DNA ladder; Lanes 2-4: 28 cycles; Lanes 5-7: 30 cycles; Lanes 8-10: 32 cycles; Lanes 11-13: 34 cycles; Lanes 14-16: 36 cycles; Lanes 17-19: 38 cycles. **Panel B** : Cycle titration for β2-microglobulin expression. Lane 1: 100bp DNA ladder; Lanes 2-4: 24 cycles; Lanes 5-7: 26 cycles; Lanes 8-10: 28 cycles; Lanes 11-13: 30 cycles; Lanes 14-16: 32 cycles; Lanes 17-18: 34 cycles.



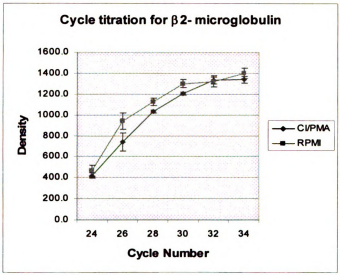


Figure 6: Determination of optimal number of cycles for amplification. The average density of the bands from each cycle was plotted on a graph. The optimal cycle number for measurement of target expression from experimentally infected samples was chosen from the linear portion of the amplification curve. IL-18 expression was measured at 31 cycles while β2-microglobulin expression was measured at 27 cycles.

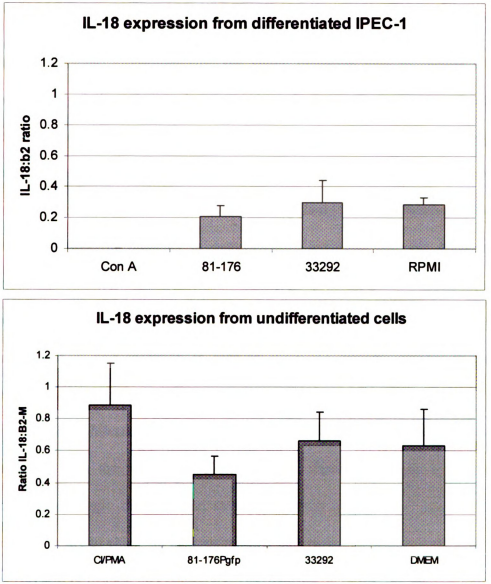


Figure 7: IL-18 expression from IPEC-1 cells. IL-18 expression was measured 8 hours after infection from both differentiated and undifferentiated IPEC-1 cells. The data from each sample was normalized to β2-microglobulin expression and is presented as the ratio of IL-18 expression over to β2-microglobulin expression. Neither IL-18 nor to β2-microglobulin were measured from Con A stimulated differentiated cells. IL-18 expression was not significantly changed in *C. jejuni* infected cells when compared to medium only controls for either cell type.

Chapter 5: Summary and Conclusions

Pigs are an excellent animal model for studying *C. jejuni* infection in humans for several reasons. Physiologically, the swine digestive tract is similar to humans and outbred populations that are heterogeneous, similar to the human population, are readily available at low cost from production facilities. Colostrum-deprived piglets become colonized and develop mucohemorrhagic diarrhea and intestinal pathology that mimics human disease after oral inoculation with *C. jejuni* [1, 2]. In addition, an extensive array of swine specific reagents is commercially available for the analysis of immune mediators. All together, these facts make pigs quite suitable for use in research on the pathogenesis of *C. jejuni* infection. We used a dual infection model of concurrent *C. jejuni* and *T. suis* infection in pigs to study the immune responses associated with mucohemorrhagic diarrhea.

Campylobacter jejuni is a commensal in the colons of healthy pigs, but upon infection with the whipworm *Trichuris suis*, these two agents synergize to produce mucohemorrhagic diarrhea and severe pathology [2-4]. The long term goals of the laboratory are to elucidate the mechanisms by which dual infection with these two agents causes severe disease and pathology. One hypothesis is that *T. suis* infection facilitates *C. jejuni* invasion by modulation of the host immune response. The central hypothesis for this dissertation project is that a proinflammatory response plays a role in swine natural resistance to *C. jejuni.* A related hypothesis is *T. suis* infection alters the protective proinflammatory response resulting in *C. jejuni* invasion in the colon and severe pathology. These hypotheses were tested, generally, by assessing local cytokine expression in the intestines of *C. jejuni* and *T. suis* infected pigs, and more specifically by

assessing IL-18 secretion from *C. jejuni* infected IPEC-1 cells, which serve as a model system for the swine intestinal epithelium.

This dissertation project was designed to address three main objectives. The first objective was to determine the nature of the local immune response to *C. jejuni* in the distal colon and LGC of pigs. To address this issue, weaned piglets were inoculated rectally with *C. jejuni* 81-176 that produced Gfp. The pigs were killed 1 hour or 24 hours after inoculation and samples from the distal colon and LGCs were taken for microscopic examination for lesions, for immunohistochemical staining with anti-*C. jejuni* antibody to localize the bacteria in the colon, and for measurement of cytokine mRNA expression by real time PCR. The second objective was to determine the nature of the local immune response in different segments of the intestine when pigs were infected with *C. jejuni, T. suis*, or both. To address this issue, weaned piglets were inoculated by oral gavage with embryonated *T. suis* eggs. Twenty one days after infection with *T. suis*, pigs were inoculated by oral gavage with *C. jejuni* strain ATCC 33292. Two days after inoculation with *C. jejuni*, all pigs were killed and samples from the jejunum, proximal colon, and distal colon were taken for microscopic examination for lesions, for immunohistochemical staining with anti-*C. jejuni* antibody to localize the bacteria, and for measurement of cytokine mRNA expression by real time PCR. The third and final objective was to determine if epithelial cell derived cytokines play a role in swine resistance to *C. jejuni* infection as determined by *in vitro* infection of swine intestinal epithelial cells. To address this issue, IPEC-1 cells in both a differentiated and undifferentiated state were infected separately with *C. jejuni* strains 81-176 and ATCC 33292. Culture supernatants were removed 0, 1, 3, 6, 8, and 24 hours after infection and

analyzed for IL-18 protein levels by ELISA. Total RNA was isolated from cells infected for 8 hours and IL-18 mRNA expression was measured by RT-PCR. Collectively the purpose of these experiments was to characterize the nature of the local immune responses to *C. jejuni* and *T. suis* and to determine whether cytokine dysregulation could play a role in the pathology and disease caused by dual infection.

Severe colonic pathology is characteristic of *C. jejuni* infection in humans, therefore characterizing the immune response in the colon is critical for understanding the pathogenesis of this disease. In a swine infection model *C. jejuni* targets distal colonic LGCs, stimulating germinal center expansion with IgA secreting B cells [2, 5]. To assess the effect of *C. jejuni* infection on cytokine expression in the distal colon and LGCs, I developed a rectal challenge model of infection. Weaned piglets inoculated rectally with $2x10^9$ cfu of *C. jejuni* had 2-3 fold increases in expression of IL-18, TNFα, and IL-8 in distal colon samples excluding lymphoid tissue 1 hour after infection, with a sustained increase in IL-8 expression 24 hours after infection. Concurrently, 3 to 20 fold increases in mRNA expression of IL-1β, IL-6, IL-8, IL-10, IL-18, GM-CSF, iNOS, MCP-1, and TNFα were measured from LGCs 1 hour after infection. Expression of IL-4, IL-5, IL-12p35, IL-12p40, and IL-13 was unaffected by *C. jejuni* infection. These data show that *C. jejuni* stimulates a proinflammatory response *in vivo*, and the more robust response in LGCs supports the hypothesis that these secondary lymphoid tissues are important sites for induction of an immune response directed against this pathogen.

C. jejuni colonizes the intestinal mucus in the colon, associating with epithelial cells, goblet cells, and invading the deep crypts [1, 2]. In this thesis, I demonstrated through immunohistochemistry and laser capture microdissection that rectally inoculated

C. jejuni is capable of migrating to the same locations as orally inoculated bacteria. This occurs despite the fact that they did not travel through the acidic pH of the stomach or the bile rich environment of the small intestine, two environmental conditions that have been shown to stimulate C. *jejuni* virulence *in vitro* [6, 7]. These data correlate with those found by Bell and Manning who showed that rectal inoculation of naïve ferrets resulted in colonization and diarrheal disease similar to their orally inoculated counterparts [8]. Although rectal inoculation in ferrets has already demonstrated the colonization and disease causing potential of *C. jejuni* infection by this nontraditional route, we used this technique in swine because they are more similar to humans physiologically and we were able to analyze the local cytokine responses in LGCs which have been characterized in pigs. Ultimately, these data show that rectal inoculation is a useful technique for studying *in vivo* effects of infectious agents with better definition of the affected area and more precise timing of responses.

The availability of a genetically modified strain of *C. jejuni* that behaved identically to the wild type parent strain facilitated detection and identification in a non germ free or specific pathogen free animal infection model. In the rectal challenge study I used a strain of *C. jejuni* that had been electroporated with a plasmid containing the *gfp* gene under the influence of a constitutively active promoter. This strain was reisolated from the feces of a pig 24 hours after rectal inoculation, and was quickly identified by examination with an epifluorescent microscope. The original goal was to use this strain to allow for easy detection of the bacteria in frozen sections of tissue by fluorescence microscopy. Mixter and colleagues used a strain of *C. jejuni* expressing gfp to infect mice intraperitoneally and were able to track the bacteria by flow cytometry for Gfp. They

proposed this as a useful technique for detecting the bacteria *in vivo* [9]. While detection of fluorescent *C. jejuni* was successful in the mouse peritoneal infection model, in the swine model examination of unstained tissue sections microscopically for the fluorescent bacteria was unsuccessful. The primary impediment to accurate visualization of the strain used for inoculation was auto fluorescence of the intestinal tissue that masked the fluorescent bacteria. I tried to overcome this problem by treating the tissues with propidium iodide to stain nucleic acids to produce background staining of a different wavelength than Gfp fluorescence to facilitate visualization of the bacteria. Unfortunately, due to the small size of the bacteria and the presence of debris in the intestine that did not stain with propidium iodide, the bacteria could not be distinguished definitively. In fact, small fluorescent particles of the same size as the bacteria were present in control pigs as well as infected animals. This phenomenon has also been encountered in cecal and intestinal samples from chicks infected with *C. jejuni* with the same *gfp* construct (Anna Bates, personal communication). The presence of fluorescent particles in the intestines severely limited the usefulness of fluorescently tagged *C. jejuni* for direct visualization in our system and necessitated the use of IHC, LCM, and DNA tests to detect and localize the inoculating strain used in the rectal challenge experiment. However, the *gfp* marker was very useful for distinguishing the inoculating strain from naturally occurring *Campylobacters* in experimentally infected pigs.

IL-18 became a focal cytokine in this work because it has been shown to play an important early role in the immune response to other enteric infections with similar pathogenesis. IL-18 is a pleiotrophic cytokine that functions in both innate and acquired immunity [10, 11]. In mammals studied to date, its mRNA and protein are both

constitutively expressed throughout intestinal tissues, including gut associated lymphoid tissues and intestinal epithelial cells [12, 13]. IL-18 production early in the response to *Shigella* and *Salmonella* infections is critical for stimulation of a Th 1 directed immune response with increased production of IFNγ, which is essential for resolution of disease [14-16]. *In vitro* studies have shown that *C. jejuni* stimulates secretion of IL-1β, IL-6, IL-8, and TNFα from swine intestinal epithelial cells (Cunningham et al, unpublished data; Parthasarathy et al, unpublished data). In addition to initiating a Th 1 directed immune response, IL-18 stimulates proinflammatory cytokine production from leukocytes *in vitro* [14-18]. Therefore, we hypothesized that *C. jejuni* stimulates secretion of IL-18 from cultured swine intestinal epithelial cells and that this functions to stimulate further immune responses that kill the bacteria. Differentiated IPEC-1 cells secreted significant amounts of IL-18 as early as 3 hours after infection. IL-18 levels remained high at 6 and 8 hours after infection, and then decreased by 24 hours. In contrast, undifferentiated IPEC-1 cells did not secrete significant amounts of IL-18 in response to *C. jejuni* at any time, despite the fact that they secreted IL-18 in response to the positive control, Con A. Additionally, mRNA expression of IL-18 was not significantly altered by *C. jejuni* 8 hours after infection of IPEC-1 cells in either differentiation state. Taken together, these data show that *C. jejuni* does induce IL-18 production from differentiated cells. Increased secretion in differentiated IPEC-1 cells does not correlate with increased mRNA expression, suggesting that transcriptional regulation does not play a role in the IL-18 response of these *C. jejuni* infected intestinal epithelial cells. The increased expression of IL-18 seen in the rectal challenge model may be due to the intraepithelial lymphocytes, macrophages, or other leukocytes present in the lamina propria and submucosa of the

distal colon and the follicles of the LGCs in live pigs with the full anatomic complexity of the gastrointestinal tract. Increased secretion from differentiated IPEC-1 cells is likely due to increased cleavage of stored immature protein by Caspase-1, facilitating its release from the cell. These data suggest that IL-18 functions in host defense against *C. jejuni* and that intestinal epithelial cells may be an important source of IL-18 in combating this infection *in vivo*.

Immunomodulation during concurrent infections can determine whether host pathology and disease are enhanced or abrogated. Pigs are naturally resistant to *C. jejuni*, which is likely due in part to a proinflammatory response initiated early in life during a primary infection. Upon infection with the swine whipworm *T. suis* in the proximal colon, *C. jejuni* invade tissues surrounding the site of worm attachment and LGCs in the distal colon [2, 3]. We hypothesized that *C. jejuni* invasion secondary to *T. suis* infection is due to downregulation of a protective proinflammatory immune response. To address this hypothesis, weaned piglets were orally inoculated with embryonated *T. suis* eggs. Twenty one days after inoculation, pigs were orally infected with *C. jejuni*. Two days after *C. jejuni* infection, pigs were killed and we measured cytokine mRNA expression in the jejunum, proximal colon, and distal colon of pigs infected with T. *suis, C. jejuni*, or both. In the proximal colons of both groups of pigs that received *T. suis*, IL-13 expression was upregulated 3 to 4 fold over uninfected controls. Concurrently, expression of cytokines known to be important for clearance of bacterial infections like IL-8 was decreased 2 to 3 fold, and IL-12 expression was decreased 2 to 4 fold compared to uninfected controls. Additionally, expression of both the chemokine MCP-1 and the Th 2 cytokine IL-5 decreased 2 fold in the dual infection group compared to uninfected

controls. At the same time, there were no significant differences in expression of any cytokines in the proximal colons of pigs infected with *C. jejuni* only compared to uninfected controls.

These data suggest a role for *T. suis* stimulated immunomodulation in the proximal colon. *T. suis* infection increased expression of IL-13 while concurrently decreasing the expression of proinflammatory cytokines in the proximal colon. IL-13 has been shown to decrease secretion of IL-8 from stimulated human intestinal epithelial cells *in vitro* [19]. Exogenous recombinant IL-13 administered to mice concurrently with a lethal dose of LPS decreases production of IL-12p40 and protects the mice from lethal toxemia [20]. Additionally, egg antigens from the intestinal helminth *Schistosoma mansoni* stimulate IL-10 production from dendritic cells, while decreasing production of IL-12 [21]. Based on *T. muris* infection studies in mice, we expected to see increased expression of IL-10 in the tissues of these *T. suis* infected pigs along with increased expression of IL-13. IL-10 expression was not increased in any tissue sampled from orally infected pigs, however a concurrent study on secreted cytokines in the feces of these pigs showed increased IL-10 in *T. suis* infected pigs on day 22 (Parthasarathy et al, unpublished results). Increased expression of IL-10 may not have been measured because we examined tissues 23 days after *T. suis* infection, which was after the increase of fecal cytokine levels was measured. Also, *T. muris* infection studies in mice generally focus on cytokine production in draining lymph nodes, which does not correlate exactly with local cytokine expression in the tissues. Nevertheless, increased production of IL-10 on day 22 likely had immunomodulatory effects on cytokine expression on day 23. The combined actions of *T. suis* stimulated IL-10 and IL-13 are likely responsible for decreased

expression of IL-8, IL-12, and possibly MCP-1 as well. Based on data from previous dual infection studies, we expected to see increased numbers of *C. jejuni* in the crypts and tissues of dual infected pigs. *C. jejuni* was detected by RFLP analysis in 4 of 6 pigs from the dual infection group, and 1 of 6 pigs from each of the single infection groups and the controls. Increased prevalence of *C. jejuni* in the proximal colon of dual infected pigs compared to pigs that received *C. jejuni* only suggests that *T. suis* facilitates its proliferation and possibly invasion. Detection of *C. jejuni* from control animals suggests that *C. jejuni* was likely present in low numbers as a commensal in these pigs. Taken together, these data suggest that decreased proinflammatory cytokine expression resulting from *T. suis* infection facilitates proliferation of *C. jejuni.*

In the distal colon, cytokine expression was only altered significantly in the *T. suis* only group. Three to four fold increases in expression of IL-1β, IL-6 and IFNγ were measured, while expression of IL-13 was upregulated 3 fold compared to uninfected controls. Increased expression of IL-1β, IL-6 and IFNγ is indicative of an inflammatory response, possibly due to secondary bacterial infection. *C. jejuni* was not detected by RFLP in the distal colons of any pigs, however, it has previously been demonstrated that *T. suis* facilitates secondary invasion of many species of resident bacteria, including *C. coli*, an unidentified *Campylobacter spp.*, and *E. coli* [3]. Invasive strains of *C. coli* stimulate IL-8 secretion from cultured human intestinal epithelial cells [22]. Analysis of distal colon tissues by RFLP of the Campylobacter 23S rRNA gene detected *C. coli* in 2 of 6 pigs, and an unidentified species of *Campylobacter* in 3 of 6 pigs. Thus it is possible that either of these species invaded tissues in the distal colon and initiated an inflammatory response. It has also been shown that oral inoculation of pigs with *E. coli*

DH5α causes increased secretion of proinflammatory cytokines in the feces (Cunningham et al, unpublished results). Larval stages of *T. suis* produce ESP *in vivo*, as is evidenced by the fact that serum antibodies directed against a 20kDa glycoprotein isolated from adult worm culture fluids can be detected as early as 21 days after inoculation with embryonated eggs [23]. In addition, *T. suis* ESP stimulates production of IL-6 and IL-10 from IPEC-1 swine intestinal epithelial cells *in vitro*. Therefore, increased expression of IL-13 in the distal colon of *T. suis* infected pigs could be attributed to the effects of excretory/secretory product (ESP) produced by the 4^{th} stage larvae in the proximal colon that have traveled to the distal colon. Taken together, these data indicate that ESP is highly stimulatory and likely has effects on intestinal epithelial cells which would increase cytokine production *in vivo* at sites distal to worm attachment.

Interestingly, although the cytokine response in the proximal colon was similar between the *T. suis* and dual infection groups, the responses diverged in the distal colon. There were no significant changes in cytokine expression in the distal colon of the dual infection group. This was unexpected, as previous studies have shown that *T. suis* facilitates invasion of *C. jejuni* and resident bacteria into the LGCs in the distal colon [2, 3], and distal colonic samples from our pigs likely included LGCs. In our rectal challenge model we showed that *C. jejuni* stimulates proinflammatory cytokines in LGCs 1 hour after infection, therefore we expected to see a strong cytokine response from the dual infected pigs. Tissues for cytokine expression from orally inoculated pigs were taken 48 hours after *C. jejuni* infection, which corresponded to 23 days after *T. suis* infection. Our rectal challenge model showed that proinflammatory cytokine responses were seen primarily 1 hour after infection, therefore it is likely that proinflammatory cytokines had

returned to baseline levels by the time samples were taken from orally infected pigs. The inability to recover *C. jejuni* from fecal culture of these pigs 48 hours after oral inoculation indicated that low levels of *C. jejuni* were present. The oral inoculum was 10^8 cfu of *C. jejuni*, while the inoculum used in the rectal challenge experiment was $2x10^9$ cfu of *C. jejuni*. Therefore the low level of *C. jejuni* present in the distal colon 48 hours may have been too low to stimulate a significant proinflammatory response. It is more likely that the absence of a measurable proinflammatory cytokine response in the distal colons of dual infected pigs is due to sampling after the response has waned. Indeed, gnotobiotic piglets orally inoculated with only 10^6 cfu of *C. jejuni* 33292 developed transient fever that lasted only for the first 24 hours after infection, suggesting that *C. jejuni* stimulated a proinflammatory response with increased production of IL-6 that caused fever [2].

Another issue that may have affected distal colonic cytokine responses is the larval stage present in the pigs. *T. suis* undergoes 4 molts from L1 larvae to L5 adults in about 41 days [24, 25]. Mucohemorrhagic diarrhea associated with *T. suis* infection can occur as early as 14 days in heavy infection, but on average occurs around 21 days [26, 27]. This corresponds to the emergence of the posterior end of L4 larvae into the lumen [24, 25]. The severity of diarrheal disease and pathology increases as worms mature to adults, reproducing and excreting significant amounts of ESP [27] (Linda Mansfield, personal communication). Progression of disease and pathology likely correlates with intensified cytokine responses. Though *T. suis* larvae were present in the proximal colons of dual infected pigs, and significant histologic changes were seen, lesions were confined to the areas where worms were present. The time that the pigs in our study were sampled

corresponded to the beginning of the molt from L3 to L4 larvae, and ESP production would have been low. Therefore, it is possible that in the dual infection groups, distal colonic effects of *T. suis* would not yet have been manifested. Analysis of cytokine expression at later time points, including 30 days and 41 days after *T. suis* infection would determine whether an inflammatory response is induced in the distal colon as disease progresses.

C. jejuni infection alone stimulated significant changes in the jejunum of orally infected animals. In the jejunum of *C. jejuni* infected pigs expression of MCP-1, TNFα, IL-12p40, IFNγ, IL-4, and IL-10 were increased 3 to 5 fold over uninfected controls. *C. jejuni* stimulates intracellular production of MCP-1, TNFα, IFNγ, IL-4, and IL-10 from cultured human intestinal epithelial cells [28, 29]. Additionally, jejunal samples likely contained samples of peyers patches as well as surrounding epithelium. It has been demonstrated in our rectal challenge model, and in an oral challenge model of germ free piglets [2] that *C. jejuni* targets LGCs and stimulates robust cytokine responses. It is likely that *C. jejuni* stimulated a similar response in the jejunal Peyers patch, which would have influenced our results. These results indicate that *C. jejuni* stimulates a proinflammatory response in the jejunum in the absence of *T. suis*. In the jejunum of pigs infected with *C. jejuni* and *T. suis* concurrently, GM-CSF is downregulated 5 fold compared to uninfected controls, and the rest of the cytokines measured were not significantly different that uninfected controls. This suggests that *T. suis* exerts immunomodulatory effects in the jejunum as well as the proximal colon, down regulating *C. jejuni* induced proinflammatory cytokines. These effects could be due to systemic IL-13 and IL-10 produced by lymphocytes stimulated by worm antigens in draining lymph

nodes. Examination of cytokine expression in draining lymph nodes and peripheral leukocytes would be necessary to confirm this.

Increased expression of proinflammatory cytokines in the distal colon and LGCs of rectally inoculated pigs and in the jejunum of orally inoculated pigs indicated that *C. jejuni* stimulated a proinflammatory response *in vivo*. However, the fact that the pigs in these studies were immunocompetent, conventionally reared animals with a complete intestinal microbial population raises the question of whether some of the cytokine responses are the result of direct actions of *C. jejuni* on intestinal tissues, or indirect actions due to perturbation of intestinal flora homeostasis by the introduction of exogenous bacteria. Pigs orally infected with a non-pathogenic laboratory strain of *E. coli* DH5α have increased secretion of IL-6 in the feces 48 hours after inoculation, but this strain does not invade swine intestinal epithelial cells *in vitro* (Parthasarathy et al, unpublished results). Introduction of exogenous bacteria could increase the total intestinal bacterial load above normal levels. Additionally, any exogenous strain could compete with resident strains for resources or provide an essential growth factor, causing an imbalance in the intestinal environment and facilitating overgrowth of resident strains. These factors could stimulate inflammatory responses in an effort by the host to reduce total bacterial numbers and possibly eliminate the exogenous strain, restoring balance to the microbial population. Taking this into account, exogenous *C. jejuni* may have stimulated a host inflammatory response indirectly. However, *in vitro* data definitively shows that *C. jejuni* infection alone stimulates secretion of a similar panel of proinflammatory cytokines from cultured intestinal epithelial cells, suggesting that the

responses seen *in vivo* can be attributed to both direct and indirect effects of *C. jejuni* on intestinal tissues.

Collectively, the data from this dissertation project support the hypothesis that cytokine dysregulation during dual infection with *T. suis* and *C. jejuni* is a major cause of the severe disease and pathology observed. From this analysis of local cytokine expression in pigs infected singly or concurrently with *C. jejuni* and *T. suis*, we propose the following model for the roles of specific cytokines in disease progression *in vivo*. A proinflammatory response is stimulated by *C. jejuni* early in the infection, from both intestinal epithelial cells and LGCs. IL-18 released from intestinal epithelial cells early in the immune response induces IL-1β, IL-8, and TNFα from leukocytes, amplifying and sustaining inflammation. Additionally, epithelial cell derived IL-18 recruits and activates neutrophils to engulf and eliminate extracellular bacteria. With the addition of *T. suis*, IL-13 is upregulated while IL-8 and IL-12 are downregulated. IL-13 and IL-4 stimulate mucin production from intestinal epithelial cells [30, 31] which provides a favorable environment for proliferation of *C. jejuni* [32, 33]. Piglets infected with a low dose of *C. jejuni*, 10^6 cfu, develop transient fever but no diarrhea, while piglets infected with high doses of *C. jejuni*, 4-5x10^9 cfu, develop diarrheal disease and pathology [1, 2, 34]. Overgrowth of *C. jejuni* in a mucus rich environment could be responsible in part for the mucohemorrhagic diarrhea seen in dual infection. Also, IL-13 downregulation of IL-8 is likely to abrogate neutrophil recruitment and containment of bacteria in the epithelial layer [35, 36], allowing invasion by significantly increased numbers of *C. jejuni* into deeper tissues.

Other cytokines may enhance invasion of *C. jejuni*. IL-4 has also been shown to decrease transepithelial electrical resistance of differentiated IPEC-1 cells and increase invasion of *C. jejuni* (Parthasarathy et al, unpublished results). Although increased IL-4 expression was not detected in our oral challenge study, concurrent analysis of secreted cytokines in the feces showed increased secretion on day 22 in dual infected pigs (Parthasarathy et al, unpublished results). *In vivo*, *T. suis* induced IL-4 may weaken tight junctions between epithelial cells allowing bacteria access to the basolateral surface of cells. These bacteria that have breached the epithelial barrier could bind fibronectin in the extracellular matrix via CadF [37] and gain access to the lamina propria and submucosa. Another factor to consider is the secreted worm products. ESP has a dose dependent cytotoxic effect on undifferentiated epithelial cells. Additionally, ESP exposed to both apical and basolateral surfaces of differentiated cells significantly decreases transepithelial electrical resistance and damages perijunctional complexes, making cellular junctions more permissive [34]. The combined effects of ESP on differentiated and undifferentiated epithelial cells would result destruction of the epithelial barrier *in vivo*, allowing invasion of *C. jejuni* into deeper tissues, increasing the severity of disease. Ultimately, the combined actions of *T. suis* stimulated IL-4 and IL-13 would provide a favorable environment for *C. jejuni* proliferation, while dampening the host response that would protect against invasion by overwhelming amounts of *C. jejuni*.

Future Directions

The analysis of local cytokine expression in intestinal tissues from *C. jejuni* and *T. suis* infected pigs expanded our knowledge of the swine immune response to these two agents and shed some light on cytokine dysregulation that can occur during infection with these two agents. These data would be enhanced by exploring several aspects of this dual infection phenomenon. In chapter 2 we analyzed cytokine expression in the distal colon and LGCs of pigs rectally inoculated with *C. jejuni.* Further analysis of cytokine expression at different times, for example 4, 8, and 12 hours after infection, would be instructive as to the *in vivo* kinetics of cytokine expression in response to *C. jejuni.* Additionally, since our data showed that the cytokine response was more robust in the LGCs compared to the distal colon, identification of cytokine producing cells by in situ hybridization or flow cytometry in these two tissues would elucidate the specific cells activated by *C. jejuni.* Concurrent analysis of intracellular protein within the cells by immunohistochemistry, and of proteins secreted into the feces by ELISA would allow comparison of changes in expression and changes in secretion.

In chapter 3 we analyzed cytokine expression in the jejunum, proximal colon, and distal colon of pigs orally infected with *C. jejuni, T. suis*, or both. As with the rectally inoculated pigs, further analysis of cytokine expression at different times with concurrent analysis of intracellular and secreted proteins would expand our knowledge of *in vivo* cytokine kinetics. Analysis of cytokine expression 4, 8, 12, and 24 hours after infection would allow us to determine if proinflammatory responses were induced in *C. jejuni* infected pigs early in the response to oral infection. In addition, examination of cytokine

expression at later time points, for example on day 30, day 41, and day 48 after *T. suis* infection, would help us to asses cytokine changes that may coincide with developmental changes in *T. suis*. Concurrent examination of pathologic changes would correlate lesion progression with cytokine changes.

Previous studies of immunoglobulin production from piglets orally inoculated with *C. jejuni* and the rectal challenge study from this dissertation demonstrated the importance of LGCs as sites for induction of immune responses to *C. jejuni*. To further characterize the cytokine response to *C. jejuni* infection, identification of specific cell types that are reactive to *C. jejuni* and characterization of their cytokine responses should be undertaken. To achieve this, multiple LGCs would be collected from pigs orally or rectally infected with *C. jejuni*. Individually, each LGC would be gently disrupted to make single cell suspensions and an aliquot would be tested for the presence of *C. jejuni* by PCR amplification of an appropriate gene, for example the QRDR of the *gyrA* gene. LGCs that do not contain *C. jejuni* would serve as internal negative controls for each pig. The cells would then be stimulated in vitro with *C. jejuni* and subsequently stained for specific cell markers to detect CD4+ cells, CD8+ cells, B-cells, and macrophages. The cells would be separated by flow cytometry and assayed for expression of a panel of proinflammatory and anti-inflammatory cytokines by real time PCR. This study would determine which cell types are involved in the immune response to *C. jejuni* and also provide information about the distribution of *C. jejuni in vivo*.

In chapter 4 we measured IL-18 secretion from differentiated and undifferentiated IPEC-1 cells infected with *C. jejuni*. The difference in responses between the two differentiation states raises the question of whether cellular maturation coincides with

intracellular signaling pathways that allow differentiated cells to secrete IL-18 in response to *C. jejuni*, while undifferentiated cells do not. This question could be addressed by examination of *C. jejuni* activated intracellular signaling molecules using western blot analysis of intracellular proteins. We may find that at some point in the signal transduction cascade a key activator of signaling molecules specific to the IL-18 pathway is absent or repressed in undifferentiated cells, indicating that the differentiation process includes synthesis of this activator or derepression of its expression. We also found that *E. coli* LPS does not stimulate IL-18 secretion from differentiated IPEC-1 cells. Previous studies have shown that adherence, invasion, and Cdt are virulence factors of *C. jejuni* that stimulate IL-8 secretion from intestinal epithelial cells [22, 38]. The effect of these and other specific virulence factors on IL-18 secretion from swine intestinal epithelial cells should be explored to determine the mechanisms that *C. jejuni* uses to stimulate host cytokine responses in the gut.

One factor that affected interpretation of the results from our *in vivo* studies, discussed in chapters 2 and 3, was the presence of other *Campylobacters* in conventionally reared pigs. It has been reported that healthy pigs can harbor *C. jejuni*, *C. coli*, and *C. lari* as commensals [4]. In my studies *C. coli* was detected by RFLP analysis of the *Campylobacter* 23S rRNA gene in both rectally and orally infected control pigs. An unidentified *Campylobacter* species that had an RFLP pattern that was different from all the other thermophilic *Campylobacters* detected by this assay was found in at least one intestinal segment from virtually 100% of orally infected pigs. Immunohistochemistry using antibody raised against whole *C. jejuni* bacteria positively stained organisms in the colons of all experimental pigs. Antibody validation tests

revealed cross reactivity with *C. lari*, but preliminary PCR analysis of tissue DNA did not detect *C. lari* in any pigs regardless of infection route. These data suggest that another *Campylobacter* species was present in the colons of our experimental animals that occupied the same niche as *C. jejuni*. A detailed analysis of the resident *Campylobacter* strains in experimental animals would help to determine whether the changes in cytokine expression were due to direct interaction of *C. jejuni* with colonic tissues, to the interaction of *C. jejuni* with the unidentified Campylobacter resulting in it invading tissues, or to the interaction of *C. jejuni* with the resident bacterial population causing a perturbation of environmental homeostasis. To address this issue we would clone and sequence 16S rRNA genes from as many *Campylobacter* species amplified from total pig intestinal DNA as possible, then compare the sequences obtained from our pigs to sequences in bacterial ribosomal databases. The results from this experiment may reveal that *C. lari* is present in the pigs, that a variant of *C. jejuni*, *C. coli*, or *C. lari* is present that gives a unique RFLP pattern using the 23S rRNA gene, or that an as yet uncharacterized *Campylobacter* is present in the pigs. This analysis is currently underway. The data gathered from these experiments could also be enhanced by quantifying *Campylobacters* in the feces of experimental animals and comparing bacterial loads between controls and infected animals. We may find that the total bacterial load is unchanged, but the ratios of different organisms are different and might indicate a dose dependent effect of *Campylobacter* infection on local intestinal cytokine production.

In summary, the data generated from this dissertation contributed to the body of research defining host immune responses to *C. jejuni* and *T. suis* infections. We

developed a swine rectal challenge model that proved to be a useful technique for studying the complexity of the host immune response to *C. jejuni*, while allowing the precise timing and specific targeting of tissues afforded by *in vitro* models. We began to define the host local immune response to *T. suis* and the *T. suis* induced immunomodulation that may decrease natural resistance to *C. jejuni*, facilitating invasion. Finally, through *in vitro* infection studies we discovered that *C. jejuni* infected intestinal epithelial cells are a source of early IL-18 production, which may be critical in initiating a protective response to infection *in vivo*. Collectively this data gave us evidence for *T. suis* downregulation of proinflammatory responses that are detrimental to swine colonized by the opportunistic pathogen *C. jejuni*.

The results obtained from this dissertation opened several avenues for further research and generated new questions to be addressed. Experimental designs of studies to address three of these questions will be discussed in detail.

Cytokine responses of intestinal epithelial cells to multi-species *Campylobacter* infection

Healthy, conventionally reared pigs can harbor multiple Campylobacter species in their colons without evidence of disease [4]. In our swine rectal challenge model, inoculation of *C. jejuni* into the distal colons of conventionally reared pigs with naturally acquired *C. coli* stimulated increased expression of proinflammatory cytokines. The hypothesis for this study is that swine intestinal epithelial cells exposed to *C. coli* will secrete proinflammatory cytokines when given a secondary challenge with *C. jejuni.* Differentiated and undifferentiated IPEC-1 cells will be exposed to *C. coli* for 0, 1, 3, 6, 8, and 24 hours. The supernatants will be removed and saved for cytokine assay by ELISA. Then the cells will be exposed to *C. jejuni* expressing Gfp, which are also resistant to kanamycin, for 1, 3, 6, 8, and 24 hours. The supernatants will be removed and saved for cytokine assay by ELISA, and then the cell monolayers will be treated with 100mg/mL gentamicin for 1 hour to kill extracellular bacteria. The gentamicin will be removed, then the cells will by lysed in 0.1% sodium deoxycholate and the cell lysates will be plated on Bolton agar with kanamycin to select for the inoculating strain of *C. jejuni*. Supernatants from *C. coli* only infected cells as well as cells infected with *C. coli*, then subsequently infected with *C. jejuni* will be assayed for IL-1β, IL-6, IL-8, IL-18,

and TNFα by ELISA. These experiments will determine 1) if *C. coli* stimulates proinflammatory cytokine production from IPEC-1 cells; 2) if *C. jejuni* stimulates a proinflammatory response in cells previously exposed to *C. coli*; and 3) if *C. jejuni* invasion into IPEC-1 cells is altered by prior exposure of the cells to *C. coli*.

The effects of IL-13 on proinflammatory cytokine production and *C. jejuni* invasion *in vivo*

T. suis induced IL-13 downregulates expression of proinflammatory cytokines in the proximal colons of pigs concurrently infected with *C. jejuni* (Jones et al, discussed in Chapter 3). In a rectal challenge model, expression of proinflammatory cytokines is significantly increased in pigs inoculated with *C. jejuni*. The hypothesis for this study is that IL-13 will downregulate proinflammatory cytokines in the distal colon and facilitate invasion of epithelial cells by *C. jejuni*. Using a rectal challenge model, the distal colons of pigs will be pretreated with recombinant IL-13, serum free media, or no treatment for 5 hours, then all pigs will be infected with *C. jejuni* expressing Gfp rectally. Three hours after *C. jejuni* infection, the pigs will be killed and the distal colons will be removed. Intestinal epithelial cells will be isolated by the enzymatic digestion technique described by Babakhani and Joens [1]. Briefly, the colonic sections will be washed with PBS, then the enterocytes will be detached by incubation with 0.25% trypsin-1mM EDTA at 37°C on a rotator. Detached cells will be washed three times with MEM supplemented with 1% FBS, then the cells will be incubated for 1 hour with 100μg/mL gentamicin in MEM supplemented with 1% FBS to kill extracellular bacteria. The cells will then be fixed with paraformaldehyde, and cells containing internalized fluorescent *C. jejuni* can be separated and counted by flow cytometry. The cells could then be stained for intracellular IL-8 protein with specific antibodies. These experiments will determine 1) if recombinant IL-13 facilitates increased invasion of intestinal epithelial cells with *C. jejuni in vivo*; 2) if *C.*

jejuni invasion stimulates IL-8 production from intestinal epithelial cells; and 3) if *C. jejuni* stimulated IL-8 secretion is decreased by IL-13 pretreatment.

The effects of IL-18 on *C. jejuni* invasion of intestinal epithelial cells *in vitro*

IL-18 is a pleiotrophic cytokine that is induced *in vivo* during infections with enteric bacteria, and is critical for host resistance to the pathogens. *C. jejuni* stimulates IL-18 secretion from differentiated IPEC-1 cells in vitro. In addition, IL-18 expression is significantly increased in the distal colons and LGCs of pigs one hour after rectal inoculation with *C. jejuni*. The hypothesis of this study is that early production of IL-18 from intestinal epithelial cells after infection with *C. jejuni* is important for resistance to invasion. Two experimental designs will be used to test this hypothesis. In the first experiment, differentiated IPEC-1 cells will be pretreated with different doses of recombinant IL-18 for 5 hours. The supernatant will be removed and saved for later cytokine assay. The pretreated cells will then be infected with *C. jejuni* for 3 hours, and then treated with 100μg/mL gentamicin for 1 hour to kill extracellular bacteria. The gentamicin will be removed, the cells will be lysed with 0.1% sodium deoxycholate, and cell lysates will be plated to enumerate internalized bacteria. In the second experiment, differentiated IPEC-1 cells will be treated with anti-IL-18 antibody and concurrently infected with *C. jejuni*. Supernatants will be removed 1, 3, 6, 8, and 24 hours after infection and saved for later cytokine assay. The infected monolayers will be treated with gentamicin to kill extracellular bacteria, then lysed and plated to enumerate internalized bacteria. The supernatants from both experiments will be analyzed by ELISA for IL-1β, IL-6, and IL-8 which can all be induced by IL-18 stimulation [17, 18]. These experiments will determine 1) if pretreatment with recombinant IL-18 enhances IPEC-1 resistance to invasion; 2) if blocking of IL-18 with antibodies during *C. jejuni* infection facilitates

invasion; and 3) if IL-18, either exogenous or recombinant, induces increased secretion of IL-1β, IL-6, and IL-8 during *C. jejuni* infection.

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