INTERPLAY OF HOST AND PATHOGEN FACTORS DETERMINES IMMUNITY AND CLINICAL OUTCOME FOLLOWING *CAMPYLOBACTER JEJUNI* INFECTION IN MICE

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ABSTRACT

INTERPLAY OF HOST AND PATHOGEN FACTORS DETERMINES IMMUNITY AND CLINICAL OUTCOME FOLLOWING *CAMPYLOBACTER JEJUNI* INFECTION IN MICE

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Campylobacter jejuni is a common cause of bacterial enteritis worldwide, and is associated with the post-infectious neuropathy Guillain-Barré syndrome (GBS). The currently accepted pathogenesis of *C. jejuni*-associated GBS involves generation of cross-reactive antibodies to the *C. jejuni* LOS and structurally similar gangliosides enriched in peripheral nerves. Complex host-pathogen interactions determine innate and adaptive immunity and corresponding disease outcomes. The overarching aim of this study was to determine how these interactions underlie contrasting immune responses to *C. jejuni* in mice, beginning with the initial interaction of *C. jejuni* with dendritic cells (DCs) *in vitro* and assessing the impact on development of adaptive immunity and disease outcome *in vivo*.

The model system was developed to exploit both *C. jejuni* strain differences and immunogenetic biases reported for C57BL/6 and BALB/c mice. *C. jejuni* strains associated with colitis (*C. jejuni* 11168) and GBS (*C. jejuni* 260.94) were used in combination with C57BL/6 mice, a reportedly Th1-biased strain, and BALB/c mice, a reportedly Th2-biased strain. *C. jejuni* strains were evaluated for invasion efficiency, intracellular survival, and elicitation of pro-inflammatory and Th-polarizing cytokine production *in vitro* using bone marrow-derived DCs (BMDCs) from C57BL/6 and BALB/c mice. *In vivo* models were used to assess how local and systemic adaptive immunity, leading to disease outcomes including colitis and production of anti-ganglioside antibodies, vary by both *C. jejuni* strain and mouse genetic background.

BALB/c wild-type and IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 were first assessed as a potential GBS model. C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 were previously shown to mount Th2-mediated immunity and produce anti-ganglioside antibodies, and BALB/c mice are

reportedly Th2-biased. BALB/c mice were therefore expected to mount strong Th2-mediated responses, produce anti-ganglioside antibodies, and develop neurological deficits. Instead, infected BALB/c mice developed systemic Th1/Th17-mediated immunity, and did not develop anti-ganglioside antibodies or neurological disease. The contrasting immune response to *C. jejuni* 260.94 demonstrated by mice of two different genetic backgrounds highlights the important and incompletely understood role of host factors in determining immunity to *C. jejuni.*

The second *in vivo* study was designed to further explore these contrasting immune responses. Both C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice were orally infected with colitogenic *C. jejuni* 11168 or GBS-associated *C. jejuni* 260.94. Immunity and disease outcome were *C. jejuni* strain-specific in BALB/c IL-10$^{-/-}$ mice: *C. jejuni* 11168 stimulated strong Th1/Th17-mediated immunity and colitis with greater transmigration of *C. jejuni* through intestinal layers and higher numbers of intracellular organisms. *C. jejuni* 260.94 was less invasive and induced less robust Th1/Th17 responses, without colitis or anti-ganglioside antibody production. These findings were mirrored *in vitro,* as *C. jejuni* 11168 demonstrated higher invasion efficiency, intracellular survival ability, and stimulated more robust production of IL-6 by BALB/c IL-10$^{-/-}$ BMDCs than *C. jejuni* 260.94. The findings in BALB/c IL-10$^{-/-}$ mice indicate that outcome of infection is dependent upon the infecting *C. jejuni* strain, and that DCs have a potential early role in driving the immune response. Interestingly, infected C57BL/6 IL-10$^{-/-}$ mice in this study colonized with *C. jejuni* but developed neither the previously reported severe colitis following *C. jejuni* 11168 infection nor the Th2-mediated immunity and anti-ganglioside antibody production after *C. jejuni* 260.94 infection. *Lactobacillus murinus* was cultured from all C57BL/6 IL-10$^{-/-}$ mice, but no BALB/c IL-10$^{-/-}$ mice, at the end of the study. Further studies evaluating *L. murinus* as a potential probiotic candidate are warranted.

Results of the current studies demonstrate that immunity and disease outcome following *C. jejuni* infection depend upon the characteristics of both the infecting *C. jejuni* strain and host genetic background, and implicate DCs as early contributors to adaptive immunity.

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KEY TO ABBREVIATIONS

µg: Microgram

µL: Microliter

µm: Micrometer

µM: Micromolar

AIDP: Acute Inflammatory Demyelinating Polyneuropathy

AMAN: Acute Motor Axonal Neuropathy

AMSAN: Acute Motor Sensory Axonal Neuropathy

ANOVA: Analysis of Variance

APC: Antigen Presenting Cells

ATCC: American Type Culture Collection

BA: Bolton Agar

BMDC: Bone Marrow-Derived Dendritic Cell

BSA: Bovine Serum Albumin

CCR: CC Chemokine Receptor

CCV: *Campylobacter jejuni*-Containing Vacuole

CD: Cluster of Differentiation

cDC: Conventional Dendritic Cells

CDT: Cytolethal Distending Toxin

CFA: Freund's Complete Adjuvant

CFU: Colony Forming Unit

Cia: *Campylobacter* Invasion Antigen

CIDP: Chronic Inflammatory Demyelinating Polyneuropathy

cm: Centimeter

CV%: Coefficient of Variation

CXCR: C-X-C Motif Chemokine Receptor

DC: Dendritic Cell(s)

deg: Degrees

DNA: Deoxyribonucleic Acid

DRG: Dorsal Root Ganglion/Ganglia

DSS: Dextran Sulphate Sodium

EAN: Experimental Autoimmune Neuritis

EDTA: Ethylenediaminetetraacetic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

FBS: Fetal Bovine Serum

Fiji: Fiji Is Just ImageJ

FITC: Fluorescein Isothiocyanate

Foxp3: Forkhead Box P3

Fr: French

g: Gauge

g: Gram

g: Gravity

GBS: Guillain-Barré Syndrome

GI: Gastrointestinal

GKA: Gentamicin Killing Assay

GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor

H&E: Hematoxylin and Eosin

HBSS: Hank's Balanced Salt Solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid

Hg: Mercury

HLA: Human Leukocyte Antigen

HMW: High Molecular Weight

IBS: Irritable Bowel Syndrome

ICJ: Ileocecocolic Junction

IFN: Interferon

Ig: Immunoglobulin

IHC: Immunohistochemistry

IL: Interleukin

iNOS: Inducible Nitric Oxide Synthetase

IP-10: IFN-Gamma-Inducible Protein 10

KO: Knock-Out

LF: Left Front

LH: Left Hind

LMW: Low Molecular Weight

LOS: Lipooligosaccharide

LPS: Lipopolysaccharide

LTβ: Lymphotoxin-Beta

MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization Time of Flight

MALP-2: Macrophage-Activating Lipopeptide 2

MAP: Mitogen Activated Protein

Mbp: Megabase Pair

MCP-1: Monocyte Chemoattractant Protein 1

Med: Medium

MFI: Median Fluorescence Intensity

MFS: Miller Fisher Syndrome

mg: Milligram

MHC: Major Histocompatibility Complex

mL: Milliliter

MLN: Mesenteric Lymph Node

mM: Millimolar

MMP: Matrix Metalloproteinase

MOI: Multiplicity of Infection

mRNA: Messenger Ribonucleic Acid

MSU: Michigan State University

MyD88: Myeloid Differentiation Primary Response 88

N: Normal

NAD: Normal Antibody Diluent

NCTC: National Collection of Type Cultures

NF-κB: Nuclear Factor Kappa B

ng: Nanogram

NLRP3: Nucleotide-Binding Domain, Leucine-Rich-Containing Family, Pyrin Domain-Containing-3

nm: Nanometer

NMR: Nuclear Magnetic Resonance

NO: Nitric Oxide

NOD: Nonobese Diabetic

NOD: Nucleotide-Binding Oligomerization Domain

ns: Non-significant

OD: Optical Density

OFT: Open Field Test

p.i.: Post Infection

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PE: R-Phycoerythrin

pg: Picogram

PI 3: Phosphoinositide 3

PRR: Pattern Recognition Receptor

rcf: Relative Centrifugal Force

RF: Right Front

RH: Right Hind

RPMI: Roswell Park Memorial Institute

s: Seconds

SAPP: Spontaneous Autoimmune Peripheral Polyneuropathy

SCID: Severe Combined Immunodeficiency

SD: Standard Deviation

SEM: Standard Error of the Mean

Siglec: Sialic Acid-Binding Ig-Like Lectins

spp: Species

ssp: Subspecies

T3SS: Type 3 Secretion System

TBS: Tris Buffered Saline

Tfh: T Follicular Helper

TGF: Transforming Growth Factor

Th: T helper

TLR: Toll-Like Receptors

TMB: Tetramethylbenzidine

TNF: Tumor Necrosis Factor

Treg: T Regulatory

TRIF: Toll-Interleukin 1 (IL-1) Receptor Domain-Containing Adaptor-Inducing Beta Interferon (IFN-β)

PAMP: Pathogen-Associated Molecular Pattern

TSAB: Tryptic Soy Agar with Blood

TSAB-CVA: Tryptic Soy Agar with Blood, containing Cefoperazone, Vancomycin, Amphotericin B

TSB: Tryptic Soy Broth

Tx Grp: Treatment Group

u: Unit

URCF: University Research Containment Facility

WT: Wild-Type

YAMC: Young Adult Mouse Colon

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

CAMPYLOBACTER JEJUNI: HISTORY AND CLASSIFICATION

Campylobacter jejuni is a leading cause of human food-borne bacterial diarrhea worldwide, and is associated with numerous post-infectious sequelae. *C. jejuni* is a member of the class *Epsilonproteobacteria* and family *Campylobacteraceae*. The genus *Campylobacter* was first described in 1886, became a separate genus from *Vibrio* in 1963,[27] and, in 1973, *C. fetus*, *C. coli*, *C. jejuni*, and *C. sputorum* species were classified within the genus.[27] Characteristics of *Campylobacter* spp. include a slender rod shape which may vary from straight, curved, or spiral; cells of most species (excepting *C. gracilis*) are motile.[27] Campylobacters are Gram-negative and non-spore-forming.

Campylobacter spp., *C. jejuni* and *C. coli* in particular, are currently considered one of the most common causes of human bacterial gastroenteritis in the United States and worldwide.[22; 102; 121] However, difficulties in isolating the organism prevented successful identification and classification for many years. Two major clinical and laboratory contributions furthering our understanding of this organism occurred in the 1970s: use of a filtration technique allowing successful isolation of "related vibrio" from human stool in addition to blood samples[28] and development of selective culture medium containing vancomycin, polymyxin B, and trimethoprim.[107] These two advancements contributed to the identification of *Campylobacter* as a hitherto unrecognized common cause of bacterial enteritis.

Since its initial discovery, classification, and rise to prominence as an important etiologic agent of diarrhea worldwide, an additional milestone in understanding the biology of *C. jejuni* was the publication of the first *C. jejuni* genome by Parkhill *et al*. in 2000.[93] Sequencing of the enteritis-associated *C. jejuni* NCTC11168 strain revealed a small (approximately 1.6 Mbp) genome with several interesting features, including hypervariable sequences found in genes involved in surface structure synthesis or modification.[93] The genome sequences of numerous other *C. jejuni* strains, including some associated with various clinical manifestations such as the human post-infectious peripheral neuropathy

Guillain-Barré syndrome,[92] abortion in livestock,[119] that of the highly virulent and commonly studied *C. jejuni* 81-176,[47] and many others have since become available as complete or draft genome sequences.

ENTERIC CAMPYLOBACTERIOSIS: EPIDEMIOLOGY AND DISEASE MANIFESTATIONS

Animal Infection*. Campylobacter* spp. are well recognized causes of bovine diarrhea, reproductive disease including abortion and infertility in cattle and sheep, and gastroenteritis in dogs and cats.[67; 89] However, many mammals and birds may be asymptomatically colonized with *C. jejuni* or *C. coli.*[114] Sources of infection to humans thus include contaminated meat (especially poultry), unpasteurized milk, and inadequately treated water. Poultry in particular are important reservoirs for human infection with *C. jejuni*, with high numbers of *C. jejuni* (10^4-10^7 CFU/g) detected in the ceca in natural[90] and experimental[9] infections. Interestingly, in chickens *C. jejuni* heavily colonizes the crypts of the cecum, large intestine, and cloaca, but appears to preferentially colonize the mucus layer, without attachment to microvilli and incites little to no pathology experimentally.[9] Domestic poultry are essentially asymptomatic carriers,[69] and the high pathogen load poses a public health risk from contamination during processing at the abattoir. The infectious dose of *C. jejuni* is as low as 800 organisms, determined in healthy human volunteers.[15]

Human Campylobacteriosis Epidemiology. The vast majority of human campylobacteriosis has historically been attributed to infection with *C. jejuni* or *C. coli.*[67] *Campylobacter* is considered by the World Health Organization as one of four key global causes of diarrheal disease, and the most common etiology of human bacterial gastroenteritis worldwide.[121] Recent estimates from surveillance data suggest that more than 800,000 cases of domestically acquired foodborne illness, resulting in over 8,000 hospitalizations and more than 70 deaths, are caused by *Campylobacter* spp. each year in the United States.[102]

Human Clinical Manifestations. Different clinical manifestations of enteric campylobacteriosis are recognized in developing and industrialized countries.[42] Repeated infections are common in children in developing countries, with clinical severity and duration of colonization and excretion decreasing with age.[16; 114] Fewer symptomatic infections occur in older children and adults, consistent with protective immunity resulting after multiple exposures.[42] In contrast, infections in patients in developed countries, while generally self-limiting, are clinically severe, with fever, diarrhea, and abdominal cramping.[42] The diarrhea is often bloody; leukocytes in stool aid in clinical diagnosis. The most common clinical features reported in community outbreaks included diarrhea, abdominal pain, fever, myalgia, and headache.[16] Histological changes are consistent with acute infectious colitis and may vary depending upon stage of infection.[112] Predominant histopathology includes neutrophilic infiltration, ulceration, presence of exudate, cryptitis and crypt abscesses, edema and inflammatory cell infiltration of the lamina propria.[112]

Animal Models of Gastrointestinal* C. jejuni *infection. The relatively recent recognition of *C. jejuni* as an important human pathogen and the lack of robust, reproducible small animal models of *C. jejuni*-induced colitis hindered our understanding of the pathogenesis and immune responses of campylobacteriosis until recently. Though chickens are natural hosts of *C. jejuni* and a significant reservoir for human infection, outcomes of infection and colonization in domestic poultry do not parallel the disease induced in humans. Several studies of *C. jejuni* infection in immunocompetent adult mice of different genetic backgrounds with conventional murine microbiotas have resulted in colonization, either transient or stable, but without induction of disease as seen in humans.[13; 18; 23; 76-78] Possible reasons immunocompetent mice appear resistant to *C. jejuni*-induced enteritis include both murine factors (cross reactive immunity against other enteric bacteria, inherent characteristics of the murine microbiome), and *C. jejuni* factors (differences in infecting strain, number of *in vitro* passages).[78]

Building on early investigations, ferret, rabbit, and murine models were subsequently developed that produce enteric symptoms and pathology mimicking human disease. Ferrets infected with *C. jejuni*

strain CG8421 developed diarrhea and colon histopathology resembling human disease, including epithelial cell loss and infiltration of neutrophils and macrophages.[86] Cimetidine-treated infant rabbits infected with *C. jejuni* NCTC 11168 developed diarrhea and swollen, red, distended intestines; histopathological and ultrastructural changes included edema, capillary congestion in cecal villi, epithelial damage, and infiltration of intraepithelial lymphocytes.[104]

Mouse Models of Human Colitis. Four landmark mouse models exploiting alterations in the immune system, microbiota, or both have furthered our understanding of *C. jejuni* colonization and pathogenesis. Mansfield *et al* (2007) showed that both wild-type (WT) C57BL/6 and congenic interleukin (IL)-$10^{-/-}$ mice infected with *C. jejuni* 11168 stably colonized; the WT mice did not develop clinical signs or pathology, while the IL-$10^{-/-}$ mice exhibited severe typhlocolitis.[76] Histopathology resembled that seen in human disease, including marked polymorphonuclear and mononuclear inflammation in the lamina propria and epithelial erosion and ulceration.[76] Fox and colleagues (2004) infected both WT and NF-κB-deficient (3X) C57BL/129 mice with *C. jejuni* 81-176 (WT and *cdtB* knockout (KO) strains).[33] They found that both WT and KO *C. jejuni* 81-176 persistently infected 3X, but not WT, mice at 2—4 months p.i. Histopathology, primarily characterized by gastritis and duodenitis, was most severe in 3X mice infected with the WT *C. jejuni* 11168.[33] Chang and Miller (2006) observed that following oral inoculation with *C. jejuni* 81-176, WT C3H mice with normal flora did not colonize as efficiently as C3H mice harboring a defined, limited enteric flora consisting of *Clostridial* species, *Lactobacillus*, and *Acinetobacter*. Furthermore, SCID mice (effectively lacking mature B and T lymphocytes) also harboring the limited flora remained persistently colonized, and in contrast to WT mice with the limited flora, exhibited severe inflammation, particularly in the cecum.[23] Notable lesions included inflammatory cell infiltration in the lamina propria and submucosa, accompanied by edema, ulceration, and hemorrhage.[23] Finally, mice with a "humanized" microbiota perorally infected with *C. jejuni* ATCC 43431 exhibited stable colonization and pronounced inflammatory responses in the colon including increased apoptotic cells,

neutrophils, T and B lymphocytes, and T regulatory (Treg) cells, while mice with a conventional murine microbiota cleared *C. jejuni* in 2-3 days.[12] These studies have greatly enhanced our understanding of the role of the immune system, specifically IL-10, NF-κB (transcription factor involved in immune cell activation) and mature T and B lymphocytes, and the mouse intestinal microbiota in relative protection of mice following *C. jejuni* infection.

C. jejuni *Virulence Factors*. *Campylobacter jejuni* has numerous virulence factors contributing to pathogenicity, survival, and modulation of the host response. Among factors shown to be important for host-pathogen interactions are motility, secretion, adhesion, a polysaccharide capsule, cytolethal distending toxin (CDT), and lipooligosaccharide (LOS).

Flagella. C. jejuni, through a combination of flagellum-mediated movement and spiral morphology, retains superior motility compared to other bacterial rods (*E. coli, V. cholerae*, and *S. enteriditis*) in solutions of high viscosity.[32] Retention of motility in highly viscous environments, such as mucus in the gastrointestinal tract, likely contributes to *C. jejuni* pathogenesis through colonization of the mucus layer. In addition to its critical role in motility, the flagellum also functions in attachment to the epithelium[81] and in secretion. Sequencing of the *C. jejuni* NCTC11168 genome did not identify type III secretion systems (T3SS) other than the flagellin export apparatus;[93] secretion of *Campylobacter* invasion antigen (Cia) proteins necessary for invasion, production of IL-8 by epithelial cells, and induction of disease occurs through the flagellar export apparatus.[65; 101] The hook, basal body, and at least one of two filaments of the apparatus must be functional for secretion.[65]

Adherence and Capsule. Adherence to host cells protects bacteria from elimination from the gastrointestinal tract by normal peristalsis, and is important for entry into host cells. *C. jejuni* are able to adhere to fibronectin, a component of the extracellular matrix, through the outer membrane protein CadF.[63] The polysaccharide capsule of *C. jejuni* also influences virulence by contributing to adherence and invasion, colonization, and modulation of the host immune response. A *C. jejuni* 81-176 mutant

lacking high molecular weight (HMW) capsular glycan (*kpsM* mutant) showed reduced invasion and adherence to INT407 (human embryo intestinal epithelial) cells *in vitro* compared to WT *C. jejuni* 81-176.[7] This same mutant strain exhibited reduced colonization in BALB/c mice[79] and lower virulence in a ferret diarrhea model[7] compared to WT *C. jejuni* 81-176. In addition, WT *C. jejuni* 81-176 induced less IL-17 expression in small intestine lamina propria lymphocytes and reduced activation of Toll-like receptor (TLR) 2 and TLR4 in HEK cells than the mutant strain,[79] suggesting the capsule is also important in mediating the host immune response to *C. jejuni*. Finally, both flagellin and "lipopolysaccharide (LPS)" (discussed below) were found to be important in adherence: LPS was able to adhere to both intestinal mucus gel and cultured INT407 epithelial cells, and flagellin could also bind epithelial cells.[81]

Cytolethal Distending Toxin. C. jejuni and other *Campylobacter* spp. produce CDT. When HeLa cells are treated with CDT extract from *C. jejuni* 81-176, cells exhibit distension, rapid and irreversible cell cycle arrest in the G_2 phase, and cell death.[120] CDT is also important for colonization and development of gastritis in mice,[33] stimulation of production of pro-inflammatory IL-8, an important chemotactic factor for neutrophils and antigen presenting cells such as macrophages and dendritic cells, from INT407 cells,[46] and mediation of apoptotic killing in cultured human monocytic cells through induction of caspases.[45]

Lipooligosaccharide. High molecular weight LPS in the outer membrane of Gram-negative bacteria is a virulence factor. LPS comprises the O-polysaccharide chain linked to a core oligosaccharide anchored in the bacterial outer membrane by lipid A.[36] Low molecular weight (LMW) LPS, lacking the O-polysaccharide chain, is referred to as LOS. Karlyshev *et al* (2000)[58] identified genes in the NCTC 11168 genome highly similar in sequence and organization to genes in *E. coli* and other Gram-negative bacteria involved in capsular polysaccharide biosynthesis and transport, and determined that what was previously thought to be HMW LPS in *C. jejuni* is actually capsular polysaccharide. It had been previously thought that all *C. jejuni* strains produce LOS, with some also producing HMW LPS.[58; 124] The current

thought is that *C. jejuni* produces both LOS (LMW) and capsular polysaccharide (HMW).[36] While the *C. jejuni* LOS is important in ganglioside mimicry and likely also the development of Guillain-Barré syndrome (discussed below), LOS also impacts *C. jejuni* invasiveness into host cells. *C. jejuni* strains bearing sialylated LOS (class A, B, or C) displayed higher invasion into Caco-2 cells than strains with non-sialylated LOS (classes D, E).[73] Interestingly, the LOS outer core of *C. jejuni* 81-176 was shown to convert between GM2 and GM3 ganglioside mimics due to variation in the *cgtA* gene.[39] Furthermore, site-specific *cgtA* mutation resulted in an LOS with a GM3 but not GM2 mimic, and enhanced invasion into INT407 epithelial cells.[39]

EXTRA-INTESTINAL COMPLICATIONS AND POST-INFECTIOUS SEQUELAE OF *CAMPYLOBACTER JEJUNI* INFECTION: EMPHASIS ON GUILLAIN-BARRÉ SYNDROME

Extraintestinal Complications and Post-Infectious Sequelae of Campylobacteriosis. In addition to enteric symptoms, extraintestinal complications including pancreatitis, hepatitis, bacteremia, nephritis, and miscarriage also occur.[16; 121] Infection with *C. jejuni* is also associated with numerous post-infectious complications. Irritable bowel syndrome (IBS), reactive arthritis and Reiter's syndrome, and Guillain-Barré syndrome (GBS) are associated with *Campylobacter* infection.[16; 121] The authors of a recent (2014) systematic literature review and meta-analysis estimated the proportion of *Campylobacter* infections associated with chronic sequelae: IBS (4.01%), reactive arthritis (2.86%), and GBS (0.07%).[59]

Guillain-Barré Syndrome. Following the near eradication of poliomyelitis, GBS is the most common cause of acute neuromuscular flaccid paralysis worldwide.[125] The authors of a recent systematic literature review estimated the overall incidence of GBS as between 1.1/100,000/year and 1.8/100,000/year.[80] This review also identified increased incidence with age, but was not able to confirm bimodality in incidence by age.[80] Up to 70% of GBS cases were associated with antecedent infection, mainly upper respiratory and gastrointestinal infections.[80] Another recent systematic literature review with meta-analysis also identified an increase in incidence with age, and reported an increased risk of

GBS in males than females, an unusual finding compared to many other autoimmune diseases.[103] Reported mortality rates vary from 3-7%.[111]

Subtypes of GBS. GBS is recognized as a heterogeneous post-infectious disorder, with variable antecedent infection, clinical course, severity, and outcome. Several subtypes of GBS are recognized; the most widely recognized forms include acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN), acute motor sensory axonal neuropathy (AMSAN), and Miller Fisher syndrome (MFS). Two features required for a diagnosis of GBS include progressive weakening of legs and arms, and areflexia or decreased tendon reflexes in the weakened limbs.[111] Beyond these two features, additional clinical symptoms and nerve conduction studies can be used to distinguish between subtypes.[111]

Clinical Manifestations of GBS. Main clinical features of AIDP include sensorimotor deficits, which may be accompanied by cranial nerve and autonomic dysfunction, and pain.[111] Physical examination findings include flaccid paralysis, reflex deficits, and variable sensory loss.[85] Nerve conduction studies reveal demyelinating polyneuropathy.[111] Histological findings include macrophage-mediated segmental demyelination and involvement of lymphocytes; T cells are presumed to be most important, with macrophage invasion occurring later.[85; 125]

The axonal forms, AMAN and AMSAN, exhibit some clinical overlap and AMAN often precedes AMSAN.[85] Patients with AMAN present with paresis or paralysis, but with intact sensory function,[85] and pain is sometimes reported.[111] There is selective loss of motor fibers with preservation of sensory fibers and normal sensory action potential.[85; 111] Histological changes include lesions at the nodes of Ranvier, including lengthening of the nodal gap corresponding to binding of IgG and complement activation, and entrance of macrophages into the periaxonal internode space.[85] In contrast to AIDP, lymphocytes are typically rare or absent and demyelination is not a feature.[85; 125] AMSAN resembles and can be considered a severe form of AMAN, with both sensory and motor deficits, abnormal sensory action

potential, and Wallerian-like degeneration in both sensory and motor fibers.[85; 111] Clinically, AMSAN patients develop widespread paralysis with a protracted recovery.[85]

Finally, MFS is characterized clinically by the typical triad of ataxia, ophthalmoplegia, and areflexia. Although clinical outcome of MFS is generally good[111] and weakness is not a prominent feature,[85] "MFS-GBS overlap syndrome" can occur, characterized clinically by additional limb weakness and respiratory insufficiency.[111]

GBS Epidemiology. Interestingly, frequency of the clinical subtypes varies by geographic region. AIDP predominates in Europe and North America, accounting for between 60-90% of GBS cases.[85; 111; 125] Conversely, the axonal forms are common in China, Japan, Mexico, and Bangladesh,[85; 125] accounting for 30-65% of GBS cases in Asia and Central and South America.[111] The basis of geographical diversity is not known, but may involve varying exposure to certain types of infections, or genetic differences between populations.[111]

Antecedent Infections. Antecedent infections are most commonly reported less than four weeks prior to the onset of GBS and are frequently grouped by "upper respiratory" or "gastrointestinal" manifestations.[80] Several infectious agents have been associated with development of GBS, including viral (cytomegalovirus, and Epstein-Barr, varicella-zoster, and influenza A viruses) and bacterial (*Haemophilus influenzae*, *Mycoplasma pneumoniae, C. jejuni*) agents.[54; 111; 125] A case-control study involving 154 GBS patients determined that *C. jejuni* was reported most frequently as the recent infection (32%), followed in frequency by cytomegalovirus, Epstein-Barr virus, and *M. pneumoniae*.[54] The presence of anti -GM1 and -GD1b antibodies was also associated with *C. jejuni* infection.[54] One large multicenter study found that *C. jejuni* was the most common cause of antecedent infection, followed by cytomegalovirus and Epstein-Barr virus.[40] Additionally, these investigators found that patients with preceding *C. jejuni* infection were more likely to have axonal neuropathy, pure motor GBS, and anti-GM1 antibodies.[40] Among other factors, *C. jejuni* infection, diarrhea, older age, severe weakness, and

abnormal motor action potentials were all associated with poor outcomes.[40] In a case-control study, 27/103 GBS or MFS patients (26%) were *C. jejuni* positive.[99] A recent systematic literature review aiming to quantify the association between *Campylobacter* and GBS determined that 31% of 2,502 GBS patients described in case-control studies were attributable to *Campylobacter* infection.[94]

Pathogenesis of GBS: Molecular Mimicry and Anti-Ganglioside Antibodies. The pathogenesis of GBS following *C. jejuni* infection is incompletely understood. Molecular mimicry-induced nerve damage initiated by cross-reactive antibodies to LOS in the *C. jejuni* outer membrane and structurally similar peripheral nerve gangliosides, including GM1 and GD1a, is accepted as a likely mechanism.[111] Gangliosides are sialic-acid containing glycosphingolipids organized in microdomains in cell membranes, playing a role in cell growth, differentiation, and signaling.[55] Gangliosides are found on most nucleated cells throughout the body but are enriched in the nervous system. The term "molecular mimicry" is used to indicate "sharing of antigens between hosts and microorganisms" and also broadly describes cross-reactivity in an immunological context.[4] Ang, Jacobs, and Laman define molecular mimicry as "dual recognition of structures of a microbe and host by a single B- or T-cell receptor" and "the mechanism by which infections trigger cross-reactive antibodies or T cells that cause symptoms of autoimmune disease".[4] GBS patients often have serum antibodies to gangliosides including GD1a, GM1a, GM1b, GQ1b, GT1a, or ganglioside complexes.[95; 111]

In 1993, Yuki and colleagues extracted LPS from *C. jejuni* strain 90-26 isolated from a GBS patient with anti-GM1 serum antibodies. Using gas-liquid chromatography mass-spectrometry and protein NMR spectroscopy, they showed that the LPS contained sugar components of GM1, the oligosaccharide structure protrudes from the LPS core, potentially exposing the GM1-oligosaccharide moiety, and that the terminal structure was identical to the GM1 terminal tetrasaccharide.[127] This study provided the first conclusive evidence of structural similarity between a ganglioside and *C. jejuni* LOS, and others have followed.[37; 39]

Several studies have reported the prevalence and type of anti-ganglioside antibodies in GBS and identified associations between these autoantibodies and preceding infection or clinical outcome. In a multicenter study of 229 GBS patients, presence of anti-GM1 IgA or IgG, but not IgM, was associated with a worse outcome.[40] Additionally, patients with preceding *C. jejuni* infection had higher frequencies of anti-GM1 IgA, IgG, and IgM, and worse outcomes than the study population as a whole.[40] A prospective case-control study of 96 GBS patients determined that of the 25 *C. jejuni* positive patients, 52% had anti-GM1 antibodies, while 15% of *C. jejuni* negative GBS patients had these antibodies.[99] *C. jejuni* positive patients with anti-GM1 antibodies were also found to have slower recoveries and greater disability than *C. jejuni* positive patients without anti-GM1 antibodies.[99]

Subtype of anti-ganglioside IgG also has been associated with type of antecedent infection and clinical outcome of GBS. Koga *et al* (2003) found that, in 42 GBS patients with anti-GM1 antibodies, the IgG1 subtype was most common and was associated with previous gastroenteritis, positive *C. jejuni* serology, and a slower clinical recovery reflected by return to independent locomotion.[62] In contrast, anti-GM1 IgG3 was associated with antecedent respiratory infection and more rapid clinical recovery.[62] In a study of 176 GBS patients, Jacobs and colleagues (2008) corroborated these findings. They determined that presence of only IgG1 subtype antibodies to motor gangliosides, without IgG3, was associated with diarrhea, positive *C. jejuni* serology and LOS cross-reactivity, and worse neurological outcomes; in contrast, anti-ganglioside antibodies of both IgG1 and IgG3 types were associated with previous upper respiratory infection, cross-reactivity with *H. influenzae* LOS, and better neurological outcomes.[55]

Role of Host Factors in GBS. Some *C. jejuni* strains isolated from patients with uncomplicated enteritis also expressed ganglioside mimics, although less frequently than strains isolated from GBS or MFS patients.[5] Furthermore, although ganglioside mimics were present in *C. jejuni* strains from some enteritis patients, antibody reactivity of enteritis patients to neural glycolipids was significantly lower

than in GBS/MFS patients, supporting a role for host-related factors in addition to *C. jejuni* ganglioside mimicry in development of neurological disease.[5] Expression of ganglioside mimics in *C. jejuni* strains isolated from both GBS and uncomplicated enteritis patients has been shown previously.[105] Moreover, anti-GM1 antibodies derived from the serum of a GBS patient did not react with autologous *C. jejuni*, suggesting a change in expression of the ganglioside mimic or involvement of a mechanism additional to molecular mimicry; binding of anti-GM1 antibodies by LPS in *C. jejuni* strains from both GBS and uncomplicated enteritis patients was also observed.[52] Associations between several human genetic factors, including MMP9, TNFα, Fc gamma receptor III, class I or II HLA types, TLR4, IL-10 and GBS incidence, severity, and outcome have been studied with variable results.[51; 55; 56] The geographic distribution of various subtypes and the rarity of GBS outbreaks also support a role of variable host susceptibility.

Animal Models of GBS. The immunopathogenesis of GBS is complex, and a robust, reproducible animal model mimicking human GBS would greatly further our understanding. As our understanding of GBS evolves, there is increasing support for contributions of both characteristics of *C. jejuni*, such as the presence of ganglioside mimics in the LOS, and host susceptibility factors. Exploration of several animal models focusing on pathogenesis and histopathological findings have been attempted.

Two published reports suggest chickens could be useful models for GBS.[70; 87] In both of these studies, chickens between 4 weeks and 6 months of age were orally infected with *C. jejuni* strains isolated from GBS patients. One study also included a spontaneous disease group, in which chickens from flocks owned by families having a member with GBS exhibited weakness and developed paralysis.[70] Two of the five chickens with spontaneous neurological disease had histological lesions resembling AMAN. In this same study, some chickens experimentally fed *C. jejuni* isolated from a patient with AMAN developed diarrhea 2—4 days later and/or paralytic neuropathy after approximately 12 days.[70] In the second study, 73% of infected chickens developed diarrhea after 3—7 days, and 17/22 of these

chickens developed paralytic neuropathy resembling GBS after 5—14 days.[87] Sciatic nerve pathology in diseased chickens included Wallerian-like degeneration, nodal lengthening, paranodal demyelination, and infiltration of lymphocytes and macrophages.[70; 87]

Rabbits have also been investigated as models for *C. jejuni*-induced GBS. Rabbits injected repeatedly for months with a mixture containing LOS isolated from GBS patient-derived *C. jejuni* strain CF 90-26 bearing a GM1 mimic, along with keyhole limpet hemocyanin and Freund's complete adjuvant (CFA) developed neurological signs sooner than control rabbits.[126] Neurological lesions in sciatic nerves of paralyzed rabbits included Wallerian-like degeneration and macrophages within periaxonal spaces.[126]

Several mouse models have been produced that reflect different forms of GBS or various aspects of pathogenesis. These include both spontaneous and experimentally induced models resembling the AIDP form of GBS. Nonobese diabetic (NOD) mice lacking the costimulatory molecule B7-2 (CD86) do not develop diabetes, but instead exhibit spontaneous autoimmune peripheral polyneuropathy (SAPP) arising at 20 weeks of age in a proportion of mice.[100] SAPP resembles chronic inflammatory demyelinating polyneuropathy (CIDP) in humans with symmetrical limb paralysis. Histopathologic features also resemble the AIDP form of GBS, including lesions with T cell infiltration into dorsal root ganglia (DRG) and peripheral nerves.[100] Another model is experimental autoimmune neuritis (EAN) that has been induced in Lewis rats and mice. SJL/J mice injected with bovine-derived peripheral nerve myelin with CFA develop a constellation of signs and lesions termed severe murine EAN, including neuromuscular weakness, nerve electrophysiology abnormalities, and demyelination and infiltration of macrophages and lymphocytes in sciatic nerves.[122]

One BALB/c mouse model of MFS involves induction of respiratory paralysis through intraperitoneal injection of antibody reacting against multiple gangliosides along with normal human serum as a complement source.[41] Anti-GQ1b antibody binding led to complement activation and subsequent transmission block at diaphragmatic neuromuscular junctions, resulting in respiratory

paralysis. In support of a role of complement in MFS pathogenesis, respiratory abnormalities were abrogated by treatment with eculizumab, a human monoclonal antibody that prevents production of terminal complement components by blocking the intermediary C5 component.[41] This model elucidates some aspects of MFS pathogenesis including molecular mimicry, but it is not a complete model of post-infectious GBS.

There are drawbacks to each of these animal models. The chicken models produce similar symptoms of diarrhea and GBS as in people, but the fundamental anatomic and physiological differences between humans and birds limit the utility of avian models. Rabbits and mice may prove to be more relevant models of human disease. While the models described above provide insight into various aspects of GBS, none of these models are induced following oral infection of *C. jejuni*, as in people; with the exception of the SAPP model, disease is induced through subcutaneous or intraperitoneal injection of LOS, anti-ganglioside antibody, or myelin and requires additional factors such as CFA or human serum for disease induction.

Mouse Models of GBS Following Oral C. jejuni *Infection*. Promising progress in developing mouse models resembling GBS through oral infection with *C. jejuni* has been made recently. Over several experiments, WT, IL-10$^{-/-}$, and CD86$^{-/-}$ NOD mice orally infected with *C. jejuni* strains associated with GBS (strains HB93-13, 260.94) or MFS (CF93-6) developed combinations of neurological abnormalities determined by phenotyping tests including DigiGait treadmill analysis and open field testing, anti-ganglioside antibodies, and nerve lesions characterized by infiltration of macrophages or T-cells into sciatic nerves and dorsal root ganglia.[108] Interestingly, treatment with the antibiotic chloramphenicol modulated the immune response in NOD IL-10$^{-/-}$ mice, reflected by significant decreases or increases in anti-*C. jejuni* or anti-ganglioside antibodies relative to *C. jejuni*- or sham-inoculated groups not receiving chloramphenicol.[108] This suggests that antibiotic-induced changes in the microbiome may be important in the immune response to *C. jejuni* infection. The importance of the microbiome in immunity to *C. jejuni*

is supported by the finding of increased anti -*C. jejuni* and -GM1 antibodies in mice with a humanized compared to a conventional mouse microbiota.[21] These two studies provide promising murine models of GBS development following oral *C. jejuni* infection.

All of these described animal models have drawbacks, including methods of induction that do not resemble post-infectious GBS and induction of only mild lesions. Both *C. jejuni* factors, such as the presence of ganglioside mimics in the LOS, and host factors, such as propensity for developing anti-ganglioside antibodies, likely contribute to susceptibility of an individual to development of GBS following *C. jejuni* infection. Therefore, a mouse model exploring both *C. jejuni* factors, such as invasiveness and pathogenicity in previous mouse models, and host factors, including reported immunological biases toward T helper (Th) 1- or Th2-mediated responses in C57BL/6 and BALB/c mice respectively, would be valuable to determine factors contributing to GBS susceptibility following oral *C. jejuni* infection. Understanding of disease outcome in *C. jejuni* infection requires understanding of the initial host-microbe interaction and development of both innate and adaptive immunity.

HOST RESPONSE IN CAMPYLOBACTERIOSIS: INITIAL HOST-MICROBE INTERACTION AND INNATE IMMUNITY

The initial host-*C. jejuni* interaction influences both non-specific innate immunity and subsequent adaptive immunity.[75] Therefore, especially in infections in which the immune response is not fully understood, knowledge of the initial interaction between the pathogen and innate barriers such as mucus in the gastrointestinal tract, and subsequent interaction with the epithelium and resident immune cells, is crucial. Studies have shown that *C. jejuni* can be found inside non-phagocytic gut epithelial cells and also within phagocytic immune cells such as macrophages and dendritic cells.

Contact with Mucus Layer. Following ingestion, *C. jejuni* is chemotactically attracted to mucins and glycoproteins present in the mucus layer, an important mechanism facilitating colonization.[19] The flagella and spiral morphology of *C. jejuni* contribute to retained motility in viscous environments.[32]

Furthermore, both *C. jejuni* flagella and LPS act as adhesins to INT 407 epithelial cells, while the LPS also bound to intestinal mucus derived from rabbits.[81] The ability of *C. jejuni* to retain motility in and adhere to mucus likely facilitates its attachment to epithelial cells and eventual colonization. Immunohistochemical and ultrastructural studies in infected humans showed that *C. jejuni* can be found not only in the glycocalyx covering surface epithelium, but also within surface epithelial cells, including goblet cells, and within histiocytes in the lamina propria.[112] Interestingly, these findings did not correlate with severity of inflammation.[112]

Interaction with Host Epithelium. Expression of the outer membrane protein CadF allows *C. jejuni* to bind to fibronectin. This is an interaction of particular importance prior to internalization into epithelial cells, as fibronectin is abundant in the basement membrane and at points of cell contact in the epithelium.[63] Further work with polarized cells, exhibiting apical and basolateral surfaces and tight intercellular junctions, of the T84 line resembling those in colonic crypts, corroborated the importance of the binding of *C. jejuni* to fibronectin.[83] This study showed that *C. jejuni* translocation occurs paracellularly, rather than intracellularly, and that invasion occurs primarily through interaction with fibronectin at the basolateral surface.[83] While there is consensus that *C. jejuni* can transmigrate across epithelium, whether the primary mechanism is transcellular, paracellular, or both, is still debated.[6]

In vitro models utilizing cultured INT407 epithelial cells and enteric *C. jejuni* strain 81-176 have helped elucidate various host cell events in invasion.[50; 88] In contrast to the microfilament-dependent invasion of other enteric pathogens including *E. coli* and *Salmonella*, invasion of *C. jejuni* 81-176 into epithelial cells instead depends primarily upon microtubules.[88] Production of bacterial, but not eukaryotic, proteins was necessary for *C. jejuni* invasion.[88] Hu *et al* (2006) proposed a model in which *C. jejuni* interaction with G-protein-coupled receptors within caveolae, small pits in the plasma membrane of many different cell types, are an early event in host cell signal transduction during invasion.[50] This

interaction leads to activation of host cell PI 3-kinase and MAP kinases, shown through inhibition studies to be important in *C. jejuni* invasion.[50]

The fate of *C. jejuni* within intestinal epithelial cells and innate immune cells such as macrophages may be different. Watson and Galán (2008) showed that *C. jejuni* 81-176 could survive within human intestinal epithelial T84 cells for at least 24 hours, although *C. jejuni* was efficiently killed by macrophages derived from mouse bone marrow.[117] This difference in survival between different cell types was mediated through different intracellular trafficking: in epithelial cells, the internalized *C. jejuni* resided in a "*C. jejuni*-containing vacuole" (CCV) which avoided delivery to lysosomes through the endocytic pathway, while in macrophages, *C. jejuni* was delivered to lysosomes and killed.[117] In human and mouse macrophages, *C. jejuni* activates the NLRP3 inflammasome, cytosolic multiprotein complexes that induce activation of caspase-1, IL-1β, and IL-18 following bacterial invasion.[20] Interestingly, cell death by pyroptosis typically follows inflammasome activation by other bacterial pathogens, but *C. jejuni* induces activation of the inflammasome without cytotoxicity.[20]

<u>*Correlating Invasion and Pathogenicity*</u>. Several studies have used models of *C. jejuni* association/adherence, invasion, and intracellular survival in cultured cells to assess correlations between these features and pathogenicity. Law *et al* (2009) examined 59 *C. jejuni* strains isolated from poultry and chose 5 with variable ability to invade HEp-2 or survive in murine macrophages (J cells). When piglets were inoculated with these strains, little correlation was found between the *in vitro* findings and development of lesions *in vivo.*[68] Everest *et al* (1992) showed that a higher proportion of colitis-associated *C. jejuni* strains were able to transcytose polarized Caco-2 cells and invade HeLa and Caco-2 cells than non-inflammatory strains.[30] These findings were difficult to interpret, as *C. jejuni* association with HeLa cells varied with time. Additionally, even though more colitis-associated strains were able to invade, similar numbers of viable intracellular *C. jejuni* from non-inflammatory strains capable of invading were seen relative to colitis strains.[30] However, Fauchere *et al* (1986) observed that

ability of *C. jejuni* or *C. coli* strains to associate with HeLa cells correlated with clinical symptoms of diarrhea and fever (although not blood in the feces), and concluded that *in vitro* association assays could be used to predict pathogenicity.[31] Similarly, Konkel and Joens (1989) showed that although *C. jejuni* strains varied considerably in ability to invade HEp-2 cells, isolates from patients with clinical campylobacteriosis were generally more invasive than non-clinical isolates.[64] Interestingly, a *C. jejuni* mutant strain deficient in the formic acid receptor Tlp7 demonstrated markedly reduced invasion into Caco-2 cells. This mutant was able to colonize mice as well as the parental strain, but did not induce immunopathology as seen by the parental strain, indicating that invasiveness and not just colonization contributed to the host immune response.[12] Thus, *in vitro* invasion may reflect pathogenicity in some strains, although technical factors such as type of cultured cells, assay used, number of laboratory passages of *C. jejuni*, and other experimental conditions should be considered. Additionally, *in vitro* assays by nature do not consider additional host factors such as microbiome and immune response, which are integral contributors to the host-microbe interaction and consequences of infection.

Interaction with innate immune cells in the lamina propria necessitates passage of *C. jejuni* through the epithelium. *C. jejuni* was shown to traverse polarized T84 human intestinal epithelial cells through the paracellular, rather than intracellular route.[83] *C. jejuni* also produces CDT, which stimulates chemotactic IL-8 production in INT 407 cells[46] but also induces cell cycle arrest.[120] These studies suggest that *C. jejuni* could interact with innate cells present in the intestinal subepithelium either after crossing the epithelium between cells, or following liberation from dying epithelial cells following invasion and CDT production. Upon entrance to the subepithelial lamina propria, *C. jejuni* subsequently interacts with resident innate immune cells, such as neutrophils and antigen presenting cells including macrophages and dendritic cells.

Dendritic Cells. In 1973, Steinman and Cohn described a novel type of cell, distinct from lymphocytes, granulocytes, and mononuclear phagocytes, isolated from murine spleen and peripheral

lymphoid tissues.[109] This cell type comprised approximately 1.0-1.6% of the total splenic nucleated cell population, exhibited numerous branching forms, and was observed to extend and retract cellular processes, leading the investigators to apply the name "dendritic cell."[109] Subsequent *in vitro* studies further distinguished dendritic cells (DC) from macrophages, including by characterization of the relatively poorer endocytic capacity of DC compared to macrophages using a variety of test substances.[110]

Current knowledge indicates that DC originate from hematopoietic precursors and comprise diverse subtypes with heterogeneous intermediate precursors, location, form, function, and expression of cell-surface antigen.[71; 106] Understanding of these different DC types is a new and evolving area of research. DC development during hematopoiesis is complex, with production of both DC and macrophages possible through both myeloid and lymphoid pathways;[106] however, myeloid- or lymphoid-origin distinction is not typically relevant even when referring to DC in lymphoid tissues, as even DC in the spleen and thymus are mostly myeloid-derived.[71] Broad categories of DC presented by Shortman include: pre-dendritic cells, such as steady state plasmacytoid DC or monocytes, capable of stimulus-induced development into DC; and conventional DC (cDC), including migratory DC and lymphoid-tissue-resident DC.[106] Migratory cDC sample the periphery, migrate to the lymph node bearing antigen, and interact with T cells; lymphoid-tissue-resident DC include cDC restricted to the thymus or spleen, where they sample and present antigen locally.[106] Inflammatory DC are an additional category, not usually present in steady state without stimulus, but can develop from cells such as inflammatory monocytes.[106]

A summary of the current understanding follows: cDC are important sentinel immune cells, positioned throughout the periphery in places such as the lamina propria of the gut to sample the local environment for potential antigens. They are extremely efficient antigen presenting cells (APC) and connect the innate and adaptive immune systems following the initial host-pathogen interaction. In the

gut, cDC surveil the environment for antigens and in the steady state, and are considered tolerogenic of innocuous antigens, maintaining symbiosis with the microbiota.[57] Uptake of antigens by DC can occur by pinocytosis, phagocytosis, or receptor-mediated endocytosis.[113] Once a differentiated but immunologically immature DC acquires an antigen, it migrates to lymph nodes and during migration, upregulates molecules important in stimulation of T cells: costimulatory molecules, cytokines, and MHC complexes.[2] As DC are considered the most important APC for the priming of naïve T cells and subsequent direction of Th differentiation into Th1, Th2, Th17, and Treg subsets,[113] DC are crucial components of the initiation of an effective and pathogen-appropriate adaptive immune response.

In a landmark 1986 study, Mosmann and Coffman described two distinct groups of Th cells based upon surface antigen expression, B cell stimulating activity, and cytokine production, designated as Th1 and Th2 subsets.[84] It is now known that Th1 cells are characterized by production of IFN-γ and control of intracellular pathogens, while Th2 cells produce IL-4, IL-5, IL-10, and IL-13 and are important in parasite control and allergic responses.[96] Additional Th subsets described to date include Treg and Th17, among others.[96] A subset of "suppressive" T cells mediating tolerance was originally described in 1970 by Gershon and Kondo[35] and since this discovery, immune suppression by Treg cells is known to be mediated in part by the anti-inflammatory actions of TGF-β and IL-10.[96] Th17 cells were relatively recently (2005) designated a distinct subset of CD4+ T cells based on characteristics of differentiation and the production of IL-17.[91] Cells producing IL-17, the signature cytokine of Th17 cells, are now understood to have a critical role in defense against extracellular bacterial and other types of pathogens at mucosal surfaces.[60; 96; 118] While differentiation of Th17 cells is closely related to that of Treg cells,[14] the production of IL-17 leads to inflammation propagated by neutrophil recruitment, enhancement of B cell function, and additional release of pro-inflammatory cytokines.[96]

Several studies with *C. jejuni* have utilized *in vitro* assays, including the use of cultured monocytes, macrophages or dendritic cells from humans or mice derived by different methods. Critical

events in the host-microbe interaction have been evaluated, including initial internalization of *C. jejuni*, intracellular survival or killing, and stimulation of cytokine production and T cell priming.

C. jejuni *Interaction with Monocytes/Macrophages*. Evaluation of internalization and intracellular survival of *C. jejuni* within monocytes and macrophages are relevant for understanding the response to *C. jejuni* during bacteremia or following invasion into the intestinal subepithelium. Studies have shown that *C. jejuni* is capable of both survival and replication in human or mouse monocytes or macrophages. Kiehlbach *et al* showed that *C. jejuni* 2964 from a diarrheic patient continued to be taken up into cultured J cells (BALB/c macrophage cell line), resident peritoneal macrophages from BALB/c mice, and human peripheral blood monocytes over the 8-hour infection period; *C. jejuni* then remained viable for 6-7 days intracellularly.[61] These observations were subsequently confirmed, as *C. jejuni* 81-176 remained viable inside human monocytic cells (28SC line) for up to 7 days.[45] CDT-mediated apoptosis was observed, and live *C. jejuni* could be visualized inside vacuoles within dead monocytes.[45] Interestingly, phagocytes from some individuals may exhibit relatively decreased ability to kill phagocytosed *C. jejuni* compared to other individuals. Macrophages derived from peripheral monocytes in human blood by cytokine stimulation were used to assess uptake and intracellular survival of *C. jejuni* by gentamycin protection assay. The investigators showed that the 16 *C. jejuni* strains tested were killed by macrophages from most donors within 48 hours, but macrophages from approximately 10% of donors were incapable of killing *C. jejuni* despite adequate uptake, instead allowing intracellular replication of *C. jejuni.*[115]

C. jejuni *Interaction with Dendritic Cells*. Despite the importance of *C. jejuni* as an enteric pathogen and the critical role of dendritic cells in bridging innate and adaptive immunity, relatively few studies in recent years have specifically evaluated *C. jejuni*-DC interaction. Both human DC derived from blood monocytes[8; 29; 48] or mouse DC derived from bone marrow[97; 98] have been used to study *C. jejuni* invasion, DC activation, and subsequent Th polarization. *C. jejuni* 81-176 was found to invade human DC

in a dose-dependent manner in up to 4 hours of infection time, although marked reduction in viable intracellular *C. jejuni* with limited DC cytotoxicity were seen by 24 hours.[48] *C. jejuni* also induced DC maturation (reflected by increased costimulatory molecule expression), activated NF-κB, and stimulated production of cytokines including IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-γ, and TNF-α.[48] Similarly, human monocyte-derived DC infected with *C. jejuni* 11168 produced IL-12, IL-23, IL-1β, and IL-6, and stimulated production of IL-17A and IFN-γ in T cells.[29] Together, these studies support *C. jejuni*-induced cytokine production and Th1/Th17 polarization by infected DC.

Sialylation of the *C. jejuni* LOS enhances invasion of epithelial cells *in vitro,*[73] and has recently been shown to impact DC function as well. Interestingly, human DC-mediated T cell polarization can vary depending upon the infecting *C. jejuni* strain, and specifically by the sialylation of the LOS: α2-3-linked sialylated LOS drove a predominant Th2 response, while α2-8-linked sialylation resulted in Th1 differentiation.[8] Expression of siglecs (sialic acid-binding Ig-like lectins) on the DC was implicated in this response, as different siglecs preferentially bind glycans with specific sialic acid linkage.[8] *C. jejuni* strains with sialylated LOS also stimulated enhanced DC activation and release of soluble factors resulting in proliferation of mucosal B cells, compared to strains with non-sialylated LOS.[66]

As with *in vitro* studies with human DC, murine bone marrow-derived DC (BMDC) internalize *C. jejuni*, stimulating DC maturation, cytokine production, and ability to differentiate T cells in co-culture systems. BMDC derived from C57BL/6 WT mice infected with *C. jejuni* 11168 showed efficient internalization and complete killing, with no viable intracellular *C. jejuni* enumerated 8 hours post infection (p.i.).[98] Furthermore, similar to human DC, *C. jejuni*-infected DC underwent maturation, reflected by increased MHC II and costimulatory molecule expression. Cytokine production and T cell polarization also mirrored that found in human DC studies: infected DC produced pro-inflammatory cytokines and induced Th1 polarization.[98] Further studies showed that TLR2, TLR4, and subsequent signaling through adaptor molecules MyD88 and TRIF are involved in events including DC maturation,

production of IL-12, and maximum Th1 polarization reflected by IFN-γ production induced by *C. jejuni* infection.[97]

Stimulation of Pattern Recognition Receptors and Downstream Events. Binding of pathogen-associated molecular patterns (PAMPs) by cell surface or intracellular pattern recognition receptors (PRRs) of host innate cells is a critical initial event in the host-pathogen interaction. TLRs are expressed by epithelial cells and immune cells including macrophages and DC, and can be located either inside or on the surface of the cell.[1] Cytoplasmic PRRs have also been identified, including nucleotide-binding oligomerization domain (NOD) proteins. Recognition of specific PAMPs by PRRs leads to initiation of the host defense by activation of complex cellular signaling pathways and stimulation of cytokine production.[1] *C. jejuni* has been shown to interact with host cells through stimulation of TLR2 and TLR4.[34; 97] Interestingly, the flagella on *C. jejuni* are able to avoid recognition by TLR5 on host cells, while retaining motility important in pathogenesis through colonization of mucus.[3] *C. jejuni* also does not efficiently stimulate TLR9, correlating with relatively low frequency of [CG] dinucleotides, compared to other bacteria.[26] The cytoplasmic NOD1 PRR in human intestinal epithelial (Caco-2) cells also recognizes *C. jejuni*, while NOD2 is apparently not as important in this system[128] or in a secondary abiotic C57BL/6 IL-10$^{-/-}$ mouse infection model.[43]

Downstream events following initial interaction of *C. jejuni* with host cells, including epithelial or antigen presenting cells, includes production of chemotactic and pro-inflammatory cytokines and subsequent initiation of the adaptive immune response. *C. jejuni* elicits secretion of chemoattractant IL-8 from cultured intestinal epithelial cells (INT407), and amount of IL-8 released was correlated with *C. jejuni* strain invasiveness.[44] Hu and Hickey (2005) subsequently showed that INT407 cells also produced pro-inflammatory chemokines, including macrophage inflammatory protein 1 and monocyte chemoattractant protein 1 (MCP-1), at least partially mediated by NF-κB, following exposure to *C. jejuni.*[49] Cultured polarized Caco-2 epithelial cells produce IL-6, a mediator of the acute phase

inflammatory response, following exposure to 8 different *C. jejuni* strains.[34] Induction of cytokines from *C. jejuni*-infected human gut explant tissue included IFN-γ, IL-12, IL-23, and IL-6.[29]

In addition to chemoattractant and pro-inflammatory cytokine production by infected epithelial cells, both human and murine DC have been shown to produce pro-inflammatory and T-cell polarizing cytokines following *C. jejuni* infection. Infected human monocyte-derived DC produced increased IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN-γ, and IL-12.[48] *C. jejuni*-infected murine BMDC also produced a similar pro-inflammatory profile, including increased TNF-α, IL-6, and IL-12p70.[98] Furthermore, the Th1 polarization predicted by Hu in response to increased IL-12 production was confirmed by this murine study, in which naïve Th cells co-cultured with infected BMDC produced IFN-γ, but not IL-4 or IL-10, indicating Th1 polarization.[98]

Collectively, these studies show that *C. jejuni* is proficient in navigating the gastrointestinal mucus layer, and subsequent colonization and invasion of the gut leads to innate immune responses by both epithelial and antigen presenting cells. Production of chemoattractant IL-8 and pro-inflammatory and T-cell polarizing cytokines leads to initiation of the adaptive immune response. Both *C. jejuni* strain characteristics and host immune responses can contribute to differences in adaptive immunity and variable disease outcomes.

ADAPTIVE IMMUNITY TO *CAMPYLOBACTER JEJUNI*

Antibody responses to *C. jejuni* infection in people have been studied in natural and experimental infection. Sera from people suffering from *Campylobacter* enteritis showed increased specific IgM, IgG, and IgA antibodies; IgM and IgG rose simultaneously and remained elevated longer than IgA, which was more transient.[17] In an experimental infection study, IgA and IgM increased more than did IgG levels, and all three Ig classes peaked at 11 days before declining.[15] Furthermore, the magnitude of serological response correlated with clinical illness.[15]

Ex vivo studies of infected human intestinal tissue and *in vivo* mouse models have helped to further characterize systemic immune responses to *C. jejuni* infection. Human intestinal biopsies were cultured and infected with *C. jejuni in vitro*; the mucosal cytokine response included increased IFN-γ, along with more modest increases in IL-23, IL-12, IL-6, and proportionally lower increases in IL-17 and IL-1β.[29] Supernatants from infected monocyte-derived DC also induced production of IFN-γ and IL-17A positive or double positive T cells.[29] Collectively these data support Th1/Th17-mediated adaptive immune responses.

In vivo mouse studies have helped further delineate systemic immune responses to *C. jejuni* infection, reflected by increases in various Th1-, Th2-, or Th17-mediated isotypes of *C. jejuni*-specific plasma IgG. Fox *et al* (2004) measured *C. jejuni*-specific plasma IgG2a (Th1-mediated) and IgG1 (Th2-mediated) in infected mice, and found that IgG2a was more frequently detected than IgG1 within the treatment groups and correlated with *C. jejuni cdtB* mutant strain clearance.[33] Similar results were found in other mouse models. C57BL/6 WT and IL-10$^{-/-}$ mice infected with colitogenic *C. jejuni* 11168 produced robust Th1/Th17-mediated plasma IgG2b, but not Th2-mediated IgG1.[76] Increased Th1-mediated plasma IgG2c and IgG3, but not IgG1, was observed in C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168; interestingly, in this study, C57BL/6 IL-10$^{-/-}$ infected with GBS patient-derived *C. jejuni* 260.94 responded with elevated IgG1 as well as IgG2c.[75] St. Charles *et al* (2017) also reported robust anti-*C. jejuni* IgG2b responses in NOD WT, IL-10$^{-/-}$, and B7-2(CD86)$^{-/-}$ mice; the magnitude of the response varied by both mouse strain and infecting *C. jejuni* strain (GBS-associated 260.94 and HB93-13 strains).[108] Considering these data and the pro-inflammatory and Th1-polarizing responses produced by infected DC,[48; 98] it appears a Th1-mediated adaptive immune response is commonly elicited in *C. jejuni* infection. However, Th2-mediated responses can also be produced, and responses vary by both infecting *C. jejuni* strain and genotype of mouse used.

The concept that development of GBS results from an aberrant adaptive immune response to *C. jejuni* antigens, involving molecular mimicry between *C. jejuni* LOS and peripheral nerve gangliosides, is a central hypothesis for the immunopathogenesis of GBS.[4; 111] It is generally thought that *C. jejuni* infection leads to Th1-mediated adaptive immunity, which leads clinically to inflammation and diarrhea, but also clearance.[124] However, antibodies specific for the LOS of *C. jejuni* can be cross-reactive with peripheral nerve gangliosides, potentially leading to antibody- and complement-mediated nerve damage. The isotype of the cross-reactive antibody also has implications, with Th2-mediated IgG1 anti-ganglioside antibodies in GBS correlating with previous *C. jejuni* infection and worse clinical outcome.[53; 62] Thus it appears that a deviation in typical immune response to *C. jejuni*, likely mediated by both *C. jejuni* and host immune factors, predisposes an individual to development of GBS following *C. jejuni* infection.

RATIONALE FOR STUDY

Overarching Aim: Examine host-microbe interaction and clinical outcome in mice of different genetic backgrounds infected with different *C. jejuni* strains to assess interplay of both microbe and host factors

In vitro, C. jejuni strains vary considerably in virulence related to specific mechanisms in pathogenesis. Adherence to intestinal mucus substrate and adherence and invasiveness in cultured epithelial cells varies by *C. jejuni* strain.[73; 81] Variation in LOS structure, such as sialylation, between strains can impact Th-polarization following interaction with DC[8] and contributes to differences in epithelial cell invasion.[73] *In vivo*, numerous *C. jejuni* strains have been used in mice of the same genetic background (C57BL/6 IL-10$^{-/-}$) in models established by Mansfield and colleagues.[10; 11; 75] In our models, *C. jejuni* strains have varied in ability to colonize and produce colitis, and also in magnitude and type of systemic immune response reflected by plasma anti-*C. jejuni* and anti-ganglioside antibodies.

In addition to *C. jejuni* strain differences, host genetic background is postulated to contribute to disease outcome, including subsequent development of GBS. Evidence supporting a potential role of host factors include geographic clustering of GBS subtypes,[111] association of specific genetic factors with development of GBS,[56] and the fact that while ganglioside mimics on the *C. jejuni* LOS are more frequent in GBS than uncomplicated enteritis, alone they are not sufficient to cause GBS following *C. jejuni* enteritis.[5; 105]

Choice of C. jejuni *Strains*. For the current study, two *Campylobacter jejuni* strains used previously in mouse models in our laboratory were chosen based upon production of different disease outcomes and immune responses. *C. jejuni* 260.94 was originally isolated from a GBS patient in South Africa. It possesses a GM1a ganglioside mimic, is LOS class A and encodes *cst*-II sialyl transferase.[74] In our mouse models, encompassing different mouse strains, it has colonized well but is not colitogenic.[10; 21; 75; 108] Malik *et al* (2014) showed that *C. jejuni* 260.94 drove a predominantly Th2-mediated immune response, including production of anti-ganglioside antibodies, in C57BL/6 IL-10$^{-/-}$ mice.[75]

In contrast, *C. jejuni* 11168 was originally isolated from an enteritis patient in the United Kingdom. It harbors both GM1 and GM2 mimics, but is not reportedly associated with GBS in people. It is LOS class C and encodes *cst*-III sialyl transferase.[74] In our mouse models, *C. jejuni* 11168 has colonized well and produced moderate to severe colitis and Th1/Th17-associated anti-*C. jejuni* plasma antibodies in C57BL/6 IL-10$^{-/-}$ mice.[10; 11; 75-77; 101] A few studies have contained experiments with less striking results: a low proportion of mice exhibited colitis on a first passage, but subsequent passages resulted in marked colitis;[11] the severity of lesions was not as marked despite most mice displaying colitis in dose-response experiments;[76] and a more recent study in which infected mice exhibited lower colonization levels and less gross pathology.[21] Aside from these exceptions, infection of C57BL/6 IL-10$^{-/-}$ mice with *C. jejuni* 11168 has produced repeatable colitis in our models.

Choice of Mouse Genetic Background. While referring to Th1 or Th2 "prototypic" mouse strains is likely an oversimplification, several reports in the literature suggest that C57BL/6 mice exhibit a Th1 bias, while BALB/c mice tend to produce Th2 responses. Differences have also been seen in relative protection or susceptibility to different pathogens or disease between these mice, or higher or lower propensities for inflammatory responses. Differences in chemokine, chemokine receptor, and cytokine mRNA expression in spleen of immunologically naïve mice have been found. C57BL/6 mice expressed higher Th1-associated IFN-γ and IP-10, while BALB/c mice expressed more regulatory and Th2-associated LTβ, IFNβ, TGFβ1, CCR3, and CXCR4.[24] When stimulated with LPS or synthetic TLR2 ligand *in vitro*, C57BL/6 peritoneal macrophages produced more TNF-α and IL-12, and had higher bacteriocidal activity than did those from BALB/c mice.[116] Similarly, when spleen cells were stimulated with concanavalin A, C57BL/6 cells produced higher levels of IFN-γ and less IL-4, while the opposite was true in BALB/c cells.[82] Studies of splenic-derived DC showed that, upon stimulation with TLR ligands LPS, lipoprotein, and CpG, DC from C57BL/6 mice produced higher IL-12 while BALB/c DC produced more MCP-1.[72] Mucosal immunity also varies between these two mouse strains. Compared to C57BL/6 mice, BALB/c mice exhibited enhanced vitamin A metabolism, leading to greater IgA production.[38] BALB/c mice also had higher percentages of Treg cells in the small intestine and were more resistant to colitis induced by dextran sulphate sodium (DSS).[38] Another study demonstrated that resistance to DSS-induced colitis by BALB/c mice was associated with a Th2/Th17/Treg-mediated response, characterized by increased production of IL-4, IL-6, IL-10, and IL-17 and more Treg cells in the regional lymph node, while susceptible C57BL/6 mice produced more TNF-α and IFN-γ.[123] Finally, Chen *et al* (2005) showed that while Treg cells were phenotypically similar between C57BL/6 and BALB/c mice, the proportion of Treg cells was higher in the spleen, lymph node, and thymus of BALB/c mice. Furthermore, C57BL/6 CD4+ "T responders" were more resistant to Treg-mediated suppression than were BALB/c CD4+ T

cells.[25] Collectively, these studies support a systemic anti-inflammatory, Th2 bias in BALB/c mice and a more pro-inflammatory, Th1 systemic bias in C57BL/6 mice.

Choice of C57BL/6 and BALB/c mouse strains for the following study was made with consideration of the differences described above between these two strains. There is a reported association between Th2-mediated anti-ganglioside IgG1 antibodies and worse clinical outcome in GBS patients,[53; 62] and Malik *et al* (2014) reported that C57BL/6 IL-10$^{-/-}$ mice mounted a Th2-mediated response to GBS patient-derived *C. jejuni* 260.94.[75] As "Th2" biased mice, BALB/c are potential candidates for studying GBS immunopathogenesis. Furthermore, comparing C57BL/6 and BALB/c mice infected with *C. jejuni* strains known to produce different clinical outcomes will allow elucidation of the contributions of both host and *C. jejuni* factors in determining the immune response and disease outcome.

Objectives of this study included 1) investigating WT and IL-10$^{-/-}$ BALB/c mice as a model of *C. jejuni*-induced GBS, 2) comparing immune response and disease outcome in C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with colitogenic and GBS-associated *C. jejuni* strains, and 3) performing parallel *in vitro* assays with dendritic cells from C57BL/6 and BALB/c mice to determine how host-pathogen interactions impacting *C. jejuni* invasion, intracellular survival, and elicitation of cytokine production contribute to immune responses *in vivo*.

Specific Aim 1: *Investigate use of BALB/c (WT and IL-10$^{-/-}$) mice infected with GBS-associated* C. jejuni *strain 260.94 as model of GBS*

Hypotheses: BALB/c mice will develop Th2-mediated responses to *C. jejuni* infection, and will produce Th2-driven anti-ganglioside antibodies. Neurological disease will manifest as gait abnormalities and neuromuscular weakness observed during neurological phenotyping tests and macrophage infiltration into dorsal root ganglia. Colitis will be mild, if present. Responses will be exacerbated in IL-10$^{-/-}$ compared to WT mice.

Specific Aim 2: ***Describe differences in immune responses and enteric and neurological outcomes in C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with colitogenic*** **C. jejuni** ***11168 and GBS-associated*** **C. jejuni** ***260.94***

Hypotheses (note: formulated after consideration of results from first Specific Aim): Both C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 will develop Th1/Th17 immune responses and colitis. C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 will develop Th2-mediated responses, including anti-ganglioside antibodies, and nerve lesions manifested by increased macrophage infiltration into dorsal root ganglia. Conversely, BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 will develop Th1/Th17 responses and be protected from neurological disease, relative to C57BL/6 IL-10$^{-/-}$ mice.

Specific Aim 3: ***Use parallel*** **in vitro** ***assays mirroring the*** **in vivo** ***model of Specific Aim 2 to assess the initial host-microbe interaction, considering both*** **C. jejuni** ***strain differences and host genetic background***

Hypotheses: Bone marrow-derived dendritic cells (BMDC) from both C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice will efficiently internalize and kill both colitogenic and GBS-associated *C. jejuni* strains. Cytokines produced by infected BMDC derived from C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice will mirror immunogenicity and pathogenicity previously observed with these *C. jejuni* strains: *C. jejuni* 11168-infected IL-10$^{-/-}$ BMDC from both mouse strains and *C. jejuni* 260.94-infected BALB/c IL-10$^{-/-}$ BMDC will produce pro-inflammatory and Th1/Th17-polarizing cytokines. C57BL/6 IL-10$^{-/-}$ BMDC infected with *C. jejuni* 260.94 will produce Th2-polarizing cytokines.

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CHAPTER 2: BALB/C MICE INFECTED WITH GUILLAIN-BARRÉ SYNDROME-ASSOCIATED *CAMPYLOBACTER JEJUNI* STRAIN 260.94 EXHIBIT TH1/TH17-MEDIATED IMMUNITY

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ABSTRACT

Campylobacter jejuni is an important cause of bacterial gastroenteritis worldwide. A small proportion of *C. jejuni* infections are associated with subsequent development of Guillain-Barré syndrome (GBS), a debilitating polyneuropathy. The immunopathogenesis of GBS is poorly understood, but is thought to involve generation of antibodies cross-reactive to lipooligosaccharide on the *C. jejuni* outer membrane and structurally similar peripheral nerve gangliosides. Both infecting *C. jejuni* strain characteristics and host factors have been implicated in development of GBS as opposed to uncomplicated enteritis. Induction of anti-ganglioside antibodies in association with T helper 2 (Th2)-mediated immune responses was reported previously in C57BL/6 interleukin (IL)-$10^{-/-}$ mice infected with GBS patient-derived *C. jejuni* strain 260.94. In the current study, we investigated BALB/c mice, a strain reported to exhibit a Th2 immunological bias, as candidates for development of a GBS model. Wild-type (WT) and IL-$10^{-/-}$ BALB/c mice were orally inoculated with *C. jejuni* 260.94 and humanely euthanized after 5 weeks. Immune response was assessed by evaluation of *C. jejuni*-specific and anti-ganglioside plasma antibodies, in addition to histological lesions and cytokine production in the proximal colon. Mice were tested for neurological deficits by weekly phenotyping tests. Peripheral nerve lesions were assessed by scoring the number of macrophages in dorsal root ganglia. *C. jejuni* 260.94 stably colonized both WT and IL-$10^{-/-}$ mice and induced a systemic Th1/Th17-mediated immune response, as reflected by significant increases in *C. jejuni*-specific IgG2a, IgG2b, and IgG3 plasma antibodies. However, *C. jejuni* 260.94 did not induce production of anti-ganglioside antibodies, colitis, or marked neurological deficits or peripheral nerve lesions in either WT or IL-$10^{-/-}$ mice. In the current study, both WT and IL-$10^{-/-}$ BALB/c mice exhibited relative protection from development of Th2-mediated immunity and anti-ganglioside antibodies as seen previously in C57BL/6 IL-$10^{-/-}$ mice. These results indicate that BALB/c mice are a

useful model for studying the immune response to *C. jejuni* and provide insight into the role of host genetic background in determining susceptibility to GBS following *C. jejuni* infection.

INTRODUCTION

Campylobacter jejuni, a spiral, motile, Gram-negative, microaerophilic bacterium, is a common cause of human bacterial gastroenteritis worldwide, with the World Health Organization considering members of the genus *Campylobacter* as one of the four key global causes of diarrheal disease.[58] While *C. jejuni* can colonize chickens in high numbers without causing apparent disease, symptoms in infected people typically include diarrhea, abdominal pain, and fever.[58; 62]

Although usually self-limiting, human campylobacteriosis is linked to multiple post-infectious complications including irritable bowel syndrome, inflammatory bowel disease, reactive arthritis, and Guillain-Barré syndrome (GBS).[26; 58] GBS is debilitating, with many patients experiencing protracted recovery and residual neurologic deficits.[55] This syndrome is clinically heterogeneous, but common characteristic symptoms include rapidly progressing limb weakness and reflex deficits. Pain, weakness, ataxia, and respiratory insufficiency are variably seen depending upon the specific GBS disease subtype present.[55] Current reported estimates of GBS incidence following *C. jejuni* infection vary from 1/1,000-5,000 to 7/10,000.[26; 55] Although several infectious agents have been associated with GBS, *C. jejuni* has been identified as the most common cause of antecedent infection in GBS in epidemiological studies.[19; 25] The author of a recent literature review estimated that of >2,500 GBS cases, 31% were attributable to previous *Campylobacter* infection.[44]

Structural similarity between the lipooligosaccharide (LOS) of some *C. jejuni* isolates and peripheral nerve gangliosides, such as GM1, has been implicated in development of anti-ganglioside antibodies and GBS.[2; 40; 64] Antibodies to gangliosides, particularly to GM1 and often of the IgG isotype, are frequently detected in GBS patient sera.[45] Furthermore, presence of anti-ganglioside antibodies is correlated with slower patient clinical recovery and increased disability, and clinical improvement was seen with decreasing anti-ganglioside antibodies in patients with *C. jejuni*-associated GBS and the Miller-Fisher syndrome (MFS) GBS subtype.[23; 47]

Ganglioside-like epitopes were found more frequently in LOS from *C. jejuni* isolates from GBS or MFS patients than in isolates from those with uncomplicated enteritis.[2] However, presence of ganglioside-like moieties alone appears insufficient to elicit GBS, as expression of ganglioside mimics can be found in *C. jejuni* isolates from enteritis patients without GBS as well.[2; 53] These findings suggest a potential role of both *C. jejuni*-specific factors such as presence of ganglioside mimics on the LOS and host factors such as immunogenetic background in susceptibility to GBS following campylobacteriosis.

Murine models resembling *C. jejuni*-induced colitis seen in humans have been developed in recent years. These models,[5; 10; 16; 35; 36] which exploit alterations in the immune system, microbiota, or both, provide robust models of disease and overcome the transient or persistent asymptomatic colonization observed in previously studied murine models.[7; 8] Of particular relevance to the current study was the development of a colitis model in C57BL/6 mice lacking anti-inflammatory interleukin (IL)-10 following infection with *C. jejuni* 11168.[36] Development of spontaneous enterocolitis with age in IL-$10^{-/-}$ mice on different genetic backgrounds is well documented, and results primarily from unchecked T helper 1 (Th1)-mediated immunological responses to intestinal microbiota in the absence of the regulatory action of IL-10.[6; 28] This cytokine has important immunosuppressive action, especially upon monocytes and macrophages, and inhibits release of pro-inflammatory mediators.[49] In our studies, absence of IL-10 has proven critical for induction of inflammatory disease following *C. jejuni* infection, as wild-type (WT) mice infected with colitogenic *C. jejuni* strain 11168 are stably colonized, but do not develop the severe colitis seen in infected IL-$10^{-/-}$ mice.[9; 36; 37]

Significant progress has been made in developing murine colitis models, but robust animal models of human GBS have proven difficult to develop. Models in species including mice,[20; 50; 60] rabbits,[63] and chicken[30; 42] bear some similarity to various aspects of human disease. However, anatomic and physiologic differences between birds and humans limit the utility of the chicken model. Aside from the spontaneous autoimmune peripheral polyneuropathy occurring in mice,[50] the aforementioned

mammalian models are experimentally induced and require injection of LOS,[63] myelin,[60] or anti-ganglioside antibody[20] with additional factors such as Freund's complete adjuvant[60; 63] or normal human serum[20] for disease induction.

Importantly, mouse models of GBS induced following oral infection with *C. jejuni* have been developed that mimic the typical route of human infection.[35 9; 54] C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94, a strain isolated from a GBS patient, developed Th2-mediated responses including production of IgG1 antibodies to gangliosides GM1 and GD1a.[35] WT non-obese diabetic (NOD) mice and congenic IL-10$^{-/-}$ and costimulatory molecule B7-2$^{-/-}$ mice infected with *C. jejuni* 260.94 showed increased generation of anti-ganglioside antibodies and macrophage and T cell infiltration into peripheral nerves, including the sciatic nerve and dorsal root ganglia (DRG), compared to controls.[54] The microbiota also contributes to anti-ganglioside antibody production following *C. jejuni* 260.94 infection in WT C57BL/6 mice, as increased anti-GM1 IgG1 antibodies were seen in mice with a human-derived microbiota compared to mice with a conventional mouse microbiota.[9] These models show that expression of ganglioside mimics in an infecting *C. jejuni* LOS elicits the production of antiganglioside antibodies and likely contributes to peripheral nerve damage observed, yet other factors may also contribute.

The importance of host factors in determining susceptibility to GBS is poorly understood. Mouse strains including C57BL/6 and NOD backgrounds have been studied, and development of additional mouse models of different genetic backgrounds would facilitate understanding of the impact of host immune response in mediating the neuropathology in GBS. Another prerequisite of such studies would be that comparisons of mice of different genetic backgrounds be carried out with a rigorously similar study design. The choice of mouse strains to compare is critical. C57BL/6 IL-10$^{-/-}$ mice infected with colitogenic *C. jejuni* 11168 developed colitis and Th1/Th17-mediated immune responses, whereas mice of the same genotype infected with GBS patient-derived *C. jejuni* 260.94 mounted Th2-mediated responses, including IgG1 reacting with GM1 and GD1a gangliosides, but did not develop colitis.[35]

Several reports in the literature indicate immunological biases between BALB/c and C57BL/6 mice. One classic example is infection with *Leishmania major*, in which susceptible BALB/c mice produce high amounts of IL-4, while resistant C57BL/6 mice down-regulate IL-4 production and instead produce IFN-γ.[48] Multiple studies have demonstrated that upon *in vitro* stimulation, peritoneal macrophages, spleen cells, and dendritic cells derived from BALB/c mice exhibit a relative Th2 bias when exposed to various stimuli compared to C57BL/6 mice, which exhibit a more inflammatory, Th1-mediated bias.[32; 33; 39; 56] In two studies of GBS patients, presence of IgG1 anti-ganglioside antibodies alone was associated with previous *C. jejuni* infection and worse clinical outcomes, while IgG3 antibodies, either alone or in combination with IgG1 anti-ganglioside antibodies, were associated with preceding respiratory infection and better outcomes.[24; 27] Thus, we sought to develop a GBS mouse model characterized by Th2-biased responses after enteric infection, resulting in strong IgG1 responses.

Considering the production of Th2-mediated responses in C57BL/6 IL-10$^{-/-}$ mice infected with GBS patient-derived *C. jejuni* 260.94, the reported relative Th2-bias in BALB/c compared to C57BL/6 mice, and the increased severity of GBS associated with anti-ganglioside IgG1 in people, we reasoned that BALB/c mice are potential candidates for a *C. jejuni*-induced GBS model. We hypothesized that WT and IL-10$^{-/-}$ BALB/c mice infected with *C. jejuni* 260.94 would 1) mount systemic and local (gastrointestinal (GI) tract) Th2-driven immune responses, including production of anti-ganglioside antibodies, without development of colitis, 2) develop gait abnormalities, neuromuscular weakness, and macrophage infiltration into peripheral nerves, reflecting *C. jejuni*-associated neuropathology; and finally, 3) that immune responses and neuropathology would be exacerbated in mice lacking anti-inflammatory IL-10.

To test these hypotheses, BALB/c mice (WT and IL-10$^{-/-}$) were orally infected with *C. jejuni* 260.94. This strain was originally isolated from a GBS patient in South Africa and harbors a GM1a ganglioside mimic. In our GBS models including mice of NOD and C57BL/6 genetic backgrounds, it

colonized well without inducing colitis[9 4; 35; 54] and has induced anti-ganglioside antibodies[35; 54] and nerve lesions.[54] ELISAs were used to measure plasma anti -*C. jejuni*, -GM1, and -GD1a IgG antibody subtypes. A flow-cytometry based multiplexed bead assay was used to measure cytokines reflecting local Th adaptive immune responses in the proximal colon. Histological lesions in the gastrointestinal tract were graded according to a previously published scale.[36] Neurological phenotyping tests included DigiGait, open field test (OFT), and hang testing. Morphometry was used to quantitate number of macrophages labeled immunohistochemically with F4/80 in lumbar dorsal root ganglia (DRG).

Results from this study indicate that *C. jejuni* 260.94 colonized infected BALB/c mice well and induced a Th1/Th17 response that was exacerbated in IL-10$^{-/-}$ mice, but did not produce colitis. Significant differences in anti-ganglioside antibody isotypes were related to mouse genotype, but not *C. jejuni* infection status, and did not correlate with increased macrophage numbers in DRG. No overt neurological phenotype was observed in any experimental mouse. Thus, surprisingly this reportedly Th2-biased mouse strain, when infected with a *C. jejuni* strain expressing ganglioside mimics in the outer core that previously induced a Th2 response in C57BL/6 IL-10$^{-/-}$ mice, mounted predominantly Th1/Th17-mediated responses. The contrasting immune responses between mice of different genetic backgrounds to the same *C. jejuni* strain seen in this study and previously[35] offer insight into the role of host factors in determining susceptibility to GBS following *C. jejuni* infection. Additionally, these results support the use of WT and IL-10$^{-/-}$ mice on the BALB/c genetic background as an additional model for studying immune responses to *C. jejuni* infection.

MATERIALS AND METHODS

***Mice*.** All mouse experiments were performed according to recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (approval numbers

06/12-107-00 and 06/15-101-00). BALB/cJ (referred to as BALB/c WT) and C.129P2(B6)-*Il10*^{tm1Cgn}/J (referred to as BALB/c IL-10$^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in house. Age matched male and female mice used in the experiment were bred and maintained in-house. Husbandry has been previously described.[36] Wild-type or IL-10-deficient genotype was verified using a PCR assay offered by the Jackson Laboratory (https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:23475,004333). DNA extracted from feces or cecal tissue collected from experimental mice used in this study was screened for enteric pathogens including *Citrobacter rodentium*, *Enterococcus faecium* and *E. faecalis*, *Helicobacter* spp., and *Campylobacter* spp. by PCR as previously described.[36] Prior to inoculation, mice were transported to the MSU's University Research Containment Facility. The mice were housed individually following acclimation. At the humane endpoint or at the end of the experiment, all mice were humanely euthanized using an overdose of CO_2 according to the guidelines of the American Veterinary Medical Association (https://www.avma.org/KB/Policies/Pages/Euthanasia-Guidelines.aspx).

Experimental Design. Prior to this study, the ability of *C. jejuni* 260.94 to colonize BALB/c WT mice was verified by inoculation of 5 mice with *C. jejuni* 260.94 (dose = 3.7×10^9 colony forming units, CFU) by gastric gavage. Three mice inoculated with tryptic soy broth (TSB; sham/vehicle) served as controls. The inoculum was prepared and confirmation of colonization of *C. jejuni* 260.94 by culture and PCR for this pilot study was performed as described below.

Forty mice, including 20 BALB/c WT and 20 BALB/c IL-10$^{-/-}$ mice, were used in this study. Mice of each genotype were randomized regarding sex, litter, treatment group, and cage position upon the rack. Mice were inoculated at 14–15 weeks of age. Ten BALB/c WT and 10 BALB/c IL-10$^{-/-}$ mice received TSB; likewise, 10 BALB/c WT and 10 BALB/c IL-10$^{-/-}$ mice received *C. jejuni* 260.94. BALB/c WT mice were

inoculated one week before BALB/c IL-$10^{-/-}$ mice. Both groups were sacrificed 5 weeks following their respective inoculation.

Campylobacter jejuni *Inoculum Preparation and Inoculation.* *Campylobacter jejuni* strain 260.94 originally obtained from ATCC (strain BAA-1234) and stored as glycerol stock cultures at -80°C was used in this study. The inocula for the BALB/c WT and BALB/c IL-$10^{-/-}$ groups were prepared as previously described.[36] Briefly, for each preparation, inoculum from frozen stock was streaked onto tryptic soy agar plates supplemented with 5% defibrinated sheep's blood (Cleveland Scientific, Bath, OH) (TSAB plates). The plates were incubated for approximately 24 hours at 37°C in vented anaerobic jars. Generation of the microaerobic environment was achieved by incubation with a Campy*Gen* sachet (Oxoid, Basingstoke, United Kingdom) or by evacuation of the jar to -25 in Hg followed by equilibration with a gas mixture comprising 80% N_2, 10% CO_2, and 10% H_2. Colonies were harvested, suspended in TSB, spread into lawns on fresh TSAB plates, and incubated overnight at 37°C in the microaerobic environment. The following day, lawns were harvested and suspended in TSB, aiming to achieve an optical density at 600 nm of approximately 1.0 in a 1:10 dilution of culture. Motility, spiral morphology, and purity of both inoculums were confirmed by wet mount and Gram stain preparations. Mice were inoculated with 0.1 mL of *C. jejuni* 260.94 or TSB via intra-gastric gavage using a 3.5 Fr red rubber catheter. Immediately before and after inoculation, the inocula were serially diluted in TSB, spread on TSAB plates, and the plates incubated at 37°C in the microaerobic environment for approximately 48 hours. Final calculated doses of *C. jejuni* 260.94 inoculum were 3.1×10^8 CFU for BALB/c WT mice, and 2.8×10^8 CFU for BALB/c IL-$10^{-/-}$ mice.

Monitoring for Clinical Signs. Mice were checked at least once daily for clinical signs starting at inoculation. Evaluated clinical parameters included observation of eating and drinking and overall

activity level, and gauging abnormalities in respiratory rate, hair coat, posture (e.g., hunching), defecation including diarrhea, and level of movement including rearing. Any mouse reaching a pre-determined humane endpoint was immediately euthanized and necropsy was performed.

Neurological Phenotyping. Mice underwent phenotyping tests weekly to evaluate clinical signs of neurological disease including gait abnormalities and loss of motor strength. Assessments included DigiGait treadmill analysis, hang test, and open field test (OFT). Prior to inoculation, mice were acclimated to these tests prior to the start of the experiment and baseline data taken prior to inoculation were included in the statistical analyses.

DigiGait Imaging System. The DigiGait (Mouse Specifics, Inc., Quincy, MA) treadmill system records numerous gait indices to allow detection of subtle gait abnormalities. The mouse was placed upon a transparent moving belt. A digital camera underneath the belt captured movement using DigiGait Video Imaging Acquisition software (Mouse Specifics, Inc., Quincy, MA). Data were recorded over a 3-second run in which the mouse ran in the center of the belt, with no or minimal leaning against the clear plastic siding or contact with the front or back bumpers. Digital images were then processed using the DigiGait Imaging Analysis software (Mouse Specifics, Inc., Quincy, MA). Numerical data reflecting multiple gait parameters are calculated for each limb and exported to an Excel spreadsheet for statistical analysis. Of the 42 available gait parameters, eight were deemed most relevant for analysis: [13; 21; 43] swing (s), propel (s), stance (s), stride (s), stride length (cm), stride frequency (steps/s), absolute paw angle (deg), and stance width (cm). In this study, the mice were recorded running at speeds of 25 cm/s, 30 cm/s, and 35 cm/s. If needed, mice were given multiple chances to achieve a 3-second run, with rest periods between attempts. The belt was cleaned with 70% ethanol between each mouse.

Hang Test. The hang test served as a measure of motor strength and balance. This test was performed as described[11; 54] with minor modifications. Briefly, the mouse was placed on a square metal grid elevated several inches above its cage and allowed to grip the metal. A stopwatch was started when the grid was flipped upside down. Mice were allowed to hang for up to 60 seconds, and the time elapsed until they fell off the grid into the cage was recorded. This was repeated 3 times, with the mice allowed to rest for 60 seconds between each trial. The grid was cleaned with 70% ethanol between mice. The average time to fall (or a maximum of 60 seconds if the mouse did not fall) over the 3 trials was used in the analyses.

Open Field Test. The OFT allows assessment of gait, posture, and activity level. The OFT was performed by allowing the mouse to move freely within a clear plastic standard 18" $\times$ 8" rat cage with 4 quadrants demarcated on the bottom. Activity of the mouse was recorded for 60 seconds, beginning with placement in the center of the cage, with a stationary video camera mounted on a tripod and set at approximately the same angle each week. At the end of each video, the mouse number was shown, with genotype and treatment group de-identified. The cage was cleaned with 70% ethanol between each mouse. Parameters intended for assessment included number of quadrants crossed, number of rears, and gait or posture abnormalities such as increased stance width or splayed toes.

Necropsy Procedures. Mice were humanely euthanized by CO_2 overdose 5 weeks following inoculation. Immediately thereafter, weight was recorded for each mouse and blood samples were collected via cardiac puncture using a 1 mL tuberculin syringe loaded with 0.1 mL 3.8% sodium citrate.

Instruments were sterilized using a hot bead sterilizer between each section of the GI tract. The cecum was excised including approximately 0.5–1 cm of adjoining proximal colon and ileum. The tip of the cecum was removed for bacteriology studies. The remaining ileocecocolic junction (ICJ) was infused with 10% neutral phosphate buffered formalin (Fisher Scientific) and placed onto a sponge in a

histological tissue cassette (Fisherbrand Histosette II Tissue Cassette, Fisher HealthCare, Pittsburgh, PA) and immersed in 10% neutral phosphate buffered formalin.

Sections of stomach, jejunum, proximal colon, and the tip of the cecum were excised and rinsed in PBS. Pieces of each tissue were divided into three sections: two were snap frozen in microfuge tubes and cryovials, and the third was streaked onto *Campylobacter* selective medium[36] (TSAB-CVA plates: TSAB plates containing cefoperazone (20 µg/mL), vancomycin (10 µg/mL), and amphotericin B (2 µg/mL); antimicrobials were obtained from Sigma-Aldrich, St. Louis, MO). Plates were placed into air-tight jars with a Campy*Gen* sachet (Oxoid, Basingstoke, Hampshire, UK). Fecal pellets were collected into microfuge tubes and placed on ice.

The sciatic nerve and 2 to 3 dorsal root ganglia (DRG) were collected from experimental mice for histological and morphological analysis in a two-step procedure. At the time of necropsy, the mouse was skinned and the muscles overlying the spine were removed. The roof of the vertebral canal was removed using Castroviejo microsurgical scissors to expose the spinal cord. Muscle and connective tissue were bluntly dissected to expose the sciatic nerve on each side. Thereafter, the carcass was submerged in 10% neutral buffered formalin in a specimen cup for further dissection of tissues at a later date.

On return to the laboratory, snap frozen tissues were stored at -80°C. Gastrointestinal samples streaked on TSAB-CVA plates in the air-tight jars were placed at 37°C. Fecal pellets were mashed with a sterile applicator stick in TSB containing 15% glycerol, vortexed, spread onto TSAB-CVA plates, and incubated at 37°C in jars with the microaerobic environment generated by evacuation and equilibration with the gas mixture. Plasma separated from whole blood by centrifugation was harvested and stored at -80°C until analysis. The ICJ cassettes and carcasses for nerve dissection were transferred from formalin to 60% ethanol after 24 and 48 hours, respectively.

Confirmation of Colonization by Culture and PCR. Colonization of stomach, jejunum, cecum, colon, and feces was reported according to a semi-quantitative grading system of plate coverage by *C. jejuni* colonies[36] following 48–72 hours of incubation: 0 = no growth; 1 = light growth (approximately 1–20 CFU); 2 = moderate growth (20–200 CFU); 3 = heavier growth (>200 CFU); 4 = confluent growth.

To confirm *C. jejuni* 260.94 colonization of infected mice at necropsy, and exclude colonization in sham-inoculated mice, *C. jejuni*-specific PCR for the *C. jejuni gyrA* gene was conducted.[36; 59] For culture-positive mice, isolates of *C. jejuni* from cecal or colon samples obtained at necropsy were used. For culture-negative infected mice and sham inoculated mice, DNA extracted from frozen cecal tissues obtained at necropsy using a commercial kit (DNEasy Blood and Tissue Kit, QIAGEN, Valencia, CA) was used.

***Pathologic Changes: Gastrointestinal Tract*.** Gross lesions in the gastrointestinal tract and changes in the ileocecocolic lymph node and spleen noted during necropsy were recorded as noted by a veterinarian and other experienced personnel. The ICJs were processed routinely by the Investigative Histopathology Laboratory, Division of Human Pathology, MSU. Briefly, tissue samples were embedded in paraffin, sectioned at 4–5 µm, stained with hematoxylin and eosin (H&E), and coverslipped. The ICJs were examined histologically by a board certified veterinary clinical pathologist (JMB) blinded to the genotype and treatment groups. Lesions were graded using a previously published scoring system,[36] with the exception that intraepithelial lymphocytes were not scored in the current study. The scale encompasses changes in the lumen (exudates, excessive mucus), epithelium (surface integrity, goblet cell hypertrophy or depletion, crypt abnormalities), lamina propria (inflammatory cell infiltrates), and submucosa (inflammation, edema, fibrosis). Raw scores (out of 42 total points) were ranked as semi-quantitative grades 0 (0–9 points; no colitis), 1 (10–19 points; mild colitis), or 2 (≥20; moderate or severe colitis).

Enzyme-Linked Immunosorbent Assay (ELISA). Aliquots of plasma were made to prevent repeated freeze-thaw cycles. Anti -*C. jejuni*, -GM1, and -GD1a antibody isotypes including IgG1, IgG2a, IgG2b, and IgG3 were evaluated. The assays were performed as previously described.[16; 36] Briefly, 96-well plates (Nunc Maxisorp, Thermo Scientific, Rochester, NY) were coated with antigen and incubated at 4°C overnight. Antigens were diluted in PBS to the following concentrations: *Campylobacter jejuni* antigen[36] 1.9 µg/mL; GM1 antigen (US Biological, Swampscott, MA) 2 µg/mL; GD1a antigen (Sigma Aldrich, St. Louis, MO) 20 µg/mL. The plates were blocked with blocking buffer (10mM PBS with 3% BSA and 0.05% Tween-20 (Sigma)) overnight at 4°C. Following three washes in wash buffer (PBS with 0.025% Tween-20), plasma samples diluted in blocking buffer (all samples were diluted 1:25, except for anti-*C. jejuni* IgG2b and IgG2a, which were 1:100) were loaded in triplicate. Positive controls included plasma from mice from previous experiments with a high OD or commercially available antibodies (anti-GD1a IgG1 (EMD Millipore, Temecula, CA)). Negative controls included anti-*Toxoplasma gondii* antibody (ViroStat, Portland, ME), and wells including only blocking buffer. Sealed plates were incubated with samples overnight at 4°C. Plates were washed, and secondary antibodies (Biotin-SP-conjugated AffiniPure Goat Anti-Mouse IgG1, IgG2a, IgG2b, or IgG3; Jackson ImmunoResearch, West Grove, PA) diluted in blocking buffer were added. Following incubation for 1 hour on a platform shaker, plates were washed again and ExtrAvidin peroxidase (Sigma-Aldrich) (diluted 1:2,000 in 10 mM PBS with 1% BSA and 0.05% Tween-20) was added. Plates were incubated for 1 hour on a platform shaker, washed, and tetramethylbenzidine (TMB substrate; Rockland Immunochemicals Inc., Gilbertsville, PA) was added. The reaction was stopped with 2N H_2SO_4. Absorbance was read at 450 nm using a Bio-Tek EL-800 Universal Microplate Reader with KC Junior software (Bio-Tek Instruments, Winooski, VT). In low numbers of samples, the CV% for triplicate values was 10% or greater. In these cases, if a clear outlier was present, that value was excluded. The absorbance generated from the diluent (blocking buffer) alone was subtracted from the mean absorbance obtained for each sample run in triplicate. This adjusted value was used in statistical

analyses. Negative values generated by subtracting the absorbance of the blocking buffer from the mean sample absorbance were treated as zero for the purpose of statistical analysis.

Measurement of Colon Cytokine Production. Rinsed samples of proximal colon collected in Eppendorf tubes and snap frozen at necropsy were stored at -80°C until analysis. Samples were thawed on ice and wet weight was recorded. Samples were homogenized on ice for one minute in 400 µL of Hank's Balanced Salt Solution (Sigma), with 0.5% Triton X-100 (Sigma) and the cOmplete Mini EDTA-free Protease Inhibitor cocktail (Roche/Sigma) using an autoclaved microtube pellet pestle rod powered by a handheld Kontes pellet pestle motor. Homogenates were centrifuged at 12,000*g* for 30 minutes at 4°C, and supernatants were aliquoted in cryovials and stored at -80°C until analysis.

Cytokines were measured using a flow cytometry based multiplexed bead assay panel (LEGENDPlex Mouse Th Cytokine Panel, BioLegend, San Diego, CA). Cytokines included in the panel are designed to characterize the adaptive immune response by delineating Th polarization. Analytes included IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, and IL-22. Prior to analysis, aliquoted supernatants of the colon homogenates were thawed on ice and centrifuged at 300*g* for 10 minutes at 4°C. The assay was performed using undiluted supernatant and a V-bottom microplate according to the manufacturer's instructions. Data were acquired on a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and analyzed using the LEGENDplex Data Analysis software (BioLegend). A standard curve was generated for each analyte. Each cytokine had a maximum standard concentration of 10,000 pg/mL. Data are presented as pg cytokine/mg tissue weight.

Assessment of Nerve Histopathology. Dissection of fixed tissue was performed using a dissecting microscope and nerves and dorsal root ganglia were embedded en bloc in a single cassette. Two to 3 lumbar dorsal root ganglia (DRG) were harvested from the left side of the mouse. Where possible and in

many cases, connections from the sciatic nerve to one or all DRG collected, including those in L3, L4, or L5 regions, were visualized prior to removal of DRG. The left sciatic nerve, brachial plexus, and lumbar DRG were placed in a cassette and stored in 60% ethanol until submission for histopathology.

The sections were labeled immunohistochemically for the mouse macrophage marker F4/80 by the Investigative Histopathology Laboratory, Division of Human Pathology, MSU. Specimens were embedded in paraffin and sectioned on a rotary microtome at 4 μm. Sections were placed on charged slides and dried at 56°C overnight, deparaffinized in xylene, and hydrated through descending grades of ethyl alcohol to distilled water. Slides were then placed in Tris Buffered Saline pH 7.4 (TBS; Scytek Labs – Logan, UT) for 5 minutes for pH adjustment. Following TBS, epitope retrieval was performed using Citrate Plus Retrieval Solution pH 6.0 (Scytek) in a vegetable steamer for 30 minutes followed by a 10 minute countertop incubation and several changes of distilled water. Following pretreatment standard avidin-biotin complex staining steps were performed at room temperature on the DAKO Autostainer. All staining steps are followed by two minute rinses in Tris Buffered Saline + Tween 20 (Scytek). After blocking for non-specific protein with Normal Rabbit Serum (Vector Labs – Burlingame, CA) for 30 minutes, sections were incubated with Avidin / Biotin blocking system for 15 minutes each (Avidin D – Vector Labs / d-Biotin – Sigma). Primary antibody slides were incubated for 60 minutes with the Monoclonal Rat anti- Mouse F4/80 diluted @ 1:100 (AbD Serotec – Raleigh, NC) in Normal Antibody Diluent (NAD) (Scytek). Biotinylated Rabbit anti-Rat IgG (H + L) Mouse Absorbed prepared at 10.0μg/mL in NAD incubated for 30 minutes, followed by R.T.U. Vector Elite Peroxidase Reagent (Vector) incubation for 30 minutes. Reaction development utilized Vector Nova Red Kit peroxidase chromogen incubation of 15 minutes followed by counterstain in Gill 2 Hematoxylin (Cancer Diagnostics – Durham, NC) for 30 seconds, differentiation, and dehydration, clearing, and mounting with Permount mounting media.

The number of F4/80 positive cells was quantified morphometrically. Images were analyzed using ImageJ software (version 2.0.0rc-49/1.51d), distributed by Fiji (Fiji Is Just ImageJ) for Windows

(http://imagej.net/Fiji/Downloads).[51; 52] The investigator (MM Cluett) was blinded to mouse genotype and treatment group. Contiguous images of each DRG section obtained at 100× magnification (10× objective) were opened in the ImageJ program. Positive cells were marked on the image using the "Cell Counter" plugin. After all positive cells were marked, the area was outlined using the "Freehand Selections" Trace Tool. When necessary, multiple areas were traced individually and the sum of the areas was recorded. Results are given as number of F4/80 positive cells/area, with area representing 100,000 pixels. The slides were finally unblinded for statistical analysis.

***Statistical Analyses*.** Analyses were performed using commercially available statistical software packages (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA; SigmaStat 3.5 for Windows, Systat Software, Inc., Point Richmond, CA) and online statistical applications (VassarStats Website for Statistical Computation; vassarstats.net).

DigiGait data generated for individual limbs were analyzed using 2-way repeated measures ANOVA, followed by Holm-Sidak's multiple comparisons test. These analyses were conducted using SigmaStat due to missing values for those weeks when a mouse was unwilling to run on the DigiGait; SigmaStat uses a general linear model to generate a best estimate of the missing values. The mice were first grouped by combined genotype and treatment group (e.g., [WT TSB], [IL-10$^{-/-}$ *C. jejuni* 260.94]) to compare all experimental mice together. Subsequent analyses evaluated differences in all parameters between control and infected groups for WT mice alone, followed by analysis of IL-10$^{-/-}$ mice alone. Data were tested for normality and equal variance. Analyses of data not meeting the assumption of equal variance are excluded from results. Data from the hang test were analyzed using 2-way repeated measures ANOVA, followed by Holm-Sidak's multiple comparisons test.

The Freeman-Halton extension of the Fisher Exact Probability Test was used to assess differences in colonization identified by culture between infected BALB/c WT and infected BALB/c IL-10^{-}

$^{/-}$ mice. For this analysis, semi-quantitative colonization grades included 0 (0 CFU), 1 (1–20 CFU), 2 (20–200 CFU) and 3 (>200 CFU; combining grades of >200 CFU and confluent growth).

Gross pathology in sham-inoculated and *C. jejuni* 260.94-infected BALB/c IL-10$^{-/-}$ mice was similarly assessed using the Freeman-Halton extension of the Fisher Exact Probability Test. For this analysis, gross pathology changes recorded at necropsy including enlarged and/or thickened proximal colon, enlarged cecum, enlarged spleen, and enlarged ileocecocolic lymph node, were graded as 0 (no changes), 1 (1 change), or 2 (2 changes).

Due to the lack of independence of observations comprising the composite score, ranks of colitis scores were analyzed using the Freeman-Halton extension of Fisher Exact Probability Test performed on overall group data, followed by 6 pair-wise comparisons. Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's post-test, was also performed on raw histopathology scores.

Data obtained from plasma ELISAs, colon cytokine measurement, and F4/80 scoring of DRG were analyzed using the non-parametric Kruskal-Wallis one-way ANOVA, followed by Dunn's multiple comparisons test when overall significance was found.

RESULTS

Confirmation of Mouse Genotype and Absence of Enteropathogens. Genotype (WT, or IL-10$^{-/-}$) was confirmed by PCR on DNA obtained from cecal tissue. At the start of the experiment, all mice were negative by PCR for all enteropathogens tested (*Citrobacter rodentium*, *Enterococcus faecium* and *E. faecalis*, *Helicobacter* spp., and *Campylobacter* spp.).

Clinical Signs. One infected BALB/c WT mouse required early humane euthanasia at 14 days post infection (p.i.) due to a progressively worsening hunched posture, mildly decreased activity, rougher coat, thinner body condition, and mildly increased respiratory effort. Necropsy revealed a large thoracic

mass. This was considered unrelated to *C. jejuni* infection, and was not further investigated. Due to morbidity unrelated to *C. jejuni* infection and the span of time between euthanasia of this mouse and remaining experimental mice (approximately 3 weeks), data for this mouse are included in results of colonization but no other experimental parameters. Questionable or mildly hunched posture, decreased activity, and hair coat quality were monitored carefully and were noted in 3/10 sham-inoculated WT mice, 7/9 infected WT mice, 5/10 sham-inoculated IL-10$^{-/-}$ mice, and 6/10 infected IL-10$^{-/-}$ mice at least once during the study. Many of these signs were attributed to characteristic BALB/c posture or hair coat appearance. No other mice required early humane euthanasia, and the remaining 39 experimental mice were humanely euthanized at 5 weeks p.i.

Neurological Phenotyping. Of all neurological phenotyping tests the results of the Digigait treadmill analysis were most informative. Data from the OFT were not analyzed, because the mice did not display enough spontaneous activity to provide sufficient information regarding rearing, quadrant crossing, or gait abnormalities.

DigiGait. Images recorded at 25 cm/s were processed as the most mice consistently ran at this speed each week. An infected male BALB/c IL-10$^{-/-}$ mouse with repeated weeks of missing data was excluded from analysis. A small number of analyses failing equal variance were excluded from results: right hind limb stride and stride length in the combined analysis; left hind limb swing in the IL-10$^{-/-}$ only analysis; left forelimb absolute paw angle in the WT only analysis. Only parameters for which $P < 0.05$ was found for either treatment group or time in the overall analysis are given (**Table 2.1**). Significant differences related to week of testing were found for multiple parameters, most frequently involving Baseline and Week 4. Significant treatment group differences were identified in the front limbs, including stance width and paw angle. When WT and IL-10$^{-/-}$ genotypes were analyzed together (combined analysis), infected IL-10$^{-/-}$ mice had wider forelimb stance width than sham-inoculated WT

mice. When the genotypes were analyzed separately, forelimb stance width was wider in infected than in sham-inoculated WT mice, but the difference was not significant within IL-10$^{-/-}$ mice. A significant effect of treatment group was also seen in the combined analysis in absolute paw angle of the right forelimb, but no pairwise comparisons were significant. Collectively, these data suggest that a relatively small proportion of gait parameters analyzed were impacted by *C. jejuni* infection.

Hang test. No significant differences between treatment groups were found (P = 0.199). However, a significant time effect (P <0.001) was found, with significant differences seen in pairwise comparisons between baseline and weeks 2, 4, and 5 (data not shown).

Colonization. At sacrifice, 9/10 infected mice from each group were culture positive in at least one area of the GI tract. Although samples from the GI tract included stomach, jejunum, cecum, colon, and feces, the cecum has been reported to be the most consistently and heavily colonized area of the GI tract[36; 37] and thus results from the cecum are shown (**Figure 2.1**). Positive *C. jejuni* 260.94 culture results in all samples from infected WT BALB/c mice were as follows: 1/10 in the stomach; 7/10 in the jejunum; 8/10 in the cecum; 9/10 in the proximal colon; 9/10 in the feces. Positive *C. jejuni* 260.94 culture results in all samples from infected BALB/c IL-10$^{-/-}$ mice were as follows: 1/10 in the stomach; 0/10 in the jejunum; 9/10 in the cecum; 9/10 in the proximal colon; 8/10 in the feces. PCR for the *C. jejuni gyrA* gene was positive on isolates cultured from necropsy samples. All 20 TSB-inoculated mice were negative for *C. jejuni* by culture in all 5 areas sampled from the GI tract. The two infected but culture-negative mice and all sham inoculated animals were negative for *C. jejuni* by *gyrA* PCR on DNA isolated from frozen cecal tissue. Results of Fisher Exact Probability Test showed that there was no significant difference in colonization of the cecum in infected BALB/c WT versus infected BALB/c IL-10$^{-/-}$ mice (P_B = 0.775).

Gross Pathology and Histological Assessment of Colitis. Gross pathological changes noted at necropsy included enlarged and/or thickened proximal colon or cecum and enlarged spleen or ileocecocolic lymph node. Gross pathology in WT mice was minimal, regardless of infection status. Nearly all mice exhibiting one or more gross pathological changes were BALB/c IL-10$^{-/-}$ mice, regardless of *C. jejuni* 260.94 infection status (**Figure 2.2A**). Nine sham-inoculated IL-10$^{-/-}$ mice had an enlarged or thickened proximal colon, and one of these mice also had an enlarged spleen. Similarly, 8/10 *C. jejuni*-infected IL-10$^{-/-}$ mice had an enlarged or thickened proximal colon; of these 8 mice, 3 also had an enlarged spleen, one also had an enlarged lymph node, and one also had a thickened cecal wall. As a single mouse from each of the WT treatment groups had only one gross pathological change and the remaining mice from both groups had no reported changes, gross pathology was not further analyzed in WT mice. Within BALB/c IL-10$^{-/-}$ mice, comparison of gross pathology between sham-inoculated and *C. jejuni* 260.94-infected mice was assessed by Fisher Exact Probability Test. No significant difference was found in gross pathology between infected and control IL-10$^{-/-}$ mice ($P_B = 0.087$).

Histological scoring of the ICJs placed all mice from all four treatment groups into grade 0 (0–9 points; no colitis) or grade 1 (10–19 points; mild colitis). As with changes noted grossly, more BALB/c IL-10$^{-/-}$ mice had histologic lesions in the ICJ relative to BALB/c WT mice, regardless of *C. jejuni* infection status (**Figure 2.2B**). All 10 sham-inoculated and all 9 *C. jejuni*-infected WT mice had scores ≤ 9, indicating no colitis. The IL-10$^{-/-}$ mice inoculated with TSB showed the greatest range of raw scores (3–17) of any of the four treatment groups. Four of 10 sham-inoculated IL-10$^{-/-}$ mice had mild colitis (scores ranging from 10–17) while the other 6 in this group had no colitis (scores ≤ 9). The most frequent changes seen in TSB-inoculated IL-10$^{-/-}$ mice were subjectively mild overall, reflecting the numerical score in the mild colitis category, and included luminal exudates comprising mucus and few neutrophils, damage to single cells in the surface epithelium, goblet cell depletion, crypt hyperplasia, increased mononuclear cells in the lamina propria, and extension of inflammation into the submucosa in 3 mice.

Similarly, 4/10 *C. jejuni*-infected IL-10$^{-/-}$ mice had mild colitis (scores ranging from 11–16), while the other 6 in this group had no colitis (scores ≤ 9). Pathological changes seen in *C. jejuni*-infected IL-10$^{-/-}$ mice with mild colitis were similar to those seen in sham-inoculated mice, including mucus and few neutrophils in the lumen, damage to single cells in the surface epithelium, goblet cell depletion, crypt hyperplasia, increased mononuclear cells in the lamina propria, and extension of inflammation into the submucosa in one mouse. While significance was seen overall using both Fisher Exact Probability Test on ranked data (P_B = 0.012) and Kruskal-Wallis on raw scores (P = 0.0265), no pairwise comparisons were significant in either analysis. Overall, these data suggest that IL-10$^{-/-}$ mice had mildly higher baseline levels of gross GI pathology and histologic lesions in the ICJ than WT mice, attributed to spontaneous colitis in IL-10$^{-/-}$ mice unrelated to *C. jejuni* infection.

Immune Response to* C. jejuni *Infection. Systemic (plasma) and local (colonic) immune responses to infection were measured.

Plasma Antibodies. Multiple antibody isotypes reacting with *C. jejuni* and gangliosides GM1 and GD1a antigens were analyzed in an indirect ELISA format. Isotypes were chosen to reflect systemic Th1 (IgG3, IgG2a, IgG2b), Th17 (IgG2b), and Th2 (IgG1) mediated responses.[1; 3; 38; 57] Plasma antibody responses to *C. jejuni* are shown in **Figure 2.3A**. Infected mice mounted primarily a Th1/Th17 response to *C. jejuni*, as shown by statistically significant increases in plasma anti-*C. jejuni* IgG2a and IgG2b levels compared to control mice, within both WT and IL-10$^{-/-}$ genotypes. Th1-mediated IgG3 was significantly elevated in *C. jejuni*-infected IL-10$^{-/-}$ mice when compared to sham-inoculated mice of either WT or IL-10$^{-/-}$ genotype. However, infection with *C. jejuni* did not produce significantly elevated IgG3 in WT mice compared to sham-inoculated WT mice, indicating that *C. jejuni* infection only stimulated a significant IgG3 response in IL-10$^{-/-}$ mice. Th2-mediated IgG1 responses were significantly increased in both sham-inoculated and infected IL-10$^{-/-}$ mice compared to sham-inoculated WT mice. Collectively, these data

indicate that BALB/c mice infected with *C. jejuni* 260.94 mounted a primary Th1/Th17 systemic response that was exacerbated by IL-10 deficiency.

Because anti-ganglioside antibodies are a hallmark of GBS and immunopathogenesis of GBS is thought to involve molecular mimicry between *C. jejuni* LOS and peripheral nerve gangliosides, anti -GM1 and -GD1a antibodies were also measured by ELISA. In general, magnitude of response and patterns of elevation in different groups were similar for anti-GM1 antibodies (**Figure 2.3B**) and anti-GD1a antibodies (**Figure 2.3C**). Significant increases in anti-GM1 and anti-GD1a IgG2b were seen in IL-10$^{-/-}$ mice, with infected IL-10$^{-/-}$ mice having significantly higher levels compared to infected WT mice. However, anti-ganglioside IgG2b was not significantly increased in *C. jejuni*-infected mice compared to control mice within either genotype. Anti-GM1 IgG3 was significantly increased in sham-inoculated WT mice compared to infected and control IL-10$^{-/-}$ mice, and infected WT mice had significantly increased IgG3 compared to sham-inoculated IL-10$^{-/-}$ mice. No other significant differences were seen between groups, including in Th2-mediated IgG1 antibodies. Collectively, these data suggest that elevations in anti-ganglioside antibodies were more closely related to presence or absence of IL-10, rather than *C. jejuni* infection status.

Colon Cytokine Production. Cytokines reflecting differentiation of Th1, Th2, Th17, Th9, Th22, and T follicular helper (Tfh) cells were measured in a panel including IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, and IL-22. Cytokines not statistically analyzed included IL-5, IL-17F, and IL-21, of which only 1/39 mice produced a detectable amount; IL-10, in which only one WT mouse and no IL-10$^{-/-}$ mice produced a detectable amount; and IL-4 and IL-13, of which no mouse of either genotype produced a detectable amount.

No significant increases related to infection status within the WT or IL-10$^{-/-}$ genotype were seen in any cytokine (**Figure 2.4**). However, sham-inoculated IL-10$^{-/-}$ mice produced significantly more TNF-α and IFN-γ than either sham-inoculated or *C. jejuni* 260.94-infected WT mice. Interestingly, although the

differences were not statistically significant, *C. jejuni* 260.94-infected IL-10$^{-/-}$ mice produced less TNF-α, IFN-γ, IL-6, IL-17A, and IL-22 than did sham-inoculated IL-10$^{-/-}$ mice. Significance overall was seen in analysis of IL-17A (Kruskal-Wallis, P = 0.0289), although no pairwise comparisons were significant. With the exception of IL-2 and IL-9, WT mice produced less of all analyzed cytokines compared to IL-10$^{-/-}$ mice, regardless of *C. jejuni* infection status, although these differences were not significant. Collectively, these data suggest that IL-10$^{-/-}$ mice trended toward increased production of cytokines reflecting Th1/Th17/Th22 differentiation than did WT mice, with a more pronounced effect seen in sham-inoculated IL-10$^{-/-}$ mice.

Nerve Histopathology. Cells positively labeled with the F4/80 macrophage marker in DRG were quantified by morphometry (**Figure 2.5**). *C. jejuni*-infected BALB/c IL-10$^{-/-}$ mice showed the most variation in number of F4/80+ cells, with scores ranging from 2–33, while F4/80+ scores in sham-inoculated WT mice were the most tightly clustered (range 5–18). No statistically significant differences between treatment groups were found (P = 0.290). These data suggest that, in this model, neither absence of IL-10 nor infection with *C. jejuni* 260.94 significantly influenced macrophage numbers in DRG after five weeks of infection.

DISCUSSION

The aim of the current study was to develop a mouse model of GBS subsequent to *C. jejuni* infection to further our understanding of the complex immunopathogenesis of this important human disease. The model aimed to exploit the reported Th2 bias of BALB/c mice that, in the absence of anti-inflammatory IL-10, were expected to mount strong Th2-mediated immunity following infection with GBS patient-derived *C. jejuni* 260.94 harboring a GM1a ganglioside mimic. Production of anti-ganglioside antibodies and associated nerve lesions were expected, as had been reported for C57BL/6 IL-10$^{-/-}$ and

WT, B7-2$^{-/-}$, and IL-10$^{-/-}$ NOD mice infected with *C. jejuni* 260.94.[35; 54] Instead, WT and IL-10$^{-/-}$ BALB/c mice orally inoculated with *C. jejuni* 260.94 mounted primarily Th1/Th17 immune responses and did not develop colitis, anti-ganglioside antibody production, an overt neurological phenotype, or nerve lesions. When considered in the context of previously published models from our group utilizing various mouse/*C. jejuni* strain combinations,[4; 35; 36; 54] the current study further highlights the importance of both infecting *C. jejuni* strain characteristics (presence of ganglioside mimics, invasiveness, colitogenic potential) and host factors (genetic background, immunological biases) in determining disease outcome.

We first hypothesized that Th2-mediated immune responses, including production of anti-ganglioside antibodies but without induction of colitis, would predominate following oral inoculation of WT and IL-10$^{-/-}$ BALB/c mice with *C. jejuni* 260.94. This reasoning was based upon reports in the literature of a relative Th2 bias exhibited by BALB/c mice in comparison to other mouse strains, including C57BL/6 mice, in *in vivo* infection models and *in vitro* studies,[33; 39; 48] and the Th2-mediated response shown in C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94.[35] Instead, infected WT and IL-10$^{-/-}$ BALB/c mice responded with significantly increased *C. jejuni*-specific plasma antibodies including IgG2a, IgG2b, and IgG3 antibody isotypes, but without significantly increased IgG1 production related to infection (**Figure 2.3**), indicating Th1/Th17-mediated class switching.[1; 3; 57] This unexpected result offers deeper insight into the diverse regulatory actions of IL-10, and the interplay of infecting pathogen and immunological bias of the host based upon genetic background. While C57BL/6 and BALB/c mice reportedly exhibit Th1- and Th2-mediated immune biases, respectively, the current study and others demonstrate that characteristics of the infecting pathogen or stimulus contribute substantially to immunological outcomes. C57BL/6 and C.B-17 (BALB/c strain congenic for C57BL/6 Ig heavy chain gene segment) mice infected with *Cryptococcus neoformans*, a fungal lung mucosal pathogen, showed differing immune responses and ability to clear the pathogen: susceptible C57BL/6 mice exhibited increasing lung burden over time, while resistant C.B-17 mice mounted an early local Th1-mediated

response correlating with superior pathogen clearance.[22] BALB/c mice are resistant to developing chemically induced (dextran sulfate sodium, DSS) colitis, while C57BL/6 mice are susceptible. Assessment of cytokine production following exposure to DSS indicated that BALB/c mice exhibited Th2/Th17/Treg (T regulatory cell) mediated immunity, characterized by IL-4, IL-6, IL-10, IL-17 production and higher numbers of Treg cells in the regional lymph node, while C57BL/6 mice responded with strong IFN-γ production.[61] C. *jejuni* is a mucosal enteric pathogen, and a Th1/Th17-mediated response has been identified in most *in vivo* and *in vitro* mouse and *ex vivo* human *C. jejuni* infection models.[4; 15; 16; 35; 36; 46; 54] Thus, WT BALB/c mice may be predisposed to Th2 responses in some models, but results of the current study suggest that Th1/Th17 responses are primarily induced in BALB/c mice following *C. jejuni* infection, even with a *C. jejuni* strain that previously induced Th2 responses in C57BL/6 IL-10$^{-/-}$ mice.[35] Th1/Th17 responses were exacerbated in IL-10$^{-/-}$ mice, but because a similar pattern was observed in WT mice, absence of IL-10 and subsequent enhancement of Th1-mediated immunity cannot be the sole reason for this outcome. These results further highlight the complexity of GBS immunopathogenesis and the interplay of both host and pathogen characteristics in induction of immunity.

Induction of anti-ganglioside antibodies is a hallmark of GBS and a partial reflection of the immune response to *C. jejuni* infection. Wild-type and IL-10$^{-/-}$ BALB/c mice infected with *C. jejuni* 260.94 were expected to produce antibodies to GM1 and GD1a gangliosides, as previously reported in other mouse genotypes.[35; 54] In the current study, Th1/Th17-mediated IgG3 and IgG2b isotypes reacting with GM1 and GD1a antigens showed significant elevations related only to presence or absence of IL-10 (**Figure 2.3**), and not to *C. jejuni* infection status with a strain known to elicit anti-ganglioside antibodies. The primary Th1/Th17 response to *C. jejuni* 260.94 in this study, instead of Th2-mediated immunity reported previously with C57BL/6 IL-10$^{-/-}$ mice,[35] apparently does not preclude generation of anti-ganglioside antibodies. In a separate study, BALB/c IL-10$^{-/-}$ mice infected with the enteritis-associated *C. jejuni* 11168 developed marked local and systemic Th1/Th17-mediated immunity with severe colitis, and

produced Th1/Th17-associated anti -GM1 and -GD1a antibodies related to *C. jejuni* infection (Brudvig *et al*., unpublished (Chapter 3)). Thus, the possibility that severe colitis or strong local colonic adaptive immune responses are required for generation of anti-ganglioside antibodies in some models cannot be excluded. It is also possible that in the current study, IL-10-mediated host-microbiota interactions influenced anti-ganglioside antibody production in response to ganglioside mimics expressed by commensal flora, but further studies would be required to establish such a connection.

An interesting observation in the current study was the similar patterns of anti -GM1 and -GD1a antibody production. *C. jejuni* 260.94 reportedly harbors a GM1a but apparently no other ganglioside mimics.[34] Closely similar production of anti -GM1 and -GD1a antibodies was noted in another study involving *C. jejuni* 11168 and 260.94 strains (Brudvig *et al*., unpublished (Chapter 3)), neither of which reportedly possess GD1a mimics.[34] GM1 and GD1a gangliosides are extremely similar in structure,[2] and the possibility that the plasma antibodies directed at GM1 also bound GD1a antigen in the ELISA format should be considered. Additionally, the *C. jejuni* strain 81-176 LOS possesses structures mimicking several gangliosides, and phase variation can result in changing expression of ganglioside mimics in both *C. jejuni* 81-176 and 11168 strains.[18; 31] Therefore, the possibility that commensal flora or *C. jejuni* 260.94 can express different or multiple ganglioside mimics *in vivo*, resulting in generation of more than one type of anti-ganglioside antibody, warrants further investigation.

Despite persistent colonization of 90% of infected mice at the end of the 5-week study, neither gross pathology nor histologic changes in the ICJ were significantly increased in either WT or IL-10$^{-/-}$ mice compared to sham-inoculated mice (**Figure 2.2**). A higher baseline of overall mild colitis in IL-10$^{-/-}$ mice regardless of *C. jejuni* infection status likely reflects the development of spontaneous colitis in IL-10$^{-/-}$ mice due to unchecked responses to the intestinal microbiota. Uncontrolled immune responses to enteric antigens may also in part explain the presence of *C. jejuni*-specific plasma IgG1 and IgG2a antibodies in some sham-inoculated IL-10$^{-/-}$ mice (**Figure 2.3**). All sham-inoculated mice were negative

for *C. jejuni* by both culture and PCR, and the *C. jejuni*-specific plasma response may reflect generation of antibodies against a structurally similar antigen in the microbiota. Colitis can occur in IL-10$^{-/-}$ mice as early as 3–4 weeks of age,[6; 28] and mice in the current study were inoculated at 14–15 weeks and humanely euthanized 5 weeks p.i. Therefore, the mild pathology observed in IL-10$^{-/-}$ mice in the current study is attributable to spontaneous disease, rather than *C. jejuni* infection. The relatively later age at inoculation compared with other studies was to allow the mice to increase in body size prior to intra-gastric gavage. Results of the current study are consistent with other mouse models demonstrating lack of colitis despite high colonization rates following infection with *C. jejuni* 260.94.[4; 9; 35; 54] *C. jejuni* strains vary widely in ability to colonize and cause colitis, with pathogenicity related to genomic content of certain open reading frames,[4] and even in IL-10$^{-/-}$ mice *C. jejuni* 260.94 has not been colitogenic in our models.

C. jejuni-specific plasma antibody isotypes reflected a systemic Th1/Th17 response in infected mice, but a significant local adaptive immune response was not identified in the proximal colon (**Figure 2.4**). Consistent with a shift toward Th1-mediated immunity in the absence of IL-10 and infection with a mucosal enteric pathogen, production of Th2 cytokines including IL-4, IL-5, and IL-13 was virtually undetectable. Significant increases were only identified in TNF-α and IFN-γ production in sham-inoculated IL-10$^{-/-}$ mice compared to WT mice, reflecting the Th1-mediated spontaneous colitis occurring in IL-10$^{-/-}$ mice. No significant difference in production of any cytokine by either WT or IL-10$^{-/-}$ mice related to *C. jejuni* infection was found. Interestingly, production of TNF-α, IFN-γ, IL-6, IL-17A, and IL-22 all tended to decrease in *C. jejuni*-infected compared to sham-inoculated IL-10$^{-/-}$ mice, suggesting that *C. jejuni* infection led to a relative dampening of local immune responses. The reason for this is not clear, but an alteration in the local microbiota or cytokine milieu induced by persistent *C. jejuni* colonization may have altered cytokine production by Th cells. Reduced intracellular survival and/or invasion efficiency of *C. jejuni* 260.94 compared to other *C. jejuni* strains has been shown by

immunohistochemistry in the ileocecocolic junction of infected BALB/c IL-10$^{-/-}$ mice (Brudvig *et al*., unpublished (Chapter 3)) and *in vitro* using murine bone marrow-derived dendritic cells (Brudvig *et al*., unpublished (Chapter 4)) and young adult mouse colon cells.[35] Persistent but relatively superficial colonization of *C. jejuni* 260.94, with heavier burdens in the mucus layer and epithelium as opposed to deeper in the lamina propria and submucosa, could be especially conducive to changes in the local microbiota and lead to altered Th responses. Further studies would be needed to confirm and determine the significance of decreased cytokine production in infected compared to sham-inoculated IL-10$^{-/-}$ mice.

The second aim of this study was to determine if *C. jejuni* 260.94 infection leads to neuropathology in BALB/c mice, manifested by gait abnormalities, neuromuscular weakness, and macrophage infiltration into DRG. Three rigorous phenotyping tests were performed prior to inoculation and weekly thereafter until sacrifice at 5 weeks p.i. to determine neurological deficits. Of these, the DigiGait treadmill analyses were most informative (**Table 2.1**). Two-way repeated measures ANOVA revealed numerous significant differences in variables with time, most of which including baseline compared to 4 weeks p.i. The only variable exhibiting significant differences related to infection status with all treatment groups analyzed together was front limb stance width, with *C. jejuni*-infected IL-10$^{-/-}$ mice having a wider stance width than sham-inoculated WT mice. The difference in stance width between infected and sham-inoculated mice remained significant when WT mice were analyzed separately, but was non-significant within IL-10$^{-/-}$ mice. The wider stance width could indirectly reflect hind limb weakness with more body weight being front-loaded to compensate, leading to wider stance width in the front. The DigiGait system is designed to detect subtle gait changes, but this single change is more difficult to interpret without concurrent gait disturbances to clarify its significance. Gait abnormalities also may have been missed when a run of sufficient quality could not be obtained for that week: at least 50% of the mice in each of the four treatment groups was missing at least one week of

DigiGait data. Similar difficulty in obtaining quality data also occurred with some NOD IL-$10^{-/-}$ mice in a separate study by our group.[54] We can therefore conclude that, with the wider stance width related to *C. jejuni* infection, mild neurological abnormalities may have been present and other gait abnormalities might have been missed due to inability to acquire a quality 3-second run.

The hang test and OFT test were not informative in the current study. The hang test was employed as a measure of motor strength and balance. Significant differences determined by two-way repeated measures ANOVA were related to time but not infection status. Subjective observations suggest that in the current study, this test was likely not adequate for detecting neurological deficits. More active mice tended to move around more on the grid, leading to a shorter hang time that was not related to weakness. In contrast, mice less interested in exploring the grid tended to hook their feet through the bars and hang on until the end of the test periods. These observations suggest that for BALB/c mice, the hang test performed with this technique did not reflect motor deficits but instead activity level, and was thus not a useful neurological indicator. Similarly, recordings of the OFT in which mice were placed in a standard rat cage in order to observe the gait, movement, and posture, were not analyzed because the mice did not consistently exhibit enough spontaneous movement to allow meaningful observation. Reduced movement and rearing by BALB/c mice compared to other mouse strains in the OFT has been reported previously,[11; 29] and may be related to photophobia in albinos such as BALB/c mice.[12] Despite the disadvantages of the OFT and hang test, the three neurological phenotyping tests combined with careful daily observation would have detected an overt neurological phenotype if present. Taken together, we conclude that infection with GBS patient-derived *C. jejuni* 260.94 did not lead to obvious or severe neurological deficits in the current study, though mild manifestations may have been missed.

Neuropathology was assessed by evaluation of macrophage numbers in lumbar DRG. Macrophages were chosen as indicators of neuropathology due to their postulated role in GBS

pathogenesis, particularly in acute motor axonal form of GBS considered by some to be the subtype most closely associated with preceding *C. jejuni* infection.[14; 41; 55] DRG were chosen for assessment as pain and sensory defects are frequently described by GBS patients.[55] Cell bodies of sensory fibers reside in DRG, which are also reported to have a particularly leaky blood-nerve barrier predisposing this area to immune-mediated damage.[41] Pathology in dorsal roots has been described following autopsy of patients succumbing to the motor-sensory axonal form of GBS,[17] and increased F4/80 positive cells were seen in the DRG, but not sciatic nerve or brachial plexus, of NOD IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94.[54]

In the current study, no significant difference in F4/80 positive cells in lumbar DRG was seen between any treatment groups (**Figure 2.5**), despite significant differences in anti-ganglioside GM1 and GD1a IgG2b and IgG3 isotypes in the plasma (**Figure 2.3**). A recent study also demonstrated a lack of correlation between significantly increased anti-GM1 IgG1 in *C. jejuni* 260.94 and 11168-infected mice with a human-derived microbiota and F4/80 positive cells in sciatic nerves and DRG.[9] The immunopathogenesis of nerve damage in GBS is incompletely understood. The lack of increased macrophages in DRG despite significantly increased plasma anti-ganglioside antibodies in some groups suggests that anti-ganglioside antibodies alone are insufficient to cause nerve lesions, the timing of higher anti-GM1 or anti-GD1a antibody levels may not coincide with cellular infiltration in the DRG, or the damage may be occurring in a different location in the peripheral nervous system. In addition to macrophages, labeling of other immune components implicated in nerve damage in GBS, such as T cells, complement, and IgG[41; 55] in peripheral nerve tissues and conducting a time-course study to determine if and when neuropathology is correlated with anti-ganglioside antibody production would provide a more complete assessment of GBS immunopathogenesis. Taken together, absence of neurological deficits identified by rigorous phenotyping tests, including DigiGait treadmill analysis, OFT, and hang testing, and the lack of increased macrophages in DRG indicate that neuropathology was not induced by *C. jejuni* 260.94 in BALB/c WT or IL-10$^{-/-}$ mice in the current model.

The final aim of this study was to determine if the absence of the regulatory actions of IL-10 resulted in exacerbation of immune responses and neuropathology. The systemic Th1/Th17 immune response to *C. jejuni* 260.94, manifested by significant increases in *C. jejuni*-specific IgG2a, IgG2b, and IgG3 antibody isotypes, was increased in magnitude in IL-10$^{-/-}$ compared to WT mice (**Figure 2.3**). Furthermore, *C. jejuni* induced significant IgG3 production only in the IL-10$^{-/-}$ group. Thus, absence of IL-10 enhanced systemic Th1/Th17 immunity in *C. jejuni* infected mice. When DigiGait parameters for WT and IL-10$^{-/-}$ mice were analyzed separately, a significantly wider forelimb stance width was associated with *C. jejuni* infection in WT but not IL-10$^{-/-}$ mice, suggesting that IL-10 deficiency was protective rather than conducive to development of *C. jejuni*-induced gait defects.

Presence or absence of IL-10 led to differences in anti-ganglioside antibody production, and absence of IL-10 induced mild colitis and increased cytokine production in the colon. However, aside from the increased *C. jejuni*-specific plasma antibodies in IL-10$^{-/-}$ relative to WT mice, IL-10 deficiency did not lead to significant exacerbation of *C. jejuni*-induced changes in immunity or neuropathology. There was no significant difference in cecal *C. jejuni* colonization between WT and IL-10$^{-/-}$ mice. An equal number of sham-inoculated and infected IL-10$^{-/-}$ mice developed mild colitis with similar histologic lesions, indicating that IL-10$^{-/-}$ mice had underlying spontaneous colitis that was not exacerbated by *C. jejuni* infection. Similarly, production of TNF-α and IFN-γ in the proximal colon was increased overall in IL-10$^{-/-}$ mice but no significant differences related to *C. jejuni* infection were seen. No significant difference in macrophage numbers in lumbar DRG was found between treatment groups. Thus, in this model, synergism between *C. jejuni* infection and IL-10 deficiency resulting in exacerbated immunity or pathology was not definitively observed. Our group has previously shown that C57BL/6 WT mice stably colonize with *C. jejuni* 11168 while IL-10$^{-/-}$ mice develop severe colitis, emphasizing the importance of IL-10 in regulating intestinal inflammatory responses.[36; 37] Further, even with IL-10 deficiency, conducive to impaired Th2- and enhanced Th1-mediated responses, C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni*

260.94 still produced Th2-mediated anti -GM1 and -GD1a IgG1 antibodies,[35] indicating that a lack of IL-10 does not always preclude a Th2-mediated response. The lack of susceptibility of *C. jejuni* 260.94-infected BALB/c WT or IL-10$^{-/-}$ mice to developing Th2-driven autoimmunity and the lack of exacerbation of pathology in IL-10$^{-/-}$ mice in the current study further emphasize the importance of both *C. jejuni* strain characteristics and host genetic background in development of pathology and autoimmunity following *C. jejuni* infection.

Results of this study indicate that WT and IL-10$^{-/-}$ BALB/c mice orally inoculated with GBS patient-derived *C. jejuni* 260.94 were stably colonized and mounted systemic Th1/Th17 responses, but did not develop colitis, produce anti-ganglioside antibodies, or develop neuropathology, including overt neurological deficits and inflammatory lesions in DRG. Systemic Th1/Th17-mediated immunity was unexpected, considering the reported Th2 immunological bias in BALB/c mice and the previous induction of Th2-mediated immunity in C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94. These results indicate BALB/c mice are a useful model for studying *C. jejuni* infection and could provide critical insights into the impact of host genetic background in polarization of the immune response and resulting pathology.

APPENDIX

Table 2.1. Results of DigiGait analysis. Gait parameters including swing, propel, stance, stride, stride length, stride frequency, absolute paw angle, and stance width were analyzed in BALB/c wild-type (WT) and IL-10$^{-/-}$ mice given either *C. jejuni* 260.94 or tryptic soy broth (sham; TSB). Gait parameters were analyzed within individual feet. Analyses included baseline (prior to inoculation) and Weeks 1-5 post infection. Two-way repeated measures ANOVA was performed on all groups together and also within individual genotypes. Where overall significance was found, Holm-Sidak post-testing was performed. P values <0.05 are reported for the factor (Week or Treatment Group) in the overall analysis, with significant pairwise comparison(s) listed. Significance was not found in any other analyses of gait parameters. Results are not shown in 4/90 total analyses, in which the assumption of equal variance was not met. LH = left hind; RH = right hind; LF = left front; RF = right front. IL-10$^{-/-}$ = BALB/c IL-10$^{-/-}$; WT = BALB/c wild-type. 260.94 = *C. jejuni* 260.94; TSB = tryptic soy broth (sham). ns = nonsignificant.

ANALYSIS INCLUDING BOTH GENOTYPES		
Gait Parameter	**P value**	**Significant Comparison**
LH Propel	0.011	Week: Baseline v. Week 4
LH Stance	0.049	Week: pairwise comparisons ns
RH Propel	<0.001	Week: Baseline v. Weeks 2, 3, 4, and 5 and Weeks 1 v. 3
RH Stance	0.017	Week: Baseline v. Week 4
RH Absolute Paw Angle	0.042	Week: pairwise comparisons ns
LF Absolute Paw Angle	0.017	Week: Baseline v. Week 4
LF Stance Width	0.003	Treatment Group: IL-10$^{-/-}$ 260.94 v. WT TSB
RF Absolute Paw Angle	0.031	Treatment Group: pairwise comparisons ns
RF Absolute Paw Angle	0.010	Week: Baseline v. Weeks 4 and 5
ANALYSIS WITHIN BALB/C WT MICE		
Gait Parameter	**P value**	**Significant Comparison**
RH Absolute Paw Angle	0.031	Week: Weeks 1 v. 3
LF Stance Width	0.034	Treatment Group: 260.94 v. TSB
ANALYSIS WITHIN BALB/C IL-10$^{-/-}$ MICE		
Gait Parameter	**P value**	**Significant Comparison**
LH Propel	0.017	Week: Baseline v. Weeks 2 and 4
LH Stance	0.035	Week: Baseline v. Week 4
RH Propel	<0.001	Week: Baseline v. Weeks 3, 4, and 5
LF Absolute Paw Angle	0.021	Week: Baseline v. Week 4
RF Absolute Paw Angle	0.004	Week: Baseline v. Weeks 3, 4 and 5

Figure 2.1. Culture results of *Campylobacter jejuni* 260.94 colonization of the cecum at the time of necropsy. Wild-type (WT) or interleukin (IL)-10 knockout (IL-$10^{-/-}$) BALB/c mice were inoculated with either *C. jejuni* 260.94 or vehicle (tryptic soy broth; TSB). Colonization rates were semi-quantitatively graded by approximate number of colony forming units (CFU) on the plate. With the exception of one infected WT mouse requiring early sacrifice unrelated to *C. jejuni* infection, mice were sacrificed 5 weeks post infection. Colonization in the cecum was not significantly different between infected WT and IL-$10^{-/-}$ mice (Fisher Exact Probability Test, $P_B = 0.775$).

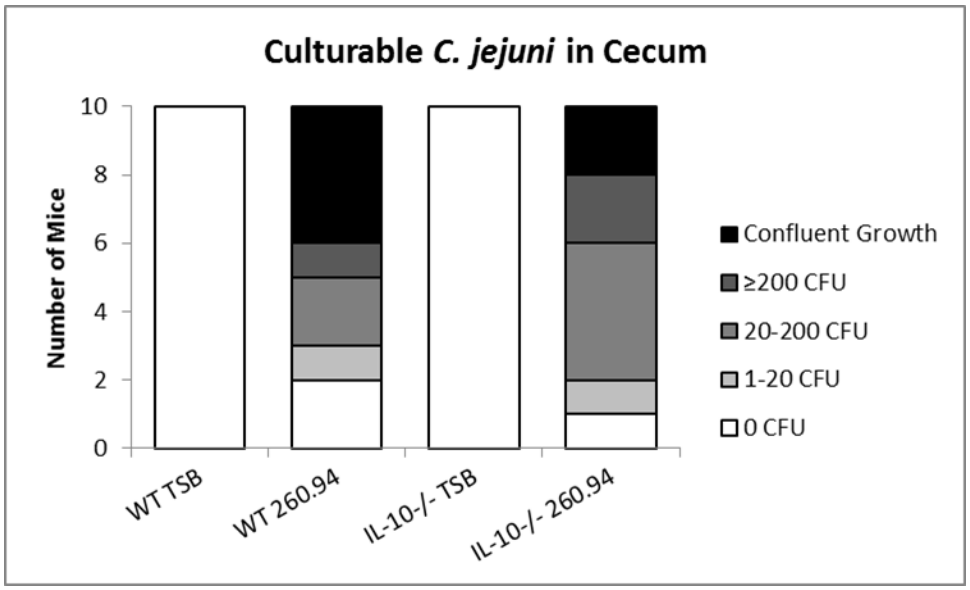


Figure 2.2. Gross pathology and ileocecocolic junction histopathology. Gross pathological changes were noted at necropsy (A) and histopathologic assessment of the ileocecocolic junction (B) was performed for 19 BALB/c wild-type (WT) and 20 BALB/c interleukin-10-deficient (BALB/c IL-$10^{-/-}$) mice receiving either *Campylobacter jejuni* 260.94 or vehicle control (tryptic soy broth; TSB). Mice were humanely sacrificed 5 weeks post infection. One infected WT mouse required early sacrifice unrelated to *C. jejuni*, and is excluded from these data. (A) Gross lesions noted at necropsy included enlarged or thickened wall of proximal colon (present in all 17 IL-$10^{-/-}$ mice with at least one change); other more sporadic changes included enlarged or thickened cecum, enlarged spleen, and enlarged ileocecocolic lymph nodes. Gross pathology scores between sham-inoculated and *C. jejuni* 260.94-infected IL-$10^{-/-}$ mice were not significantly different (Fisher Exact Probability Test, $P_B = 0.087$). (B) Histologic assessment of colitis in the ileocecocolic junction involved assigning a raw score out of 42 possible points, and subsequent ranking of raw scores for statistical purposes. Whiskers span minimum to maximum values, the box extends from 25^{th} – 75^{th} percentiles, and the line in the middle of the box denotes the median. Mice with scores ≤ 9 points are considered to have no colitis, while scores of 10—19 indicate mild colitis. Significance was seen overall using both Fisher Exact Probability Test on ranked data ($P_B = 0.012$) and Kruskal-Wallis on raw scores ($P = 0.0265$), but no pairwise comparisons were significant in either analysis.

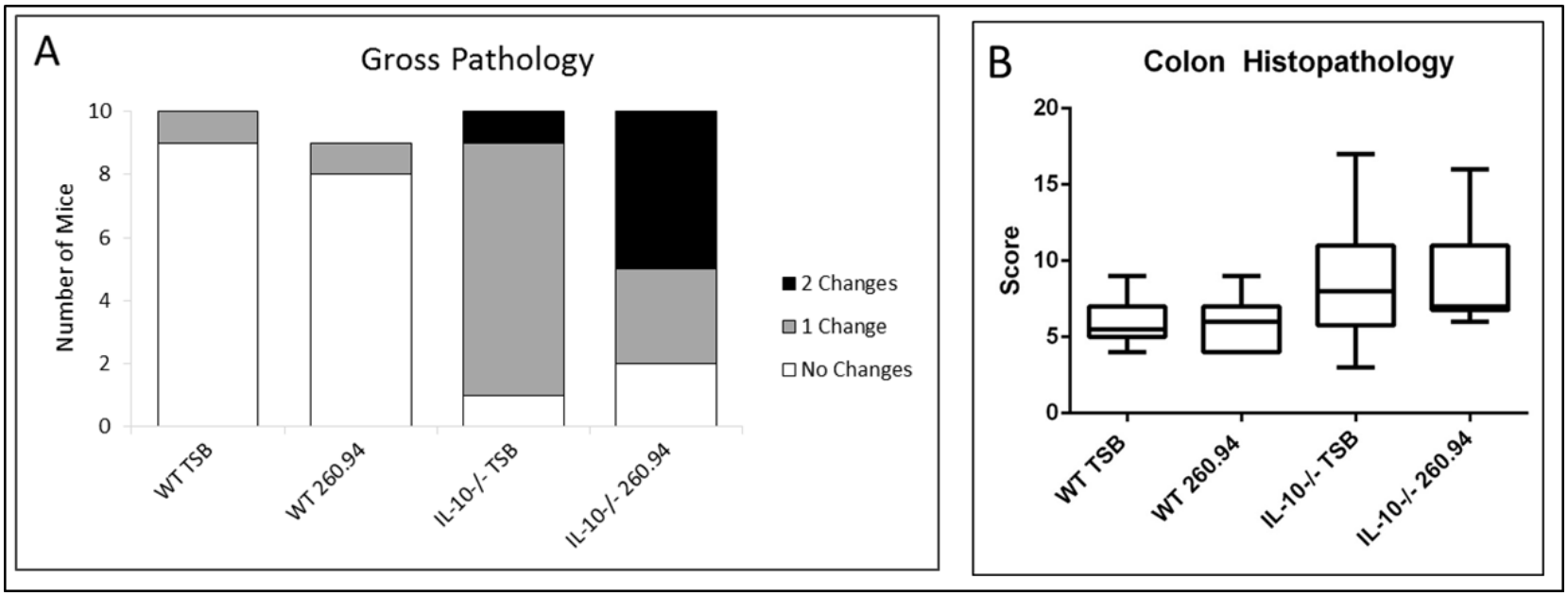


Figure 2.3. Assessment of plasma anti-*C. jejuni* and anti-ganglioside IgG isotypes. Quantification of plasma IgG1, IgG2a, IgG2b, and IgG3 specific for *C. jejuni* (panel A) and gangliosides GM1 (panel B) and GD1a (panel C) was performed by indirect ELISA. BALB/c wild-type (WT) and interleukin (IL)-$10^{-/-}$ mice were inoculated with either *C. jejuni* 260.94 or vehicle (tryptic soy broth, TSB), and sacrificed 5 weeks post infection. One infected WT mouse required early sacrifice unrelated to *C. jejuni*, and is excluded from these data. P values <0.05 were considered significant. Lines above indicate significant differences between groups (Kruskal-Wallis, followed by Dunn's post-test). Mean ± SEM.

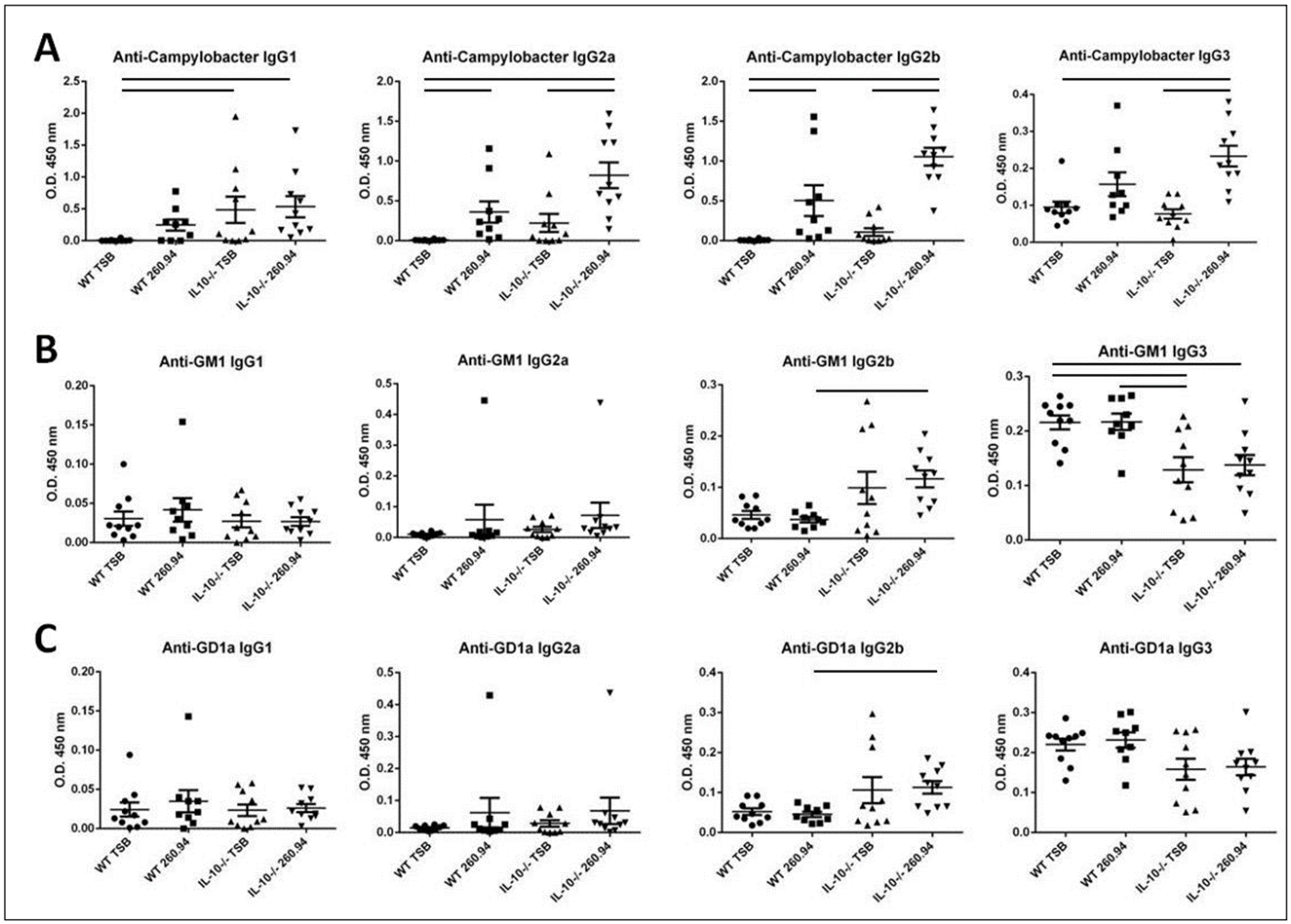


Figure 2.4. Th cytokine production in the proximal colon. Cytokine production, presented as picogram of cytokine per milligram of colon tissue, was measured in supernatants of homogenized colon samples using a multiplexed, flow cytometry-based bead array. Treatment groups included wild-type (WT) and interleukin (IL)-10 deficient (IL-$10^{-/-}$) BALB/c mice, either sham inoculated (tryptic soy broth, TSB) or orally infected with *C. jejuni* 260.94. Mice were sacrificed 5 weeks post infection. One infected WT mouse requiring early sacrifice unrelated to *C. jejuni* was excluded. P values <0.05 were considered significant. Lines above groups indicate significant differences (Kruskal-Wallis ANOVA on ranks, followed by Dunn's post-test). Overall significance was found in analysis of IL-17A (P = 0.0289), although no pairwise comparisons were significant. Mean ± SEM.

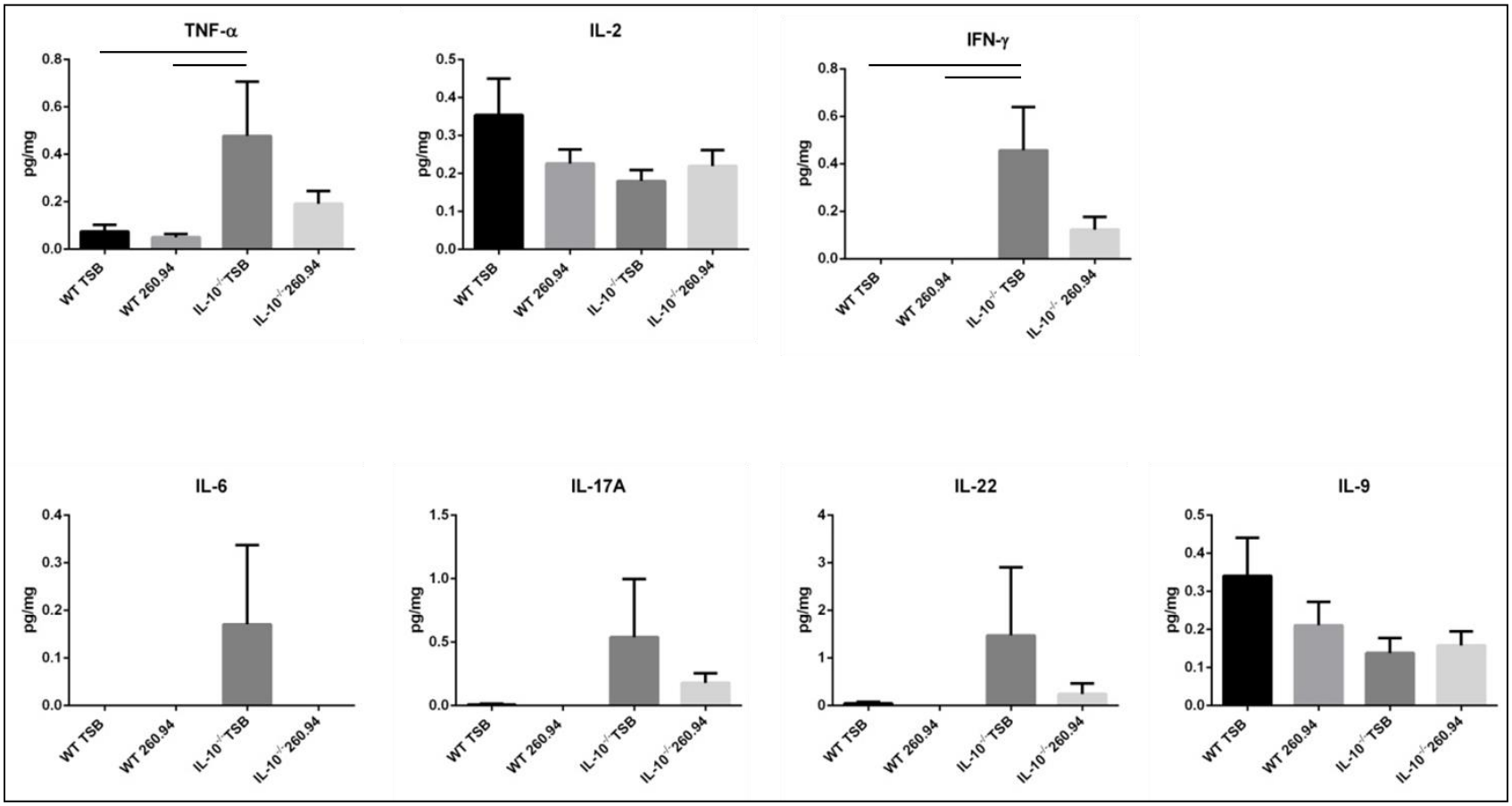
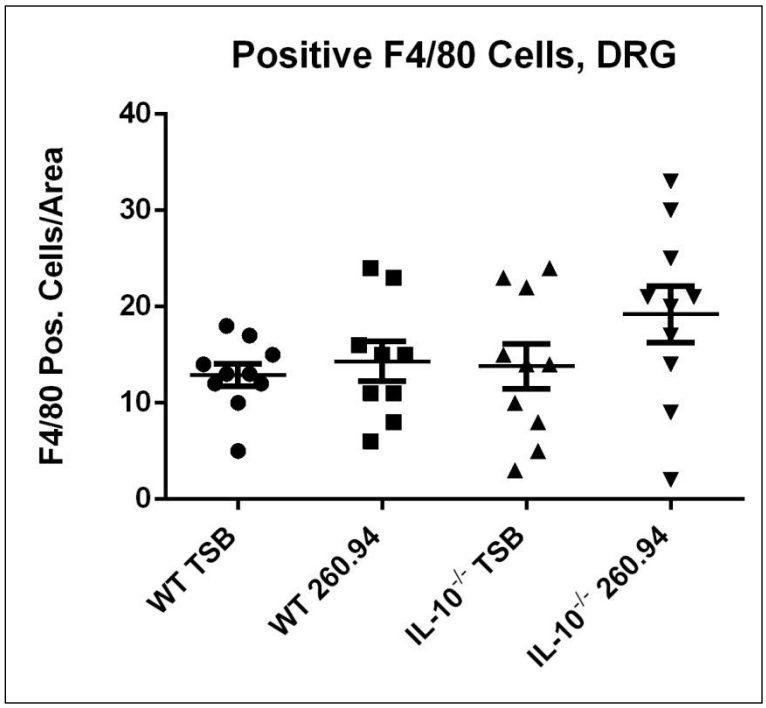


Figure 2.5. Quantification of macrophages in dorsal root ganglia. Lumbar dorsal root ganglia (DRG) were dissected from 19 BALB/c wild-type (WT) and 20 BALB/c interleukin (IL)-$10^{-/-}$ mice given either *C. jejuni* 260.94 or TSB (sham; tryptic soy broth). Mice were sacrificed at 5 weeks post infection. One infected WT mouse required early sacrifice unrelated to *C. jejuni*, and was excluded from these data. Macrophages identified by immunohistochemical labeling with F4/80 were quantitatively scored using morphometry in Image J. Results are given as number of F4/80 positive cells per unit area (100,000 pixels). No significant differences between groups were seen (Kruskal-Wallis, P = 0.290). Mean ± SEM.



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CHAPTER 3: INFECTING *CAMPYLOBACTER JEJUNI* STRAIN DETERMINES TH1/TH17-MEDIATED IMMUNITY AND COLITIS IN INTERLEUKIN-10-DEFICIENT BALB/C MICE

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ABSTRACT

Campylobacter jejuni is an important cause of bacterial enteritis worldwide and is associated with the post-infectious neuropathy Guillain-Barré syndrome (GBS). The immunopathogenesis of GBS is incompletely understood, but both infecting *C. jejuni* strain characteristics and host genetic background are thought to contribute. The purpose of this study was to develop an *in vivo* mouse model characterizing the differences in immune response to colitogenic *C. jejuni* strain 11168 and GBS patient-derived *C. jejuni* strain 260.94 in orally inoculated C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice. We hypothesized that 1) infection with *C. jejuni* 11168 would induce significant systemic and local Th1/Th17 immune responses in both mouse genotypes, culminating in severe colitis, and 2) infection with *C. jejuni* 260.94 would lead to Th2-mediated anti-ganglioside antibody production and nerve lesions mimicking GBS in C57BL/6 IL-10$^{-/-}$ mice, while BALB/c IL-10$^{-/-}$ mice would mount Th1/Th17 responses with protection from autoimmunity. Following a 4-week infection period, *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice exhibited strong Th1/Th17-mediated immunity, severe colitis, and anti -GM1 and -GD1a ganglioside antibody production. *C. jejuni* 260.94-infected BALB/c IL-10$^{-/-}$ mice also exhibited Th1/Th17 responses, but of lesser magnitude than following *C. jejuni* 11168 infection and without colitis or anti-ganglioside antibody production. C57BL/6 IL-10$^{-/-}$ mice infected with either *C. jejuni* strain exhibited less colonization, no colitis, and overall milder immune responses than in previously published models from our group. The unexpected finding that C57BL/6 IL-10$^{-/-}$ but not BALB/c IL-10$^{-/-}$ mice carried *Lactobacillus murinus,* a potential probiotic organism, combined with the atypical results for C57BL/6 IL-10$^{-/-}$ mice presents the possibility of a protective probiotic effect exerted by the resident strain of *L. murinus.* Results of the current study indicate that BALB/c IL-10$^{-/-}$ mice are a useful model for studying mucosal

enteric pathogens such as *C. jejuni*, which stimulates a marked Th1/Th17 response in mouse strain that is reportedly Th2-biased. Furthermore, colitogenic and GBS patient-derived *C. jejuni* strains stimulate Th1/Th17 responses of different magnitude and result in distinct disease outcomes in BALB/c IL-10$^{-/-}$ mice.

INTRODUCTION

Campylobacter spp., spiral, microaerophilic, Gram-negative rods, are considered to be the most frequent cause of bacterial gastroenteritis worldwide.[65] While *C. jejuni* colonizes chickens in high numbers without inducing disease,[36] infection in humans leads to inflammatory enteritis including symptoms of abdominal cramping, fever, diarrhea, and myalgia.[12] *C. jejuni* is also associated with numerous post-infectious sequelae, including reactive arthritis, irritable bowel syndrome, and Guillain-Barré syndrome (GBS).[30]

GBS is a peripheral neuropathy associated with an antecedent infection with viral or bacterial agents, including Epstein-Barr virus, cytomegalovirus, *Mycoplasma*, or *C. jejuni*.[25; 29] *C. jejuni* is the most commonly reported antecedent infection,[25; 29; 61] associated with approximately 30% of GBS cases.[50] The pathogenesis of GBS subsequent to *C. jejuni* infection is incompletely understood, but is thought to be mediated by generation of cross-reactive antibodies to lipooligosaccharide (LOS) in the *C. jejuni* outer membrane and structurally similar gangliosides on peripheral nerves.[4; 46; 61; 69] Antibodies to gangliosides, especially GM1 and often of the IgG subtype, are frequently found in GBS patients[51] and correlate with clinical severity in GBS patients following *C. jejuni* infection.[27; 52] Subtype of anti-ganglioside IgG also impacts patient prognosis: IgG1 subtypes are associated with preceding *C. jejuni* infection and worse clinical outcome, while IgG3 alone or in combination with IgG1 is associated with preceding respiratory infection and improved outcome.[28; 32]

Both the infecting *C. jejuni* strain and the patient genetic background and immune response are thought to contribute to susceptibility to GBS following *C. jejuni* infection. Structural similarity between the LOS of some *C. jejuni* strains and GM1 and GD1a gangliosides,[22; 69] in addition to the overrepresentation of certain *C. jejuni* serotypes preceding GBS,[2; 34] support a role for *C. jejuni* strain characteristics in GBS pathogenesis. However, while expression of ganglioside mimics in LOS of *C. jejuni* isolates from GBS and Miller Fisher syndrome (MFS; a subtype of GBS) patients was more common than

in isolates from uncomplicated enteritis patients,[4] ganglioside mimics can also be found in *C. jejuni* isolates causing only enteritis.[4; 58] The low proportion (0.07%) of *C. jejuni* infections associated with subsequent GBS, the rarity of GBS outbreaks, and the geographic clustering of GBS subtypes[30; 47] further suggest differences in host genetic background also influence susceptibility to GBS. Thus, bacterial factors such as ganglioside mimicry in the LOS, in addition to patient immune response, likely both contribute to a disease outcome of uncomplicated enteritis versus development of GBS.

Recent establishment of murine models exploiting alterations in host immune system, microbiota, or both have been critical in furthering our understanding of pathogenesis and immune response in *C. jejuni*-induced colitis.[9; 15; 21; 42] One landmark model developed by Mansfield *et al*. utilizes infection of C57BL/6 mice lacking interleukin (IL)-10 with *C. jejuni* 11168.[42] IL-10 is an essential anti-inflammatory mediator and a critical regulator of immune responses to intestinal flora.[33] Enterocolitis developing in IL-10$^{-/-}$ mice is thought to be mediated by unchecked Th1 responses, can manifest as early as 3-4 weeks of age, and was reportedly more severe in 3-month-old BALB/c IL-10$^{-/-}$ than C57BL/6 IL-10$^{-/-}$ mutants.[10; 33] *C. jejuni* 11168 was originally isolated from an enteritis patient in the United Kingdom. This strain harbors both GM1 and GM2 mimics,[37] but based on the literature has not been associated with GBS in humans. *C. jejuni* 11168 has an LOS class C and encodes *cst*-III sialyl transferase, leading to monosialylation rather than the disialylated form most often associated with high anti-ganglioside activity.[22] *C. jejuni* 11168 has colonized C57BL/6 IL-10$^{-/-}$ mice well, producing enterocolitis and primarily Th1/Th17 systemic responses identified by anti-*C. jejuni* plasma IgG2c, IgG3, and IgG2b isotypes[6; 7; 41-43; 54] and increased colonic expression of IFN-γ and IL-17.[41] The few exceptions in which marked colitis did not result in C57BL/6 IL-10$^{-/-}$ mice following infection with *C. jejuni* 11168 include: development of colitis in only 1/5 mice in an initial passage, but followed by severe colitis with subsequent passages;[7] more mice in a dose-response experiment displaying mild than severe colitis, although the majority of mice exhibited some colitis;[42] and lower than typical colonization rates and gross pathological changes in a

pilot experiment.[13] Aside from these examples, infection of C57BL/6 IL-10$^{-/-}$ mice with *C. jejuni* 11168 has provided a repeatable colitis model.

C. jejuni strains can vary widely in ability to colonize and cause disease in mice[6; 7] and can also elicit contrasting immune responses in mice of the same genetic background.[41] *C. jejuni* 260.94 was originally isolated from a GBS patient in South Africa. It possesses a GM1a ganglioside mimic, has an outer core classified as an LOS class A and encodes a *cst*-II sialyl transferase.[39] *C. jejuni* 260.94 has colonized different mouse strains at high prevalence with high enteric colony-forming units (CFU), is not colitogenic and can induce anti-ganglioside antibodies in mice.[6; 13; 41; 59] Interestingly, C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 did not exhibit colitis, but produced Th2-mediated responses, including plasma anti -*C. jejuni*, -GM1, and -GD1a ganglioside IgG1 antibodies and upregulated colonic Gata-3 and IL-4 expression.[41] *C. jejuni* 260.94 also induced production of anti-ganglioside antibodies in non-obese diabetic (NOD) mice with and without knockout of the IL-10 gene.[59]

Murine models of campylobacteriosis assessing both pathogen and host factor interactions would further our understanding of the complex GBS immunopathogenesis. Several reports suggest that mice of C57BL/6 and BALB/c genetic backgrounds develop polarized immune responses to some well-studied pathogens, including in aspects of innate and adaptive immunity. The intracellular protozoan pathogen *Leishmania major* provides an excellent example: upon infection, resistant C57BL/6 mice down-regulate Th2-associated IL-4 production and produce Th1-associated IFN-γ; in contrast, susceptible BALB/c mice produce IL-4 upon infection leading to non-healing lesions.[53] Similarly, when stimulated with various ligands *in vitro*, peritoneal macrophages, spleen cells, and dendritic cells derived from C57BL/6 mice produced higher levels of pro-inflammatory cytokines including TNF-α, IL-12, IFN-γ, while cells from BALB/c mice produced more IL-4 and MCP-1.[38; 45; 63] These reports suggest that BALB/c mice predominantly produce Th2-polarized responses that may enhance the molecular mimicry driving GBS.

Thus, our rationale for this study design was based on the contrasting immune responses and disease outcomes elicited in C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 or *C. jejuni* 260.94 strains,[41] and contrasting immune responses of C57BL/6 and BALB/c mice to the same stimulus or pathogen.[38; 45; 53; 63; 68] We expected that infection of C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice with colitogenic and GBS patient-derived *C. jejuni* strains would elicit polarized immune and disease responses and could further elucidate the underlying adaptive immune mechanisms. We hypothesized that 1) both C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with colitogenic *C. jejuni* 11168 would develop colitis and primarily Th1/Th17 immune responses, with Th2 responses also contributing in BALB/c IL-10$^{-/-}$ mice; 2) C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 would be protected from colitis and would develop both Th2-mediated responses, including anti-ganglioside antibodies, and nerve lesions manifested by increased macrophage infiltration into dorsal root ganglia; 3) BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 would similarly be protected from colitis but would mount primarily Th1/Th17-mediated responses with lesser Th2 responses, without developing anti-ganglioside antibodies or nerve lesions.

To test these hypotheses, C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice were orally infected with *C. jejuni* strains 11168 and 260.94 and sacrificed after four weeks. Enzyme-linked immunosorbent assays (ELISAs) were used to measure anti -*C. jejuni*, -GM1, and -GD1a specific IgG subtypes in plasma and *C. jejuni*-specific IgA in fecal supernatants. Histological lesions in the gastrointestinal tract were graded according to a previously published scale.[42] Production of 13 cytokines reflecting adaptive immune responses in the proximal colon was assessed by a flow-cytometry based multiplexed bead assay. Morphometry was used to quantitate number of macrophages labeled immunohistochemically (IHC) with F4/80 in dorsal root ganglia (DRG).

Results from this study show that BALB/c IL-10$^{-/-}$ mice mounted Th1/Th17-mediated immune responses to *C. jejuni* infection that varied in magnitude and disease manifestation depending upon the

infecting *C. jejuni* strain. Infection of C57BL/6 IL-10$^{-/-}$ mice with either *C. jejuni* strain did not produce adaptive immune responses or disease consistent with previous experiments in our laboratory. All C57BL/6 IL-10$^{-/-}$, but no BALB/c IL-10$^{-/-}$, mice were unexpectedly found to harbor *Lactobacillus murinus* in the gastrointestinal tract, and we speculate that *L. murinus* may have conferred protection against *C. jejuni*-induced pathology in C57BL/6 IL-10$^{-/-}$ mice.

MATERIALS AND METHODS

Experimental Animals. All mouse experiments were performed according to recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (approval numbers 06/12-107-00 and 06/15-101-00). B6.129P2-*IL-10*^{tm1Cgn}/J (referred to as C57BL/6 IL-10$^{-/-}$) and C.129P2(B6)-*IL-10*^{tm1Cgn}/J (referred to as BALB/c IL-10$^{-/-}$) mice were originally obtained from the Jackson Laboratories (Bar Harbor, ME). Experimental animals were bred and maintained in-house in a specific pathogen-free colony. DNA was extracted from pooled fecal samples from experimental mice prior to the start of the experiment, and PCR was used to screen for enteropathogens including *Helicobacter* spp., *Campylobacter* spp., *Enterococcus faecalis*, *E. faecium*, and *Citrobacter rodentium* as previously described.[42] Absence of IL-10 was assessed by a PCR assay based on the method of the Jackson Laboratories (https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:23475,004333) using DNA extracted from cecal tissue from experimental mice taken at necropsy. Prior to inoculation, mice were transported to MSU's University Research Containment Facility (URCF) and allowed to acclimate before placement in individual cages. At the humane endpoint or at the end of the experiment, all mice were humanely euthanized using an overdose of CO_2 according to the guidelines of

the American Veterinary Medical Association (https://www.avma.org/KB/Policies/Pages/Euthanasia-Guidelines.aspx).

Experimental Design. Prior to this study, a 15-mouse pilot experiment was performed to verify ability of experimental *C. jejuni* strains to colonize mice following oral inoculation with sterile pipette tip, in contrast to the gastric gavage method used previously. Five wild-type (WT) BALB/c mice were each orally inoculated with *C. jejuni* 11168 (3.3×10^9 CFU), *C. jejuni* 260.94 (4.3×10^9 CFU), or tryptic soy broth (TSB; sham/vehicle). Stable colonization of mice throughout and at the end of the 3-week pilot experiment was verified by culture and *C. jejuni gyrA* gene-specific PCR as described below.

Sixty mice were included in the present study: 30 C57BL/6 IL-$10^{-/-}$ mice and 30 BALB/c IL-$10^{-/-}$ mice. Of the 30 mice of each genotype, 10 were inoculated with *C. jejuni* 11168, *C. jejuni* 260.94, or TSB (**Table 3.1**). Age matched male and female mice were randomized by sex, litter, rack and slot position, and treatment group as previously described.[42] Mice were inoculated at 6 weeks of age and sacrificed 4 weeks post-infection.

Campylobacter jejuni *Inocula Preparation and Inoculation*. *C. jejuni* inocula were prepared as previously described.[42] *C. jejuni* strains were originally obtained from the American Type Culture Collection (ATCC; Manassas, VA) and stored as glycerol stock cultures at -80°C. *C. jejuni* ATCC 700819 (referred to as *C. jejuni* 11168) and *C. jejuni* ATCC BAA-1234 (referred to as *C. jejuni* 260.94) were streaked onto tryptic soy agar supplemented with 5% defibrinated sheep's blood (Cleveland Scientific, Bath, OH) (TSAB plates). The plates were incubated for approximately 42 hours at 37°C in sealed jars containing a single Campy*Gen* sachet (Oxoid, Basingstoke, United Kingdom). Growth was harvested using a sterile swab, resuspended in TSB to an optical density (OD) 600 nm of 0.262 for *C. jejuni* 11168 and 0.255 for *C. jejuni* 260.94, and 100 µL aliquots were spread on TSAB plates. Following incubation for

approximately 18 hours at 37°C with a Campy*Gen* sachet, growth was harvested and resuspended in TSB so that a 1:10 dilution of the culture exhibited an OD_{600} 1.031 for *C. jejuni* 11168 and 0.956 for *C. jejuni* 260.94. Gram stain and wet mount preparations of each strain were used to verify purity, spiral morphology, and motility. The cultures were placed on ice and transported to the URCF for immediate inoculation. Each mouse received 100 µL of its assigned treatment (*C. jejuni* 11168, *C. jejuni* 260.94, or TSB) by oral inoculation using a sterile 200 µL pipette tip. Serial dilutions of the inocula were made pre- and post-inoculation. CFU in the inocula were determined following approximately 72 hours' incubation at 37°C with a Campy*Gen* sachet. Each infected mouse received 5.2×10^9 CFU of *C. jejuni* 11168, or 5.9×10^9 CFU of *C. jejuni* 260.94.

Monitoring for Clinical Signs. Following infection mice were checked at least once daily by a veterinarian, in addition to checks by MSU University Laboratory Animal Resources personnel for clinical signs of illness including rough hair coat, lethargy, hunched posture, dehydration, and soft feces or diarrhea. Mice reaching a pre-determined humane endpoint based on a previously developed standardized scoring sheet[42; 59] were promptly euthanized and necropsied.

Necropsy Procedures. Mice were humanely euthanized by CO_2 overdose when an early humane endpoint was reached, or at the scheduled end of the 4-week experiment. Weight was recorded for each mouse. Blood was collected via cardiac puncture with a 25g needle on a 1 mL tuberculin syringe pre-loaded with 0.1 mL 3.8% sodium citrate. Mice were necropsied in a hood using instruments sterilized with a hot bead sterilizer between isolations from each section of the gastrointestinal (GI) tract.

One fecal pellet was collected into an Eppendorf tube and placed on ice for culture. The remaining fecal pellets were placed into a second Eppendorf tube containing 1 mL of 1% Protease

Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) per 100 mg feces and placed on ice for fecal IgA determination. The cecum was excised including approximately 0.5—1 cm of adjoining proximal colon and ileum. The tip of the cecum was removed. The remaining ileocecocolic junction (ICJ) was injected with 10% neutral buffered formalin before placing onto a sponge in a histological tissue cassette (Fisherbrand Histosette II Tissue Cassette, Fisher HealthCare, Hampton, NH) and immersing in 10% phosphate buffered formalin (Fisher Scientific).

Sections of stomach, jejunum, proximal colon, and the tip of the cecum were excised and rinsed in sterile PBS in separate Petri dishes. Pieces of each tissue were divided into three sections: two were snap frozen in microcentrifuge tubes and cryovials, and the third was streaked onto TSAB plates containing cefoperazone (20 µg/mL), vancomycin (10 µg/mL), and amphotericin B (2 µg/mL) (TSAB-CVA plates; all antimicrobials obtained from Sigma-Aldrich, St. Louis, MO). Plates were placed into air-tight jars with a Campy*Gen* sachet.

Finally, the mice were skinned and muscle overlying the spine was removed. The roof of the vertebral canal was removed using Castroviejo scissors to expose the spinal cord. Muscle and connective tissue were bluntly dissected to expose the sciatic nerve on each side. Thereafter, the carcass was immersed in a specimen cup containing 10% phosphate buffered formalin for further dissection and retrieval of tissue at a later date.

On return to the laboratory, snap frozen tissues were stored at -80°C. Jars with the Campy*Gen* sachet containing TSAB-CVA plates were placed at 37°C. Fecal pellets were crushed with a sterile applicator stick in 300 µL TSB containing 15% glycerol, vortexed, and a loopful was spread onto TSAB-CVA plates. Plates were incubated at 37°C in a microaerobic environment generated by evacuation of anaerobic jars to -25 in Hg and equilibration with a gas mixture comprising 80% N_2, 10% CO_2, and 10% H_2. The remainder of the fecal suspension was frozen at -80°C. Plasma separated from whole blood by centrifugation was harvested and stored at -80°C until analysis. Fecal pellets collected in PBS with

protease inhibitor were vortexed for 30 seconds and centrifuged at 16,000 rcf for 10 minutes. Supernatants were collected and stored at -80°C. The ICJ cassettes and carcasses for nerve dissection were transferred from phosphate buffered formalin to 60% ethanol after 24 and 48 hours, respectively.

Confirmation of Colonization.

Culture. Colonization of the stomach, jejunum, cecal tip, proximal colon, and presence of *C. jejuni* in the feces sampled at necropsy were reported following a semi-quantitative grading system of plate coverage by *C. jejuni* colonies.[42] After 72 hours of incubation, *C. jejuni* growth was enumerated as follows: 0 = no growth; 1 = 1-20 CFU; 2 = 20-200 CFU; 3 = over 200 CFU; 4 = confluent growth. *C. jejuni* colonies were harvested and stored in 15% glycerol in TSB at -80°C.

Colonies inconsistent with *C. jejuni* morphology were swabbed and observed microscopically. In cases where another organism grew on the same plate as *C. jejuni*, only numbers of *C. jejuni* were used in semi-quantitative grading. Colonies of different morphology were harvested and stored separately. Following conclusion of the experiment, an isolate of the second organism was streaked onto TSAB and TSAB-CVA plates and incubated in the microaerophilic environment at 37°C for 72 hours. Growth was observed on plates with and without antibiotics. Colony morphology was recorded, growth was swabbed from the two types of plates, and Gram staining was performed. Colonies subsequently grown on TSAB-CVA plates were suspended and serially diluted in TSB and spread upon both TSAB and TSAB-CVA plates. Colony morphology and Gram staining were again assessed, and the two types of plates with growth were submitted to the Diagnostic Center for Population and Animal Health (currently the Veterinary Diagnostic Laboratory), MSU, for analysis by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry.

PCR. *C. jejuni gyrA* gene-specific PCR[66] was performed to confirm colonization of infected mice at sacrifice and exclude colonization in control mice. Isolates of *C. jejuni* cultured from samples obtained

at necropsy were used for culture-positive mice; DNA extracted from cecal tissue obtained during necropsy was used for TSB-inoculated and infected but culture-negative mice.

Immunohistochemical Labeling of C. jejuni *in Ileocecocolic Junctions*. IHC was performed by the Investigative Histopathology Laboratory, MSU. Specimens were processed, embedded in paraffin and sectioned on a rotary microtome at 4 μm. Sections were placed on slides coated with 2% 3-aminopropyltriethoxysilane and dried at 56°C overnight. The slides were subsequently deparaffinized in xylene, hydrated through descending grades of ethyl alcohol to distilled water, and placed in Tris Buffered Saline pH 7.4 (TBS; Scytek Labs, Logan, UT) for 5 minutes for pH adjustment. Following TBS, slides underwent heat induced epitope retrieval utilizing Scytek Citrate Plus Retrieval pH 6.0 in a Vegetable Steamer for 30 minutes, were allowed to cool on the counter at 25° for 10 minutes, and were rinsed in several changes of distilled water. Endogenous Peroxidase was blocked utilizing a 3% Hydrogen Peroxide/Methanol bath for 30 minutes followed by running tap and distilled water rinses. Following pretreatment, standard micro-polymer complex staining steps were performed at room temperature on the IntelliPath™ Flex Autostainer. All staining steps were followed by rinses in TBS Autowash buffer (Biocare Medical, Concord, CA). After blocking for nonspecific protein with Background Punisher (Biocare) for 5 minutes, sections were incubated with Rabbit Polyclonal anti – *Campylobacter jejuni* (US Biological Canada) (Cat# C1037-08) @ 1:500 diluted in Normal Antibody diluent (NAD-Scytek) for 60 minutes. Rabbit anti – Rodent™ Micro-Polymer (Biocare) was applied for 60 minutes followed by reaction development with Romulin AEC™ (Biocare) for 10 minutes and counterstaining with Cat Hematoxylin (Biocare) for 1 minute.

Presence, abundance, and location of positive labeling within the ileum, cecum, and colon were graded using a semi-quantitative scale by a single investigator (LS Mansfield) who was blinded to mouse identity and experimental group. The grading system included semi-quantitative scoring for positive labeling in the lumen (associated with mucus, contents, crypts, and crypt abscesses); epithelium (apical

or basolateral surfaces, within paracellular junctions or effacing lesions); lamina propria (between cells, or intracellularly within polymorphonuclear cells or macrophages/dendritic cells); and submucosa (within connective tissue, intracellularly within polymorphonuclear cells or macrophages/dendritic cells, associated with vasculature, lymphoid tissues, or histiocytes).

Pathologic Changes: Gastrointestinal Tract. Gross lesions in the gastrointestinal tract, such as thickening, enlargement, or watery or soft contents in the cecum or colon and changes in the ileocecocolic lymph node and spleen, noted by a veterinarian and other experienced personnel during necropsy were recorded. The fixed ICJ sections were embedded in paraffin, finely sectioned at 4-5 µm, routinely stained with Hematoxylin and Eosin (H&E), and coverslipped by the Investigative Histopathology Laboratory at MSU. The sections were evaluated by a board-certified veterinary clinical pathologist (JM Brudvig) who was blinded to mouse identity and experimental group. With the exception that intraepithelial lymphocytes were not scored in the current study, a previously published grading scale[42] was used to evaluate changes in the lumen (exudates, excessive mucus), epithelium (surface integrity, goblet cell hypertrophy or depletion, crypt abnormalities), lamina propria (inflammatory cell infiltrates), and submucosa (inflammation, edema, fibrosis). Raw scores (out of 42 total points) were subsequently ranked into semi-quantitative grades as 0 (0-9 points; no colitis), 1 (10-19 points; mild colitis), or 2 (≥20; moderate or severe colitis).

ELISAs.

Plasma ELISAs. Plasma stored at -80°C was thawed on ice and aliquoted to prevent repeated freeze-thaw cycles. ELISAs were used to quantify anti -*C. jejuni*, -GM1, and -GD1a ganglioside IgG1, IgG2a (present in BALB/c mice),[44] IgG2c (present in C57BL/6 mice),[44] IgG2b, and IgG3 subtypes. Assays were performed as previously described.[42] Nunc Maxisorp (Thermo Scientific, Waltham, MA) 96-well plates

were coated with antigen and incubated at 4°C overnight. Antigens were diluted in PBS to the following concentrations: *Campylobacter jejuni* antigen 1.9 µg/mL;[42] GM1 antigen (US Biological, Swampscott, MA) 2 µg/mL; GD1a antigen (Sigma Aldrich, St. Louis, MO) 20 µg/mL. The plates were blocked with blocking buffer (10mM PBS with 3% BSA and 0.05% Tween-20 (Sigma)) overnight at 4°C. Following three washes in wash buffer (PBS with 0.025% Tween-20), plasma samples diluted in blocking buffer (all samples were diluted 1:25, except for anti-*C. jejuni* IgG2b and IgG2a, which were 1:100) were loaded in triplicate. Positive and negative controls and wells containing only blocking buffer were run on each plate. Positive controls included plasma from mice from previous experiments with a high OD or commercially available antibodies (anti-*Campylobacter* IgG1 (Virostat, Portland, ME)). Anti-*Toxoplasma gondii* (ViroStat, Portland, ME) antibody was used as a negative control. Sealed anti -GM1 and -GD1a antibody plates were incubated with samples overnight at 4°C, while anti-*C. jejuni* antibody plates were incubated for 1 hour at room temperature on a platform shaker. Plates were washed, and secondary antibodies (Biotin-SP-conjugated AffiniPure Goat Anti-Mouse IgG1, IgG2a, IgG2b, IgG2c, or IgG3; Jackson ImmunoResearch, West Grove, PA) diluted in blocking buffer were added. Following incubation for 1 hour on a platform shaker, plates were washed again and ExtrAvidin peroxidase (Sigma-Aldrich, St. Louis, MO) diluted 1:2,000 in 10 mM PBS with 1% BSA and 0.05% Tween-20 was added. Plates were incubated for 1 hour on a platform shaker, washed, and tetramethylbenzidine (TMB substrate; Rockland Immunochemicals Inc., Limerick, PA) was added. The reaction was stopped with 2N H_2SO_4. Absorbance was read at 450 nm using a Bio-Tek EL-800 Universal Microplate Reader with KC Junior software (Bio-Tek Instruments, Winooski, VT). The absorbance generated from the diluent (blocking buffer) alone was subtracted from the mean absorbance obtained for each sample run in triplicate. This adjusted value was used in statistical analyses. Negative values generated by subtracting the absorbance of the blocking buffer from the mean sample absorbance were treated as zero for the purpose of statistical analysis.

*Fecal Supernatant (anti-*C. jejuni *IgA) ELISA*. Measurement of *C. jejuni*-specific IgA from fecal supernatants was performed in similar fashion to plasma antibodies. Nunc Maxisorp (Thermo Scientific, Waltham, MA) 96-well plates were coated with *C. jejuni* antigen diluted in PBS to 1.9 µg/mL[42] and incubated at 4°C overnight. The plates were then blocked with blocking buffer overnight. Following washing, 100 µL of undiluted supernatants were loaded onto the plate in duplicate. Fecal supernatants from the pilot experiment with high ODs were used as positive controls on each plate. Wells containing only blocking buffer (used to dilute the secondary antibody) and PBS (for background of fecal supernatant) were included on each plate. Anti-*Toxoplasma gondii* antibody (ViroStat, Portland, ME) was used as a negative control on each plate. Following 1 hour of incubation at room temperature, the plates were washed and biotin-conjugated goat anti-mouse IgA (Sigma) was added. After 1 hour incubation, plates were washed and ExtrAvidin peroxidase was added. Plates were incubated for 1 hour, washed, and TMB substrate was added. The reaction was stopped with 2N H_2SO_4 and absorbance was read at 450 nm. The absorbance generated from the PBS alone was subtracted from the mean absorbance obtained for each sample run in duplicate. This adjusted value was used in statistical analyses. For purposes of statistics, negative values generated by subtracting the absorbance of the PBS from the mean sample absorbance were treated as zero, and the two samples with absorbances returning a value of "OUT" (out-of-range; set to ≥3.000 on plate reader) were converted to 3.000 before subtracting the blank.

Measurement of Colon Cytokine Production. At necropsy, rinsed proximal colon samples were collected in Eppendorf tubes and snap frozen. Tissues were stored at -80°C until analysis. Subsequently, samples were thawed on ice and wet tissue weight was recorded. Tissue pieces were homogenized using autoclaved microtube pellet pestle rods attached to a handheld Kontes pellet pestle motor for one minute, on ice, in 400 µL of Hank's Balanced Salt Solution (Sigma), with 0.5% Triton X-100 (Sigma) and

the cOmplete Mini EDTA-free Protease Inhibitor cocktail (Roche/Sigma). Homogenates were centrifuged at 12,000 × *g* for 30 minutes at 4°C. Supernatants were aliquoted in cryovials and stored at -80°C until further analysis.

Cytokines were measured using a commercially available flow cytometry based, multiplexed, bead assay panel (LEGENDPlex Mouse Th Cytokine Panel, BioLegend, San Diego, CA). Cytokines included in the panel are designed to characterize the Th adaptive immune response by delineating specific Th polarization. Analytes included IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, and IL-22. Just before analysis, aliquoted supernatants of the colon homogenates were thawed on ice and centrifuged at 300 × *g* for 10 minutes at 4°C to further pellet any debris and ensure clarity. The assay was performed according to the manufacturer's instructions using undiluted supernatant and a V-bottom microplate. Data were acquired on a BD FACSCanto II flow cytometer and analyzed using the LEGENDplex Data Analysis software. Standard curves were generated for each analyte. Each cytokine had a maximum standard concentration of 10,000 pg/mL. Data are presented as pg cytokine/mg tissue weight.

Assessment of Nerve Histopathology. Dissection of fixed tissue was performed using a dissecting microscope. Two to 3 lumbar dorsal root ganglia (DRG) were harvested from the left side of the mouse. Where possible and in many cases, connections from the sciatic nerve to one or all DRG collected, including those in L3, L4, or L5 regions, were visualized prior to removal of DRG. The left sciatic nerve, brachial plexus, and lumbar DRG were placed en bloc in a cassette and stored in 60% ethanol until submission for histopathology.

The sections were labeled by IHC for the mouse macrophage marker F4/80 by the Investigative Histopathology Laboratory, Division of Human Pathology, MSU. Specimens were embedded in paraffin and sectioned on a rotary microtome at 4 μm. Sections were placed on charged slides and dried at 56°C

overnight, deparaffinized in xylene, and hydrated through descending grades of ethyl alcohol to distilled water. Slides were then placed in Tris Buffered Saline pH 7.4 (TBS; Scytek Labs – Logan, UT) for 5 minutes for pH adjustment. Following TBS, epitope retrieval was performed using Citrate Plus Retrieval Solution pH 6.0 (Scytek) in a vegetable steamer for 30 minutes followed by a 10 minute countertop incubation and several changes of distilled water. Following pretreatment standard avidin-biotin complex staining steps were performed at room temperature on the DAKO Autostainer. All staining steps are followed by two minute rinses in Tris Buffered Saline + Tween 20 (Scytek). After blocking for non-specific protein with Normal Rabbit Serum (Vector Labs – Burlingame, CA) for 30 minutes, sections were incubated with Avidin / Biotin blocking system for 15 minutes each (Avidin D – Vector Labs / d-Biotin – Sigma). Primary antibody slides were incubated for 60 minutes with the Monoclonal Rat anti-Mouse F4/80 diluted @ 1:100 (AbD Serotec – Raleigh, NC) in Normal Antibody Diluent (NAD) (Scytek). Biotinylated Rabbit anti-Rat IgG (H + L) Mouse Absorbed prepared at 10.0 μg/mL in NAD incubated for 30 minutes, followed by R.T.U. Vector Elite Peroxidase Reagent (Vector) incubation for 30 minutes. Reaction development utilized Vector Nova Red Kit peroxidase chromogen incubation of 15 minutes followed by counterstain in Gill 2 Hematoxylin (Cancer Diagnostics – Durham, NC) for 30 seconds, differentiation, and dehydration, clearing, and mounting with Permount mounting media.

Number of F4/80 positive cells was quantified by morphometry. Images were analyzed using ImageJ software (version 2.0.0rc-49/1.51d), distributed by Fiji (Fiji Is Just ImageJ)[56; 57] for Windows (http://imagej.net/Fiji/Downloads). The investigator (MM Cluett) was blinded to mouse genotype and treatment group. Contiguous images of each DRG section obtained at 100× magnification (10× objective and ocular) were opened in the ImageJ program. Positive cells were marked on the image using the "Cell Counter" plugin. After all positive cells were marked, the area was outlined using the "Freehand Selections" Trace Tool. When necessary, multiple areas were traced individually and the sum of the

areas was recorded. Results are given as number of F4/80 positive cells/area, with area representing 100,000 pixels. The slides were finally unblinded for statistical analysis.

Statistical Analyses. Analyses were performed and figures generated using commercial statistical software packages (SigmaStat 3.5, Systat Software, San Jose, CA; and GraphPad Prism 6, GraphPad Software, San Diego, CA) or through online applications (VassarStats: Website for Statistical Computation; vassarstats.net). P-values ≤0.05 were considered statistically significant. Data were tested for normality and equal variance.

Survival curves were compared by log-rank (Mantel-Cox) testing.

The Freeman-Halton extension of the Fisher Exact Probability Test was used to assess differences in *C. jejuni* colonization depending upon mouse genotype and infecting *C. jejuni* strain. Colonization was assessed between all infected C57BL/6 IL-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice (regardless of infecting *C. jejuni* strain), along with testing for (1) differences in colonization of each of the two *C. jejuni* strains within each mouse genotype, and (2) differences in colonization of the two mouse genotypes with each of the two *C. jejuni* strains considered individually. Semi-quantitative colonization grades were entered as 0, no CFU; 1, 1-20 CFU; 2, 21-200 CFU; and 3, >200 CFU (a single grade, combining grades 3 and 4 used in assigning values of plate coverage by *C. jejuni* colonies).

Differences in gross pathology in infected C57BL/6 IL-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice were similarly assessed by the Freeman-Halton extension of the Fisher Exact Probability Test. Gross pathology was assessed between all infected C57BL/6 IL-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice (regardless of infecting *C. jejuni* strain), along with testing for (1) differences in pathology resulting from of each of the two *C. jejuni* strains within each mouse genotype, and (2) differences in pathology in the two mouse genotypes with each of the two *C. jejuni* strains considered individually. For these analyses, gross pathology recorded at necropsy was graded as 0 changes; 1 change; 2 changes; and 3-4 changes as a single grade.

Kruskal-Wallis analysis followed by Dunn's post-testing was performed on semi-quantitative data following ranking of raw scores for assessment of colon histopathology. The non-parametric ANOVA and analysis of semi-quantitative grades, rather than raw scores, were chosen to account for non-independence of some parameters of the grading system.

One- or two-way ANOVA and Kruskal-Wallis ANOVA on ranks with appropriate post-hoc testing (Holm-Sidak, Tukey, Dunn's) were used to evaluate impact of mouse genotype and infecting *C. jejuni* strain on production of anti -*C. jejuni* and -ganglioside plasma antibodies, *C. jejuni*-specific IgA in fecal supernatant, cytokines reflecting Th polarization in the proximal colon, and number of F4/80 positive cells in DRG. Data not meeting the assumption of equal variance were analyzed non-parametrically by Kruskal-Wallis.

RESULTS

Confirmation of Mouse Genotype and Absence of Enteropathogens. Absence of IL-10 was confirmed by PCR in all 60 experimental mice (data not shown). Additionally, pooled fecal samples (at least one fecal pellet per group-housed cage) obtained pre-inoculation were negative by PCR for *Helicobacter* spp., *Campylobacter* spp., *Enterococcus faecalis*, *E. faecium*, and *Citrobacter rodentium* (data not shown).

Clinical Signs and Survivorship. Clinical signs of illness, including a slight hunch, decreased activity, roughened hair coat, and soft or sticky stool were first noted on day 16 post-infection in BALB/c IL-$10^{-/-}$ mice infected with *C. jejuni* 11168. In total, 5/10 *C. jejuni* 11168-infected BALB/c IL-$10^{-/-}$ mice required early humane euthanasia. In the C57BL/6 IL-$10^{-/-}$ groups, 4 mice infected with *C. jejuni* 11168 showed mild intermittent clinical signs including slight hunching, slightly decreased activity, and a questionably slight rough hair coat starting on day 20; one of these mice required early humane euthanasia because the clinical signs score was greater than 10. In all other experimental mice, no clinical signs aside from

questionable and intermittent findings including slight hunching, decreased activity levels, or roughened hair coat were noted. In all mice, signs were monitored carefully using our scoring sheet by at least one observer and least once per day, and mice were promptly euthanized if humane endpoint was determined.

In total, 5 BALB/c IL-10$^{-/-}$ mice and one C57BL/6 IL-10$^{-/-}$ mouse, all infected with *C. jejuni* 11168, required early euthanasia between days 17 and 25 post infection because their clinical signs scores exceeded the limit. No mouse inoculated with TSB or *C. jejuni* 260.94 required early humane euthanasia. Survival curves for all treatment groups are shown in **Figure 3.1A**; curves were significantly different from each other when assessed by log-rank (Mantel-Cox) testing (P = 0.0001).

C57BL/6 IL-10$^{-/-}$ Mice: Clinical Signs and Survivorship. Clinical signs in C57BL/6 IL-10$^{-/-}$ mice included mild intermittent hunching, lethargy, or roughened hair coat. One mouse infected with *C. jejuni* 11168 was humanely sacrificed on day 25 due to a hunched posture. No mouse in the TSB or *C. jejuni* 260.94 groups required early sacrifice. Survival curves for C57BL/6 IL-10$^{-/-}$ mice are shown in **Figure 3.1B**; curves were not significantly different from each other (Mantel-Cox, P = 0.3679).

BALB/c IL-10$^{-/-}$ Mice: Clinical Signs and Survivorship. Clinical signs in BALB/c IL-10$^{-/-}$ mice were first observed in *C. jejuni* 11168-infected mice on day 16, and included slight hunching, soft or sticky stools with wet or matted fur around the anus, decreased activity, and slightly roughened hair coat. Two of these mice were euthanized and necropsied on day 17, one on day 21, and two on day 25. No mouse in the TSB or *C. jejuni* 260.94 groups required early sacrifice. Survival curves for BALB/c IL-10$^{-/-}$ mice are shown in **Figure 3.1C**; when compared by Mantel-Cox analysis, curves were significantly different from each other (P = 0.002).

Colonization.

Culture. Colonization data, represented by culturable *C. jejuni* isolated from the cecum, are shown in **Figure 3.2**. Although feces and sections of stomach, jejunum, and proximal colon taken at necropsy were also assessed by culture for presence of *C. jejuni*, the cecum was previously shown to be the most consistently and heavily colonized location in the GI tract.[42; 43] Colonization for all experimental mice together is shown in **Figure 3.2A**. When all 20 infected BALB/c IL-10$^{-/-}$ mice were compared with all 20 infected C57BL/6 IL-10$^{-/-}$ mice, a significantly higher proportion of BALB/c IL-10$^{-/-}$ mice were more heavily colonized than C57BL/6 IL-10$^{-/-}$ mice in the cecum at the time of necropsy ($P_B < 0.0001$). Differences in colonization between C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 were also compared, as were differences between mouse genotypes infected with *C. jejuni* 260.94. A significantly higher proportion of BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 had higher cecal colonization scores than *C. jejuni* 11168-infected C57BL/6 IL-10$^{-/-}$ mice ($P_B = 0.001$). Similarly, BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 had significantly higher cecal colonization rates than C57BL/6 IL-10$^{-/-}$ mice also infected with *C. jejuni* 260.94 ($P_B = 0.015$).

When all 5 sampled areas of the gastrointestinal tract are included, a total of 9/10 BALB/c IL-10$^{-/-}$ mice inoculated with *C. jejuni* 260.94 were culture positive, 5/10 C57BL/6 IL-10$^{-/-}$ mice inoculated with *C. jejuni* 260.94 were positive, 10/10 BALB/c IL-10$^{-/-}$ mice inoculated with *C. jejuni* 11168 were positive, and 9/10 C57BL/6 IL-10$^{-/-}$ mice inoculated with *C. jejuni* 11168 were positive. *C. jejuni gyrA* gene-specific PCR confirmed positive culture results, and PCR performed on extracted cecal DNA from infected but culture negative mice also confirmed negative culture results. All TSB inoculated mice were culture negative for *C. jejuni* in all areas sampled; *C. jejuni gyrA* gene specific PCR performed on extracted cecal DNA from TSB mice was also negative in all 20 control mice.

Immunohistochemical Labeling of C. jejuni *in Ileocecocolic Junctions.* Labeling of *C. jejuni* by IHC in ileocecocolic junctions of control and infected mice showed that 9/10 mice in the BALB/c IL-10$^{-/-}$, *C.*

jejuni 11168-infected group exhibited positive intracellular labeling within macrophage/dendritic cell types within the submucosa, versus between 0-3/10 mice in the other five treatment groups. Similarly, 4/10 *C. jejuni* 11168-infected BALB/c IL-$10^{-/-}$ mice exhibited intracellular labeling within macrophage/dendritic cell types within the lamina propria; this was not seen in any other mice in any group. Non-specific labeling does occur with this method. Positive staining was noted in at least one area of the epithelium or submucosa in 6/10 sham-inoculated BALB/c IL-$10^{-/-}$ mice and 3/10 sham-inoculated C57BL/6 IL-$10^{-/-}$ mice, along with staining of luminal contents in virtually all experimental mice, regardless of treatment group.

C57BL/6 IL-$10^{-/-}$ Mice: Colonization.

Culture. Cecal colonization by *C. jejuni* is shown for C57BL/6 IL-$10^{-/-}$ groups in **Figure 3.2B**. C57BL/6 IL-$10^{-/-}$ mice infected with *C. jejuni* 11168 did not have significantly different colonization scores in the cecum than those infected with *C. jejuni* 260.94 ($P_B = 0.184$). Positive *C. jejuni* 260.94 culture results in all samples were as follows: 0/10 in the stomach; 4/10 in the jejunum; 3/10 in the cecum; 4/10 in the proximal colon; 3/10 in the feces. Positive *C. jejuni* 11168 culture results were as follows: 1/10 in the stomach; 5/10 in the jejunum; 7/10 in the cecum; 5/10 in the proximal colon; 3/10 in the feces.

Immunohistochemical Labeling of C. jejuni *in Ileocecocolic Junctions of C57BL/6 IL-$10^{-/-}$ Mice*. In the epithelium, 3/10 TSB mice exhibited labeling associated with the apical surface. In the *C. jejuni* 260.94 and *C. jejuni* 11168 infection groups, 2/10 mice in each group were positive for labeling in the epithelium, one in each group in association with the apical surface and another in each group associated with an effacing lesion. No C57BL/6 IL-$10^{-/-}$ mouse exhibited labeling associated with the lamina propria. The single C57BL/6 IL-$10^{-/-}$ mouse with positive labeling intracellularly in the submucosa was infected with *C. jejuni* 260.94.

BALB/c IL-10$^{-/-}$ Mice: Colonization.

Culture. Cecal colonization by *C. jejuni* in BALB/c IL-10$^{-/-}$ mice is shown in **Figure 3.2C**. BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 did not have significantly different colonization scores in the cecum than those infected with *C. jejuni* 260.94 (P_B = 0.195). Positive *C. jejuni* 260.94 culture results in all samples were as follows: 2/10 in the stomach; 2/10 in the jejunum; 9/10 in the cecum; 9/10 in the proximal colon; 9/10 in the feces. Positive *C. jejuni* 11168 culture results were as follows: 8/10 in the stomach; 8/10 in the jejunum; 10/10 in the cecum; 10/10 in the proximal colon; 10/10 in the feces.

Immunohistochemical Labeling of C. jejuni *in Ileocecocolic Junctions of BALB/c IL-10$^{-/-}$ Mice*. In the epithelium, 5/10 sham-inoculated BALB/c IL-10$^{-/-}$ mice exhibited positive labeling at the apical surface, with one of these also staining positively in an effacing lesion. In BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94, 6/10 mice exhibited positive labeling on the apical surface. Two of these 6 also stained positively in an effacing lesion and one of these also was positive on the basolateral surface. Within the *C. jejuni* 11168-infected mice, 8/10 showed positive labeling on the apical surface. Of these 8, staining in an effacing lesion was noted in 3, and additional staining in paracellular junctions was seen in one. Additionally, only the *C. jejuni* 11168 group had mice exhibiting positivity within macrophages/dendritic cells in the lamina propria (4/10 in this group). Positive labeling in the submucosa was seen in all three groups of BALB/c IL-10$^{-/-}$ mice. 3/10 TSB mice had positive staining intracellularly in the macrophage/dendritic cell types, and one of these also had staining identified in regional lymphoid tissue. BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 also were positive in the macrophage/dendritic cell types in the submucosa (3/10; one of these also had vascular tissue involvement). Finally, in the 9/10 *C. jejuni* 11168-infected mice showing positive submucosal staining, *C. jejuni* was found intracellularly in macrophages/dendritic cells in all 9, in the lymphoid tissue in 2, and associated with vasculature in 2.

Presence of* Lactobacillus murinus *in C57BL/6 IL-10$^{-/-}$, but not BALB/c IL-10$^{-/-}$, Mice. When assessing colonization of *C. jejuni* by culture, TSAB-CVA plates from all 30 C57BL/6 IL-10$^{-/-}$ mice (irrespective of treatment group), but no BALB/c IL-10$^{-/-}$ mice, were noted to exhibit growth of a second organism in all five samples of the gastrointestinal tract (stomach, jejunum, cecum, colon, and feces). These colonies had a small, dry, flat, puckered appearance, were hemolytic, and exhibited robust growth on all plates, with or without concurrent growth of *C. jejuni* colonies. A greenish-orange hue was observed on the swab while harvesting these colonies. Wet mount preparation revealed non-motile rods larger than *C. jejuni*. When a stored isolate from the cecum of a TSB-inoculated mouse was later streaked onto TSAB and TSAB-CVA plates, differences in colony morphology and microscopic appearance were observed. On plates without antibiotics, colonies appeared more mucoid and on Gram staining, the organisms appeared as mostly fat, Gram-positive rods. In contrast, colonies grown on plates with antibiotics had a more dry and puckered appearance and microscopically the organisms appeared as mostly Gram-positive filamentous rods with occasional fat rods.

Colonies from the stored isolate grown on TSAB-CVA plates were diluted and spread onto plates with and without antibiotics. Gram staining revealed a near-pure growth of filamentous rods from plates containing antibiotics, and near-pure growth of fatter rods from plates without antibiotics. The fatter rods were identified by MALDI-TOF mass spectrometry as *Lactobacillus murinus* with a score of 2.3, and the filamentous rods also were identified as *L. murinus* with a score of 2.2.

The unexpected presence of *L. murinus* only in C57BL/6 IL-10$^{-/-}$ mice, combined with the atypical results for infected C57BL/6 IL-10$^{-/-}$ mice compared to previous studies from our group, complicated the comparison of C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice as originally intended. Data are thus described first with C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice together, followed by analyses of C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice separately.

Gross Pathology and Histological Assessment of Colitis. Gross pathological data are shown in **Figure 3.3**. Pathology in the cecum, colon, mesenteric lymph nodes (MLN), or spleen at the time of necropsy was recorded. Possible changes included thickening, enlargement, or watery or soft contents in the cecum or colon and enlarged MLN or spleen. Pathology for all experimental mice together is shown in **Figure 3.3A**. When all 20 infected BALB/c IL-10$^{-/-}$ mice were compared with all 20 infected C57BL/6 IL-10$^{-/-}$ mice, a significantly higher proportion of BALB/c IL-10$^{-/-}$ mice had greater pathology than C57BL/6 IL-10$^{-/-}$ mice at the time of necropsy ($P_B < 0.0001$). Differences in gross pathology between C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 were also compared, as were differences between mouse genotypes infected with *C. jejuni* 260.94. A significantly higher proportion of BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 had greater pathology scores than *C. jejuni* 11168-infected C57BL/6 IL-10$^{-/-}$ mice ($P_B = 0.001$). Similarly, BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 had greater pathology scores than *C. jejuni* 260.94-infected C57BL/6 IL-10$^{-/-}$ mice ($P_B < 0.01$).

Assessment of histopathologic lesions in the ileocecocolic junctions (ICJ) is shown in **Figure 3.4**. Raw scores are displayed graphically for all mice together in **Figure 3.4A**. Raw scores were subsequently placed into grades, and statistical analysis was performed on these data using the non-parametric Kruskal Wallis one-way ANOVA on ranks to account for non-independence within some parameters of the previously published[42] grading system. A raw score of 0-9 indicates no colitis is present (semi-quantitative grade 0), raw scores of 10-19 indicate mild colitis (grade 1), and scores ≥20 reflect moderate to severe colitis (grade 2). BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 had significantly higher colitis scores than all other treatment groups, excepting BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94. The most pronounced changes in BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 compared to other treatment groups included higher frequency of mucus and cellular exudate within the lumen, higher numbers of mice with groups of damaged surface epithelial cells, more severe goblet cell depletion, more severity in crypt changes including irregular architecture, dilation, and

cryptitis/abscesses, increased cellular infiltrates in the lamina propria, and more inflammation in the submucosa.

C57BL/6 IL-10$^{-/-}$ Mice: Gross Pathology and Histological Assessment of Colitis. Gross pathology in C57BL/6 IL-10$^{-/-}$ mice is shown in **Figure 3.3B**. Within the *C. jejuni* 11168-infected mice, with the exception of one mouse with a thickened proximal colon, the changes were limited to mildly enlarged MLN and/or spleen. Similarly, within the C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94, one mouse had a slightly thickened proximal colon and the remaining changes were attributable to mildly to moderately enlarged MLN. C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 did not have significantly different gross pathology scores than those infected with *C. jejuni* 260.94 ($P_B = 0.262$). No gross pathology was found in any mouse receiving TSB.

Assessment of histopathology in the ICJ of C57BL/6 IL-10$^{-/-}$ mice is shown in **Figure 3.4B**. One sham-inoculated mouse had a score of 11, indicating mild colitis. Of the *C. jejuni* 260.94-infected mice, one had a score of 10 (mild colitis), and one other had a score of 32, reflecting severe colitis; no other mice in this group had raw scores above 8. In the *C. jejuni* 11168-infected group, a single mouse had a score of 13, indicating mild colitis, and all other mice had no colitis (scores ranging from 3—8). There was no statistical difference between groups ($P = 0.715$).

BALB/c IL-10$^{-/-}$ Mice: Gross Pathology and Histological Assessment of Colitis. Gross pathology in BALB/c IL-10$^{-/-}$ mice is shown in **Figure 3.3C**. Pathology was noted in 9/10 *C. jejuni* 11168-infected mice, with 7 of these 9 mice exhibiting 3 or 4 changes. Pathology was noted in 6/10 *C. jejuni* 260.94-infected mice; 4 of these mice exhibited changes in the cecum and/or colon, and all 6 had enlarged MLN, spleen, or both. BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 did not have significantly different gross pathology scores than those infected with *C. jejuni* 260.94 ($P_B = 0.084$). No gross pathology was found in any mouse receiving TSB.

Assessment of histopathology in the ICJ of BALB/c IL-10$^{-/-}$ mice is shown in **Figure 3.4C**. In the TSB group, 3 mice exhibited mild colitis (raw scores 10—19), while all other mice in this group had no colitis (raw score range 6–9). In the *C. jejuni* 260.94 group, 7/10 mice had scores between 10–17 indicating mild colitis, and the other 3 mice had no colitis. Pathology was most severe in the *C. jejuni* 11168-infected group. The majority (8/10) of mice had moderate or severe colitis, with scores ranging from 23—33; one mouse had mild colitis and the last had a raw score of 9, indicating no colitis. Statistically significant differences were found between the *C. jejuni* 11168 and 260.94 groups (P = 0.024) and between *C. jejuni* 11168 and sham-inoculated groups (P = 0.0004). The colon of a sham-inoculated BALB/c IL-10$^{-/-}$ mouse with a grade of 0 (no colitis) is shown in **Figure 3.4D**, compared to the severe colitis in a *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mouse (**Figure 3.4E**).

Systemic and Local Immune Response to* C. jejuni *Infection.

Plasma ELISAs. Systemic immune responses to *C. jejuni* infection were evaluated by measurement of *C. jejuni*-specific IgG1, IgG2b, IgG3, and IgG2c (in C57BL/6 IL-10$^{-/-}$ mice) or IgG2a (in BALB/c IL-10$^{-/-}$ mice)[44] antibodies in plasma by indirect ELISA (**Figure 3.5**). Additionally, as antibodies cross-reacting to LOS on the *C. jejuni* outer membrane and gangliosides on peripheral nerves are a hallmark of GBS and thought to be involved in GBS immunopathogenesis, anti -GM1 antibodies (**Figure 3.6**) and -GD1a antibodies (**Figure 3.7**) also were assessed by indirect ELISA.

When anti-*C. jejuni* specific IgG1, IgG2b, and IgG3 antibody levels were compared between all 6 groups (**Figure 3.5A**), the most pronounced responses were seen in *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice. This group had significantly higher production of Th2-mediated IgG1 than BALB/c IL-10$^{-/-}$ mice receiving TSB or *C. jejuni* 11168-infected C57BL/6 IL-10$^{-/-}$ mice. A robust Th1/Th17 response, evidenced by production of IgG2b and IgG3, also was most pronounced in BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168. Mice in this group had significantly higher plasma IgG2b than TSB-inoculated mice of either

mouse genotype. BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 also produced the most robust IgG3 response, which was significantly higher than all other treatment groups excepting the BALB/c IL-10$^{-/-}$ *C. jejuni* 260.94 group. Collectively, these results suggest that of C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 or 260.94 strains, BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 mount the most pronounced immune response and that this response is mixed Th1/Th2/Th17 in character.

Production of anti-ganglioside GM1 (**Figure 3.6A**) and GD1a (**Figure 3.7A**) antibodies was compared between all 6 treatment groups. IgG1, IgG2b, and IgG3 isotypes reacting with GM1 were nearly identical in pattern and magnitude of response to those isotypes reacting with GD1a. Significant differences between groups were found only in IgG2b production. C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 produced the strongest IgG2b response, although a low-level background production in all C57BL/6 IL-10$^{-/-}$ groups may be present, as suggested by the background in C57BL/6 IL-10$^{-/-}$ TSB-inoculated mice.

Local Response: Fecal IgA and Colon Cytokine Production. Local immune responses in the gastrointestinal tract were assessed by measurement of *C. jejuni*-specific IgA in fecal supernatants (**Figure 3.8**) and evaluation of the production of cytokines reflecting character of Th adaptive immunity in the proximal colon (**Figure 3.9**). When IgA production was compared in all 6 treatment groups (**Figure 3.8A**), results were similar to the systemic response assessed by plasma anti-*C. jejuni* antibodies. The strongest response was seen in BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168. These mice produced significantly higher amounts of *C. jejuni*-specific IgA than all other treatment groups, excepting the *C. jejuni* 260.94-infected BALB/c IL-10$^{-/-}$ group.

Measurement of colon cytokine production was performed with a panel including IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, and IL-22. Detectable amounts of IL-4, IL-5, IL-17F, and IL-21 were found in only 1/60 mice, and IL-13 production was not detected in any mouse. As expected, no detectable IL-10 was produced by any mouse. Therefore, only production of IFN-γ, TNF-α,

IL-2, IL-6, IL-9, IL-17A, and IL-22 was analyzed. Comparison of cytokine production in the proximal colon between all 6 groups (**Figure 3.9**) similarly showed that BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 produced significantly higher amounts of IFN-γ, TNF-α, IL-17A, and IL-22, and although non-significant, BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 had the highest IL-6 levels. Collectively, these results show that of C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 or *C. jejuni* 11168, the most potent mucosal immunity and robust Th1/Th17/Th22-mediated gastrointestinal responses are elicited in BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168.

C57BL/6 IL-10$^{-/-}$ Mice: Immune Response to* C. jejuni *Infection.

Plasma ELISAs. Production of anti-*C. jejuni* specific IgG1, IgG2b, IgG3, and IgG2c is compared between C57BL/6 IL-10$^{-/-}$ infection groups in **Figure 3.5B**. Infected mice produced slightly higher levels of IgG2b, IgG3, and IgG2c anti-*C. jejuni* antibodies than TSB control mice, but these responses were mild and no significant difference was found between any C57BL/6 IL-10$^{-/-}$ groups in any of the four anti-*C. jejuni* antibody isotypes tested.

Production of anti-GM1 antibodies (**Figure 3.6B)** and anti-GD1a antibodies (**Figure 3.7B**) by mice in different C57BL/6 IL-10$^{-/-}$ infection groups was compared. Again, production of IgG1, IgG2b, IgG3, and IgG2c antibodies reacting with GM1 closely mirrored those reacting with GD1a. IgG2b production by mice infected with *C. jejuni* 11168 represented the strongest response, although no significant differences were seen between any treatment groups, in any of the four isotypes tested reacting with GM1 or GD1a.

Local Response: Fecal IgA and Colon Cytokine Production. Comparison of *C. jejuni*-specific IgA production between mice in C57BL/6 IL-10$^{-/-}$ treatment groups is shown in **Figure 3.8B**. Although both *C. jejuni* 260.94- and *C. jejuni* 11168-infected C57BL/6 IL-10$^{-/-}$ mice produced slightly higher levels of IgA than control mice, these responses were mild overall and no significant difference was found between any treatment groups. Local cytokine production in the proximal colon of C57BL/6 IL-10$^{-/-}$ mice was

similarly mild (**Figure 3.10**), and no statistically significant difference was found between any treatment groups. IL-6 and IL-17A were not produced in detectable amounts in any group. Although the differences were not significant, there appeared to be mild relative up- or down-regulation of three cytokines in the panel depending upon infection status. *C. jejuni* 11168-infected mice produced the greatest amount of IFN-γ, while this group produced no IL-22 or IL-9. Mice infected with *C. jejuni* 260.94 also produced no IL-22. Collectively, the data support a mild overall local response to *C. jejuni* infection in C57BL/6 IL-$10^{-/-}$ mice, reflected by *C. jejuni*-specific IgA and colonic cytokine production, that was not significantly different between infected and control mice.

BALB/c IL-$10^{-/-}$ Mice: Immune Response to* C. jejuni *Infection.

Plasma ELISAs. Production of anti-*C. jejuni* specific IgG1, IgG2b, IgG3, and IgG2a is compared between BALB/c IL-$10^{-/-}$ infection groups in **Figure 3.5C**. The most pronounced responses were elicited in mice infected with *C. jejuni* 11168. The most robust response was Th1/Th17-mediated IgG2b: mice infected with either *C. jejuni* 11168 or *C. jejuni* 260.94 had significantly increased production compared to sham-inoculated mice. Additionally, *C. jejuni* 11168-infected mice had significantly higher IgG2b production than *C. jejuni* 260.94-infected mice. Th1-mediated IgG3 and IgG2a isotypes mirrored each other, with significantly higher production of both isotypes in *C. jejuni* 11168-infected mice than in *C. jejuni* 260.94- or sham-inoculated groups. Overall significance was identified in IgG1 production by one-way ANOVA (P = 0.044), although no significant pairwise comparisons were identified following Holm-Sidak post-testing. Together, these data suggest a primary Th1/Th17-mediated immune response to *C. jejuni* 11168 and a similar response of lesser magnitude to *C. jejuni* 260.94 by infected BALB/c IL-$10^{-/-}$ mice.

Production of anti-GM1 antibodies (**Figure 3.6C**), and anti-GD1a antibodies (**Figure 3.7C**) was compared between BALB/c IL-$10^{-/-}$ infection groups. The pattern of production of IgG1, IgG2b, IgG3, and IgG2a antibody isotypes reacting with GM1 again closely resembled those reacting with GD1a. Mice

infected with *C. jejuni* 11168 produced the most robust responses, manifested mainly by significantly increased levels of IgG2b and IgG2a isotypes to both GM1 and GD1a gangliosides in *C. jejuni* 11168-infected mice, compared to the other two BALB/c IL-10$^{-/-}$ treatment groups. No significant differences between treatment groups were found in production of anti -GM1 or -GD1a IgG1 or IgG3. Collectively, these data suggest that BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 produce Th1/Th17-mediated anti-ganglioside antibodies.

Local Response: Fecal IgA and Colon Cytokine Production. Production of *C. jejuni*-specific IgA measured in fecal supernatants in BALB/c IL-10$^{-/-}$ mice was compared (**Figure 3.8C**). Similar to *C. jejuni*-specific IgG3 and IgG2a antibodies measured in the plasma, the most pronounced IgA response was elicited in *C. jejuni* 11168-infected mice. These mice showed significantly increased IgA production compared to both sham-inoculated and *C. jejuni* 260.94-infected mice. Colon cytokines reflecting adaptive immune responses also were compared between BALB/c IL-10$^{-/-}$ treatment groups (**Figure 3.11)**. Mice infected with *C. jejuni* 11168 produced significantly higher amounts of IFN-γ and TNF-α than the other two treatment groups, and significantly more IL-22 than *C. jejuni* 260.94-infected mice. *C. jejuni* 11168-infected mice also trended toward increased production of IL-6 and IL-17A compared to the other groups. Similar to comparisons within the C57BL/6 IL-10$^{-/-}$ group, production of IL-9 was decreased, although non-significantly, in *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice compared to the sham- or *C. jejuni* 260.94-inoculated groups. Collectively, these data support the conclusion that *C. jejuni* 11168 elicits the strongest mucosal immunity and primarily a Th1/Th17/Th22-mediated local adaptive response in BALB/c IL-10$^{-/-}$ mice.

Nerve Histopathology. Macrophage densities in the lumbar dorsal root ganglia were identified by labeling of cells by IHC with the F4/80 macrophage marker. Number of macrophages in DRG were quantified by morphometry and presented as F4/80 positive cells per unit area of DRG. Results of

comparisons between all 6 treatment groups are shown in **Figure 3.12A**. No statistically significant difference was found between any treatment groups (two-way ANOVA; P-values for mouse genotype, treatment group, and interaction were all >0.05). The widest range of scores was seen in sham-inoculated BALB/c IL-$10^{-/-}$ mice (7–39). Overall these data suggest that, in this model, production of anti -GM1 and -GD1a IgG2b antibodies was alone not sufficient to mediate increased macrophage infiltration or proliferation in the DRG.

C57BL/6 IL-$10^{-/-}$ Mice: Nerve Histopathology. Numbers of macrophages in DRG were compared within C57BL/6 IL-$10^{-/-}$ treatment groups (**Figure 3.12B**). *C. jejuni* 260.94-infected mice showed the greatest range (7–28) of F4/80 positive cells per unit area. Although mice infected with *C. jejuni* 11168 had higher scores than the other two groups, the difference between the three groups was not statistically significant (Kruskal-Wallis, P = 0.157).

BALB/c IL-$10^{-/-}$ Mice: Nerve Histopathology. Comparison of F4/80-positive cells in DRG of BALB/c IL-$10^{-/-}$ mice is shown in **Figure 3.12C**. TSB-inoculated mice showed the greatest variability in scores, while *C. jejuni* 11168-infected mice had the most tightly clustered scores. No significant differences were seen between groups (one-way ANOVA, P = 0.641). As in the all-group comparisons, these data suggest that production of anti -GM1 and -GD1a IgG2a and IgG2b antibodies in *C. jejuni* 11168-infected BALB/c IL-$10^{-/-}$ mice was alone not sufficient to mediate increased macrophage numbers in DRG.

DISCUSSION

The purpose of this study was to develop a mouse model to evaluate the contributions of both infecting *C. jejuni* strain characteristics and host genetic background in determining disease outcome, specifically colitis and susceptibility to GBS. Results of this study indicate that BALB/c IL-$10^{-/-}$ mice infected with the colitogenic *C. jejuni* 11168 strain provide an additional model of colitis mimicking

human disease, presenting opportunities for studying the pathogenesis of campylobacteriosis in mice of an additional genetic background; to our knowledge, BALB/c IL-10$^{-/-}$ mouse models of *C. jejuni*-induced colitis have not been previously reported. Infection of BALB/c IL-10$^{-/-}$ mice with GBS-associated *C. jejuni* 260.94 produced a less robust Th17 systemic response than that of *C. jejuni* 11168, without anti-ganglioside antibody production, significantly increased IgA production, or development of colitis. These results indicate that in BALB/c IL-10$^{-/-}$ mice, magnitude of mucosal immunity and Th1/Th17-mediated immune responses vary with infecting *C. jejuni* strain, providing an additional model to further study mechanisms by which *C. jejuni* induces a variable immune response.

In contrast, C57BL/6 IL-10$^{-/-}$ mice infected with either *C. jejuni* 11168 or the GBS patient-derived *C. jejuni* strain 260.94 remained clinically healthy, with the exception of one *C. jejuni* 11168-infected mouse deemed to require early humane sacrifice. These results contrast with previously published reports indicating C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 develop severe colitis, while infection with *C. jejuni* 260.94 stimulates anti-ganglioside antibody production.[6; 7; 41-43; 54] C57BL/6 IL-10$^{-/-}$ mice mounted only weak immune responses and did not develop the expected colitis or anti-ganglioside antibodies with the respective *C. jejuni* strains reported in previous studies. The original intent of the study was to compare *C. jejuni* infection in C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice to evaluate the impact of immune bias due to host genetic background in determining immunity. However, the atypical lack of immune response or colitis despite *C. jejuni* colonization of infected C57BL/6 IL-10$^{-/-}$ mice, with the concurrent unexpected finding of *L. murinus* carriage, necessitated the additional interpretation of results within C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ genotypes separately.

The first aim of this study involved determining polarization of the adaptive immune response and susceptibility to colitis depending upon mouse genotype and infecting *C. jejuni* strain. We hypothesized that C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with colitogenic *C. jejuni* 11168 would develop colitis and primarily Th1/Th17 immune responses, with a mixed Th1/Th17/Th2 response

in BALB/c IL-10$^{-/-}$ mice due to the reported Th2 bias in this mouse strain. This hypothesis was proposed based upon previous studies demonstrating colitis, systemic (plasma antibodies) or local colonic Th1/Th17 responses in *C. jejuni* 11168-infected mice, including C57BL/6 IL-10$^{-/-}$ [6; 7; 41-43; 54] and NOD IL-10$^{-/-}$ genotypes.[59]

Indeed, when all treatment groups were compared together in the current study, BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 mounted the most robust systemic *C. jejuni*-specific IgG2b and IgG3 responses, along with significantly increased IgG2a (**Figure 3.5**), reflecting Th1/Th17-mediated class switching.[1; 5; 64] A robust Th1/Th17 cytokine response also was identified locally in the proximal colon: *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice again showed the most pronounced IFN-γ, TNF-α, IL-6, IL-17A, and IL-22 production of any treatment group (**Figure 3.9**). Interestingly, when C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice were compared together, BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 also had a significant Th2-mediated IgG1 plasma response, but when BALB/c IL-10$^{-/-}$ mice were analyzed individually, no pairwise comparisons were significant (**Figure 3.5**). In contrast to the results presented in Malik *et al*., in which C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 exhibited upregulated colonic expression of Th2-associated Gata-3, IL-4, and IL-13 measured by real-time PCR,[41] mice in the current study were not producing detectable amounts of colonic Th2-associated IL-4, IL-5, or IL-13 at the time of euthanasia. These results are consistent with a shift to Th1-mediated immunity in the absence of IL-10[10] and with primary systemic and local Th1/Th17 responses to *C. jejuni* infection. The robust Th1/Th17 and essentially negligible Th2 response in BALB/c IL-10$^{-/-}$ mice, especially those infected with *C. jejuni* 11168, reflects a consistent Th1/Th17 response to *C. jejuni* as seen in previous *in vivo* mouse[6; 21; 41; 42; 59] and *ex vivo* human models.[18] Interestingly, production of Th17-related cytokines IL-17A and IL-22 by *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice was significantly higher than all three C57BL/6 IL-10$^{-/-}$ treatment groups (**Figure 3.9**), but when BALB/c IL-10$^{-/-}$ groups were compared alone production was not significantly different than in sham inoculated BALB/c IL-10$^{-/-}$ mice (**Figure 3.11**). This suggests a higher

baseline production of Th17-related cytokines in BALB/c IL-10$^{-/-}$ than C57BL/6 IL-10$^{-/-}$ mice in the current study, regardless of infection status, reflecting a stronger default to Th17- as well as Th1-mediated immunity in the absence of IL-10. While WT BALB/c mice may be predisposed to Th2 responses in various models, in the absence of IL-10, a shift to a heightened combined Th1/Th17 response was induced with *C. jejuni* infection in the current and previous studies (Brudvig *et al*., unpublished, Chapter 2)). This primary Th1/Th17 response with apparent suppression of a Th2 response is exacerbated in IL-10$^{-/-}$ mice, but an absent or muted Th2 response is likely not only due to absence of regulatory IL-10 but also due to *C. jejuni* itself: in the previous study, infected WT BALB/c mice also mounted Th1/Th17, but not Th2, responses (Brudvig et al., unpublished (Chapter 2)).

Th17 cells are a relatively recently described subset of Th cells distinct from Th1 and Th2 subsets. Induction of Th17 cells is closely related to that of Treg cells but differentiation of these two subsets is mutually exclusive: TGF-β in steady-state induces Foxp3^{+} Treg cells, but with concurrent production of IL-6 by innate cells during infection or inflammation, TGF-β and IL-6 together induce Th17 cell differentiation.[11] Th17 cells play a primary role in control of extracellular bacteria at mucosal surfaces, including through recruitment of inflammatory cells such as neutrophils, and in maintaining mucosal homeostasis in the gut.[31; 64] Interestingly, the chemically induced (dextran sulfate sodium) colitis model is characterized by resistance to colitis in BALB/c mice, which mount a primarily Th2/Th17/Treg response characterized by higher levels of IL-4, IL-6, IL-10, and IL-17, compared to susceptibility in C57BL/6 mice producing more IFN-γ and TNF-α.[68] These data suggest that, in the current study, the absence of the regulatory actions of IL-10 compounded with infection with an enteric mucosal pathogen such as *C. jejuni* 11168 led to an unchecked Th1/Th17-mediated response in BALB/c IL-10$^{-/-}$ mice. This response is apparently dependent upon characteristics of the infecting *C. jejuni* strain, as infection with *C. jejuni* 260.94 induced significant plasma IgG2b production but did not result in significant colitis or increased colon cytokine production (**Figures 3.4**, **3.5**, and **3.11**). Differences in

colitogenic potential and immune responses induced by infection with *C. jejuni* 11168 and 260.94 strains may be explained in part by the enhanced invasion efficiency and intracellular survival of *C. jejuni* 11168 compared to *C. jejuni* 260.94, demonstrated by IHC in the current study and *in vitro* using dendritic cells (Brudvig *et al*., unpublished (Chapter 4)).

BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 exhibited the most severe colitis of any treatment group, and compared to sham-inoculated and *C. jejuni* 260.94-infected BALB/c IL-10$^{-/-}$ groups alone (**Figure 3.4**). The severe colitis seen in this group was characterized by inflammatory exudates in the lumen consisting primarily of mononuclear cells and neutrophils; damage to groups of surface epithelial cells; marked goblet cell depletion; crypt abnormalities including irregular architecture, dilation, and cryptitis/abscesses; diffuse and marked inflammatory infiltrates consisting of mononuclear cells, neutrophils, and plasma cells in the lamina propria; and frequent extension of inflammatory infiltrates into the submucosa and mesenteric fat. These lesions are similar to those described previously in C57BL/6 IL-10$^{-/-}$ mice,[42] although severe colitis was not observed in *C. jejuni* 11168-infected C57BL/6 IL-10$^{-/-}$ mice in the current study. BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 also exhibited the most marked production of *C. jejuni*-specific IgA in fecal supernatants (**Figure 3.8**), reflecting a strong mucosal immune response. Although direct comparison between BALB/c IL-10$^{-/-}$ and C57BL/6 IL-10$^{-/-}$ mice in the current study is complicated by atypical results and carriage of *L. murinus* in C57BL/6 IL-10$^{-/-}$ mice, it is worth noting that BALB/c mice were reported to have enhanced vitamin A metabolism in the intestine and increased ability to induce IgA class switching compared to C57BL/6 mice.[23]

Evaluation of systemic immunity reflected by *C. jejuni*-specific plasma IgG antibody isotypes, local immunity assessed by colonic cytokines reflecting Th polarization, and mucosal immunity gauged by IgA production all suggest that BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 mounted strong Th1/Th17 responses resulting in unchecked inflammatory pathology in the colon. Thus, the hypothesis

that Th1/Th17 responses mediated pathology in BALB/c IL-10$^{-/-}$ mice, although without a significant Th2 component, can be accepted. In the current study, the virtual lack of immune response or pathology as typically reported in *C. jejuni* 11168-infected C57BL/6 IL-10$^{-/-}$ mice precludes accepting or rejecting this hypothesis for C57BL/6 IL-10$^{-/-}$ mice. Though additional experiments will be necessary to rigorously validate BALB/c IL-10$^{-/-}$ mice as a model of *C. jejuni*-induced colitis, these results suggest that a bias toward Th1/Th17-mediated immunity in the absence of IL-10 makes BALB/c IL-10$^{-/-}$ mice a useful model for studying enteric mucosal pathogens such as *C. jejuni*.

The second aim of this study involved assessing the importance of host genetic background in determining susceptibility to *C. jejuni*-induced GBS, using production of anti-ganglioside antibodies, a hallmark of GBS, and increased numbers of macrophages in DRG as indicators. We hypothesized that mice of both genetic backgrounds infected with *C. jejuni* 260.94 would not develop colitis, but Th-mediation of immunity would vary between mouse strains. We reasoned that based upon previous models by Malik *et al*[41] and Brudvig *et al* (unpublished (Chapter 2)), the Th2 response in C57BL/6 IL-10$^{-/-}$ mice would lead to anti -GM1 and -GD1a antibodies and nerve lesions, while a mixed Th1/Th17/Th2 response would protect BALB/c IL-10$^{-/-}$ mice from neurological manifestations.

Histopathological scoring of the ICJ is shown in **Figure 3.4**. Seven of 10 *C. jejuni* 260.94-infected BALB/c IL-10$^{-/-}$ mice had mild colitis (raw scores 10—17; the remaining 3 in this group were <10, indicating no colitis). Despite higher numbers of *C. jejuni* 260.94-infected BALB/c IL-10$^{-/-}$ mice with raw colitis scores ≥10, this group was not statistically different from sham-inoculated BALB/c IL-10$^{-/-}$ mice, likely owing to 3 uninfected mice also having mild colitis. Similarly, within the C57BL/6 IL-10$^{-/-}$ group, one mouse in the *C. jejuni* 260.94-infected group had severe colitis (score = 32), but overall *C. jejuni* 260.94 did not induce significant colitis in C57BL/6 IL-10$^{-/-}$ mice. Lack of colitis induced with *C. jejuni* 260.94 in these mouse strains is consistent with previous studies[6; 41] and with Brudvig *et al*. (unpublished, (Chapter

2)), indicating that despite colonization and stimulation of immunity, this strain is less colitogenic than *C. jejuni* 11168.

Following assessment of colitis, susceptibility to *C. jejuni* 260.94-induced GBS was evaluated. Measurement of anti-ganglioside antibodies by ELISA (**Figures 3.6** and **3.7**) demonstrated significant differences in anti-GM1 and anti-GD1a IgG2b between many groups when all treatment groups were compared. C57BL/6 IL-10$^{-/-}$ mice showed the most robust responses, especially those infected with *C. jejuni* 11168; however, when analyzed separately, C57BL/6 IL-10$^{-/-}$ mice showed no significant increases in any anti-GM1 or anti-GD1a IgG isotype. Clearly, C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 produced high levels of anti-GM1 and anti-GD1a IgG2b antibodies, although some TSB-inoculated mice also had detectable levels of these antibodies indicating that C57BL/6 IL-10$^{-/-}$ mice had a higher baseline production. *C. jejuni* 11168 also induced the most pronounced production of Th1/Th17-mediated anti-ganglioside IgG2a and IgG2b isotypes in BALB/c IL-10$^{-/-}$ mice. When only BALB/c IL-10$^{-/-}$ groups were compared, *C. jejuni* 11168-induced increases were statistically significant when compared to both sham- and *C. jejuni* 260.94-inoculated groups. This Th1/Th17 mediated production of anti-ganglioside IgG2a and IgG2b in BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168, compared with Th2-mediated production of anti -GM1 and -GD1a IgG1 in *C. jejuni* 260.94-infected C57BL/6 IL-10$^{-/-}$ mice previously reported,[41] further highlights the potential importance of both host and *C. jejuni* strain characteristics in disease outcomes. Though associated with enteritis and not GBS, *C. jejuni* 11168 produces GM1 and GM2 ganglioside mimics and thus production of anti-ganglioside antibodies could occur, possibly associated with the strong Th1/Th17 response to a mucosal enteric pathogen. Persistent colonization of *C. jejuni* 11168 and resulting severe inflammation may have led to alterations in the microbiota; expression of ganglioside mimics by commensal flora leading to anti-ganglioside antibody production cannot be excluded. The lack of anti -GM1 or -GD1a antibody production in *C. jejuni* 260.94-infected BALB/c IL-10$^{-/-}$ mice is consistent with a previous study, in which increased anti-ganglioside antibodies related to

presence or absence of IL-10 but not to infection status (Brudvig *et al*., unpublished (Chapter2)). Lack of anti-ganglioside antibody production in *C. jejuni* 260.94-infected C57BL/6 IL-10$^{-/-}$ mice in the current study is consistent with relatively low colonization rates identified by culture, lack of invasion into deeper layers in the proximal colon reflected by IHC, and overall mild and non-significant responses to *C. jejuni* infection in these mice.

A second indicator of GBS susceptibility chosen for this study was number of macrophages in lumbar DRG. Macrophages were chosen due to their postulated role in pathogenesis of GBS, particularly in the acute motor axonal neuropathy pattern of GBS reported by some to be the subtype most closely associated with *C. jejuni* infection.[17; 47; 61] Dorsal root ganglia were chosen because some GBS patients describe pain and sensory defects, and the cell bodies of sensory fibers are located in DRG.[47] The blood-nerve barrier is also reportedly particularly leaky within DRG, increasing vulnerability to immune attack.[47] Furthermore, pathology was described in dorsal roots following autopsies of patients with motor-sensory axonal GBS[24] and a significant increase in F4/80 positive cells was seen in the DRG, but not sciatic nerve or brachial plexus, of *C. jejuni* 260.94-infected NOD IL-10$^{-/-}$ mice.[59]

There was no significant increase in F4/80 positive cells in DRG in any treatment group in the current study (**Figure 3.12**) despite significantly increased production of anti-ganglioside antibodies by *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice. Similarly, significantly increased anti-GM1 IgG1 in *C. jejuni* 260.94 and 11168-infected wild type C57BL/6 mice with a humanized microbiota did not result in increased F4/80 positive cells in sciatic nerves and DRG.[13] The lack of correlation between macrophage infiltration or proliferation and increased plasma anti -GM1 and -GD1a antibodies may indicate that anti-ganglioside antibodies alone are insufficient to cause nerve lesions. The exact immunopathogenesis of nerve damage in the AMAN form of GBS is incompletely understood, but macrophages, complement, and IgG have all been implicated.[47; 61] Labeling of these other components may reveal that nerve pathology in GBS is mediated by multiple factors. Nerve lesions in this study were assessed in tissues

obtained at the scheduled necropsy 4 weeks post-infection or when earlier sacrifice was necessary; a time-course study would more clearly delineate if and when inflammatory nerve lesions occur in relation to elevations in anti-ganglioside antibodies.

In considering susceptibility to GBS, we hypothesized that infection with GBS patient strain *C. jejuni* 260.94 would not lead to colitis in either C57BL/6 IL-10$^{-/-}$ or BALB/c IL-10$^{-/-}$ mice, but would drive Th2-mediated immunity with susceptibility to GBS in C57BL/6 IL-10$^{-/-}$ mice, while predominant Th1/Th17 responses in BALB/c IL-10$^{-/-}$ mice would result in protection from neurological manifestations. Considering colon histopathology, anti-ganglioside antibody production, and macrophage numbers in DRG, we conclude that *C. jejuni* 260.94 was not colitogenic in either mouse strain, but also did not induce production of anti -GM1 and -GD1a antibodies. Surprisingly, significant anti-ganglioside antibody production was induced only by infection with the colitogenic *C. jejuni* 11168 strain in BALB/c IL-10$^{-/-}$ mice, albeit without corresponding nerve lesions. The lack of nerve lesions in mice with anti-ganglioside antibodies resulting from infection may also indicate IgG2a and IgG2b isotypes do not mediate nerve damage. The carriage of *L. murinus* and atypically mild immune responses in *C. jejuni* infected C57BL/6 IL-10$^{-/-}$ mice precludes the rejection of C57BL/6 IL-10$^{-/-}$ mice as a suitable GBS model. Here *L. murinus* colonization of C57BL/6 IL-10$^{-/-}$ mice was associated with a lack of significant disease manifestations and immune responses, including IgG1 anti-ganglioside antibodies and nerve lesions following *C. jejuni* infection. It will be necessary to validate the possibility that *L. murinus* acts as a protective probiotic preventing GBS.

The severe colitis, marked IgA production, and upregulation of Th1/Th17 related cytokines in the colon of *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice compared to all other treatment groups correlated with enhanced intracellular labeling and subepithelial presence of *C. jejuni* 11168 in this group as evaluated by IHC in the ICJ. *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice had the highest proportion of mice exhibiting intracellular labeling within macrophage/dendritic cell types in both the lamina propria

and the submucosa. These results are consistent with *in vitro* studies demonstrating enhanced invasion efficiency and intracellular survival of *C. jejuni* 11168 compared to *C. jejuni* 260.94 in cultured dendritic cells assessed by gentamicin killing assay (Brudvig *et al*., unpublished (Chapter 4)). Studies attempting to correlate *C. jejuni* invasiveness or association with cultured cells and clinical symptoms or pathology have shown conflicting results. Association of *C. jejuni* and *C. coli* strains with cultured HeLa cells was related to symptoms of fever and diarrhea, but not blood in the feces.[20] A subsequent study determined that association of *C. jejuni* and *C. coli* strains with HeLa cells was not adequate in discriminating between isolates from patients with colitis versus non-inflammatory diarrhea, although a higher proportion of isolates causing colitis displayed transcytosis through polarized Caco-2 monolayers compared to isolates from patients with non-inflammatory diarrhea.[19] Invasion and survival ability of *C. jejuni* strains *in vitro* did not impact intestinal lesion development in a pig model,[35] but *C. jejuni* invasion was positively correlated with immunopathology in mice by comparison with mutant strains capable of colonization but showing reduced invasion capacity *in vitro*.[9] In the current studies, the enhanced invasion efficiency and intracellular survival ability of *C. jejuni* 11168 shown *in vitro* (Brudvig *et al*., unpublished, Chapter 4)) fits with *in vivo* IHC assessment of intracellular organisms, subepithelial location of *C. jejuni* 11168, and severity of colitis in BALB/c IL-10$^{-/-}$ mice. The IHC labeling method of *C. jejuni* in intestinal tissues exhibits non-specific staining, as evidenced by positive labeling in the epithelium or submucosa in some sham-inoculated mice. All 20 sham inoculated mice in this study were culture negative for *C. jejuni* in all sampled areas of the gastrointestinal tract, and negative status was confirmed by *C. jejuni gyrA* gene-specific PCR performed on DNA extracted from cecal tissue. This non-specific labeling precludes the use of this method for more than identification of broad pattern. Despite the disadvantage of non-specific staining, this method was chosen as a way to assess *C. jejuni* load, location within the mucosa, submucosa, and extraintestinal areas such as the lymph node, and presence of intracellular organisms.

Colonization of C57BL/6 IL-10$^{-/-}$ mice with *C. jejuni* 11168 or *C. jejuni* 260.94 did occur, but these mice displayed virtually none of the pathology or immune responses reported in previous studies involving these *C. jejuni* strains and C57BL/6 IL-10$^{-/-}$ mice.[6; 7; 41-43; 54] Ninety percent of *C. jejuni* 11168-infected C57BL/6 IL-10$^{-/-}$ mice were colonized at the end of the study, but the gross and histopathological lesions expected in the cecum and colon were not seen. In contrast to previous models, no significant *C. jejuni*-specific IgG2c, IgG2b, or IgG3 plasma responses were seen, and these mice exhibited virtually no *C. jejuni*- specific mucosal IgA or cytokines reflecting a local adaptive immune response in the colon.

Similarly, 50% of the *C. jejuni* 260.94-infected C57BL/6 IL-10$^{-/-}$ mice were colonized at the end of the 4-week study, but this group did not exhibit any significant increase in *C. jejuni*-specific plasma antibodies of any isotype or mucosal IgA, and no significant differences in production of cytokines reflecting adaptive immunity in the colon were seen. No GM1 or GD1a ganglioside-specific antibodies were made, in contrast to significant production of anti-GM1 and anti-GD1a IgG1 previously reported.[41] Additionally, *C. jejuni* 260.94 has stably colonized high percentages of inoculated mice, including C57BL/6 IL-10$^{-/-}$ mice, for up to several weeks in previous studies; (Brudvig *et al*., unpublished (Chapter 2))[6; 13; 41; 59] colonization with *C. jejuni* 260.94 in the current study is comparably lower.

Despite colonization determined by culture and PCR methods in a total of 14/20 infected C57BL/6 IL-10$^{-/-}$ mice (70%) at the end of the 4-week study or at humane endpoint for one mouse at day 25, *in vivo* assessment of intracellular organisms and location beyond the surface epithelium into the lamina propria and submucosa by IHC labeling of *C. jejuni* in the ICJ suggested decreased migration or invasiveness of *C. jejuni* in C57BL/6 IL-10$^{-/-}$ compared to BALB/c IL-10$^{-/-}$ mice: no C57BL/6 IL-10$^{-/-}$ mouse exhibited labeling in the lamina propria, and only a single C57BL/6 IL-10$^{-/-}$ mouse infected with *C. jejuni* 260.94, and none infected with *C. jejuni* 11168, showed positive staining intracellularly in the submucosa. Interestingly, despite a lack of detectable colitis or systemic or local immune responses in

infected mice, 12/20 infected C57BL/6 IL-10$^{-/-}$ mice exhibited an enlarged regional lymph node and/or spleen at necropsy. This suggests that the persistent colonization, though present more superficially compared to BALB/c IL-10$^{-/-}$ mice, was stimulating a local, but relatively mild, immune response that did not lead to systemic morbidity or pathology in these mice. It was recently shown that a *C. jejuni* mutant strain lacking the formic acid receptor Tlp7 was able to colonize mice with similarly high loads to the parental WT strain, although the mutant displayed five times reduced invasion capacity *in vitro* and did not induce immunopathology *in vivo* in the mice compared to the WT strain.[9] Thus, the results of the current study indicating that infected C57BL/6 IL-10$^{-/-}$ mice exhibited virtually no colitis and no systemic or local immunity may reflect not only decreased colonization rates compared to previous studies, but also decreased invasiveness or translocation of the colonized *C. jejuni* beyond the epithelium.

The relative protection of C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 or *C. jejuni* 260.94 in this study, compared with previously published outcomes and those of BALB/c IL-10$^{-/-}$ mice in the current study receiving the same inocula, warrants further exploration to determine if carriage of the *L. murinus* isolated from C57BL/6 IL-10$^{-/-}$ mice may have contributed to this unexpected outcome. Members of the *Lactobacillus* genus can be found in the intestinal tract of humans, though many species are apparently not stable inhabitants but instead originate in the oral cavity or from exogenous sources such as food.[62] In contrast, lactobacilli form stable populations in the gastrointestinal tracts of animals, including mice, where colonization of the stomach occurs with epithelial associations resembling biofilms.[62] Presence of *L. murinus* in mice, including C57BL/6 substrains, can reportedly vary by vendor.[26] *L. murinus* has also been found in healthy BALB/c mice, colonizing the upper gastrointestinal tract in an arrangement described as mimicking a biofilm.[3] *Lactobacillus* spp. may therefore increase colonization resistance by forming a physical barrier inhibiting colonization of pathogenic bacteria. Beyond colonization resistance, commensal bacteria have also demonstrated local immunomodulatory effects.[14; 60] Interestingly, through interactions with dietary components in the gut, *L. reuteri* contributed

to differentiation of CD4+ T cells into CD4+CD8αα^{+} intraepithelial lymphocytes, T cells with regulatory functions similar to Tregs.[14] *L. murinus* did not have the same effect as *L. reuteri* in that study,[14] but an unidentified immunomodulatory effect of *L. murinus*, in addition to possible colonization resistance, cannot be excluded in C57BL/6 IL-10$^{-/-}$ mice in our study.

Studies evaluating the potential effect of lactobacilli on colitis in mice report differences in *Lactobacillus* spp. and strains present in mice with and without colitis, and suggest a potential protective role of lactobacilli in preventing development of spontaneous colitis in IL-10 deficient mice. *L. murinus* and other *Lactobacillus* spp. were isolated from Swiss Webster and inducible nitric oxide synthetase (iNOS)-deficient C57BL/6 mice without colitis, while only *L. johnsonii* was isolated from C57BL/6 IL-10$^{-/-}$ mice with colitis.[49] Prior to development of colitis, 129 Sv/Ev IL-10$^{-/-}$ mice showed increased mucosal invasion of aerobic bacteria with a concurrent decrease in *Lactobacillus* spp., and repopulation with *L. reuteri* reduced aerobic bacterial invasion and attenuated the colitis.[40] A subsequent study corroborated these findings, demonstrating that IL-10$^{-/-}$ mice on C57BL/6 and C57BL/10 backgrounds fed *L. salivarius* ssp*. salivarius* showed reduction in *C. perfringens* load, development of colitis, neoplasia, and death compared to control mice receiving placebo.[48] In the current study, 3/10 sham-inoculated BALB/c IL-10$^{-/-}$ mice exhibited mild spontaneous colitis and 1/10 sham-inoculated C57BL/6 IL-10$^{-/-}$ mice had mild colitis. This may only reflect the relative severity of spontaneous enterocolitis in BALB/c IL-10$^{-/-}$ compared to C57BL/6 IL-10$^{-/-}$ previously reported,[10] but an effect of the *L. murinus* in C57BL/6 IL-10$^{-/-}$ mice in this study should be considered. Standardized experiments specifically designed to examine the prevalence of spontaneous colitis in both BALB/c IL-10$^{-/-}$ and C57BL/6 IL-10$^{-/-}$ mice with and without *L. murinus* carriage would be necessary to further test the possibility that development of spontaneous colitis may have been ameliorated by *L. murinus* in the current study.

Both *in vitro* and *in vivo* studies also have demonstrated antagonism of *Lactobacillus* spp. against *C. jejuni*. Several lactobacilli, including *L. pentosus*, 6 strains of *L. plantarum*, and *L.*

pseudomesenteroides exhibited *in vitro* inhibitory activity against 3 different *C. jejuni* strains; however, despite repeated administration of the chosen *L. plantarum* strain to chickens in attempt to estimate the effect on *C. jejuni* populations *in vivo*, colonization could not be established.[55] Cell-free extracts of milk fermented by two *Lactobacillus* spp., *L. acidophilus* and *L. rhamnosus,* inhibited growth and down-regulated the *flaA* σ^{28} promoter activity of two *C. jejuni* strains.[16] Invasion of *C. jejuni* into cultured epithelial cells was inhibited by *L. helveticus* and *L. rhamnosus*, but probiotic activity varied depending upon *Lactobacillus* spp., *C. jejuni* strain, and epithelial cell line.[67] Protective effects, including immunomodulation, of lactobacilli against *C. jejuni*-induced colitis in mice have also been shown. While *L. johnsonii* did not reduce colonization of abiotic C57BL/6 mice with *C. jejuni* 81-176, reduction in colonic apoptosis, number of colonic B cells, and secretion of IL-6, MCP-1, TNF, and NO was observed.[8] Considered together, lactobacilli may exhibit both *in vitro* and *in vivo* inhibitory activity against *C. jejuni*, but protective effects vary depending on *C. jejuni* strain and *Lactobacillus* spp. or strain, and *in vitro* inhibition may be difficult to reproduce *in vivo* due to difficulty in establishing stable colonization.

Because of the inhibitory activity of *Lactobacillus* spp. against *C. jejuni* shown *in vitro* and *in vivo,* the carriage of *L. murinus* by C57BL/6 IL-$10^{-/-}$ mice exhibiting abnormally mild responses despite colonization with *C. jejuni* warrants further study. Detection of *L. murinus* in the current study was unexpected, and the source is unknown. Direct comparison to outcomes in BALB/c IL-$10^{-/-}$ mice without *L. murinus* cannot be made, as BALB/c IL-$10^{-/-}$ mice are of a different genetic background and are not yet an established and repeatedly validated model *C. jejuni*-induced colitis as are the C57BL/6 IL-$10^{-/-}$ mice. The possibility that the mild responses of *C. jejuni*-infected C57BL/6 IL-$10^{-/-}$ mice and the presence of *L. murinus* were coincidental cannot be excluded. TSAB-CVA plates are considered selective for *C. jejuni* growth, but *L. murinus* is vancomycin resistant[49] and can be found in laboratory mice;[3; 26] thus *L. murinus* present in the gut would be expected to grow simultaneously with *C. jejuni* on this medium. Furthermore, competition in culture medium resulting in reduced *C. jejuni* growth, if this occurred, does

not necessarily equate to inhibition of *C. jejuni in vivo*. *L. murinus* may have occupied a relatively small fraction of the gut microbiota but exhibited disproportionately robust growth in culture with reduced competition from other members of the gut microbiota; levels of *L. murinus* were significantly expanded during vancomycin treatment in a mouse study.[26] Finally, in contrast to established outcomes of severe colitis in repeated experiments involving C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168,[6; 7; 41-43; 54] anomalous results of relatively milder colitis have occurred in individual experiments.[7; 13; 42] Therefore, the possibility that the *L. murinus* harbored by C57BL/6 IL-10$^{-/-}$ mice conferred protection from *C. jejuni* in this study is intriguing but will require additional rigorous experiments to prove or disprove.

An essential step preceding further *in vitro* or *in vivo* experiments testing the inhibitory effect of the cultured *L. murinus* against *C. jejuni* is the further assessment of the different colony morphologies and appearance on Gram staining observed following growth on plates with and without antibiotics. Furter testing is needed to confirm both morphologies as different phenotypes of the same organism, rather than *L. murinus* in close association with a second organism. MALDI-TOF mass spectrometry identified organisms with both morphologies as *L. murinus* with high accuracy scores, and further confirmation of the identity of the two types of rods by 16S sequencing is in progress. If the two colony types are indeed both *L. murinus*, exhibiting change in morphology due to antibiotic-induced pressure, the single genome will be sequenced. Analysis of the sequenced genome will include evaluation of genes involved in antibiotic resistance and known protective probiotic effects including bacteriocin production. In addition to genome sequencing, *in vitro* inhibition assays to definitively evaluate the *L. murinus* interaction with *C. jejuni* strains 11168 and 260.94 are planned. Assays may include assessment of direct inhibition of *C. jejuni* growth on agar plates or in wells, using live *L. murinus* cells or supernatants, respectively.[55] Pretreatment of isolated dendritic cells or epithelial cells with *L. murinus* prior to infection with *C. jejuni* can be used to evaluate any reduction in *C. jejuni* invasion by gentamicin killing assay.[67] If *in vitro* results support the possibility of *in vivo* inhibition of *C. jejuni* by the isolated strain of *L.*

murinus, an *in vivo* mouse experiment involving *L. murinus*-negative BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* with and without *L. murinus* inoculation(s) will be performed. Ability of *L. murinus* to stably colonize BALB/c IL-10$^{-/-}$ mice following inoculation would first have to be verified.

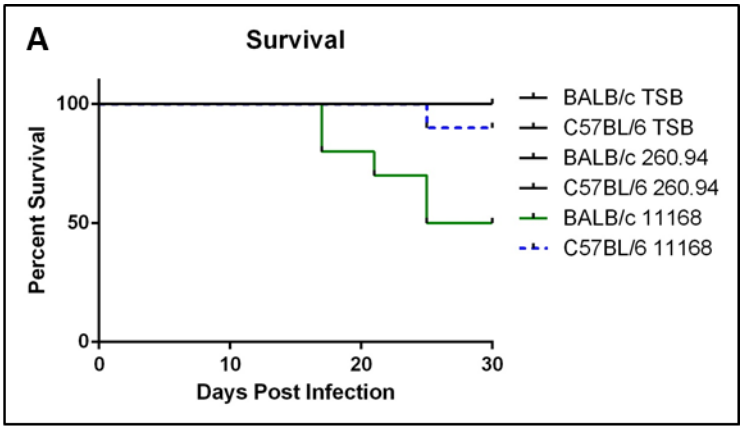
The present study was designed to develop an *in vivo* mouse model characterizing the differences in immune response to colitogenic and GBS-associated *C. jejuni* strains in mice of two genetic backgrounds. Models like these are needed to further our understanding of the complex determinants of disease outcomes, such as colitis or GBS, following *C. jejuni* infection. In this study, infected C57BL/6 IL-10$^{-/-}$ mice mounted only mild immune responses and did not develop colitis or produce anti-ganglioside antibodies as previously reported. The unexpected finding of *L. murinus* carriage, coupled with atypically mild results in C57BL/6 IL-10$^{-/-}$ mice, presents a promising area of future research to determine whether the *L. murinus* can be an effective and safe probiotic conferring protection against *C. jejuni*-mediated pathology. In contrast, *C. jejuni*-infected BALB/c IL-10$^{-/-}$ mice mounted Th1/Th17-mediated immunity, without a significant Th2 component. Magnitude of this Th1/Th17 response, induction of mucosal immunity, and disease outcome including colitis and production of anti-ganglioside antibodies depended upon infecting *C. jejuni* strain. BALB/c IL-10$^{-/-}$ mice thus present an additional model mimicking human colitis, and provide an additional avenue for further study of the mechanisms by which different *C. jejuni* strains induce a variable immune response.

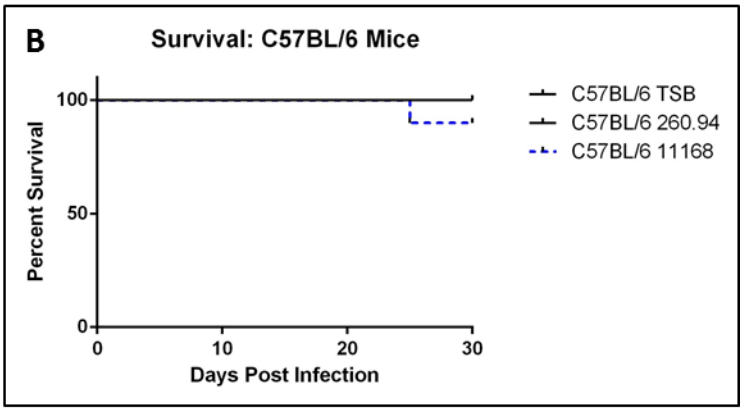
APPENDIX

Table 3.1. Experimental design. Sixty mice were included, 30 each of C57BL/6 interleukin (IL)-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ genotypes. Ten of each mouse strain were orally inoculated with *Campylobacter jejuni* strain 260.94, *C. jejuni* 11168, or vehicle (tryptic soy broth, TSB). Mice were inoculated at 6 weeks of age and humanely euthanized after 4 weeks of infection, or earlier when necessary.

Mouse Genotype	Treatment Group	Number of Mice
C57BL/6 IL-$10^{-/-}$	TSB	10
C57BL/6 IL-$10^{-/-}$	*C. jejuni* 260.94	10
C57BL/6 IL-$10^{-/-}$	*C. jejuni* 11168	10
BALB/c IL-$10^{-/-}$	TSB	10
BALB/c IL-$10^{-/-}$	*C. jejuni* 260.94	10
BALB/c IL-$10^{-/-}$	*C. jejuni* 11168	10

Figure 3.1. Survival of C57BL/6 interleukin (IL)-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice inoculated with *C. jejuni* 260.94, *C. jejuni* 11168, or vehicle (tryptic soy broth; TSB). Mice were inoculated at 6 weeks of age and humanely euthanized at approximately 4 weeks post-infection, or earlier if humane endpoint was reached. Survival curves were significantly different from each other when (A) all 6 treatment groups were compared (log-rank [Mantel-Cox]; P = 0.0001) and when (C) BALB/c IL-10$^{-/-}$ mice alone were compared (log-rank [Mantel-Cox]; P = 0.002), but not when (B) C57BL/6 IL-10$^{-/-}$ mice alone were compared (log-rank [Mantel-Cox]; P = 0.3679).





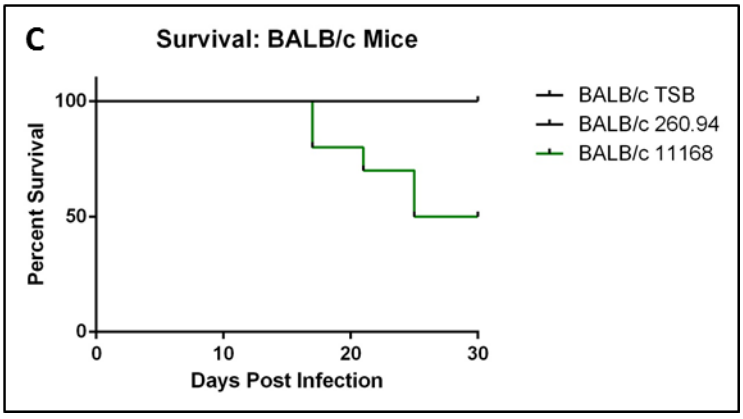
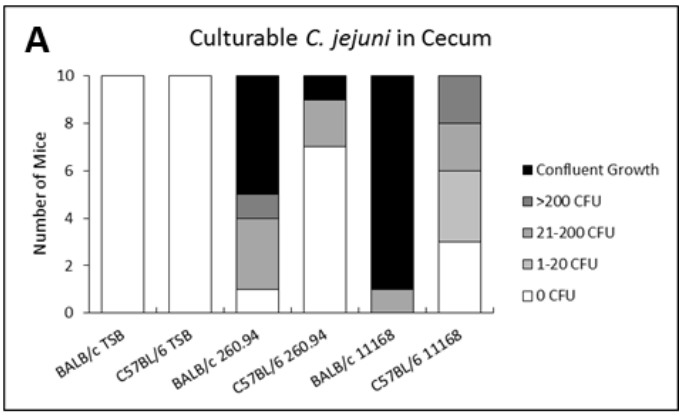
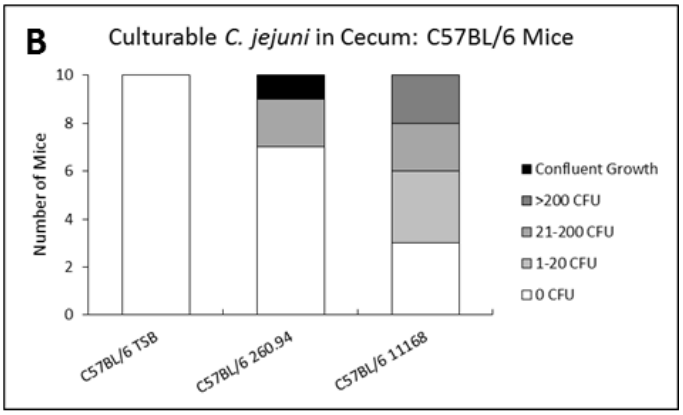


Figure 3.2. Culture results, cecum. Culturable *C. jejuni* strains 11168 and 260.94 isolated from C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice at the time of necropsy for (A) all mice in the experiment, (B) C57BL/6 IL-10$^{-/-}$ mice only, and (C) BALB/c IL-10$^{-/-}$ mice only. While samples of stomach, jejunum, cecum, proximal colon, and the feces were cultured, the cecum was chosen for graphic representation as *C. jejuni* typically colonizes the cecum most heavily. In total (out of all 5 sampled areas of the gastrointestinal tract), 9/10 BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 were culture positive; 5/10 C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 were positive, 10/10 BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 were positive, and 9/10 C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 were positive. All sham (tryptic soy broth, TSB) inoculated mice were culture negative for *C. jejuni* in all areas sampled.





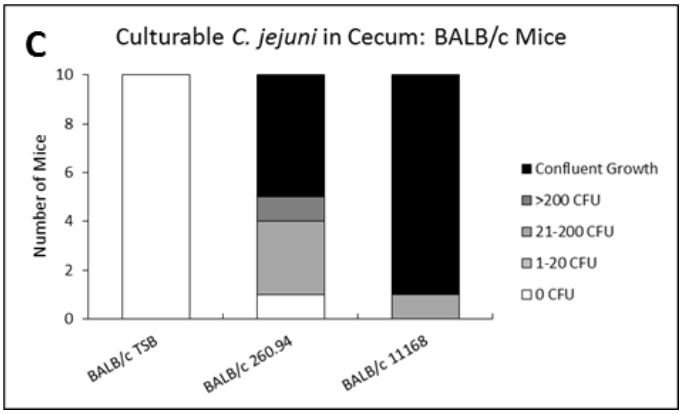
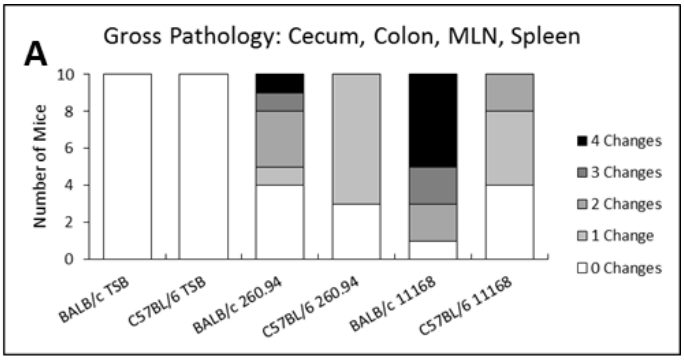
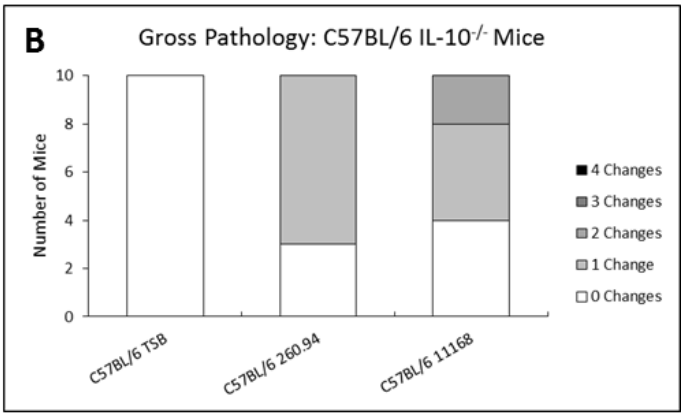


Figure 3.3. Gross pathology noted in the cecum, colon, mesenteric lymph nodes (MLN), or spleen at the time of necropsy in C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice following inoculation with *C. jejuni* 11168, *C. jejuni* 260.94, or sham inoculation (tryptic soy broth; TSB). Pathology is shown for (A) all mice together, (B) C57BL/6 IL-10$^{-/-}$ mice only, and (C) BALB/c IL-10$^{-/-}$ mice only. Possible changes included thickening, enlargement, or watery or soft contents in the cecum or colon, and enlarged lymph nodes or spleen. In C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 11168, with the exception of one mouse with a thickened proximal colon, the changes were due to mildly enlarged MLN and/or spleen. Similarly, in C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94, one mouse had a thickened proximal colon and the remaining changes were attributable to mildly to moderately enlarged MLN.





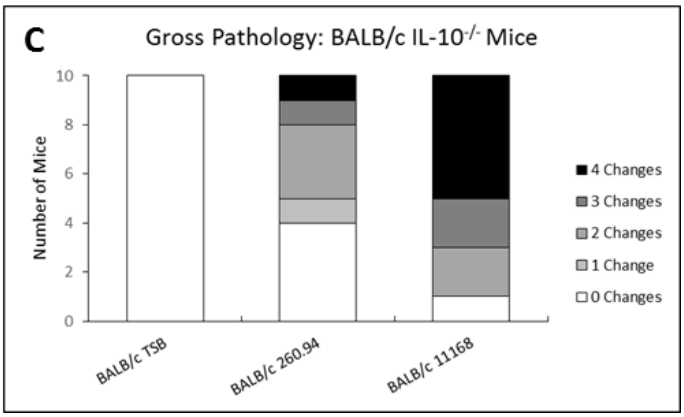


Figure 3.4. Colon histopathology. C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice were inoculated with *C. jejuni* 260.94, *C. jejuni* 11168, or vehicle (tryptic soy broth, TSB). (A-C) Histopathologic scoring of the ileocecocolic junction. Sections were blinded and given a raw score (0-42 point scale, displayed graphically). Scores were then further graded semi-quantitatively for statistical analysis as Grade 0 (no colitis; 0-9 points), Grade 1 (mild colitis; 10-19 points), or Grade 2 (moderate or marked colitis; ≥20 points). All six treatment groups were analyzed together (A), followed by analysis of C57BL/6 IL-10$^{-/-}$ mice (B) and BALB/c IL-10$^{-/-}$ mice (C) separately. Kruskal-Wallis was performed on semi-quantitative scores, followed by Dunn's multiple comparisons test. Brackets indicate statistically significant differences between groups. Data are represented by box and whisker, with the box extending from 25th-75th percentiles and the line plotted at the median. Whiskers represent minimum to maximum values. (D) Colon of a sham-inoculated BALB/c IL-10$^{-/-}$ mouse (Grade 0; 10× magnification, H&E). (E) Colon of a *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mouse; severe pathologic changes, including inflammatory cell infiltrate into the lamina propria and submucosa, mucus and necrotic debris in the lumen, and crypt abscesses, are evident (Grade 2; 10× magnification, H&E).

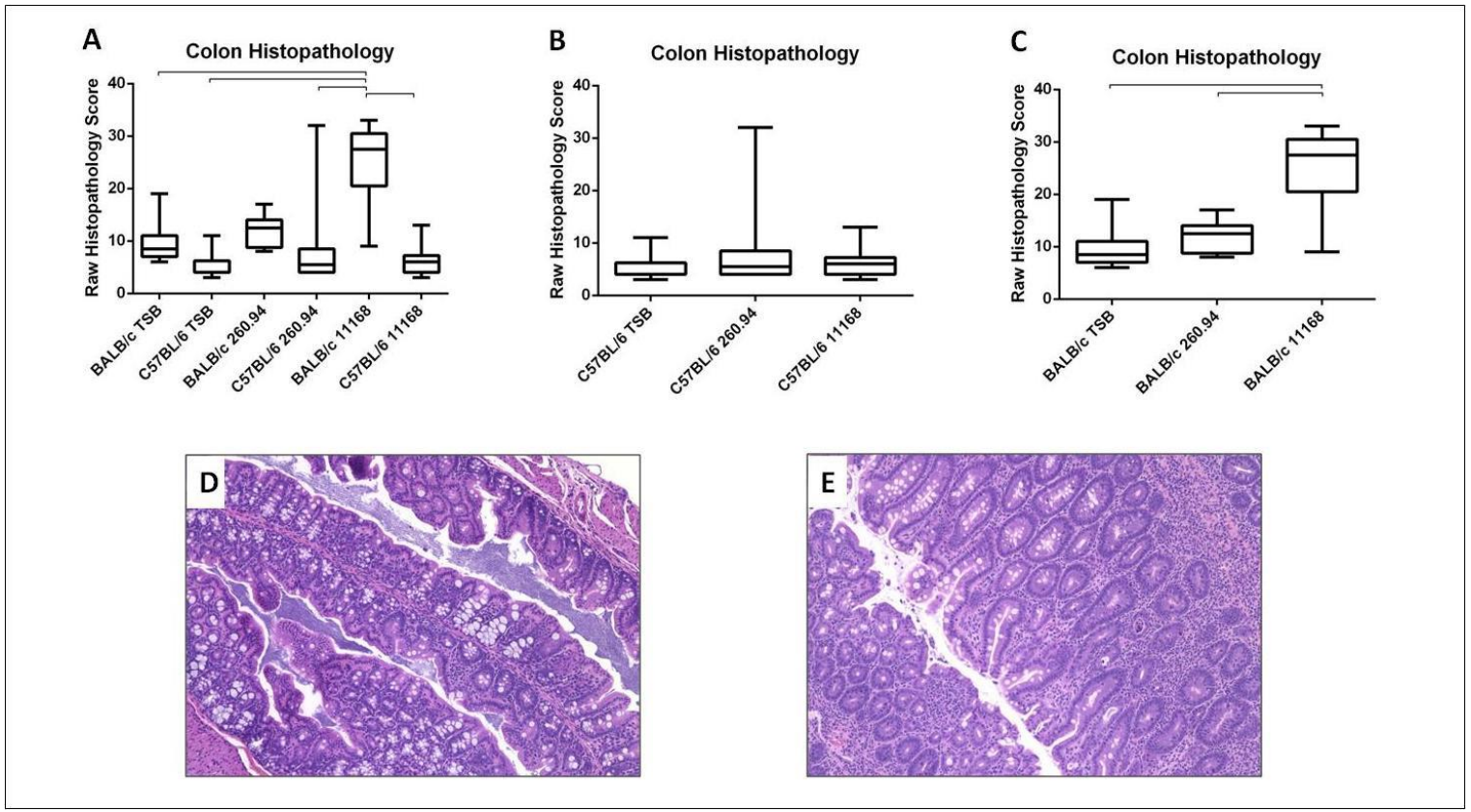


Figure 3.5. Plasma anti-*C. jejuni* IgG1, IgG2b, IgG3 for all mice, IgG2c (C57BL/6 IL-10$^{-/-}$ only), and IgG2a (BALB/c IL-10$^{-/-}$ only) antibodies measured by ELISA in samples taken at necropsy. C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice were inoculated with *C. jejuni* 260.94, *C. jejuni* 11168, or vehicle (tryptic soy broth; TSB). All six treatment groups were analyzed together (panel A), followed by analysis of C57BL/6 IL-10$^{-/-}$ (panel B) and BALB/c IL-10$^{-/-}$ (panel C) mice separately. Statistical analyses included two- or one-way ANOVA followed by Holm-Sidak post-testing, and when the assumption of equal variance was not met, Kruskal-Wallis followed by Tukey's post-test. Brackets above groups indicate significant pairwise comparisons; $P \leq 0.05$ was considered statistically significant. Significance was found overall in analysis of IgG1 production in BALB/c IL-10$^{-/-}$ mice (one-way ANOVA, $P = 0.044$); however, no significant pairwise comparisons were found by Holm-Sidak post-testing. Mean ± SEM.

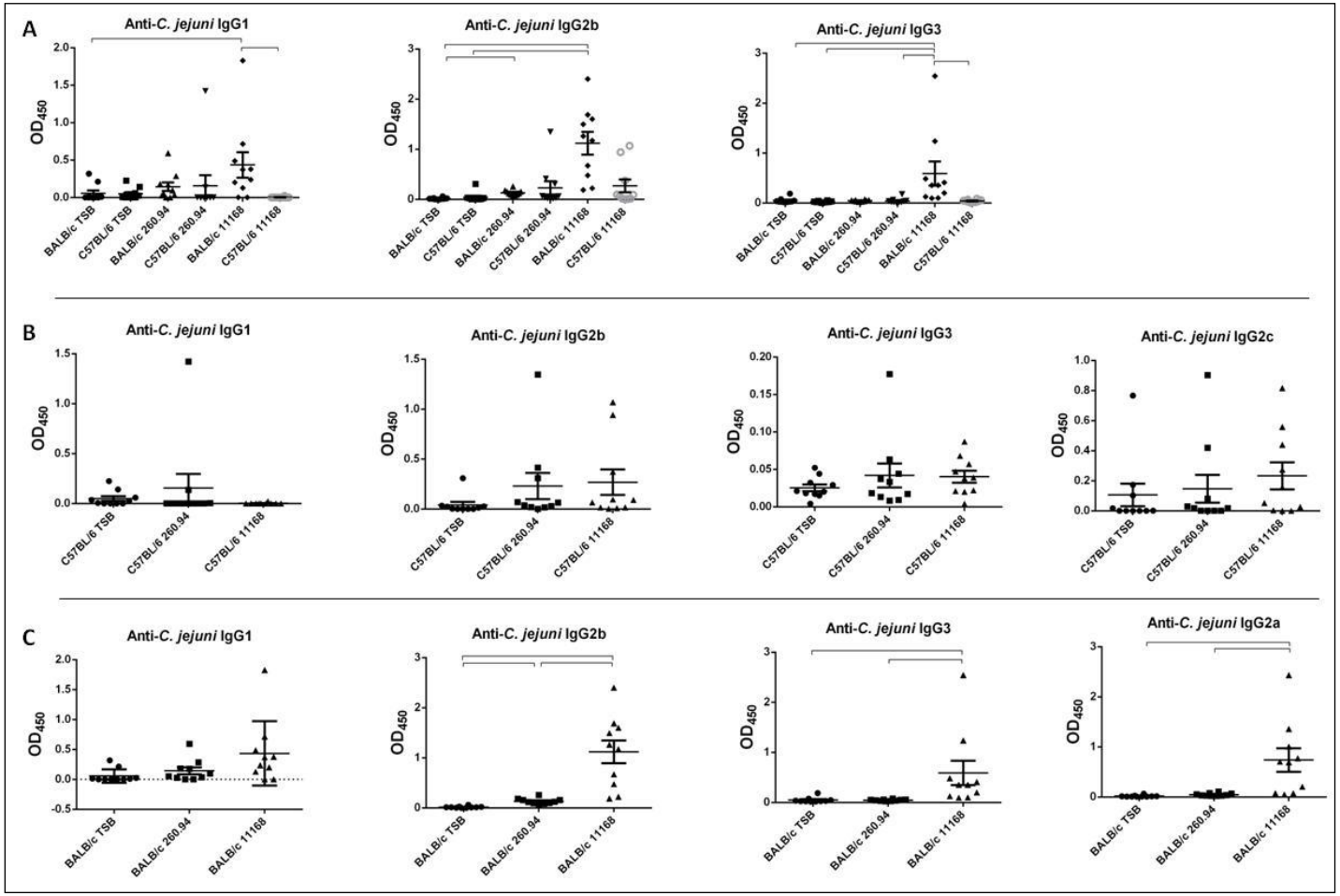


Figure 3.6. Plasma anti-GM1 IgG1, IgG2b, IgG3 for all mice, and IgG2c (C57BL/6 IL-10$^{-/-}$ only), and IgG2a (BALB/c IL-10$^{-/-}$ only) antibodies measured by ELISA in samples taken at necropsy. C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice were inoculated with *C. jejuni* 260.94, *C. jejuni* 11168, or vehicle (tryptic soy broth; TSB). One C57BL/6 IL-10$^{-/-}$ mouse inoculated with *C. jejuni* 260.94 did not have enough plasma for analysis and is excluded. All six treatment groups were analyzed together (panel A), followed by analysis of C57BL/6 IL-10$^{-/-}$ (panel B) and BALB/c IL-10$^{-/-}$ (panel C) mice separately. Statistical analyses included one- and two-way ANOVA, and when the assumption of equal variance was not met, Kruskal-Wallis followed by Dunn's or Tukey's post-testing. Brackets above groups indicate significant pairwise comparisons; $P \leq 0.05$ was considered statistically significant. Mean ± SEM.

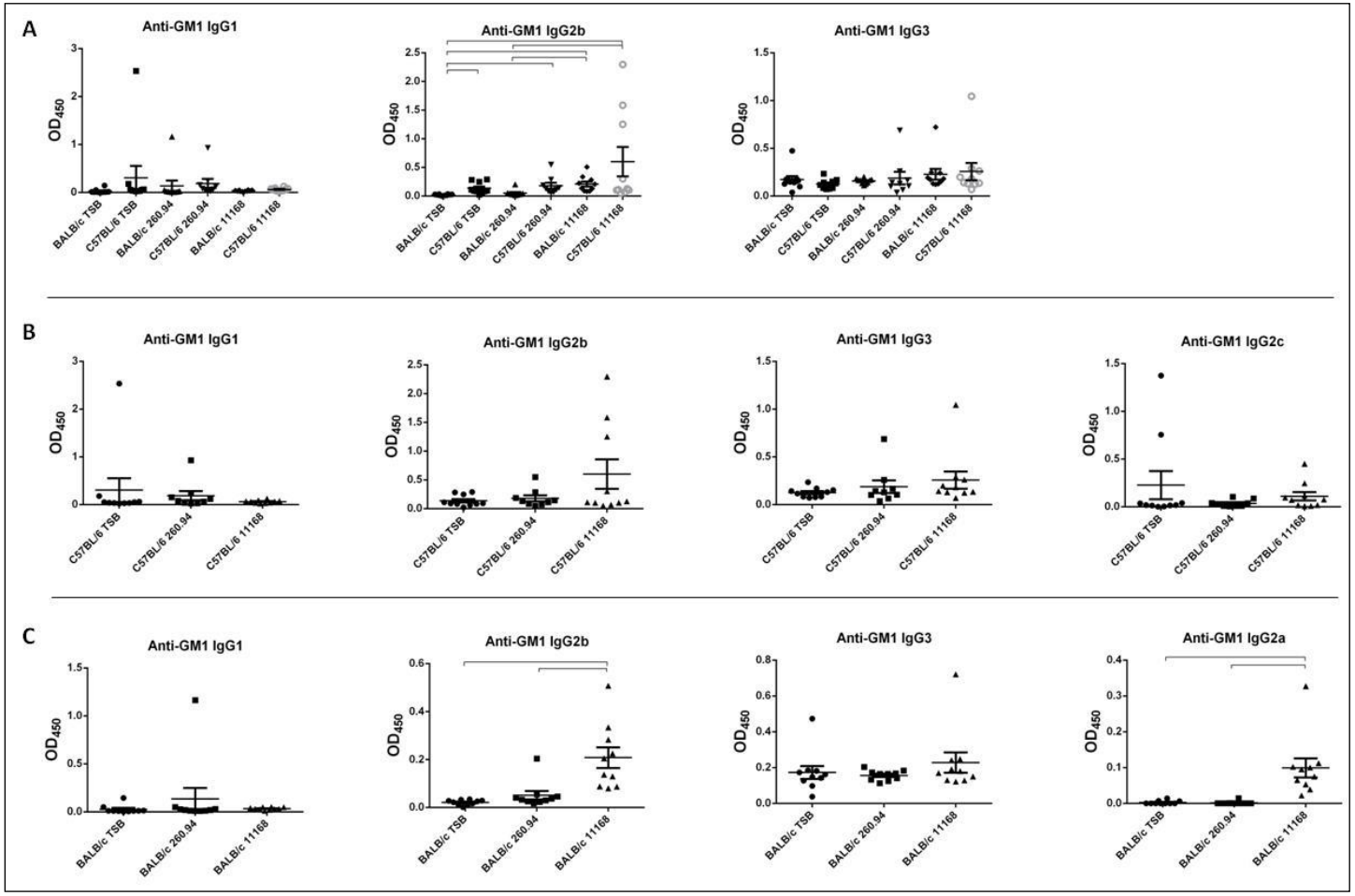


Figure 3.7. Plasma anti-GD1a IgG1, IgG2b, IgG3 in all mice, and IgG2c (C57BL/6 IL-10$^{-/-}$ only), and IgG2a (BALB/c IL-10$^{-/-}$ only) antibodies measured by ELISA in samples taken at necropsy. C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice were inoculated with *C. jejuni* 260.94, *C. jejuni* 11168, or vehicle (tryptic soy broth; TSB). One C57BL/6 IL-10$^{-/-}$ mouse inoculated with *C. jejuni* 260.94 did not have enough plasma for analysis and is excluded. All six treatment groups were analyzed together (panel A), followed by analysis of C57BL/6 IL-10$^{-/-}$ (panel B) and BALB/c IL-10$^{-/-}$ (panel C) mice separately. Statistical analyses included one- or two-way ANOVA, or when the assumption of equal variance was not met, Kruskal-Wallis followed by Dunn's or Tukey's post-test. Brackets above groups indicate significant pairwise comparisons; $P \leq 0.05$ was considered statistically significant. Mean ± SEM.

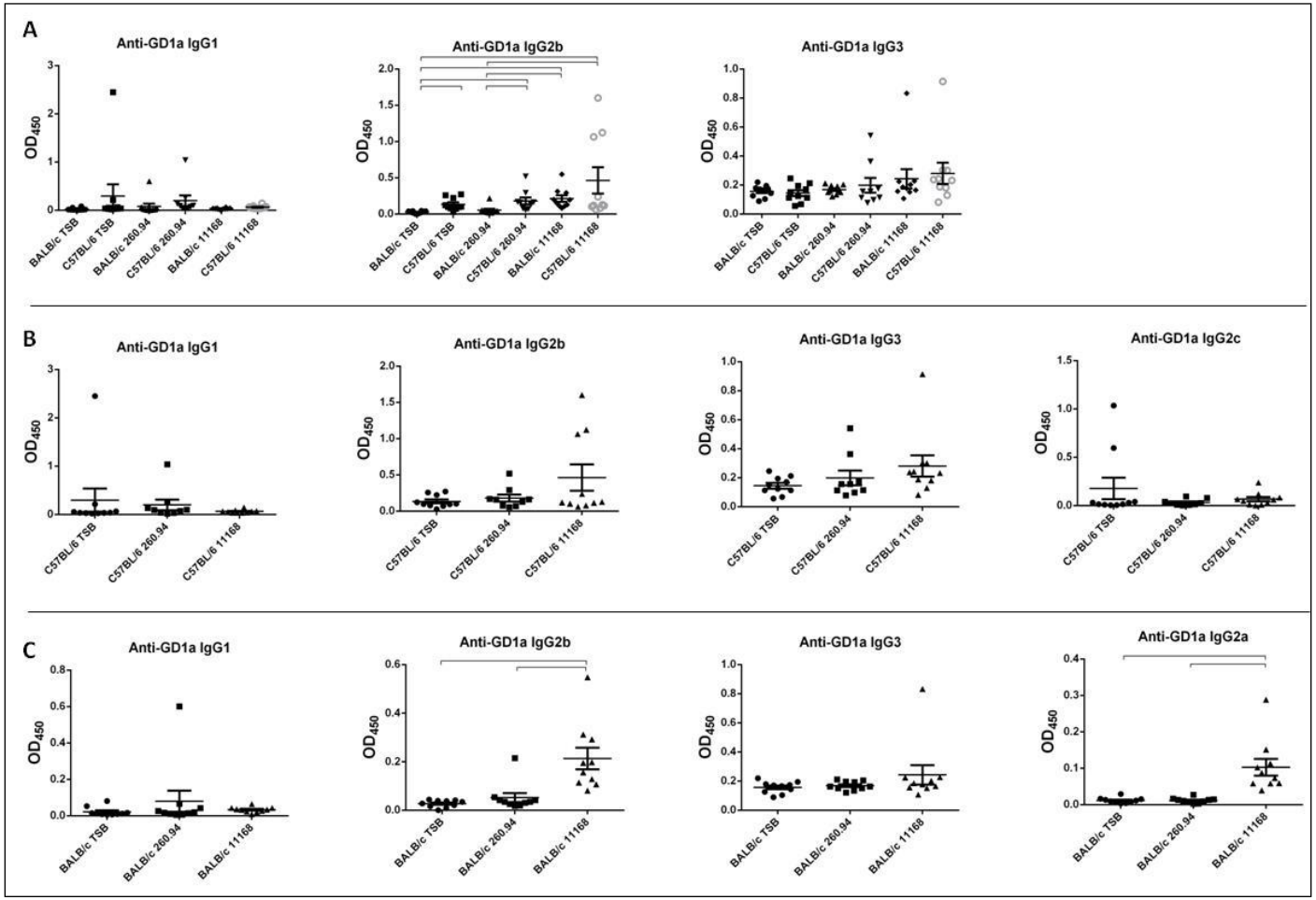


Figure 3.8. Measurement of anti-*C. jejuni* specific IgA in supernatants of feces collected at necropsy from C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice inoculated with *C. jejuni* 260.94, *C. jejuni* 11168, or vehicle (tryptic soy broth; TSB). All six treatment groups were analyzed together (A), followed by analysis of C57BL/6 IL-10$^{-/-}$ (B) and BALB/c IL-10$^{-/-}$ (C) mice separately. Because the assumption of equal variance was not met, Kruskal-Wallis followed by Tukey post-hoc testing was performed. Brackets above groups indicate significant pairwise comparisons; $P \leq 0.05$ was considered statistically significant. Mean ± SEM.

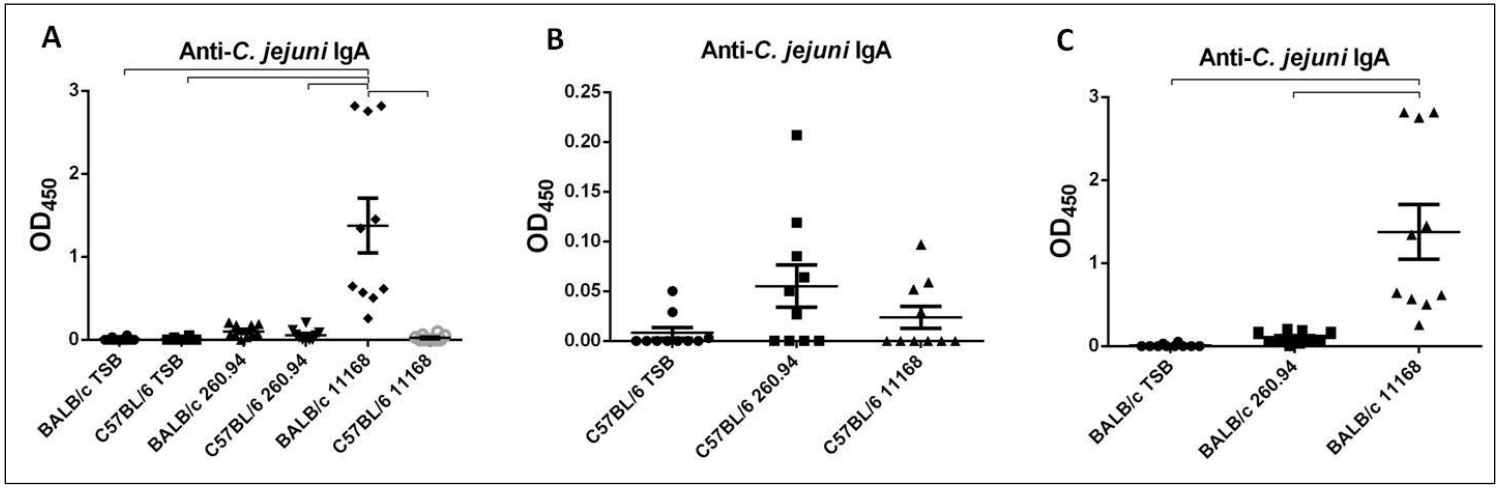


Figure 3.9. Assessment of colon cytokine production in C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice inoculated with *C. jejuni* 11168, *C. jejuni* 260.94, or sham (tryptic soy broth, TSB) by flow cytometry-based multiplexed bead assay. Production of IL-4, IL-5, IL-10, IL-13, IL-17F, and IL-21 was undetectable in 59-60/60 mice. Statistical analyses included two- and one-way ANOVA with Holm-Sidak post-testing, or when the assumption of equal variance was not met, Kruskal-Wallis followed by Tukey post-testing. Brackets above groups indicate statistically significant pairwise comparisons; $P \leq 0.05$ was considered statistically significant. Although a significant genotype effect (BALB/c IL-10$^{-/-}$ > C57BL/6 IL-10$^{-/-}$) was found by two-way ANOVA in IL-9 production, further analysis by one-way ANOVA did not determine any overall significance. Mean ± SEM.

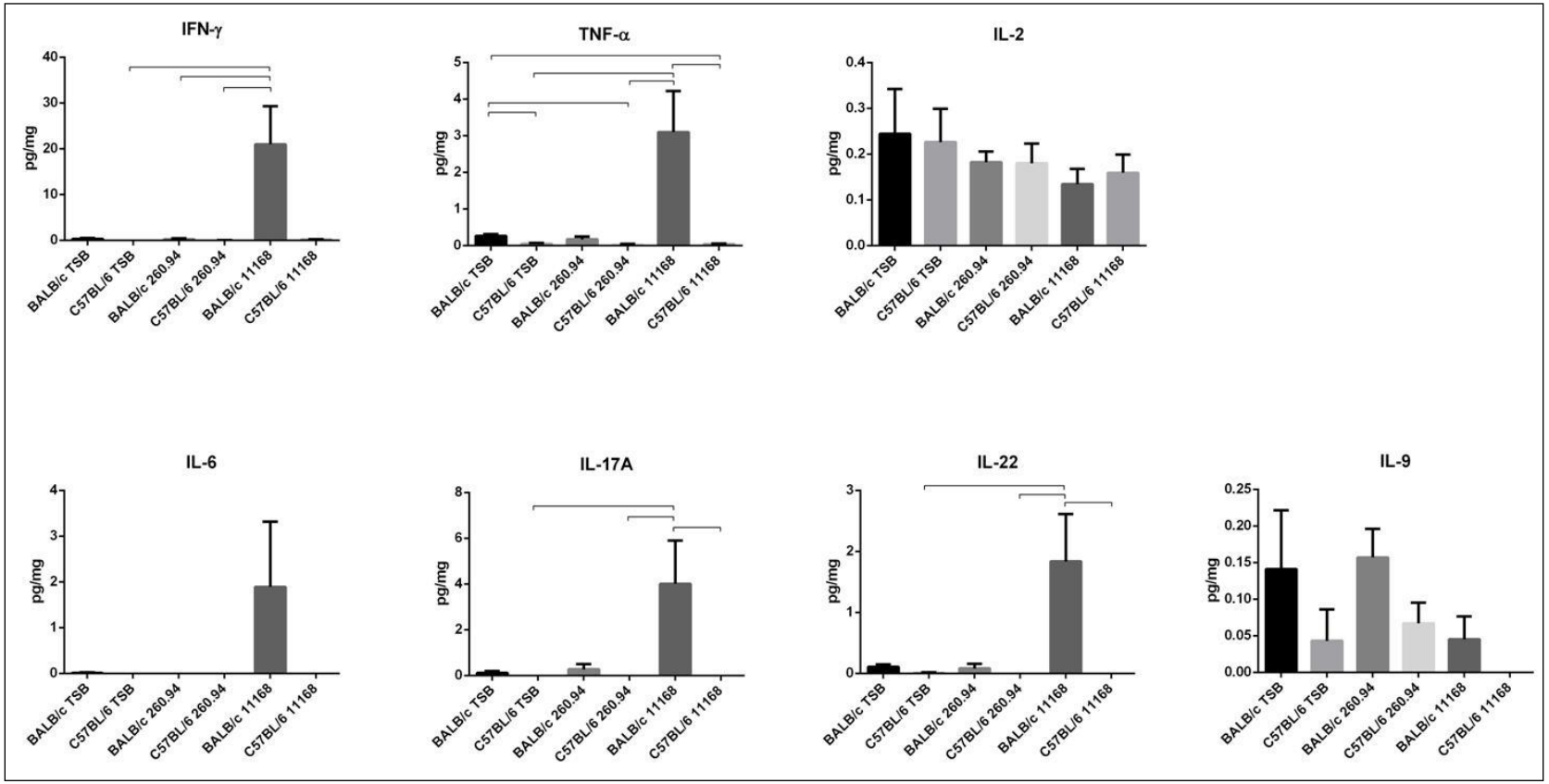


Figure 3.10. Assessment of colon cytokine production in C57BL/6 IL-10$^{-/-}$ mice inoculated with *C. jejuni* 11168, *C. jejuni* 260.94, or sham (tryptic soy broth, TSB) by flow cytometry-based multiplexed bead assay. Data were analyzed by one-way ANOVA. No statistically significant differences ($P \leq 0.05$) between groups were found. IL-6 and IL-17A production was undetectable in all C57BL/6 IL-10$^{-/-}$ mice. Mean ± SEM.

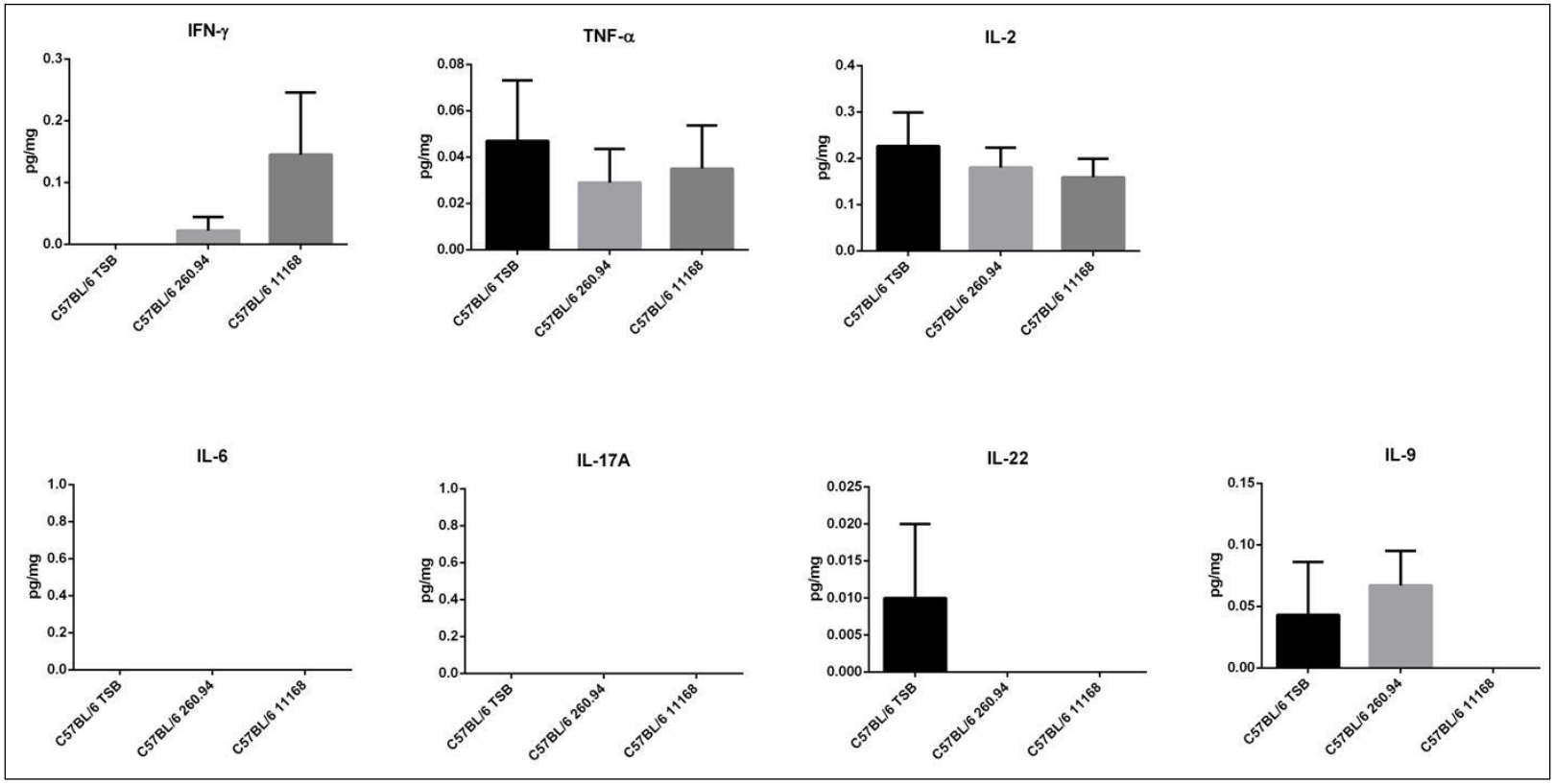


Figure 3.11. Assessment of colon cytokine production in BALB/c IL-10$^{-/-}$ mice inoculated with *C. jejuni* 11168, *C. jejuni* 260.94, or sham (tryptic soy broth, TSB) by flow cytometry-based multiplexed bead assay. Data were analyzed by one-way ANOVA, or when assumption of equal variance was not met, by Kruskal-Wallis followed by Tukey's post-testing. Brackets above groups indicate statistically significant pairwise comparisons; P ≤ 0.05 was considered statistically significant. Mean ± SEM.

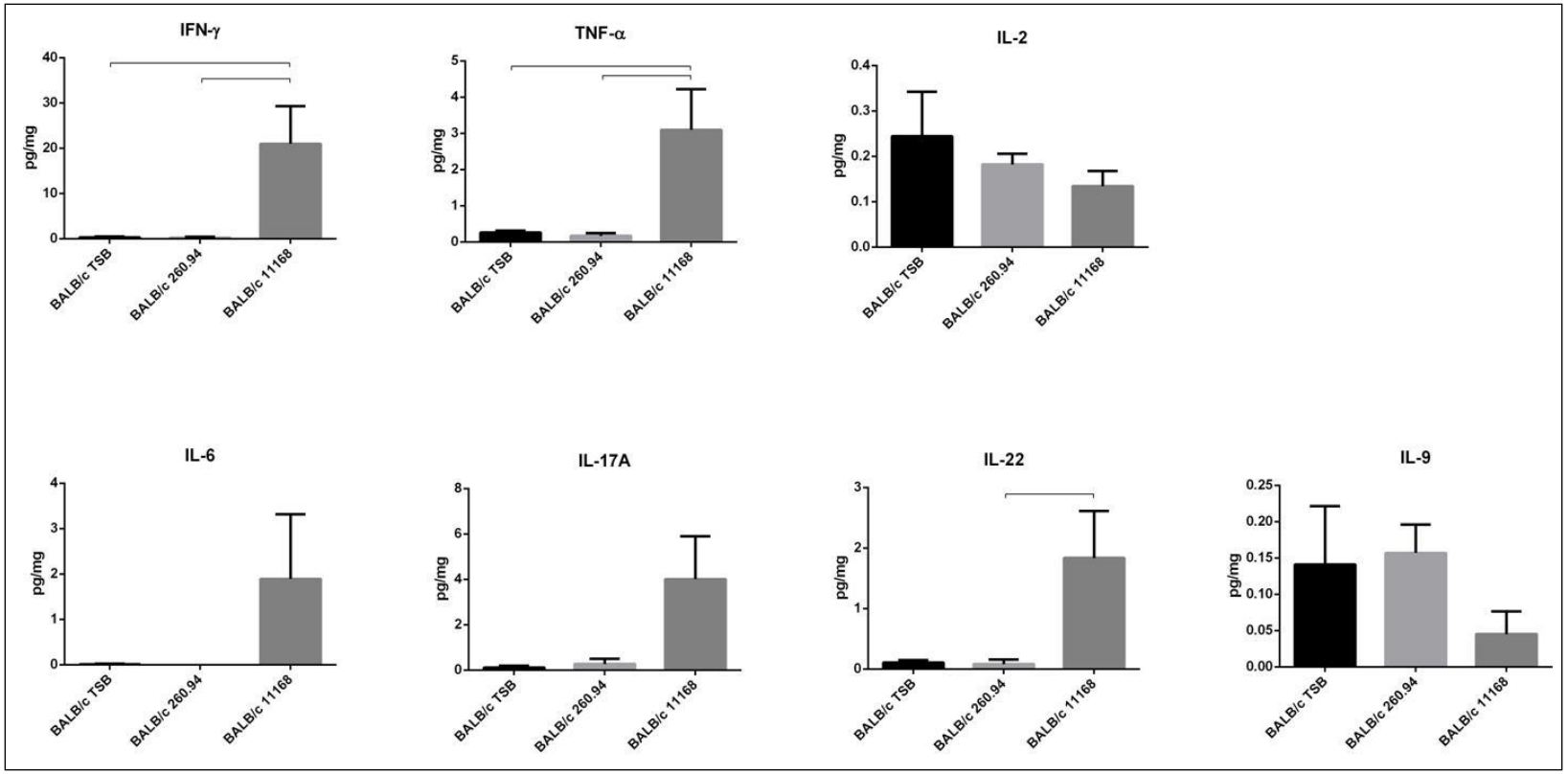
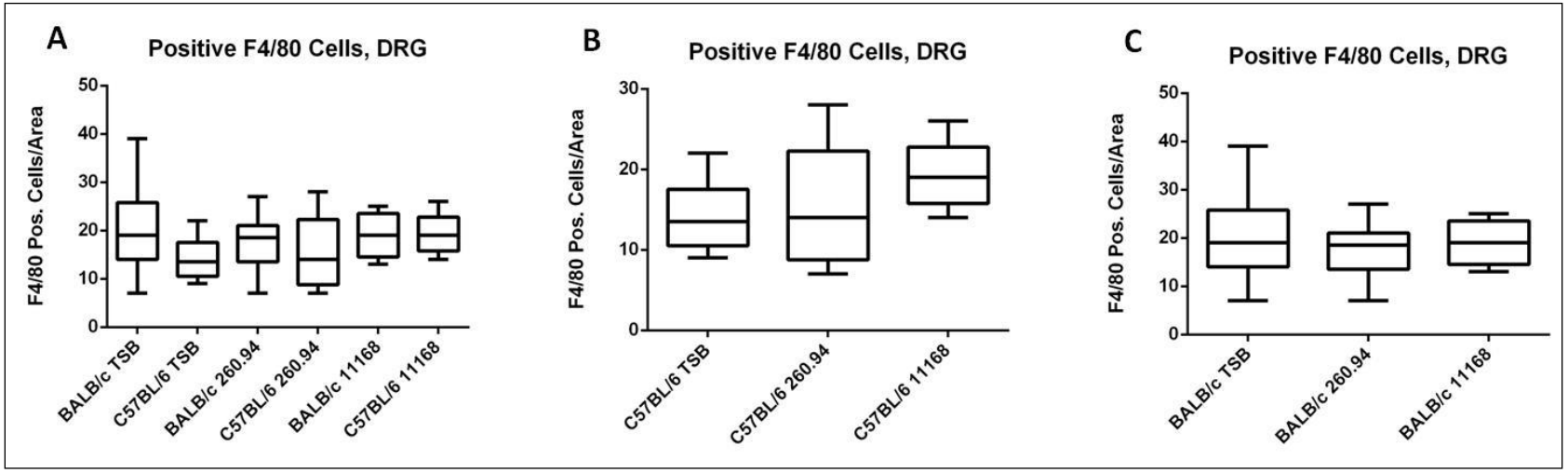


Figure 3.12. Assessment of cells positively labeled with the F4/80 macrophage marker in lumbar dorsal root ganglia (DRG) of C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice at the time of necropsy. Mice were inoculated with *C. jejuni* 260.94, *C. jejuni* 11168, or vehicle (tryptic soy broth; TSB) and sacrificed 4 weeks post-infection, or earlier when humane endpoint was reached. The DRG were labeled immunohistochemically for F4/80. Positive cells were scored by morphometry on contiguous images using the Image J program. Results are given as number of F4/80 positive cells/area, with area representing 100,000 pixels. All six treatment groups (A) were analyzed by two-way ANOVA. C57BL/6 IL-10$^{-/-}$ mice (B) were analyzed by Kruskal-Wallis, as the assumption of equal variance was not met. BALB/c IL-10$^{-/-}$ mice (C) were analyzed by one-way ANOVA. No statistically significant differences ($P \leq 0.05$) were found between any treatment groups. Data are represented by box and whisker, with the box extending from 25th-75th percentiles and the line plotted at the median. Whiskers represent minimum to maximum values.



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CHAPTER 4: INVASION EFFICIENCY, INTRACELLULAR SURVIVAL, AND ELICITATION OF CYTOKINE PRODUCTION IN MURINE DENDRITIC CELLS IS DETERMINED BY BOTH *CAMPYLOBACTER JEJUNI* STRAIN CHARACTERISTICS AND MOUSE GENOTYPE

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ABSTRACT

Campylobacter jejuni is an important cause of bacterial diarrhea worldwide and is associated with post-infectious Guillain-Barré syndrome (GBS). Immunological mechanisms underlying different disease outcomes are incompletely understood, but both infecting *C. jejuni* strain characteristics and host genetic background are thought to contribute. Dendritic cells (DCs) play a critical role in pathogen recognition and initiation of adaptive immunity by the polarization of naïve T helper (Th) cells. The objective of this study was to evaluate the interaction of colitogenic *C. jejuni* 11168 and GBS patient-derived *C. jejuni* 260.94 with murine bone-marrow derived DCs (BMDCs) from mice of two inbred genotypes. C57BL/6 and BALB/c mice were chosen due to reported Th1 and Th2 immunological biases, respectively. We hypothesized that BMDCs from wild-type (WT) and IL-10$^{-/-}$ mice on both C57BL/6 and BALB/c backgrounds would efficiently internalize and kill both *C. jejuni* strains, and that cytokines produced by infected IL-10$^{-/-}$ BMDCs would mirror adaptive immunity seen in previous *in vivo* mouse models: *C. jejuni* 11168-infected IL-10$^{-/-}$ BMDCs from both mouse genotypes and BALB/c IL-10$^{-/-}$ BMDCs infected with *C. jejuni* 260.94 would produce Th1/Th17-polarizing cytokines, while *C. jejuni* 260.94 would stimulate Th2-polarizing cytokine production in infected C57BL/6 IL-10$^{-/-}$ BMDCs. BMDC populations from C57BL/6 and BALB/c mice differed significantly: flow cytometry showed a more diverse population of differentiating DCs, with lower MHC II expression and higher proportions of macrophages, in C57BL/6 compared to BALB/c BMDCs. *C. jejuni* invasiveness and intracellular survival within BMDCs assessed by gentamicin killing assay differed significantly based on *C. jejuni* strain. Infection time with *C. jejuni* strains 11168 or 260.94 prior to gentamicin treatment and IL-10$^{-/-}$ BMDC lysis was varied to assess combined invasion efficiency and intracellular survival; to test intracellular survival alone, gentamicin was applied

after 1 hour of infection and WT or IL-10$^{-/-}$ BMDCs were lysed after 24 hours. *C. jejuni* 11168 showed significantly higher combined invasion/survival over time and intracellular survival alone compared to *C. jejuni* 260.94. A consistent mouse genotype difference in *C. jejuni* recovery was not found across all time points, but when a difference was identified, more *C. jejuni* was recovered from C57BL/6 BMDCs. BMDC cytokine profiles indicate that C57BL/6 IL-10$^{-/-}$ cells produced more MCP-1, especially following infection with *C. jejuni* 260.94, while BALB/c IL-10$^{-/-}$ cells produced more IL-6, especially when infected with *C. jejuni* 11168. Enhanced invasion and intracellular survival of *C. jejuni* 11168 *in vitro* correlated with higher *in vivo* pathogenicity and invasion than *C. jejuni* 260.94 in our mouse models. Production of MCP-1 and IL-6 similarly mirrored Th2 and Th17 immune responses seen *in vivo*. These results support an important role of DCs in the initial host-microbe interaction and implicate invasiveness, intracellular survival, and elicitation of cytokine production of infecting *C. jejuni* strains as determinants of *in vivo* disease outcomes.

INTRODUCTION

Campylobacter spp. are Gram negative, motile, spiral rods, and are considered the most frequent cause of bacterial gastroenteritis worldwide.[61] Estimates from active surveillance data indicate that *Campylobacter* spp. cause over 800,000 cases of foodborne illness, and more than 8,000 hospitalizations and 70 deaths, annually in the United States.[53] In addition to causing symptoms of enteritis including diarrhea and abdominal pain, *Campylobacter* infection has been associated with numerous severe sequelae, including inflammatory bowel disease and Guillain-Barré syndrome (GBS).[30] GBS is a debilitating post-infectious neuropathy, with approximately 25% of patients requiring artificial ventilation, and many suffering from long-lasting residual pain and neurologic deficits.[56] While several viral and bacterial agents are associated with GBS, campylobacteriosis is the most frequently reported preceding illness.[21; 26]

The immunopathogenesis of GBS subsequent to *C. jejuni* infection is incompletely understood, but is thought to involve generation of cross-reactive antibodies to the lipooligosaccharide (LOS) on the *C. jejuni* outer membrane and structurally similar peripheral nerve gangliosides.[2; 43; 47; 56; 63] Patients with GBS frequently have antibodies to gangliosides GM1a, GM1b, GD1a, GT1a, and GQ1b, among others.[48; 56] Rise in anti-ganglioside antibodies coincides with onset of paralysis, with subsequent waning of antibodies occurring with clinical improvement.[56] GBS patients with preceding *C. jejuni* infection have been shown to have higher frequencies of antibodies to ganglioside GM1 and suffer worse clinical outcomes.[21; 50] Subtype of anti-ganglioside IgG may also be related to type of preceding infection and clinical outcome of GBS. Anti-GM1 IgG1 and IgG1 antibodies to motor gangliosides have been associated with preceding gastroenteritis/diarrhea, positive *C. jejuni* serology, and slower recovery; in contrast, anti -GM1 or -ganglioside IgG3, alone or with IgG1, was associated with antecedent respiratory infection and better clinical outcome.[25; 32]

Current analyses estimate that GBS develops subsequent to *Campylobacter* infection in approximately 0.07% of cases.[30] *C. jejuni* strain characteristics are thought to play a role in development of GBS, particularly due to the confirmed structural similarity between the LOS of some *C. jejuni* strains and gangliosides, including GM1 and GD1a,[17; 63] and the overrepresentation of certain *C. jejuni* serotypes in GBS patients.[1; 33] Multiple lines of epidemiological evidence suggest that host factors also contribute to GBS susceptibility, including the rarity of GBS outbreaks and geographic clustering of GBS subtypes, low proportion of *C. jejuni* infections resulting in GBS, isolation of *C. jejuni* strains harboring ganglioside mimics from both GBS and uncomplicated enteritis patients, and lack of identification of ganglioside mimics in some GBS-associated *C. jejuni* strains.[3; 17; 30; 46; 47; 54; 56] Taken together, the evidence of *C. jejuni* molecular mimicry and host genetic features associated with GBS onset provides a rationale for further study into these mechanisms.

Studies in mice and humans suggest that T helper (Th)1/Th17-driven immune responses predominate following *C. jejuni* infection, although Th2 responses can also occur. Multiple studies have demonstrated increased *C. jejuni*-specific plasma or serum IgG2a/IgG2c and IgG3 (Th1-mediated), along with IgG2b (Th1/Th17-mediated) in mice of various genetic backgrounds infected with different *C. jejuni* strains.[16; 39; 40; 55] Th1/Th17 immune responses were similarly found in human intestinal biopsies infected with colitogenic *C. jejuni in vitro*; IFN-γ, along with more modest increases in IL-23, IL-12, IL-6, IL-17 and IL-1β, characterized the mucosal cytokine response.[12] However, Th2-mediated plasma anti -*C. jejuni*, -GM1, and -GD1a IgG1 antibodies and colonic expression of Gata-3 and IL-4 were significantly increased in C57BL/6 IL-10$^{-/-}$ mice infected with GBS patient-derived *C. jejuni* 260.94,[39] indicating that characteristics of the infecting *C. jejuni* strain impact Th polarization.

Dendritic cells (DCs) are sentinel immune cells, bridging the innate and adaptive immune systems.[57] DCs are important antigen presenting cells (APCs) that surveil peripheral tissues, and upon activation by antigen, migrate to a regional lymph node. DCs undergo several changes in this process in

preparation for interaction with naïve T cells, including increased expression of costimulatory molecules and MHC II, along with decreased phagocytosis of antigen. DCs contribute to Th polarization by direct interaction and cytokine production.[57] Thus, DCs are a target for understanding the initial host-microbe interaction in *C. jejuni* infection and subsequent initiation of adaptive immunity. However, relatively few studies to date have investigated the role of DCs in *C. jejuni* pathogenesis.

In vitro studies using DCs derived from human peripheral blood monocytes and mouse bone marrow have demonstrated efficient internalization and killing of *C. jejuni* by DCs, along with increased costimulatory molecule expression and production of pro-inflammatory and Th1-polarizing cytokines.[23; 49] Furthermore, co-culture of *C. jejuni*-infected DCs with CD4+ T cells, or exposure of T cells to the supernatant of infected DCs, confirmed both Th1 and Th17 T cell polarization by production of IFN-γ and IL-17A.[12; 49] Collectively, these studies show that Th1/Th17 responses can be initiated by DCs following interaction with *C. jejuni*, consistent with the isotypes of *C. jejuni*-specific antibodies described in mouse infection studies.[16; 40; 55] However, Th1/Th17 responses are not the sole outcome of the adaptive response to *C. jejuni*, as demonstrated by a predominant Th2 response produced by C57BL/6 IL-$10^{-/-}$ mice infected with *C. jejuni* 260.94.[39] Noteworthy is that the type of sialic acid linkage in the *C. jejuni* LOS can influence Th1 or Th2 polarization by DCs through binding of specific siglecs.[5] These studies showed that *C. jejuni* adaptive responses are not limited to Th1/Th17 polarization, but that Th2 responses may also be triggered in the initial interaction of *C. jejuni* with DCs.

Th1/Th17 adaptive immune responses to infection with colitogenic *C. jejuni* strains described in mouse and human studies[12; 16; 40] are thought to correlate with *C. jejuni* clearance.[16] However, production of antibodies cross-reacting to gangliosides is a hallmark of GBS. Anti-ganglioside, especially GM1, antibodies of the Th2-mediated IgG1 isotype have been identified in mice infected with GBS-associated *C. jejuni* 260.94[39] and in GBS patients, correlate with poor clinical outcome.[25; 32] Collectively, these studies suggest that contrary to a protective Th1/Th17 immune response, “aberrant” Th2

responses resulting from *C. jejuni* infection contribute to GBS development and that both *C. jejuni* strain characteristics and host factors contribute to susceptibility.

Our rationale is that adaptive immunity is initiated following the host-microbe interaction and innate immune response to *C. jejuni*. Contrasting immune responses, likely influenced by both *C. jejuni* strain characteristics and host factors, can produce different disease outcomes following infection.[39] Because DCs are potent APCs and contributors to Th cell polarization, a model system designed to evaluate the interaction of different *C. jejuni* strains with DCs derived from mice of different genetic backgrounds would further our understanding of how the interplay of bacterial and host factors influences disease outcome.

The current study evaluated the interaction of colitogenic and GBS patient-derived *C. jejuni* strains with bone-marrow derived DCs (BMDCs) from mice of C57BL/6 and BALB/c backgrounds, with and without IL-10, to examine potential differences in cellular uptake, killing ability, and cytokine production. *C. jejuni* 11168 was originally isolated from an enteritis patient in the United Kingdom, is LOS class C, and encodes *cst*-III sialyl transferase. This strain bears both GM1 and GM2 mimics,[37] but has not been reported to be associated with GBS. *C. jejuni* 11168 has repeatedly produced colitis mimicking human disease with induction of Th1/Th17-associated anti-*C. jejuni* antibodies in our C57BL/6 IL-$10^{-/-}$ mouse models.[6; 39; 40; 52] Absence of anti-inflammatory IL-10 has been shown to be critical for induction of colitis, as *C. jejuni* 11168 stably colonizes wild-type (WT) mice without causing disease.[9; 40; 41]

The second strain included in these studies is *C. jejuni* 260.94. This strain was isolated from a GBS patient in South Africa. It is LOS class A, possesses a GM1a ganglioside mimic, and encodes *cst*-II sialyl transferase.[37] This strain has colonized well in our mouse models without producing colitis,[6; 9; 39; 55] and, in contrast to *C. jejuni* 11168, has induced a Th2-mediated response, including production of anti-ganglioside antibodies, in C57BL/6 IL-$10^{-/-}$ mice.[39]

C57BL/6 and BALB/c mice are two commonly used laboratory strains. Reports in the literature suggest that mice of these genetic backgrounds vary in innate, adaptive, and mucosal immune responses to various stimuli. One classic example is experimental infection studies with *Leishmania major*, in which susceptible BALB/c mice fail to suppress and even display overabundant IL-4 production, while resistant C57BL/6 mice downregulate IL-4 production and instead produce IFN-γ.[51] BALB/c mice also reportedly harbor greater proportions of immunosuppressive T regulatory (T reg) cells in the small intestine and lymphoid organs than C57BL/6 mice,[10; 18] and exhibit comparatively enhanced vitamin A metabolism, IgA production, and resistance to chemically induced colitis.[18; 62]

In vitro studies have shown important differences between the responses of stimulated spleen cells, peritoneal macrophages, and DCs derived from C57BL/6 and BALB/c mice. C57BL/6-derived spleen cells stimulated with concanavalin A produced more IFN-γ, while those from BALB/c mice produced higher levels of IL-4.[42] Similarly, peritoneal macrophages from C57BL/6 mice stimulated with LPS and MALP-2 (TLR-4 and TLR-2 ligands, respectively) produced more TNF-α and IL-12 compared to BALB/c-derived macrophages.[59] DCs derived from the spleens of naïve C57BL/6 and BALB/c mice differed in expression of various TLRs, and when stimulated with TLR-4, -2, and -9 ligands, C57BL/6-derived DC produced more IL-12p40 while those from BALB/c mice produced more MCP-1.[35]

Studies comparing C57BL/6 and BALB/c mice utilizing *in vivo* infection models combined with assessment of DC responses help to more completely elucidate differences in immune responses and disease outcomes. Similar to *L. major* infection, C57BL/6 mice are resistant to infection with *Listeria monocytogenes* whereas BALB/c mice are susceptible. Infected C57BL/6 mice showed increased survival, enhanced pathogen clearance, higher baseline and infection-induced IL-12 production in splenic DC, and increased IFN-γ production by splenic T cells compared to BALB/c mice.[36] Similarly, C57BL/6 mice infected with the respiratory pathogen *Chlamydia muridarum* showed reduced morbidity, increased pathogen clearance, and greater production of IFN-γ in the lung and spleen compared to infected

BALB/c mice, while BALB/c mice produced more IL-17.[28] When BMDCs from C57BL/6 and BALB/c mice were infected with *C. muridarum*, no difference was shown in endocytosis but BMDCs from BALB/c mice produced less IL-12 and more IL-23, IL-6, IL-10, and TNF-α than those from C57BL/6 mice.[28] These studies highlight discrepancies in immune responses between C57BL/6 and BALB/c mice to various infectious agents, and suggest that these differences may begin with DCs.

We hypothesized that 1) BMDCs from WT and IL-10$^{-/-}$ mice on both C57BL/6 and BALB/c genetic backgrounds would efficiently internalize and kill both colitogenic and GBS-associated *C. jejuni* strains, and 2) cytokines produced by infected BMDCs derived from IL-10$^{-/-}$ mice would mirror immunogenicity and pathogenicity previously observed in our mouse models: specifically, *C. jejuni* 11168-infected BMDCs from both BALB/c IL-10$^{-/-}$ and C57BL/6 IL-10$^{-/-}$ mice, and *C. jejuni* 260.94-infected BMDCs from BALB/c IL-10$^{-/-}$ mice, would produce pro-inflammatory and Th1/Th17-polarizing cytokines, while C57BL/6 IL-10$^{-/-}$ BMDCs infected with *C. jejuni* 260.94 would produce Th2-polarizing cytokines.

Differentiation of bone marrow cells from both WT and IL-10$^{-/-}$ mice of C57BL/6 and BALB/c backgrounds into DCs was compared by flow cytometry. The gentamicin killing assay (GKA) was used to identify broad patterns in invasion efficiency and intracellular survival of the two *C. jejuni* strains in experiments using BMDCs derived from WT and IL-10$^{-/-}$ mice on both C57BL/6 and BALB/c backgrounds. BMDCs derived from WT mice were used as a comparison group to assess the impact of IL-10 on ability of BMDCs to effectively kill internalized *C. jejuni*. Finally, production of pro-inflammatory and Th-polarizing cytokines, including TNF-α, IFN-γ, IL-12p70, IL-4, MCP-1, TGF-β, and IL-6, by infected BMDC derived from IL-10$^{-/-}$ mice was assessed. Our results show that DCs differentiated from bone marrow precursors of C57BL/6 mice comprise a more diverse population of maturing DCs and a higher proportion of macrophages than those from BALB/c mice. *C. jejuni* 11168 exhibited increased invasion efficiency and intracellular survival in BMDCs compared to *C. jejuni* 260.94, but mouse genetic background did not have a consistent effect in each replicate on *C. jejuni* uptake or killing. BMDC

cytokine profiles indicated that pro-inflammatory and Th-polarizing cytokine production depends on both infecting *C. jejuni* strain and host genetic background, and that DCs are important early contributors to adaptive immunity following *C. jejuni* infection.

MATERIALS AND METHODS

Experimental Animals. Wild-type (WT) C57BL/6J, B6.129P2-IL-10^{tm1Cgn}/J (referred to as C57BL/6 IL-10$^{-/-}$), WT BALB/cJ, and C.129P2(B6)-*Il10*^{tm1Cgn}/J (referred to as BALB/c IL-10$^{-/-}$) mice were originally purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were bred and maintained in-house; husbandry has been previously described.[40] Briefly, mice were housed in sterile cages on a rack with filtered air and received sterile chow and water. The colony is monitored for enteric pathogens, and is free of *Helicobacter* spp. All protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (approval numbers 06/12-107-00 and 06/15-101-00). All mice were humanely euthanized using an overdose of CO_2 according to the guidelines of the American Veterinary Medical Association (https://www.avma.org/KB/Policies/Pages/Euthanasia-Guidelines.aspx).

Generation of Bone Marrow-Derived Dendritic Cells (BMDCs). Bone marrow stem cells were derived from the femurs of WT C57BL/6, WT BALB/c, C57BL/6 IL-10$^{-/-}$, and BALB/c IL-10$^{-/-}$ mice of 2—5.5 months of age. The cells were isolated and differentiated according to published methods[49] with modifications. Briefly, both femurs were removed, soaked in RPMI 1640 medium (Gibco; Waltham, MA), cleaned of muscles and tendons, surface sterilized in 70% ethanol, and rinsed in Hank's Balanced Salt Solution (HBSS; Sigma, St. Louis, MO). Cells were flushed from the femur and through a 70 µm cell strainer, pelleted, treated with ACK Lysing Buffer (Gibco) to lyse erythrocytes, and washed twice with HBSS. Trypan Blue (Sigma, St. Louis, MO) staining was used to assess viability and cell numbers were estimated using a hemocytometer. Finally, cells were seeded onto polystyrene 100 × 15 mm sterile Petri dishes

(VWR; Radnor, PA) at a density of approximately 2.5×10^6 cells in 10 mL of R10 medium (RPMI 1640 with L-glutamine (Gibco), supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, 50 µM 2-mercaptoethanol, 100 u/mL penicillin, and 100 µg/mL streptomycin with 20 ng/mL recombinant murine granulocyte-macrophage colony stimulating factor (GMCSF; Peprotech, Rocky Hill, NJ)) and incubated at 37°C with 5% CO_2. On day 3, non-adherent cells were discarded and fresh medium was added. On day 6, half of the medium was exchanged for fresh medium and non-adherent cells were again discarded. Half of the medium was again exchanged for fresh medium on day 8, but without discarding any cells. Morphology of the cells, including size, shape, adherence, and presence and size of cellular processes was observed until they were collected on days 9, 10, or 11 for assessment by flow cytometry, use in GKAs, or for assessment of cytokine production. Collection of adherent and non-adherent populations was achieved by collecting the medium and incubating the cells remaining in the Petri dish in 10 mL of Accutase (Sigma) for 20 minutes at 37°C with 5% CO_2.

Flow Cytometry. Multiple batches of cultured cells were analyzed by flow cytometry on days 9, 10, or 11 for confirmation of CD11c and MHC II expression over the course of time in which GKAs were performed. Flow cytometry was performed on cells derived from C57BL/6 IL-$10^{-/-}$ mice (once on days 9 and 11 of culture, twice on day 10), C57BL/6 WT mice (once each on days 9 and 10), BALB/c IL-$10^{-/-}$ mice (once on days 9 and 11, twice on day 10), and BALB/c WT mice (once on day 9, twice on day 10). In one preliminary experiment, cells derived from C57BL/6 IL-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice on days 10 and 9 of culture, respectively, were analyzed with and without stimulation with lipopolysaccharide (LPS). Flow cytometry was performed on extra cells one day prior to the GKA in two experiments. Cells from C57BL/6 $10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice used in cytokine production experiments on day 10 were also assessed for expression of the F4/80 macrophage marker, in addition to CD11c and MHC II, on the day of the experiment.

For flow cytometric analysis, cells were harvested, resuspended in flow staining buffer (phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.09% sodium azide), and incubated in cocktails including LIVE/DEAD Fixable Far-Red Stain (Life Technologies, Carlsbad, CA), rat anti-mouse CD16/CD32 (Mouse BD Fc Block, clone 2.4G2; BD Biosciences, San Jose, CA), and antibodies including PE-conjugated anti-mouse CD11c (N418; eBioscience, Waltham, MA), FITC-conjugated anti-mouse I-A/I-E (2G9; BD Biosciences), and Pacific Blue-conjugated anti-mouse F4/80 (BM8; BioLegend, San Diego, CA). Controls included incubation of cells in flow staining buffer only (unstained controls) or with appropriate antibody isotypes. Following incubation for 30 minutes covered on ice, the cells were washed twice with flow staining buffer and analyzed with a BD FACSCanto II (BD Biosciences) flow cytometer. Compensation matrices were established and populations were gated using FlowJo software version 10.2 (Becton, Dickinson & Company, Franklin Lakes, NJ).

Preparation of **Campylobacter jejuni** ***Inocula***. Frozen glycerol stock cultures of minimally passaged *C. jejuni* strain 11168 (ATCC 700819) and *C. jejuni* strain 260.94 (ATCC BAA-1234) were streaked onto Bolton agar (BA) plates and grown in microaerophilic conditions for approximately 48 (range: 41-49.5) hours at 37°C. The microaerophilic environment was achieved by evacuation of vented anaerobic jars to -25 in Hg and equilibration with a gas mixture comprising 80% N_2, 10% CO_2, and 10% H_2. Colonies were suspended in tryptic soy broth (TSB) to an optical density of approximately 0.2-0.3 at 560 nm (OD_{560}). Lawns (100 µL) made from this suspension were grown on BA plates for approximately 18-24 hours in microaerophilic conditions at 37°C. Immediately prior to use, bacterial growth was suspended in R10.1 medium (RPMI 1640 with L-glutamine, supplemented with 10% FBS and 20 mM HEPES) to an OD_{560} of ~0.120, corresponding to approximately 2×10^8 colony forming units (CFU)/mL. Wet mounts of both cultures were performed with each experiment to assess purity, morphology, and motility. CFU in the inocula were verified by limiting dilution in TSB and plating in single or duplicate on BA plates.

Gentamicin Killing Assays. GKAs were performed using published methods[49; 58] with modifications. BMDCs were harvested on days 10 or 11 and resuspended in R10.1 medium at a concentration of 2×10^5 cells/mL. Cells were kept on ice until just prior to use, when 1 mL (2×10^5 cells) of cell suspension was added to each well of a 24-well cell culture plate. One hundred microliters (aiming to achieve approximately 2×10^7 CFU) of *C. jejuni* culture was immediately added to appropriate wells to achieve a multiplicity of infection (MOI) of approximately 100. Cells were incubated at 37°C with 5% CO_2 throughout the assay. BMDCs were not removed from the original 24-well plate at any point during the assay until lysis.

The experimental design for the GKAs was as follows. Each mouse genotype/*C. jejuni* strain combination was assayed in triplicate wells, with four experimental groups: 1) C57BL/6 BMDCs, *C. jejuni* 11168; 2) C57BL/6 BMDCs, *C. jejuni* 260.94; 3) BALB/c BMDCs, *C. jejuni* 11168; 4) BALB/c BMDCs, *C. jejuni* 260.94. Killing of both *C. jejuni* strains by gentamicin was verified by addition of 100 µL of *C. jejuni* 11168 or 260.94 inocula to separate wells containing only R10.1 medium, with and without subsequent gentamicin treatment. Additional duplicate control wells to monitor any cross-contamination during the assay included sham inoculation (R10.1 medium only) of wells containing BMDCs, with and without subsequent application of gentamicin. Aliquots of cell culture suspensions were spread onto BA plates at the beginning of the assay to screen for any initial contamination. The cells were visualized at multiple time points throughout the assay to assess morphology, viability, and adherence, and following lysis and harvesting to verify removal of well contents. A control experiment to test viability of both *C. jejuni* strains in 0.1% Triton X-100 (Sigma) in PBS in a closed Eppendorf tube at room temperature showed relatively constant viability at 30, 60, 90, and 120 minutes of incubation.

To assess combined invasion efficiency and intracellular survival of the two *C. jejuni* strains, cells derived from IL-$10^{-/-}$ mice of both C57BL/6 and BALB/c genetic backgrounds were infected for 1, 2, 3, or 23 hours. Following incubation at 37°C with 5% CO_2, medium was discarded and replaced with R10.1

medium containing 250 µg/mL of gentamicin (Life Technologies) for 1 hour. Medium was discarded again, and cells were washed twice with R10.1 medium to remove gentamicin before lysis. Each time point was assessed by individual experiments.

To assess intracellular survival alone, cells derived from IL-$10^{-/-}$ or WT mice were infected for 1 hour. Medium was removed and replaced with R10.1 medium containing 250 µg/mL of gentamicin for 1 hour. Cells were washed twice and incubated with fresh R10.1 medium at 37°C with 5% CO_2 until lysis at 24 hours post-infection (p.i.).

In both invasion and survival assays, cells were lysed by incubation in 500 µL of PBS containing 0.1% Triton X-100 for 15 minutes at room temperature. Well contents were removed by scraping the bottom with a pipette tip, pipetting up and down, and transferring the contents to a microfuge tube. Undiluted lysate and/or appropriate dilutions made in R10.1 medium were then spread on BA plates. *C. jejuni* CFU were enumerated following approximately 72 hours incubation in microaerophilic conditions at 37°C.

Assessment of Cytokine Production by* C. jejuni-*Infected BMDCs Derived From IL-$10^{-/-}$ Mice. Cells derived from C57BL/6 IL-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice were harvested on day 10 of differentiation and resuspended in R10.1 medium to a concentration of 2×10^5 cells/mL. Cells were kept on ice until just prior to use, when 1 mL (2×10^5 cells) of cell suspension was added to each well of a 24-well cell culture plate. The experimental design was as follows: BMDCs from each mouse strain were infected with *C. jejuni* 11168 or 260.94 (MOI of approximately 100), or treated with 1 µg/mL LPS from *Salmonella enterica* serotype Typhimurium (Sigma) (positive control), or R10.1 medium (negative control). The eight groups were as follows: 1) C57BL/6 BMDCs, *C. jejuni* 11168; 2) C57BL/6 BMDCs, *C. jejuni* 260.94; 3) BALB/c BMDCs, *C. jejuni* 11168; 4) BALB/c BMDCs, *C. jejuni* 260.94; 5) C57BL/6 BMDCs, R10.1 medium; 6) C57BL/6 BMDCs, LPS; 7) BALB/c BMDCs, R10.1. medium; and 8) BALB/c BMDCs, LPS. Each mouse

genotype/*C. jejuni* strain combination was performed in triplicate, as were positive and negative controls. CFU in the inocula were verified by limiting dilution. Wet mount preparations to evaluate purity, morphology, and motility were performed with each experiment. Infected BMDCs were incubated for 1 hour at 37°C with 5% CO_2. Medium was then removed, and replaced with 1 mL R10.1 medium containing 250 μg/mL of gentamicin. Following 1 hour of incubation at 37°C with 5% CO_2, medium was again removed. The plate was washed once to remove gentamicin by adding and removing 1 mL of R10.1 medium to each well. Finally, 1 mL of fresh R10.1 was added to each well, and the plate was incubated at 37°C with 5% CO_2 until approximately 24 hours p.i. Medium was collected from wells, aliquoted into cryovials, and stored at -80°C until analysis.

Cytokine production by IL-$10^{-/-}$ BMDCs was assessed by a custom multiplex bead-based assay panel (LEGENDplex Custom Panel; BioLegend, San Diego, CA) according to the manufacturer's instructions. The panel was designed to measure pro-inflammatory cytokines and those involved in polarization of Th1, Th2, and Th17 cells by stimulated dendritic cells. The seven analytes included TNF-α, IFN-γ, IL-12p70, IL-4, MCP-1, IL-6, and TGF-β. Data were acquired using a BD FACSCanto II flow cytometer and analyzed using LEGENDplex Data Analysis Software (BioLegend) according to the manufacturer's guidelines.

Statistical Analyses.

Analyses were performed and figures generated using commercial statistical software packages (SigmaStat 3.5, Systat Software, Inc., San Jose, CA; and GraphPad Prism 6, GraphPad Software, La Jolla, CA). Assumptions of normality and equal variance were tested prior to each analysis. P-values ≤0.05 were considered significant.

Flow Cytometry. Statistical analyses were performed on data from cells used in the 3 independent cytokine production experiments, which included CD11c, MHC II, and F4/80 markers. t-

tests were performed to determine if there was a difference between C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ cells in %CD11c(+) cells; %CD11c(+)/MHC II^{high} cells; %CD11c(+)/MHC II^{low} cells; %CD11c(+)/F4/80(+) cells; and the median fluorescence intensity (MFI) of MHC II in populations including all MHC II(+) cells and within CD11c(+)/F4/80(+) cells. For percentage data, t-tests were performed on Arcsin Square Root transformed data.

Gentamicin Killing Assays. Statistical analyses were performed on data from individual experiments from each time point. In experiments with both *C. jejuni* strains and both mouse strains, two-way ANOVAs were performed. When normality or equal variance assumptions were not met or a significant interaction was identified, a Kruskal-Wallis ANOVA on ranks or one-way ANOVA was also performed. Post-hoc testing, including Holm-Sidak, Tukey, Dunn's, Bonferroni t-test, and Student-Newman-Keuls methods for pairwise multiple comparisons, was performed as appropriate. Data from experiments with two mouse strains but only one *C. jejuni* strain were tested for normality and equal variance and subjected to a t-test or Mann Whitney rank sum test as appropriate.

Cytokine Production. The seven analytes assessed in each experiment were analyzed individually by 2-way ANOVA. Holm-Sidak pairwise testing was implemented when overall statistical significance in mouse genotype (C57BL/6, or BALB/c), treatment group (positive or negative control, *C. jejuni* 11168, or *C. jejuni* 260.94), or an interaction was identified. One-way ANOVA followed by Holm-Sidak pairwise testing was additionally performed in some cases to further evaluate pairwise comparisons. Data not meeting equal variance assumptions were analyzed by Kruskal-Wallis one-way ANOVA on ranks followed by Tukey's post-test.

RESULTS

Bone Marrow-Derived Dendritic Cell Cultures. Cells were analyzed by flow cytometry on days 9, 10, or 11 of culture, used in GKAs on day 10 (all experiments except 2, which were performed on day 11), or

used to measure cytokine production on day 10 (3 experiments). Cells were occasionally seeded at lower densities in 10 mL of medium at day 0 if sufficient numbers were not obtained from bone marrow. Cultures used in most GKAs analyzed statistically had a starting density of isolated bone marrow stem cells of approximately 2.5×10^6 in 10 mL medium, with a range of 1.5×10^6 - 3.2×10^6 (see **Tables 4.2A-D** and **Tables 4.3A-B**). A concentration of 2×10^5 cells/mL was always used on the day of the assay. Morphology was observed on days 3, 6, and 8 of differentiation and the day of the experiment. Subjectively, cells from C57BL/6 and BALB/c mice were typically observed to have slightly different morphology during differentiation, culminating in cells from C57BL/6 appearing to have a greater proportion of larger, firmly adherent cells, and a lower proportion of smaller, round, semi-adherent cells when fully differentiated than cells from BALB/c mice. Cells from BALB/c mice also had a layer of larger, firmly adherent cells, but these cells typically appeared smaller than the corresponding population of adherent cells in C57BL/6 mice. Cells from BALB/c mice also had a relatively greater proportion of smaller, round, semi-adherent cells than those from C57BL/6 mice. Presence or absence of IL-10 within a mouse strain did not obviously change cellular morphology.

Criteria for Statistical Analyses.

Flow Cytometry. Flow cytometry data generated from analysis of CD11c, MHC II, and F4/80 expression in BMDCs from C57BL/6 IL-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice used in the 3 independent cytokine production experiments were analyzed statistically (**Table 4.1, Figure 4.2**). Scatter plots from day 10 of differentiation of IL-$10^{-/-}$ and WT BMDCs from C57BL/6 and BALB/c mice are presented for descriptive purposes (**Figure 4.1**), but comparisons of differentiation in WT and IL-$10^{-/-}$ cells or differences over days 9-11 of culture were not performed due to insufficient replicates of these experiments for statistical analyses.

Gentamicin Killing Assays: Invasion and Intracellular Survival. A total of 17 experiments analyzed statistically had the following characteristics: performance on day 10 or 11 of BMDC culture; inclusion of BMDCs from both mouse genotypes, with starting densities (isolation from marrow on day 0) above 8×10^5 cells in 10 mL (the lowest starting density of cells assessed by flow cytometry in other experiments); and confirmation of MOIs by limiting dilution between approximately 60-175 calculated from a dilution factor of 10^{-6} (MOI ranges from limiting dilution plates given with data in **Tables 4.2** and **4.3**). When an MOI of ~60-175 was achieved for only one *C. jejuni* strain, data regarding the other *C. jejuni* strain were excluded from analysis. An exception to the MOI criterion was made regarding the two GKAs assessing 23 hours of invasion time, in which the MOI for *C. jejuni* 11168 was ~44 and ~33. The exception was made due to the prolonged incubation in medium with a protein source (10% FBS) and conditions (37°C, 5% CO_2) favorable for *C. jejuni* growth. Masses of clumped structures exhibiting movement, interpreted to be *C. jejuni* organisms, were visualized in inoculated wells at the end of the 23-hour invasion period in both of these experiments. Additionally, *C. jejuni* 11168 exhibited a several-fold increase in growth in similar conditions over 8 hours.[49]

Cytokine Production. Eight independent experiments including BMDCs derived from both C57BL/6 IL-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice and including both *C. jejuni* strains were performed; medium was collected and stored pending evaluation of MOI and flow cytometric data. The three experiments selected for measurement of cytokine production and statistical analysis all had confirmation of MOIs of both *C. jejuni* strains between 60-175 by limiting dilution and flow cytometric analysis on the day of the experiment for CD11c, MHC II, and F4/80 expression (**Table 4.1, Tables 4.4-4.6**).

Flow Cytometry.

CD11c and MHC II Expression in BMDCs Derived from WT and IL-$10^{-/-}$ Mice. Cells derived from WT and IL-$10^{-/-}$ mice on C57BL/6 and BALB/c genetic backgrounds were analyzed by flow cytometry

between days 9-11 of culture. Representative plots of CD11c and MHC II expression from one WT and one IL-10$^{-/-}$ mouse of each background, all on day 10 of differentiation, are shown in **Figure 4.1**. Presence or absence of IL-10 did not markedly influence patterns of differentiation. Percentage of CD11c(+) cells was generally similar between genotypes (WT or IL-10$^{-/-}$, C57BL/6 or BALB/c). Within the CD11c(+) positive cells, C57BL/6 mice showed 4 distinct smaller populations, while BALB/c mice had 2 distinct larger populations. The Median Fluorescence Intensity of MHC II, in all MHC II positive cells (MFI MHC II), was consistently higher in BALB/c than C57BL/6 mice, with or without IL-10 (**Figure 4.1**, and **Table 4.1**). A preliminary experiment assessing LPS stimulation of cells derived from C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice on days 10 and 9 of culture, respectively, showed that LPS increased the percentage of CD11c(+)/MHC II^{high} cells in both mouse strains, although the increase was proportionately greater in cells from C57BL/6 IL-10$^{-/-}$ than BALB/c IL-10$^{-/-}$ mice (data not shown).

CD11c, MHC II, and F4/80 Expression in BMDCs Derived from IL-10$^{-/-}$ Mice. Flow cytometry was performed on cells derived from C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice used in the 3 independent cytokine experiments. Plots from one experiment are shown in **Figure 4.2**. Percentages of CD11c(+) cells, CD11c(+)/F4/80(+) cells, CD11c(+)/MHC II^{high} cells, CD11c(+)/MHC II^{low} cells, and MHC II MFI were analyzed by t-test, following transformation of percentage data. Descriptive statistics (mean ± SD) and results of statistical analyses are given in **Table 4.1**. Cells derived from C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice did not differ significantly in percentage of CD11c(+) cells (P = 0.111), or CD11c(+)/MHC II^{high} cells (P = 0.063). However, C57BL/6 IL-10$^{-/-}$ mice had significantly more CD11c(+)/MHC II^{low} cells than BALB/c IL-10$^{-/-}$ mice (57% versus 26% respectively; P = 0.03). Differences were also identified between mouse strains in proportion of macrophages in the differentiated populations, indicated by CD11c and F4/80 double positivity. Although not statistically significant, C57BL/6 IL-10$^{-/-}$ mice trended toward producing higher percentages of CD11c(+)/F4/80(+) cells than BALB/c IL-10$^{-/-}$ mice (26% versus 16% respectively; P = 0.054). Despite a trend toward lower numbers of macrophages in BALB/c IL-10$^{-/-}$ cultures, the

difference in MHC II MFI of CD11c(+)/F4/80(+) cells was significant, with BALB/c IL-10$^{-/-}$ mice having higher MHC II MFIs in this population than C57BL/6 IL-10$^{-/-}$ mice (1,169 versus 557 respectively; P = 0.05). Finally, a significant difference was found (P < 0.001) in the MFI of MHC II within all MHC II(+) cells, with BALB/c IL-10$^{-/-}$ cells exhibiting higher expression than C57BL/6 IL-10$^{-/-}$ populations (11,933 versus 3,202 respectively). These data show that there may be subtle differences in the differentiation of stem cells derived from bone marrow of C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice with regard to proportion of macrophages and expression of MHC II. The differentiated cells are considered to comprise a majority of DCs with fewer macrophages, and are referred to as BMDCs with acknowledgement that lower numbers of macrophages are also present.

Invasion Assays: BMDCs Derived from IL-10$^{-/-}$ Mice. Individual experiments assessing combined invasion efficiency and intracellular survival of the *C. jejuni* strains within BMDCs derived from IL-10$^{-/-}$ mice on a C57BL/6 or BALB/c genetic background were performed with 1, 2, 3, and 23 hours of infection prior to application of gentamicin. Statistically analyzed experiments are described in **Tables 4.2A-D**. One representative, independent experiment from each time point that includes both *C. jejuni* strains is shown graphically (**Figure 4.3**). Because each time point was assessed in a separate experiment, differences in *C. jejuni* recovery over time could not be assessed statistically.

C. jejuni *Strain Differences*. No statistically significant difference was identified between recovery of *C. jejuni* 11168 and *C. jejuni* 260.94 after 1 hour of infection time (P = 0.466, **Table 4.2A**). However, significantly more *C. jejuni* 11168 than *C. jejuni* 260.94 was recovered from BMDCs derived from both IL-10$^{-/-}$ mouse genotypes after 2, 3, and 23 hours of infection time (P ≤ 0.001 for each time point; repeatable between independent experiments; **Tables 4.2B-D**). The magnitude of difference between *C. jejuni* strain recoveries became more pronounced with increased infection time (**Figure 4.3**).

Mouse Genotype Differences. A statistically significant difference in *C. jejuni* recovery was identified between BMDCs derived from the two mouse genotypes after 1 hour of infection time, with more of both *C. jejuni* strains recovered from BMDCs obtained from C57BL/6 IL-10$^{-/-}$ than BALB/c IL-10$^{-/-}$ mice (P = 0.003; **Table 4.2A**). Significantly more *C. jejuni* 11168 but not 260.94 was similarly recovered from C57BL/6 IL-10$^{-/-}$ than BALB/c IL-10$^{-/-}$ BMDCs infected for 3 hours (P = 0.002; **Table 4.2C**). There was a trend of more *C. jejuni* 260.94 recovered from BALB/c IL-10$^{-/-}$ than C57BL/6 IL-10$^{-/-}$ BMDCs after 23 hours of infection (**Table 4.2D**), but the difference was not statistically significant in both experiments and repeatable mouse genotype differences in *C. jejuni* 260.94 were not identified at other time points.

Collectively, these data show that *C. jejuni* 11168 has higher invasion efficiency and/or intracellular survival compared to *C. jejuni* 260.94, beginning at 2 hours of infection and becoming more marked with increased infection time. A mouse genotype difference was identified early in infection, but with the exception of differences in *C. jejuni* 11168 recovery between genotypes at 3 hours of infection, a clear difference in mouse genotypes was not found at later time points.

Intracellular Survival Assays: BMDCs Derived from WT and IL-10$^{-/-}$ Mice. Experiments assessing intracellular survival of the *C. jejuni* strains within BMDCs derived from WT and IL-10$^{-/-}$ mice on a C57BL/6 or BALB/c genetic background were performed with 1 hour of infection prior to gentamicin application. Cells were then washed and incubated with fresh medium until lysis and enumeration of *C. jejuni* CFU at 24 hours p.i. Experiments involving BMDCs from WT and IL-10$^{-/-}$ mice were performed separately. Details of the experiments and statistical results are described in **Table 4.3A** (WT mice) and **Table 4.3B** (IL-10$^{-/-}$ mice). Results of one representative experiment with WT BMDCs and both *C. jejuni* strains are shown in **Figure 4.4A**, and results of one representative experiment with IL-10$^{-/-}$ BMDCs and both *C. jejuni* strains are shown in **Figure 4.4B**. The experiments are combined graphically in **Figure 4.4C**.

C. jejuni *Strain Differences*. In BMDCs derived from WT mice, significantly more *C. jejuni* 11168 was recovered than *C. jejuni* 260.94 ($P < 0.001$) within BMDCs derived from both mouse genotypes (**Table 4.3A**, **Figure 4.4A**). A similar pattern was identified in IL-$10^{-/-}$ mice, with significantly more *C. jejuni* 11168 recovered than *C. jejuni* 260.94 ($P < 0.001$); however, this *C. jejuni* strain difference was only significant in BMDCs from C57BL/6 IL-$10^{-/-}$ mice, while BALB/c IL-$10^{-/-}$ BMDCs showed a nonsignificant trend toward higher *C. jejuni* 11168 than 260.94 recovery (**Table 4.3B**, **Figure 4.4B**).

Mouse Genotype Differences. No difference in *C. jejuni* recovery between cells derived from WT C57BL/6 and WT BALB/c mice was identified in the experiment shown in **Figure 4.4A** ($P = 0.637$; **Table 4.3A**). This experiment showing no difference in genotype was chosen as representative in part because of conflicting results for *C. jejuni* 11168 recovery between genotypes in the other two experiments (**Table 4.3A**). In contrast, a genotype effect was found to be significant ($P < 0.001$; **Table 4.3B**) in cells derived from IL-$10^{-/-}$ mice, with significantly more *C. jejuni* 11168 recovered from C57BL/6 IL-$10^{-/-}$ than BALB/c IL-$10^{-/-}$ derived BMDCs (**Figure 4.4B**). Recovery of *C. jejuni* 260.94 was not significantly different between mouse genotypes, as virtually no *C. jejuni* 260.94 CFU were recovered from BMDCs of either C57BL/6 IL-$10^{-/-}$ or BALB/c IL-$10^{-/-}$ mice in either experiment (**Table 4.3B**).

Taken together, these data show that *C. jejuni* 11168 exhibits enhanced intracellular survival within BMDCs compared to *C. jejuni* 260.94. This was observed in cells derived from both WT and IL-$10^{-/-}$ mice on both C57BL/6 and BALB/c genetic backgrounds. A difference in *C. jejuni* recovery between mouse genotypes was observed in recovery of *C. jejuni* 11168 only in BMDC derived from IL-$10^{-/-}$ mice. Although *C. jejuni* survival cannot be compared statistically between WT and IL-$10^{-/-}$ derived cells, the patterns suggest that *C. jejuni* exhibits reduced survival capacity within IL-$10^{-/-}$ cells, a difference that was more marked in *C. jejuni* 260.94.

Cytokine Production: BMDCs Derived from IL-10$^{-/-}$ Mice. Three independent experiments were performed to measure pro-inflammatory and Th-polarizing cytokine production by BMDCs derived from C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 or *C. jejuni* 260.94. Statistical results of the three experiments are detailed in **Table 4.4** (Experiment 1), **Table 4.5** (Experiment 2), and **Table 4.6** (Experiment 3). Results of Experiment 2 are displayed graphically (**Figure 4.5**) and described below.

Levels of neither IFN-γ nor TGF-β were significantly different between any treatment groups, including negative (medium only) and positive (LPS) controls, in BMDCs derived from either C57BL/6 IL-10$^{-/-}$ or BALB/c IL-10$^{-/-}$ mice. Production of pro-inflammatory TNF-α was significantly increased in BMDCs derived from both C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94, but not *C. jejuni* 11168, compared to the negative control. The Th1-polarizing cytokine IL-12 was not significantly increased in BMDCs derived from either mouse genotype infected with either *C. jejuni* 11168 or 260.94; however, BMDCs derived from C57BL/6 IL-10$^{-/-}$ mice exhibited a significantly higher baseline production of IL-12 in uninfected cells compared to BMDCs derived from BALB/c IL-10$^{-/-}$ mice. Similarly, BMDCs derived from C57BL/6 IL-10$^{-/-}$ mice exhibited a significantly higher baseline production of the Th2-polarizing cytokine IL-4, compared to BMDCs derived from BALB/c IL-10$^{-/-}$ mice. IL-4 production was significantly decreased in C57BL/6 IL-10$^{-/-}$ BMDCs infected with *C. jejuni* 11168 compared to the negative control; in contrast, *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ BMDCs trended toward increased production of IL-4 compared to the negative control. BMDCs from C57BL/6 IL-10$^{-/-}$ mice showed significantly increased MCP-1 production following exposure to LPS and infection with *C. jejuni* 260.94 compared to BALB/c IL-10$^{-/-}$ BMDCs receiving the same treatments. Within C57BL/6 IL-10$^{-/-}$ BMDCs, production of MCP-1 in *C. jejuni* 260.94-infected cells was significantly increased compared to exposure to medium only or infection with *C. jejuni* 11168; infection with either *C. jejuni* strain did not lead to significantly increased MCP-1 levels in infected BALB/c IL-10$^{-/-}$ BMDCs. Finally, IL-6 production was more marked in

BALB/c IL-10$^{-/-}$ than C57BL/6 IL-10$^{-/-}$ BMDCs: BALB/c-IL-10$^{-/-}$-derived cells exposed to LPS or infected with *C. jejuni* 11168 showed significantly higher IL-6 levels than C57BL/6 IL-10$^{-/-}$ cells receiving these treatments. Additionally, production of IL-6 following infection with *C. jejuni* 11168 was significantly increased in BALB/c IL-10$^{-/-}$ BMDCs compared to the negative control, but the difference was not significant in C57BL/6 IL-10$^{-/-}$ cells. Collectively, these data indicate that production of pro-inflammatory and Th-polarizing cytokines by *C. jejuni*-infected IL-10$^{-/-}$ BMDCs is determined by both infecting *C. jejuni* strain and mouse genetic background.

DISCUSSION

This study was conducted to evaluate the role of dendritic cells in initiation of innate and adaptive immunity following *C. jejuni* infection. The model was designed to elucidate the potential impacts of both *C. jejuni* strain characteristics and host genetic background on early DC-mediated determinants of eventual disease outcome, such as colitis or development of GBS following *C. jejuni* infection. Results of this study suggest that differentiation of cultured bone marrow stem cells into dendritic cells varies between C57BL/6 and BALB/c mice, most notably in MHC II expression and proportion of macrophages present. The colitogenic *C. jejuni* 11168 strain displayed enhanced invasion efficiency and intracellular survival compared to the Guillain-Barré syndrome patient-derived strain *C. jejuni* 260.94 in cultured BMDCs, while differences in *C. jejuni* uptake or killing were less consistent between mouse genotypes. Cytokine production by infected IL-10$^{-/-}$ BMDCs varied by both mouse genotype and infecting *C. jejuni* strain: a more marked response in MCP-1 from C57BL/6 IL-10$^{-/-}$ cells was seen, especially following infection with *C. jejuni* 260.94, while IL-6 production was more pronounced in BALB/c IL-10$^{-/-}$ cells, especially when infected with *C. jejuni* 11168.

Flow cytometric analysis of BMDCs on days 9, 10, and 11 of culture from WT and IL-10$^{-/-}$ mice on both C57BL/6 and BALB/c backgrounds was performed with the original intent of verifying the BMDC

phenotype by CD11c and MHC II expression. These analyses revealed unexpected variation in differentiated populations related to genetic background, and assessment of F4/80 expression was subsequently evaluated. While not different from BALB/c IL-10$^{-/-}$ cells in overall CD11c positivity, cells derived from C57BL/6 IL-10$^{-/-}$ mice trended toward fewer CD11c(+)/MHC II^{high} cells, with correspondingly more CD11c(+)/MHC II^{low} cells, and a greater proportion of CD11c(+)/F4/80(+) cells in differentiated populations compared to BALB/c IL-10$^{-/-}$ cells. Macrophages can be found intermixed with immature and mature DCs in populations of bone marrow stem cells cultured with GM-CSF,[24; 38] and the proportion of macrophages compared to DCs generated in culture can be increased with higher concentrations of GM-CSF.[45] However, to our knowledge, differences in proportions of macrophages in DC populations from the bone marrow of C57BL/6 and BALB/c mice cultured by the same method has not been previously reported. Subjective observations of morphology during differentiation are consistent with discrepancies in MHC II and F4/80 expression identified by flow cytometry. Both WT and IL-10$^{-/-}$ C57BL/6 cells appeared to have a relatively greater proportion of larger, firmly adherent cells (macrophage type morphology) and a relatively smaller proportion of smaller, rounder, semi-adherent cells (dendritic cell type morphology),[38] compared to both WT and IL-10$^{-/-}$ BALB/c cultures. Although CD11c expression was until recently or is still used to distinguish DCs from macrophages,[44] data from the current study corroborate the relatively recent realization that CD11c expression is not limited to DCs; F4/80+ monocytes in the intestine also acquire CD11c expression during maturation to macrophages, while retaining F4/80 expression.[29]

The other notable difference between cultured populations derived from WT and IL-10$^{-/-}$ mice on both C57BL/6 and BALB/c backgrounds was the higher expression of MHC II in BALB/c populations. As MHC II expression increases with DC maturation, this likely reflects greater numbers of more mature DCs in BALB/c than C57BL/6 cultures. This finding is inconsistent with that reported for DCs derived from spleens of naïve C57BL/6 and BALB/c mice, in which C57BL/6 mice contained more mature DC subsets

based upon expression of CD40 and Stat4.[35] This discrepancy may be attributable to isolation methods and origin of DC, as culture of hematopoietic precursors from the marrow is likely to produce DCs with a different phenotype than those already fully differentiated in a naïve spleen. Differences in diet or microbiome may also contribute.

In the current study, a pattern in which four smaller populations can be distinguished within WT and IL-10$^{-/-}$ C57BL/6 CD11c(+) cells, while two distinct larger populations are evident in WT and IL-10$^{-/-}$ BALB/c CD11c(+) cells (**Figure 4.1**), was repeatedly observed. Considering the F4/80 and MHC II expression together with the patterns exhibited in representative scatter plots, we speculate that bone marrow precursors from the two mouse genotypes exhibit varying kinetics of maturation under the influence of GM-CSF. F4/80 and MHC II expression suggest that populations from C57BL/6 IL-10$^{-/-}$ mice develop a higher proportion of macrophages and immature DCs in varying stages of maturation, while BALB/c IL-10$^{-/-}$ cells differentiate with a higher proportion of mature DCs following 10 days of culture with GM-CSF. Haase *et al* (2002) similarly found a predominance of immature BMDCs derived from C57BL/6 mice in unstimulated day 8 cultures; these cells responded to LPS stimulation with a conversion to MHC II expression in nearly all cells.[20] In the current study, results of a preliminary experiment showed relatively greater upregulation of MHC II expression in BMDCs derived from C57BL/6 IL-10$^{-/-}$ than BALB/c IL-10$^{-/-}$ mice following stimulation with LPS. This finding also supports a higher baseline of more mature DCs in cultures from BALB/c IL-10$^{-/-}$ mice, but further replicates of this experiment are warranted to confirm this result.

In populations of differentiated but unstimulated cells from C57BL/6 and BALB/c mice, the presence or absence of IL-10 did not markedly affect CD11c or MHC II expression (**Figure 4.1**). Previous *in vitro* studies have demonstrated potent effects of IL-10 on DC maturation, cytokine secretion, and T-cell stimulating ability. Neutralization of IL-10 resulted in enhanced maturation and TNF-α and IL-12 production by LPS-stimulated human peripheral blood monocyte-derived DC.[11] Furthermore, these DCs

exhibited spontaneous maturation *in vitro* in the absence of IL-10.[11] Similarly, administration of IL-10 effectively inhibited LPS-induced DC maturation, production of pro-inflammatory cytokines, and ability to drive T cell proliferation in BMDCs derived from C57BL/6 mice.[20] These studies indicate that IL-10 has potent inhibitory effects on DC maturation and T-cell stimulating ability following LPS stimulation. It is possible that a difference in MHC II expression between WT and IL-10$^{-/-}$ BMDCs could be induced with LPS or other stimulation, but in unstimulated cells the presence or absence of IL-10 did not affect MHC II expression.

Because DCs are potent antigen presenting cells and play a major role in initiation of the adaptive immune response through T cell activation, we next wanted to determine the effects of *C. jejuni* strain characteristics and host genetic background in the initial *C. jejuni*-DC interaction. The GKA was used to test the original hypothesis that BMDCs from WT and IL-10$^{-/-}$ mice on both C57BL/6 and BALB/c genetic backgrounds would efficiently internalize and kill both *C. jejuni* strains; this method was chosen as a way to identify broad patterns in invasion and survival for further study in more detail.

In assays with increasing infection time of IL-10$^{-/-}$ BMDCs, the colitogenic *C. jejuni* 11168 showed enhanced combined invasion efficiency and intracellular survival compared to GBS patient-derived *C. jejuni* 260.94, beginning at 2 hours of infection time and becoming more marked as infection time increased up to 23 hours (**Figure 4.3**). Recovery of viable intracellular *C. jejuni* 11168 from IL-10$^{-/-}$ BMDCs was not significantly different than that of *C. jejuni* 260.94 when the infection time of 1 hour was followed by incubation with gentamicin and immediate lysis. In addition to enhanced invasion and/or intracellular survival, the possibility that *C. jejuni* 11168 may survive and replicate more efficiently than *C. jejuni* 260.94 in the extracellular medium or *in vivo* in the host, producing more bacteria over a longer time frame capable of invading host cells, cannot be excluded.

Intracellular survival assays were performed with gentamicin treatment following 1 hour of infection and enumeration of viable intracellular *C. jejuni* at 24 hours p.i. In BMDCs derived from both

C57BL/6 WT and BALB/c WT mice, both *C. jejuni* strains were viable intracellularly at 24 hours p.i. with *C. jejuni* 11168 exhibiting significantly increased intracellular survival compared to *C. jejuni* 260.94 (**Figure 4.4A**). Significantly higher recovery of *C. jejuni* 11168 than *C. jejuni* 260.94 was also found in C57BL/6 IL-$10^{-/-}$ BMDCs (**Figure 4.4B**). There was not a significant difference in viable *C. jejuni* 11168 versus 260.94 strains in the 1 hour invasion assay in IL-$10^{-/-}$ cells (**Figure 4.3**). Therefore, higher recovery of *C. jejuni* 11168 after the same invasion time but at 24 hours p.i. suggests that intracellular survival and perhaps replication contributed to increased recovery of *C. jejuni* 11168 over time in the invasion assays. Assays of increasing infection time in WT cells, as performed with IL-$10^{-/-}$ BMDCs, would be required to further characterize *C. jejuni* invasion and survival in WT cells.

In contrast to intracellular survival within WT cells, virtually no *C. jejuni* 260.94 survived until 24 hours p.i. in BMDCs from IL-$10^{-/-}$ mice of either genotype in either of two replicates (**Table 4.3B**). However, intracellular survival of *C. jejuni* 11168 was significantly higher than that of *C. jejuni* 260.94 only in C57BL/6 IL-$10^{-/-}$ derived cells (**Figure 4.4B**). While more *C. jejuni* 11168 than 260.94 was recovered from BALB/c IL-$10^{-/-}$ BMDCs, the difference was not significant. Possible explanations for the discrepancy in survival between *C. jejuni* strains in IL-$10^{-/-}$ BMDCs from C57BL/6 vs. BALB/c mice include enhanced killing of *C. jejuni* 11168 or relatively fewer internalized *C. jejuni* organisms during the invasion period in BALB/c IL-$10^{-/-}$ BMDCs. The latter explanation is supported by the 1 hour invasion efficiency assay, in which significantly more *C. jejuni* was recovered in BMDCs from C57BL/6 IL-$10^{-/-}$ than BALB/c IL-$10^{-/-}$ mice (**Figure 4.3**). These observations are consistent with a higher proportion of mature DCs and fewer macrophages in BALB/c IL-$10^{-/-}$ populations, leading to decreased phagocytic efficiency.

Representative experiments of one intracellular survival assay using WT cells, and the other using IL-$10^{-/-}$ cells, are graphed together in **Figure 4.4C**.The patterns of viable intracellular *C. jejuni* recovered suggest that IL-10 deficiency decreases *C. jejuni* survival within BMDCs, although statistical comparisons between WT and IL-$10^{-/-}$ cells could not made as experiments were performed

independently. A pattern of increased killing of *C. jejuni* in the absence of IL-10 is consistent with reported enhancement of DC function, including expression of costimulatory molecules and production of TNF-α and IL-12, in human LPS-treated DCs following IL-10 neutralization.[11] Experiments designed specifically to test the impact of IL-10 on killing ability of BMDCs are warranted, especially considering the severity of disease in IL-10$^{-/-}$ compared to WT mice infected with *C. jejuni* 11168.[40; 41] The possibility that enhanced killing of *C. jejuni in vivo* by DCs in IL-10$^{-/-}$ mice contributes to exacerbated immune responses warrants further exploration.

While significant differences were consistently found between *C. jejuni* strain recoveries, a difference between C57BL/6 and BALB/c mice was not consistent in all replicates at all time points (**Tables 4.2A-4.2D** and **Tables 4.3A-4.3B**). Significantly more *C. jejuni* was recovered from C57BL/6 IL-10$^{-/-}$ than BALB/c IL-10$^{-/-}$ derived BMDCs following 1 hour of invasion. Other repeatable genotype differences were seen only following 3 hours' invasion time and in intracellular survival assays involving IL-10$^{-/-}$ BMDCs; in both cases, significantly more viable *C. jejuni* 11168 was enumerated in C57BL/6 IL-10$^{-/-}$ than BALB/c IL-10$^{-/-}$ cells. Possible explanations for these results include the enhanced intracellular survival shown by *C. jejuni* 11168, combined with the higher proportion of macrophages and immature DCs in C57BL/6 IL-10$^{-/-}$compared to BALB/c IL-10$^{-/-}$ cultures. While both macrophages and DCs are antigen presenting cells, macrophages are more efficient phagocytes while DCs specialize in processing the antigen in preparation for presentation to T cells. Indeed, bone marrow-derived MHC II^{low}F4/80^{high} macrophages generated along with MHC II^{high}F4/80^{low} DCs using GM-CSF demonstrated superior phagocytosis of latex beads compared to DCs.[45] C57BL/6 IL-10$^{-/-}$ BMDCs with a higher proportion of macrophages may have enhanced phagocytosis, and BALB/c IL-10$^{-/-}$ BMDCs with more mature DCs may lead to less efficient phagocytosis but increased killing. Therefore, increased recovery of the *C. jejuni* strain with enhanced intracellular survival, and also perhaps higher invasion efficiency, from cultured cells with a higher proportion of cells characterized by higher phagocytosis but less efficient killing,

represents key differences in characteristics of both *C. jejuni* strains and of populations of cultured cells in this model system. These findings suggest that the original hypothesis that both *C. jejuni* strains would be efficiently internalized and killed by BMDCs from both mouse genotypes should be rejected, as complexities in the host-microbe interaction preclude this predicted clear-cut result.

In vitro assays reported in the literature evaluating invasion and survival of *C. jejuni* within cultured cells have varied substantially, including differences in cultured cell types, *C. jejuni* strains, use and concentration of gentamicin, MOI, and infection times.[4; 27] Therefore, unsurprisingly, a variety of results have been reported concerning ability of *C. jejuni* to survive within phagocytic cells derived from mice and humans. Several studies have reported efficient killing of *C. jejuni* by macrophages and DCs. *C. jejuni* 81-176 was killed by mouse bone marrow-derived macrophages within 24 hours,[60] and by human monocyte-derived DCs over 24-48 hours.[23] Similarly, no *C. jejuni* 11168 was recovered from BMDCs derived from WT C57BL/6 mice after 8 hours.[49] The last finding is inconsistent with our results (**Table 4.3A**, **Figure 4.4A**). This difference could be attributed to methodological variations or the time in which BMDCs were lysed and viable intracellular *C. jejuni* enumerated. That *C. jejuni* was present intracellularly in undetectable amounts by culture at 8 hours, but replicated sufficiently to allow detection at 24 hours, is a viable explanation; *C. jejuni* surviving within human macrophages began to multiply after 8 hours in one study.[58]

Similar to results of the current study, *C. jejuni* also has been reported to avoid intracellular killing, even up to several days. The majority of macrophages derived from human peripheral blood monocytes killed multiple strains of phagocytized *C. jejuni*, although ineffective killing and intracellular multiplication in macrophages derived from approximately 10% of donors was observed.[58] Despite efficient phagocytosis, *C. jejuni* enteritis strains persisted within both BALB/c murine macrophages and human blood monocytes for 6 days,[31] a finding confirmed with *C. jejuni* 81-176 in the human monocyte 28SC line.[22] Persistent intracellular survival has implications for transit to other organs *in vivo*.

While methodological variation should not be discounted, discrepant reports in the literature regarding the intracellular fate of *C. jejuni* used in *in vitro* infection studies undoubtedly also result from infecting *C. jejuni* strain characteristics and type of cultured cell. To our knowledge, BMDCs from IL-10$^{-/-}$ mice on different genetic backgrounds have not been used to assess invasion and survival differences between *C. jejuni* strains associated with different disease outcomes. In the current study, the GKA was employed in two different ways to identify broad patterns of *C. jejuni* invasion efficiency and intracellular survival in BMDCs. A secondary goal was to determine if invasion and survival correlate with *in vivo* murine *C. jejuni* infection models, as studies reporting associations between invasiveness and clinical disease have shown conflicting results. No correlation was found between *C. jejuni* invasion and survival in murine macrophages and development of intestinal lesions in piglets.[34] While invasive but non-inflammatory diarrhea-associated *C. jejuni* and *C. coli* isolates did not differ from colitis strains in internalization into HeLa cells, a higher number of colitis strains exhibited transcytosis through polarized Caco-2 monolayers than non-inflammatory strains.[14] Association of *C. jejuni* and *C. coli* strains with HeLa cells correlated with symptoms of febrile diarrhea in patients, although association was not related to blood in the feces.[15] Interestingly, *C. jejuni* mutant strains with deficiencies in formic acid metabolism shown to have reduced invasion capacity *in vitro* were able to colonize mice in similar abundance to the parent WT strain B2, but did not induce immunopathology in the colon as did the WT strain.[7]

In the current study, GKA results indicate superior invasion efficiency and intracellular survival of colitogenic *C. jejuni* 11168 compared to GBS-associated *C. jejuni* 260.94. These results are consistent with identification of several virulence-associated genes in *C. jejuni* 11168 with putative functions involving adherence, colonization, invasion, acid resistance, LOS, and motility that are either divergent or altogether absent in *C. jejuni* 260.94.[6] *C. jejuni* 11168 also showed increased invasion into cultured young adult mouse colon (YAMC) epithelial cells compared to *C. jejuni* 260.94, although the difference was relatively smaller compared to differences between these strains and *C. jejuni* HB-9313.[39] The

current GKA results also support findings in a BALB/c IL-10$^{-/-}$ mouse infection model, in which immunohistochemical labeling of *C. jejuni* in the ileocecocolic junction of sham-inoculated mice and those infected with *C. jejuni* 11168 or 260.94 strains was used to discern invasiveness *in vivo*. In this study, 9/10 *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice exhibited positive intracellular labeling within macrophage/dendritic cell types within the submucosa, in contrast to 3/10 mice in each of the sham-inoculated and *C. jejuni* 260.94 groups. Similarly, 4/10 *C. jejuni* 11168-infected BALB/c IL-10$^{-/-}$ mice, but no mice in other treatment groups, exhibited intracellular labeling within macrophage/dendritic cell types within the lamina propria. In addition to heightened intracellular labeling of *C. jejuni* 11168 in the lamina propria and submucosa, BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 exhibited the most severe colitis and lowest survivorship (Brudvig *et al*., unpublished; Chapter 3). These studies suggest that patterns observed *in vitro* mimic *in vivo* interactions, and suggest that BMDCs are a useful *in vitro* model for further study of the initial host-microbe interaction in *C. jejuni* infection.

A final objective of this study was to address the hypothesis that cytokines produced by *C. jejuni*-infected BMDCs derived from IL-10$^{-/-}$ mice would mirror immunogenicity previously observed in our IL-10$^{-/-}$ mouse models: *C. jejuni* 11168-infected IL-10$^{-/-}$ BMDCs from both mouse genotypes and BALB/c IL-10$^{-/-}$ BMDCs infected with *C. jejuni* 260.94 would produce pro-inflammatory and Th1/Th17-polarizing cytokines, while C57BL/6 IL-10$^{-/-}$ BMDCs infected with *C. jejuni* 260.94 would produce Th2-polarizing cytokines. Production of pro-inflammatory and Th-polarizing cytokines was assessed by a multiplexed flow cytometry-based bead assay (**Figure 4.5**).

The most striking findings included enhanced production of MCP-1 by C57BL/6 IL-10$^{-/-}$ cells, especially those infected with *C. jejuni* 260.94. Because the chemokine MCP-1 is thought to contribute to Th2 polarization,[19] this finding supports the Th2-mediated immune response of C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 reported previously.[39] It was previously shown that splenic-derived DC from WT BALB/c mice produced more MCP-1 than those from WT C57BL/6 mice when exposed to TLR-2,

-4, and -9 ligands.[35] This finding contrasts those in the current study, in which neither *C. jejuni* strain elicited significant MCP-1 production from BALB/c IL-10$^{-/-}$ mice. This discrepancy may be due to different DC subsets and stimulating ligands between these studies, or the absence of IL-10 in the current study. Additionally, because monocytes and macrophages are a main cellular source of MCP-1, the increased production by C57BL/6 IL-10$^{-/-}$ cells may reflect the higher number of macrophages in the culture.

BALB/c-derived IL-10$^{-/-}$ BMDCs infected with *C. jejuni* 11168 exhibited significantly higher production of IL-6 compared to the negative control, and also to *C. jejuni* 11168-infected C57BL/6 IL-10$^{-/-}$ derived cells. IL-6 has pro-inflammatory effects and is critical in polarization of Th17 cells.[8] This finding thus suggests that early events including the initial interaction of *C. jejuni* with DCs in the gut contribute to Th17 responses seen *in vivo*, including production of plasma anti-*C. jejuni* IgG2b and increased colonic IL-17 and IL-22 in BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 (Brudvig *et al*, unpublished; Chapter 3).

The enhanced production of TNF-α in *C. jejuni* 260.94, but not *C. jejuni* 11168, infected IL-10$^{-/-}$ BMDCs from either mouse genotype in this experiment was surprising. Enteritis isolates *C. jejuni* 81-176 and *C. jejuni* 11168 both induced robust TNF-α production in human monocyte-derived DC and murine BMDCs, respectively.[23; 49] TNF-α is a pro-inflammatory cytokine and the lack of significant production with *C. jejuni* 11168 infection was unexpected, considering the heightened inflammatory responses induced in *in vivo* IL-10$^{-/-}$ mouse models by *C. jejuni* 11168 compared to *C. jejuni* 260.94.[6; 39] Possible explanations for the lack of robust production in *C. jejuni* 11168-infected IL-10$^{-/-}$ BMDCs include timing of collection of supernatants following infection, as perhaps without IL-10, maximal increases in TNF-α production stimulated by *C. jejuni* 11168 occur earlier or later than 24 hours p.i. as assessed in the current study.

Because C57BL/6 mice have been reported to exhibit a Th1 immunological bias, production of IL-12p70 was of particular interest. In contrast to previous studies indicating that *C. jejuni* elicits robust

IL-12p70 production from infected DCs,[23; 49] in the current study neither LPS nor either *C. jejuni* strain elicited significant IL-12 production in IL-10$^{-/-}$ BMDCs from either mouse genotype at 24 hours p.i. IL-12p70 concentration was significantly higher only in uninfected (negative control) C57BL/6 IL-10$^{-/-}$ than BALB/c IL-10$^{-/-}$ BMDCs, suggesting a higher baseline production of IL-12 in C57BL/6 IL-10$^{-/-}$ mice and also complicating interpretation of responses in other treatment groups. IL-12p70, a heterodimer comprising p35 and p40 subunits, was chosen for measurement as the bioactive form of IL-12. Interestingly, LPS and IFN-γ stimulated blood monocytes isolated from human sepsis patients showed higher production of regulatory IL-12p40 and lower IL-12p70 production; the authors concluded that the balance between the two forms of IL-12 may represent a check on excessive inflammation.[13] A possible explanation for the unexpected lack of production of bioactive IL-12p70 by infected IL-10$^{-/-}$ BMDCs is that, in the absence of IL-10, kinetics of production are altered or other immunoregulatory checks such as enhanced regulatory IL-12p40 production are activated to prevent uncontrolled inflammation.

Production of IL-4 was also of particular interest as BALB/c mice are reported to have a Th2 immunological bias. IL-4 is a signature cytokine produced by Th2 cells, but IL-4 itself is also thought to be a key differentiation factor for Th2 cells.[57] However, IL-4 is inconsistently produced by DCs and there is now evidence that other factors, such as affinity of T cell receptor in antigen presentation and cytokines produced by cells other than DCs, contribute to Th2 polarization.[57] Nevertheless, IL-4 was included in the panel to determine if IL-10$^{-/-}$ BMDCs infected with *C. jejuni*, especially strain 260.94, would elicit IL-4 production. As with IL-12p70 production, infection with neither *C. jejuni* strain stimulated significant IL-4 production in IL-10$^{-/-}$ BMDCs from either C57BL/6 or BALB/c mice at 24 hours p.i. Surprisingly, baseline production in uninfected IL-10$^{-/-}$ BMDCs was significantly higher in C57BL/6 than BALB/c cells. As with IL-12p70, the higher IL-4 production in uninfected C57BL/6 IL-10$^{-/-}$ BMDCs complicates interpretation of the response in other treatment groups. The lack of IL-4 production in uninfected BALB/c IL-10$^{-/-}$ cells suggests that in this model, absence of IL-10 dampens the reported Th2 bias in BALB/c mice. In C57BL/6

IL-10$^{-/-}$ BMDCs, infection with *C. jejuni* 11168 resulted in significant suppression of IL-4 production, supporting the earlier finding that this strain preferentially drives Th1/Th17 instead of Th2 responses in C57BL/6 IL-10$^{-/-}$ mice.[39]

The original hypotheses concerning cytokine production were formulated based upon systemic and local colonic immune responses seen in previous mouse *C. jejuni* infection models. As C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 exhibited primarily Th2 responses,[39] the enhanced production of MCP-1 from BMDCs of C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 suggests that production of this cytokine by DCs may contribute to early polarization of the immune response. Similarly, Th17 responses were observed in BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 (Brudvig *et al*, unpublished, Chapter 3), suggesting that early production of IL-6 by infected DCs likewise contributes to polarization of adaptive immunity.

Although novel in many aspects, this study has several limitations and presents numerous opportunities for future research. While no striking differences between differentiation of BMDCs in WT and IL-10$^{-/-}$ mice were seen, further replicates with flow cytometric analysis especially on days 9, 10, and 11 of culture when the cells are used experimentally would allow more complete evaluation. Furthermore, while no difference was obvious in unstimulated cells, treatment of WT and IL-10$^{-/-}$ cells from both mouse genotypes with LPS would further elucidate the effects of IL-10 on maturation, including expression of costimulatory CD40, CD80, and CD86 molecules and MHC II, and function including cytokine production. Increasing the number of replicate experiments assessing expression of CD11c, MHC II, and F4/80 in C57BL/6 and BALB/c cells, and including a wider time frame of differentiation such as days 6-12 of culture, would help confirm or refute the hypothesis that C57BL/6 cultures represent a more diverse population of differentiating cells. Finally, the realization that differentiated BMDCs comprise different populations of DCs and macrophages between mouse genotypes complicates comparison of invasion and survival data and cytokine production between

mouse genotypes in this model, yet likely also represents how host genetics combined with *C. jejuni* strain variations can induce different host responses.

The GKAs used in this study were designed to detect major differences in invasion efficiency and intracellular survival, accounting for both *C. jejuni* strain characteristics and host genetic background. In order to statistically evaluate both *C. jejuni* strain and mouse genotype as factors, a comparable MOI had to be attained for both *C. jejuni* strains in the same assay. While spectrophotometry allows estimation of CFU/mL in culture, achieving the desired OD_{560} did not consistently yield the predicted CFU when serially diluted and plated. This may have been due to several factors, including clumping together of the *C. jejuni* cells by their flagella, despite vortexing. Additionally, *C. jejuni* cultures were prepared in RPMI-based medium. The blank measurement used in determining OD of the *C. jejuni* culture was observed to drift over repeated readings, likely due to color change of medium upon exposure to air, and this may have led to a skewed OD of the culture. Difficulty in achieving precisely 2×10^8 CFU/mL for both *C. jejuni* cultures in all assays for an MOI of 100 led to inclusion of a broader range of approximate MOI (60-175) for the results presented in the body of the dissertation (**Tables 4.2A-D** and **Tables 4.3A-B**). The number of replicates at each time point is also a limitation; additional replicates would have strengthened statistical conclusions and reinforced observed patterns. [Additional experiments assessing intracellular survival using BMDCs derived from both WT and IL-$10^{-/-}$ mice were performed following completion of experiments included in the body of the dissertation. Results from these additional replicates are not discussed, but are presented as supplemental data in Appendix B, **Table A.1**.] However, numbers of *C. jejuni* recovered between replicates were fairly consistent despite lack of an MOI of precisely 100, and use of the GKA served the purpose of identifying broad patterns in invasion and survival.

Statistical analyses also were limited by the experimental designs, which were complicated by logistical limitations. Each individual time point was assessed by independent experiments; ideally, *C.*

jejuni recovery over time would have been more precisely evaluated in time course assays, eliminating the variability of different MOIs and batches of BMDCs between time points. However, an assay that large, including four *C. jejuni* strain/mouse genotype combinations over multiple time points with sufficient replicate wells, would have been logistically challenging with this method. Thus, statistical analysis of results at each time point and observation of patterns over time was chosen as the best possible method of evaluating invasion and survival. Experiments performed assessing one mouse genotype/*C. jejuni* strain combination at different time points would allow statistical comparisons within that combination to more clearly evaluate changes in intracellular *C. jejuni* survival.

Similarly, an interesting pattern of enhanced killing of *C. jejuni* by IL-10$^{-/-}$ compared to WT BMDCs was observed, but could not be compared statistically because IL-10$^{-/-}$ and WT cells were not evaluated in the same assays. Therefore, further assessment of the impact of IL-10 on intracellular *C. jejuni* survival can be compared in future experiments including BMDCs from a WT and IL-10$^{-/-}$ mouse of the same genetic background; again, the logistics of including WT and IL-10$^{-/-}$ mice from different genetic backgrounds infected with two *C. jejuni* strains would be logistically prohibitive by this method.

Finally, measurement of cytokine production by infected IL-10$^{-/-}$ BMDCs provided insight on putative Th-polarization induced by colitogenic and GBS-associated *C. jejuni* strains in mice of different genetic backgrounds. Further experiments should include co-culture of infected BMDCs with naïve CD4+ T cells, followed by measurement of cytokines such as IFN-γ, IL-4, and IL-17 to further confirm induction of Th1, Th2, or Th17 polarization. Additionally, inclusion of BMDCs derived from WT mice to investigate the impact of IL-10 on cytokine production and Th polarization would be informative. Inclusion of WT cells would help confirm or refute the hypothesis that DC-derived IL-10 directs Th2 polarization, and determine if IL-10 alters kinetics of TNF-α or IL-12p70 production. Measurement of IL-12p40 along with IL-12p70 production in IL-10$^{-/-}$ cells would be warranted to evaluate if a similar anti-inflammatory check by IL-12p40 as seen in human monocytic cells is also present in DCs.

The main objectives of this study included characterizing the initial host-microbe interaction, and determining the interplay of *C. jejuni* strain characteristics and host genetic background in initiation of the immune response. Results of this study suggest that DCs differentiated from bone marrow precursors are a useful model for studying invasion and intracellular survival of *C. jejuni*. Intracellular survival of colitogenic *C. jejuni* 11168 correlates with enhanced invasion and pathogenicity observed *in vivo,* and DC cytokine responses elicited by the different *C. jejuni* strains are in general agreement with adaptive immune responses observed in previous mouse models. Future studies, including more precise characterization of differences in BMDCs derived from C57BL/6 and BALB/c mice and the effect of IL-10 during differentiation, are warranted. Additional studies to expand upon patterns observed in the GKAs and further discern the relative contributions of invasion and intracellular survival, especially in *C. jejuni* 11168, by methods such as fluorescent or electron microscopy, would be informative.

APPENDICES

APPENDIX A: TABLES AND FIGURES

Table 4.1. Mean ± Standard Deviation of parameters determined by flow cytometric analysis of three independent experiments, BMDCs. Stem cells derived from the bone marrow of C57BL/6 interleukin (IL)-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice were differentiated for 10 days with granulocyte-macrophage colony stimulating factor. t-tests were performed on each parameter to determine if discrepancies in differentiation between C57BL/6 IL-$10^{-/-}$ and BALB/c IL-$10^{-/-}$ mice were statistically significant; P-values are listed in bottom row for each parameter. Percentage data were subjected to Arcsine transformation prior to analysis. MFI, Median Fluorescence Intensity; BMDC, Bone Marrow-derived Dendritic Cell.

	% CD11c Positive	% CD11c Positive, MHC II^{high}	MFI MHC II (All MHC II Positive)	% CD11c Positive, MHC II^{low}	% CD11c, F4/80 Double Positive	MFI MHC II (CD11c, F4/80 Double Positive)
C57BL/6 IL-$10^{-/-}$	90.4 ± 3.96	35.9 ± 11.8	3,202 ± 293	56.8 ± 15.6	25.7 ± 5.8	557 ± 96
BALB/c IL-$10^{-/-}$	80.5 ± 7.27	55.5 ± 6.1	11,933 ± 344	25.5 ± 5.7	16.4 ± 2.5	1,169 ± 369
P-value	0.111	0.063	<0.001	0.03	0.054	0.05

Table 4.2A. Results of gentamicin killing assays evaluating combined invasion efficiency and intracellular survival of *C. jejuni* 11168 and 260.94 strains in cultured dendritic cells derived from the bone marrow of interleukin (IL)-$10^{-/-}$ mice on C57BL/6 (C57) and BALB/c (BALB) genetic backgrounds; 1 hour invasion time followed by 1 hour exposure to gentamicin and immediate lysis. MOI = multiplicity of infection; SD = standard deviation; CFU = colony forming units; ns = non-significant.

Exposure Time to *C. jejuni,* Prior to Gentamicin Treatment	Replicate (*Indicates Presented in Graph)	Starting Density (No. Cells in 10 mL)	MOI (Approximate Range Derived from Limiting Dilution Results)	Mean ± SD, CFU/mL	Statistical Analyses and Results
1 Hour	1	C57 2.6 x 10^6 BALB 2.5 x 10^6	11168: 99-105	C57 11168: 4.7 ± 0.35 x 10^3 BALB 11168: 4.3 ± 0 x 10^3	Assumption of equal variance not met. Mann-Whitney Rank Sum Test: ns (P = 0.333)
	2	C57 2.5 x 10^6 BALB 2.4 x 10^6	260.94: 86-165	C57 260.94: 2.88 ± 0.536 x 10^3 BALB 260.94: 1.5 ± 0.96 x 10^3	t-test; ns (P = 0.114)
	3*	Both 2.5 x 10^6	11168: 65-105 260.94: 75-190	C57 11168: 3.7 ± 1.5 x 10^3 BALB 11168: 1 ± 0.9 x 10^3 C57 260.94: 2.97 ± 0.585 x 10^3 BALB 260.94: 1.13 ± 0.071 x 10^3	**Two-way ANOVA: Genotype significant (P = 0.003)**, but not *C. jejuni* strain (P = 0.466). Holm-Sidak pairwise comparisons: **Genotype significant, [C57 > BALB]. One-way ANOVA** was also performed to further examine pairwise comparisons. Significance overall (**P = 0.017**); significant Holm-Sidak pairwise comparisons: **[C57 11168 > BALB 260.94]; [C57 11168 > BALB 11168].** Results of the two-way ANOVA are shown graphically.
Conclusions for one hour invasion, IL-$10^{-/-}$ BMDCs: No *C. jejuni* strain difference detected, but a significant genotype difference was found (supported by pattern in the two replicates not graphed): More *C. jejuni* recovered from C57 than BALB mice.					

Table 4.2B. Results of gentamicin killing assays evaluating combined invasion efficiency and intracellular survival of *C. jejuni* 11168 and 260.94 strains in cultured dendritic cells derived from the bone marrow of interleukin (IL)-$10^{-/-}$ mice on C57BL/6 (C57) and BALB/c (BALB) genetic backgrounds; 2 hours invasion time followed by 1 hour exposure to gentamicin and immediate lysis. MOI = multiplicity of infection; SD = standard deviation; CFU = colony forming units.

Exposure Time to *C. jejuni,* Prior to Gentamicin Treatment	Replicate (*Indicates Presented in Graph)	Starting Density (No. Cells in 10 mL)	MOI (Approximate Range Derived from Limiting Dilution Results)	Mean ± SD, CFU/mL	Statistical Analyses and Results
2 Hours	1	C57: 3.2 x 10^6 BALB: 2.8 x 10^6	11168: 90-96	C57 11168: 1.66 ± 0.157 x 10^4 BALB 11168: 2.87 ± 0.465 x 10^4	**t-test: significantly more *C. jejuni* 11168 recovered from BALB than C57 mice (P = 0.013)**
	2*	C57: 2.8 x 10^6 BALB: 2.1 x 10^6	11168: 60-63 260.94: 118-160	C57 11168: 5.0 ± 0.23 x 10^3 BALB 11168: 5.0 ± 1.1 x 10^3 C57 260.94: 1 ± 0.7 x 10^3 BALB 260.94: 1 ± 0.2 x 10^3	**Two-way ANOVA**: significance found in ***C. jejuni* strain (P < 0.001)**, but not in Genotype (P = 0.867). Holm-Sidak pairwise comparisons: **significantly more *C. jejuni* 11168 than 260.94 recovered.**
	3	C57: 2.3 x 10^6 BALB: 2.5 x 10^6	11168: 70-92 260.94: 144-190	C57 11168: 8.0 ± 1.6 x 10^3 BALB 11168: 4.4 ± 1.9 x 10^3 C57 260.94: 1 ± 0.6 x 10^2 BALB 260.94: 70 ± 60	**Two-way ANOVA**: **Genotype (P = 0.032), *C. jejuni* strain (P <0.001), and interaction (P = 0.037) all significant**. The following Holm-Sidak pairwise comparisons were significant: **[C57 11168 > C57 260.94]; [BALB 11168 > BALB 260.94]; [C57 11168 > BALB 11168]**; recovery of *C. jejuni* 260.94 was not significantly different between mouse genotypes. Because the assumption of normality was not met, **Kruskal-Wallis was also performed, with overall significance (P = 0.018)**. Dunn's and Tukey's post-hoc testing: **[C57 11168 > BALB 260.94]** was significant. Significance was found in the following comparisons using Student-Newman-Keuls method: **[C57 11168 > BALB 260.94]; [C57 11168 > C57 260.94]; [C57 11168 > BALB 11168]; [BALB 11168 > BALB 260.94]; and [BALB 11168 > C57 260.94]**. Recovery of *C. jejuni* 260.94 was not different between mouse genotypes.
	4	C57: 2.5 x 10^6 BALB: 2.1 x 10^6	11168: 104-170 260.94: 115-145	C57 11168: 7.1 ± 2.4 x 10^3 BALB 11168: 6.4 ± 3.9 x 10^3 C57 260.94: 70 ± 20 BALB 260.94: 2.4 ± 1.0 x 10^2	**Two-way ANOVA**: ***C. jejuni* strain significant (P = 0.001)**, but not Genotype (P = 0.848). Holm-Sidak pairwise comparisons determined **significantly more *C. jejuni* 11168 than 260.94 was recovered**. Because the assumption of normality was not met, **Kruskal-Wallis was also performed, with overall significance (P = 0.024)**. Tukey, Dunn's, and Student-Newman-Keuls post-hoc testing all found significance in only **[C57 11168 > C57 260.94]**.
Conclusions for two hour invasion, IL-$10^{-/-}$ BMDCs: Consistent significant difference in *C. jejuni* strain: more 11168 than 260.94 was recovered. A mouse genotype difference was not consistent between replicates.					

Table 4.2C. Results of gentamicin killing assays evaluating combined invasion efficiency and intracellular survival of *C. jejuni* 11168 and 260.94 strains in cultured dendritic cells derived from the bone marrow of interleukin (IL)-$10^{-/-}$ mice on C57BL/6 (C57) and BALB/c (BALB) genetic backgrounds; 3 hours invasion time, followed by 1 hour exposure to gentamicin and immediate lysis. MOI = multiplicity of infection; SD = standard deviation; CFU = colony forming units; ns = non-significant.

Exposure Time to *C. jejuni,* Prior to Gentamicin Treatment	Replicate (*Indicates Presented in Graph)	Starting Density (No. Cells in 10 mL)	MOI (Approximate Range Derived from Limiting Dilution Results)	Mean ± SD, CFU/mL	Statistical Analyses and Results
3 Hours	1*	C57: 2.5 x 10^6 BALB: 1.7 x 10^6	11168: 126-150 260.94: 95-192	C57 11168: 4.91 ± 0.436 x 10^4 BALB 11168: 3.36 ± 0.421 x 10^4 C57 260.94: 5.3 ± 1.9 x 10^3 BALB 260.94: 4.8 ± 0.72 x 10^3	**Two-way ANOVA: Genotype (P = 0.002), *C. jejuni* strain (P < 0.001) and interaction (P = 0.004)** all significant. Holm-Sidak significant pairwise comparisons: **[C57 11168 > C57 260.94]; [BALB 11168 > BALB 260.94]; [C57 11168 > BALB 11168]**; recovery of *C. jejuni* 260.94 was not significant between mouse genotypes. Because of the significant interaction, **one-way ANOVA also was performed. Significance found overall (P <0.001)**. Post-hoc testing included Bonferroni t-test, Holm-Sidak, Tukey, and Student-Newman-Keuls methods, with the following significant comparisons found with all methods: **[C57 11168 > BALB 260.94]; [C57 11168 > C57 260.94]; [C57 11168 > BALB 11168]; [BALB 11168 > BALB 260.94]; [BALB 11168 > C57 260.94].** Recovery of *C. jejuni* 260.94 was not significantly different between mouse genotypes. Results of the two-way ANOVA shown graphically.
	2	Both 2.5 x 10^6	260.94: 43-75	C57 260.94: 5.8 ± 1.6 x 10^2 BALB 260.94: 3.7 ± 0.44 x 10^2	t-test: ns (P = 0.092)
	3	Both 2.5 x 10^6	11168: 106-127	C57 11168: 6.7 ± 1.1 x 10^4 BALB 11168: 2.73 ± 0.318 x 10^4	**t-test: significantly more *C. jejuni* 11168 recovered from C57 than BALB mice (P = 0.003)**
Conclusions for three hour invasion, IL-$10^{-/-}$ BMDCs: Significantly more *C. jejuni* 11168 than 260.94 was recovered from mice of both genotypes, and significantly more *C. jejuni* 11168 was recovered from C57 than BALB mice. *C. jejuni* 260.94 recovery was not different between mouse genotypes.					

Table 4.2D. Results of gentamicin killing assays evaluating combined invasion efficiency and intracellular survival of *C. jejuni* 11168 and 260.94 strains in cultured dendritic cells derived from the bone marrow of interleukin (IL)-10$^{-/-}$ mice on C57BL/6 (C57) and BALB/c (BALB) genetic backgrounds; 23 hours invasion time, followed by 1 hour exposure to gentamicin and immediate lysis. MOI = multiplicity of infection; SD = standard deviation; CFU = colony forming units.

Exposure Time to *C. jejuni,* Prior to Gentamicin Treatment	Replicate (*Indicates Presented in Graph)	Starting Density (No. Cells in 10 mL)	MOI (Approximate Range Derived from Limiting Dilution Results)	Mean ± SD, CFU/mL	Statistical Analyses and Results
23 Hours	1	C57: 2.5 x 10^6 BALB: 1.5 x 10^6	11168: 35-60 260.94: 109-150	C57 11168: 3.00 ± 0 x 10^5 BALB 11168: 3.00 ± 0 x 10^5 C57 260.94: 3.2 ± 1.0 x 10^3 BALB 260.94: 1.4 ± 0.51 x 10^4	**Two-way ANOVA: Genotype (P = 0.006), *C. jejuni* strain (P <0.001), and interaction (P = 0.006)** all significant. Significant Holm-Sidak pairwise comparisons: **[C57 11168 > C57 260.94]; [BALB 11168 > BALB 260.94]; [BALB 260.94 > C57 260.94]**. Because assumption of normality was not met and a significant interaction was present, **Kruskal-Wallis was also performed. Overall significance was found (P = 0.014)**, but Tukey, Dunn's, and Student-Newman-Keuls post-hoc methods did not identify any significant pairwise comparisons. [Note: *C. jejuni* 11168 colony numbers were too numerous to count (>300) with a dilution of 10^{-3}; for statistical purposes, these results were treated as 3.00 x 10^5 CFU/mL recovered for both C57 and BALB mice.]
	2*	Both 2.5 x 10^6	11168: 27-39 260.94: 142-185	C57 11168: 8.4 ± 3.5 x 10^5 BALB 11168: 1.1 ± 0.42 x 10^6 C57 260.94: 9 ± 10 x 10^2 BALB 260.94: 3.0 ± 2.8 x 10^3	**Two-way ANOVA: *C. jejuni* strain was significant (P < 0.001)**, but not Genotype (P = 0.361). **Holm-Sidak pairwise comparisons: significantly more *C. jejuni* 11168 than 260.94 was recovered.** Because normality and equal variance assumptions were not met, **Kruskal-Wallis was also performed with overall significance found (P = 0.030)**, followed by three post-hoc tests. Significant comparisons listed. **Tukey: [BALB 11168 > C57 260.94];** Dunn's: none; **Student-Newman-Keuls: [BALB 11168 > C57 260.94]; [BALB 11168 > BALB 260.94]; [C57 11168 > C57 260.94]; [C57 11168 > BALB 260.94].** Results of the two-way ANOVA are shown graphically.
Conclusions for 23 hour invasion, IL-10$^{-/-}$ BMDCs: Significantly more *C. jejuni* 11168 was recovered than 260.94. There was also a trend for more *C. jejuni* to be recovered from BALB than C57 mice, but this was not confirmed statistically in both replicates. The second replicate was chosen as representative since more precise enumeration of *C. jejuni* 11168 recovery was possible.					

Table 4.3A. Results of gentamicin killing assays evaluating intracellular survival of *C. jejuni* 11168 and 260.94 strains in cultured dendritic cells derived from the bone marrow of wild-type (WT) mice on C57BL/6 (C57) and BALB/c (BALB) genetic backgrounds; one hour of invasion followed by gentamicin treatment, with lysis at 24 hours post-infection. MOI = multiplicity of infection; SD = standard deviation; CFU = colony forming units.

Replicate (*Indicates Presented in Graph)	Starting Density (No. Cells in 10 mL)	MOI (Approximate Range Derived from Limiting Dilution Results)	Mean ± SD, CFU/mL	Statistical Analyses and Results
1*	Both 2.5 x 10^6	11168: 117-175 260.94: 146-175	C57 11168: 2.48 ± 0.603 x 10^4 BALB 11168: 2.13 ± 0.590 x 10^4 C57 260.94: 1.2 ± 0.58 x 10^3 BALB 260.94: 2.22 ± 0.673 x 10^3	**Two-way ANOVA**: Genotype was not significant (P = 0.637), but ***C. jejuni* strain was significant (P <0.001). Holm-Sidak post-hoc testing: significantly more *C. jejuni* 11168 than 260.94 was recovered. One-way ANOVA** was also run to further examine pairwise comparisons. There was **significance overall** (**P <0.001**), and Holm-Sidak testing indicated signficant differences in the following: **[C57 11168 > C57 260.94]; [C57 11168 > BALB 260.94]; [BALB 11168 > C57 260.94]; [BALB 11168 > BALB 260.94].** Results of the two-way ANOVA are shown graphically.
2	Both 2.5 x 10^6	11168: 154-215 260.94: 103-152	C57 11168: 6.6 ± 1.2 x 10^3 BALB 11168: 2.6 ± 0.92 x 10^3 C57 260.94: 4.2 ± 2.8 x 10^2 BALB 260.94: 80 ± 70	**Two-way ANOVA**: Significance in **Genotype (P = 0.001)**, ***C. jejuni* strain (P < 0.001), and interaction (P = 0.004)** were found. Holm-Sidak pairwise testing showed signficance in the following: **[C57 11168 > C57 260.94]; [BALB 11168 > BALB 260.94]; [C57 11168 > BALB 11168]**. Recovery of *C. jejuni* 260.94 was not significantly different between mouse genotypes. Because a signficant interaction was identified, **one-way ANOVA was also performed. Significance overall was found (P < 0.001**). Holm-Sidak post-hoc testing revealed significance in the following comparisons: **[C57 11168 > BALB 260.94]; [C57 11168 > C57 260.94]; [C57 11168 > BALB 11168]; [BALB 11168 > BALB 260.94]; [BALB 11168 > C57 260.94]**.
3	Both 2.5 x 10^6	11168: 147-160	C57 11168: 1.46 ± 0.532 x 10^4 BALB 11168: 8.5 ± 1.8 x 10^4	**t-test: Significantly more *C. jejuni* 11168 recovered from BALB than C57 mice (P = 0.003)**
WT mice: Conclusions for intracellular survival: Significantly more *C. jejuni* 11168 than 260.94 was recovered, but differences between mouse genotypes were not consistent. The first replicate was chosen as representative because no genotype difference was identified, and numbers of *C. jejuni* 11168 recovered were similar to those in the third replicate.				

Table 4.3B. Results of gentamicin killing assays evaluating intracellular survival of *C. jejuni* 11168 and 260.94 strains in cultured dendritic cells derived from the bone marrow of interleukin (IL)-10$^{-/-}$ mice on C57BL/6 (C57) and BALB/c (BALB) genetic backgrounds; one hour of invasion followed by gentamicin treatment, with lysis at 24 hours post-infection. Wet mount preparations of inocula were not possible in the first replicate. MOI = multiplicity of infection; SD = standard deviation; CFU = colony forming units; ns = non-significant.

Replicate (*Indicates Presented in Graph)	Starting Density (No. Cells in 10 mL)	MOI (Approximate Range Derived from Limiting Dilution Results)	Mean ± SD, CFU/mL	Statistical Analyses and Results
1	C57: 2.5 x 10^6 BALB: 2.3 x 10^6	11168: 40-88 260.94: 50-107	C57 11168: 4 ± 7 x 10^2 BALB 11168: 3 ± 3 x 10^2 C57 260.94: 0 ± 0 BALB 260.94: 3 ± 6	Two-way ANOVA: Genotype (P = 0.685), *C. jejuni* strain (P = 0.157) both ns. Because assumption of normality was not met, **Kruskal-Wallis was also performed. Significance was found overall (P = 0.027)**, but Tukey, Dunn's and Student-Newman-Keuls post-hoc testing did not determine significance in any pairwise comparison. [*Note: Uneven distribution of* C. jejuni *11168 recovery between triplicate wells*.]
2*	Both 2.5 x 10^6	11168: 162-190 260.94: 58-61	C57 11168: 1.41 ± 0.267 x 10^3 BALB 11168: 2 ± 0.9 x 10^2 C57 260.94: 10 ± 20 BALB 260.94: 0 ± 0	**Two-way ANOVA: Genotype (P <0.001), *C. jejuni* strain (P < 0.001), and interaction (P <0.001) all significant. Significant comparisons by Holm-Sidak testing: [C57 11168 > C57 260.94]; [C57 11168 > BALB 11168].** Because a significant interaction was found, **one-way ANOVA was also performed and was significant overall (P < 0.001)**. Holm-Sidak post-hoc testing indicated significance in the following comparisons: **[C57 11168 > BALB 260.94]; [C57 11168 > C57 260.94]; and [C57 11168 > BALB 11168]**. Because assumption of normality was not met, **Kruskal-Wallis was also performed. Significance was found overall (P = 0.017)**. Pairwise testing with Tukey, Dunn's methods indicated **[C57 11168 > BALB 260.94]** was significant; Student-Newman Keuls pairwise comparisons indicated significance in the following: **[C57 11168 > BALB 260.94]; [C57 11168 > C57 260.94]; [C57 11168 > BALB 11168]; [BALB 11168 > BALB 260.94]; [BALB 11168 > C57 260.94]**. Results of the 2-way ANOVA are shown graphically.
IL-10$^{-/-}$ mice: Conclusions for intracellular survival: In mice of either genotype, more *C. jejuni* 11168 than 260.94 was recovered, but this difference was only significant within C57 mice. Significantly more *C. jejuni* 11168 was recovered from C57 than from BALB mice, but the difference in *C. jejuni* 260.94 recovered between mouse genotypes was not significant. The second replicate was chosen as representative due to less variation between triplicate wells in recovery of *C. jejuni* 11168, in addition to identification of the same patterns as in the first replicate.				

Table 4.4. Cytokine analysis, first independent experiment. This table depicts the results of statistical analyses of cytokine production by *C. jejuni*-infected BMDCs derived from IL-$10^{-/-}$ mice on a C57BL/6 or BALB/c genetic background, Experiment 1. Multiplicities of infection for this assay were: *C. jejuni* 11168: 144; *C. jejuni* 260.94: 115. C57, C57BL/6 IL-$10^{-/-}$; BALB/c, BALB/c IL-$10^{-/-}$; LPS, lipopolysaccharide (positive control); Med, medium only (negative control); 11168, *C. jejuni* 11168; 260.94, *C. jejuni* 260.94; Ns, non-significant (P > 0.05); Treatment group refers to LPS, *C. jejuni* strain, or medium.

Analyte	Statistical Test	Normality/Equal Variance	Significance?	Significant Comparison(s)?	P-value (for Factor or Interaction)
IFN-γ	2-way ANOVA	Passed Both	No	None	Ns
IL-12p70	2-way ANOVA	Passed Both	No	None	Ns
TGF-β	2-way ANOVA	Failed Normality	No	None	Ns
MCP-1	**2-way ANOVA**	Failed Normality	Yes		
				Mouse Genotype	<0.001
				Treatment Group	<0.001
				Interaction	0.003
		MCP-1, significant pairwise comparisons (Holm-Sidak)			
		Within C57:	**Within BALB/c:**	**Within 260.94:**	**Within LPS:**
		LPS > Med	LPS > Med	C57 > BALB/c	C57 > BALB/c
		LPS > 11168	LPS > 11168		
		LPS > 260.94	LPS > 260.94		
		260.94 > Med			
		260.94 > 11168			
IL-4	2-way ANOVA	Failed Normality	No	None	Ns
IL-6	**2-way ANOVA**	Failed Normality	Yes		
				Treatment Group	0.002
				Interaction	0.05
		IL-6, significant pairwise comparisons (Holm-Sidak)			
		Within C57:	**Within BALB/c:**	**Within LPS:**	
		None	LPS > Med	BALB/c > C57	
			LPS > 260.94		
			LPS > 11168		
TNF-α	2-way ANOVA	Failed Normality	No	None	Ns

Table 4.5. Cytokine analysis, second independent experiment. This table depicts the results of statistical analyses of cytokine production by *C. jejuni*-infected BMDCs derived from IL-10$^{-/-}$ mice on a C57BL/6 or BALB/c genetic background, Experiment 2. Multiplicities of infection for this assay were: *C. jejuni* 11168: 170; *C. jejuni* 260.94: 138. Graphs of these results are shown in **Figure 4.5**. C57, C57BL/6 IL-10$^{-/-}$; BALB/c, BALB/c IL-10$^{-/-}$; LPS, lipopolysaccharide (positive control); Med, medium only (negative control); 11168, *C. jejuni* 11168; 260.94, *C. jejuni* 260.94; Ns, non-significant (P > 0.05); Treatment group denotes LPS, *C. jejuni* strain, or medium.

Analyte	Statistical Test	Normality/Equal Variance	Significance?	Significant Comparison(s)?	P-value (for Factor or Interaction)
IFN-γ	2-way ANOVA	Passed Both	No	None	Ns
IL-12p70	2-way ANOVA	Failed Normality	Yes		
				Interaction	0.039
		IL-12p70: significant pairwise comparisons (Holm-Sidak)			
		Within C57:	**Within BALB/c:**	**Within Medium:**	
		None	None	C57 > BALB/c	
TGF-β	2-way ANOVA	Failed Normality	No	None	Ns
MCP-1	**2-way ANOVA**	Failed Normality	Yes		
				Mouse Genotype	<0.001
				Treatment Group	<0.001
				Interaction	<0.001
		MCP-1: significant pairwise comparisons (Holm-Sidak)			
		Within C57:	**Within BALB/c:**	**Within 260.94:**	**Within LPS:**
		LPS > Med	LPS > Med	C57 > BALB/c	C57 > BALB/c
		LPS > 11168	LPS > 260.94		
		LPS > 260.94	LPS > 11168		
		260.94 > Med			
		260.94 > 11168			
IL-4	**2-way ANOVA**	Failed Normality	Yes		
				Interaction	0.029
		IL-4: significant pairwise comparisons (Holm-Sidak)			
		Within C57:	**Within BALB/c:**	**Within Medium:**	
		Med > 11168	None	C57 > BALB/c	

Table 4.5 (cont'd)

Analyte	Statistical Test	Normality/Equal Variance	Significance?	Significant Comparison(s)?	P-value (for Factor or Interaction)
IL-6	**2-way ANOVA**	Failed Normality	Yes		
				Mouse Genotype	<0.001
				Treatment Group	<0.001
				Interaction	0.001
		IL-6: significant pairwise comparisons (Holm-Sidak)			
		Within C57:	**Within BALB/c:**	**Within 11168:**	**Within LPS:**
		LPS > Med	LPS > Med	BALB/c > C57	BALB/c > C57
		LPS > 11168	LPS > 260.94		
		LPS > 260.94	LPS > 11168		
			11168 > Med		
TNF-α	**2-way ANOVA**	Failed Normality	Yes		
				Treatment Group	<0.001
		TNF-α: significant pairwise comparisons (Holm-Sidak) (2-way ANOVA)			
		All within Treatment Group:			
		LPS > Med			
		LPS > 11168			
		LPS > 260.94			
		260.94 > Med			
	1-way ANOVA	Failed Normality	Yes	Overall	<0.001
		TNF-α: significant pairwise comparisons (Holm-Sidak) (1-way ANOVA)			
		BALB/c LPS > BALB/c Med		BALB/c LPS > BALB/c 260.94	
		BALB/c LPS > C57 Med		BALB/c LPS > BALB/c 11168	
		BALB/c LPS > C57 11168		C57 LPS > C57 11168	
		C57 LPS > BALB/c Med		C57 LPS > BALB/c 260.94	
		C57 LPS > C57 Med		C57 LPS > BALB/c 11168	
				BALB/c LPS > C57 260.94	

Table 4.6. Cytokine analysis, third independent experiment. This table depicts the results of statistical analyses of cytokine production by *C. jejuni*-infected BMDCs derived from IL-10$^{-/-}$ mice on a C57BL/6 or BALB/c genetic background, Experiment 3. Multiplicities of infection for this assay were: *C. jejuni* 11168: 107; *C. jejuni* 260.94: 148. C57, C57BL/6 IL-10$^{-/-}$; BALB/c, BALB/c IL-10$^{-/-}$; LPS, lipopolysaccharide (positive control); Med, medium only (negative control); 11168, *C. jejuni* 11168; 260.94, *C. jejuni* 260.94; Ns, non-significant (P > 0.05); Tx Grp, Treatment Group (LPS, *C. jejuni* strain, medium).

Analyte	Statistical Test	Normality/Equal Variance	Significance?	Significant Comparison(s)?	P-value (for Factor or Interaction)
IFN-γ	**2-way ANOVA**	Passed Both	Yes		
				Treatment Group	0.012
				Interaction	<0.001
		IFN-γ: significant pairwise comparisons (Holm-Sidak)			
		Within C57:	**Within BALB/c:**	**Within Med:**	**Within 11168:**
		Med > 260.94	None	C57 > BALB/c	BALB/c > C57
		Med > 11168			
		Med > LPS			
IL-12p70	**2-way ANOVA**	Failed Normality	Yes		
				Interaction	0.009
		IL-12p70: significant pairwise comparisons (Holm-Sidak)			
		Tx Grp w/in C57:	**Tx Grp w/in BALB/c:**	**Within Med:**	**Within LPS:**
		None	None	C57 > BALB/c	BALB/c > C57
TGF-β	**2-way ANOVA**	Passed Both	Yes		
				Genotype	<0.001
		TGF-β: significant pairwise comparisons (Holm-Sidak) (2-way ANOVA)			
		C57 > BALB/c; 1-way ANOVA performed for further comparison			
	1-way ANOVA	Passed Both	Yes	Overall	0.003
		TGF-β: significant pairwise comparisons (Holm-Sidak) (1-way ANOVA)			
		C57 260.94 > BALB/c 260.94			
		C57 260.94 > BALB/c 11168			

Table 4.6 (cont'd)

Analyte	Statistical Test	Normality/Equal Variance	Significance?	Significant Comparison(s)?	P-value (for Factor or Interaction)
MCP-1	**2-way ANOVA**	Failed Normality	Yes		
				Mouse Genotype	<0.001
				Treatment Group	<0.001
				Interaction	<0.001
	MCP-1: Significant pairwise comparisons (Holm-Sidak)				
	Within C57:	**Within BALB/c:**	**Genotype within Treatment Groups:**		
	LPS > Med	LPS > Med	C57 > BALB/c for: Med, 11168, 260.94, LPS		
	LPS > 11168	LPS > 11168			
	LPS > 260.94	LPS > 260.94			
	260.94 > Med				
	260.94 > 11168				
	11168 > Med				
IL-4	2-way ANOVA	Failed Normality	No	None	Ns
IL-6	**2-way ANOVA**	Failed Both	Yes		
				Mouse Genotype	<0.001
				Treatment Group	<0.001
				Interaction	<0.001
		IL-6: significant pairwise comparisons (Holm-Sidak) (2-way ANOVA)			
		Within C57:	**Within BALB/c:**	**Within 11168:**	**Within LPS:**
		LPS > Med	LPS > Med	BALB/c > C57	BALB/c > C57
		LPS > 260.94	LPS > 260.94		
		LPS > 11168	11168 > Med		
			11168 > 260.94		
			LPS > 11168		
	Kruskal-Wallis		Yes	Overall	0.002
		IL-6: significant pairwise comparisons (Tukey) (Kruskal-Wallis)			
		BALB/c LPS > C57 Med			
		BALB/c LPS > BALB/c Med			
TNF-α	**2-way ANOVA**	Failed Normality	Yes		
				Mouse Genotype	0.003
				Treatment Group	<0.001
				Interaction	<0.001
		TNF-α: significant comparisons (Holm-Sidak)			
		Within C57:	**Within BALB/c:**	**Within 11168:**	
		LPS > Med	11168 > Med	BALB/c > C57	
		11168 > Med	11168 > 260.94		
		LPS > 260.94	LPS > Med		
			LPS > 260.94		
			11168 > LPS		

Figure 4.1. Analysis of CD11c and MHC II expression by flow cytometry. This figure represents scatter plots of bone marrow stem cells isolated from (A) one C57BL/6 wild-type (WT) mouse, (B) one C57BL/6 IL-$10^{-/-}$ mouse, (C) one BALB/c WT mouse, and (D) one BALB/c IL-$10^{-/-}$ mouse each after 10 days of culture with granulocyte-macrophage colony stimulating factor. Figure (A) is representative of two analyses of C57BL/6 WT mice, one each from days 9 and 10 of culture; the Median Fluorescence Intensity of MHC II, in all MHC II-positive cells (MHC II MFI), of the C57BL/6 WT mouse was 5,640. Figure (C) is representative of three analyses of BALB/c WT mice, one from day 9 and the other two from day 10 of culture; the MHC II MFI of the BALB/c WT mouse was 14,175. (B) and (D) are also shown in **Figure 4.2**, and each figure is from one experiment representative of three independent experiments. The MHC II MFI of the C57BL/6 IL-$10^{-/-}$ mouse (B) was 2,987, while that of the BALB/c IL-$10^{-/-}$ mouse (D) was 11,554.

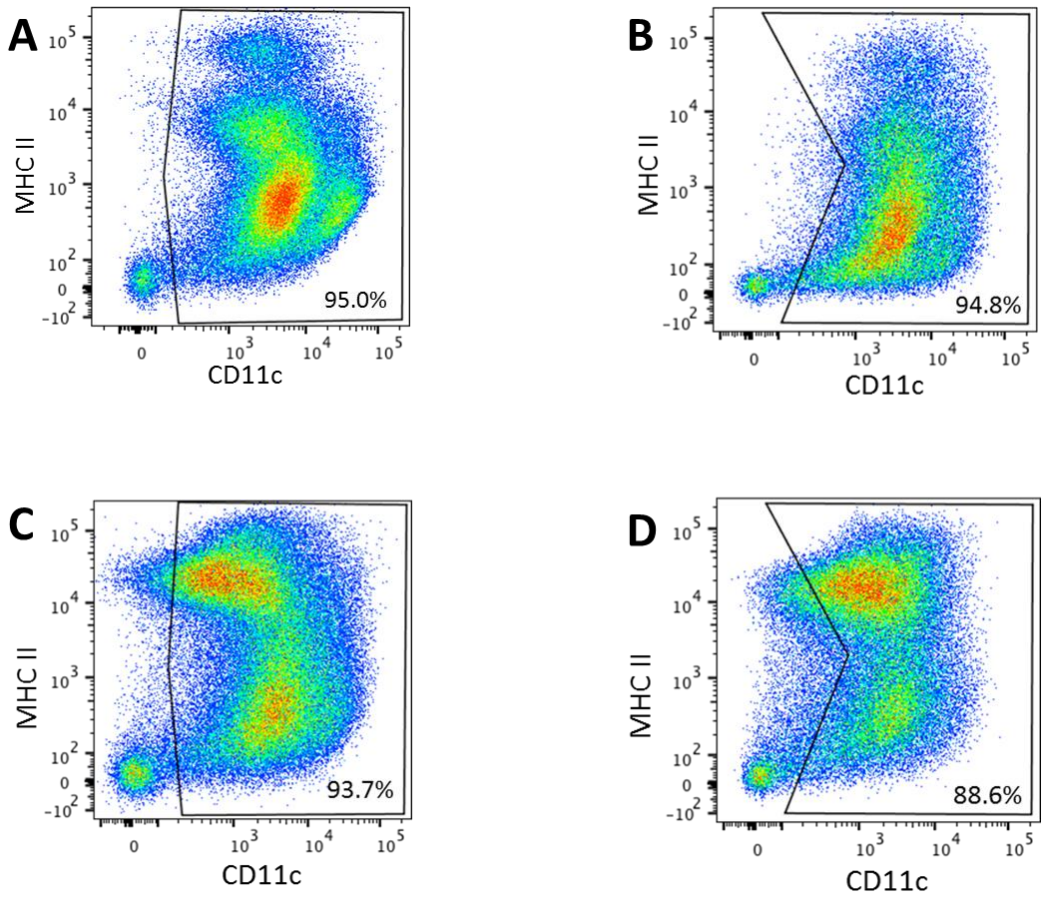


Figure 4.2. Analysis of CD11c, MHC II, and F4/80 expression by flow cytometry. This figure represents scatter plots of bone marrow stem cells isolated from (A) one C57BL/6 IL-$10^{-/-}$ mouse, and (B) one BALB/c IL-$10^{-/-}$ mouse following 10 days of culture with granulocyte-macrophage colony stimulating factor. The left figure in each panel shows the percentage of CD11c+ cells within the single viable cell population. The right figure in each panel shows the percentage of F4/80+ cells within the CD11c+ population. Results of one experiment representative of three independent experiments are shown. Details regarding these and additional parameters in the three independent experiments are shown in **Table 4.1**. The plots depicting CD11c and MHC II expression are also shown in **Figure 4.1**.

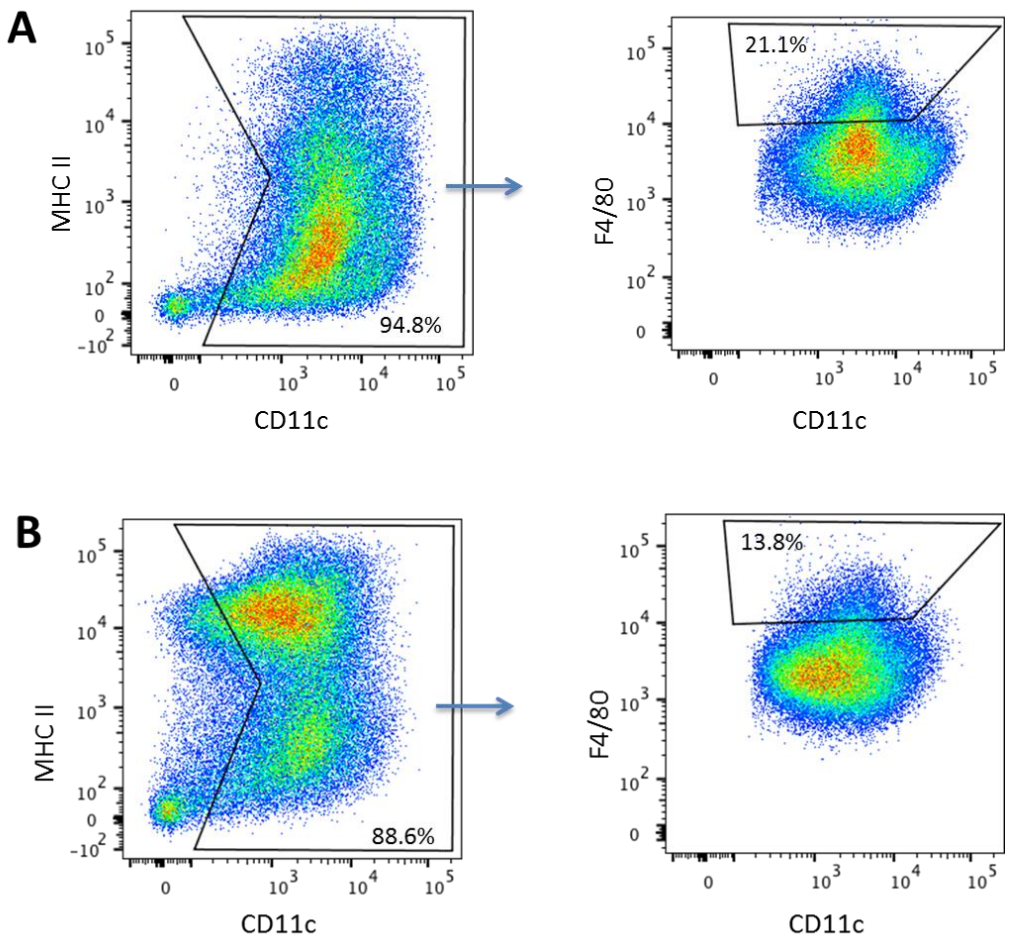


Figure 4.3. Differences in invasion efficiency and intracellular survival of two *Campylobacter jejuni* strains in bone marrow-derived dendritic cells (BMDCs) from C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice assessed by gentamicin killing assay. Cells were infected with either colitogenic *C. jejuni* 11168, or Guillain-Barré syndrome patient-derived *C. jejuni* 260.94, for 1, 2, 3, or 23 hours prior to gentamicin treatment to kill extracellular bacteria. BMDCs were then lysed, and lysates were spread (diluted when needed) and cultured on Bolton agar plates for 72 hours to enumerate viable intracellular *C. jejuni,* presented as colony forming units (CFU)/mL. Data are from three-wells in an experiment, expressed as mean ± SEM. One experiment representative of the 2-4 independent experiments performed at each time point is shown. Colored, dotted lines connect the data between time points for each treatment group and are added to help identify pattern over time; no statistical analyses were performed between time points, since experiments at each time point were performed separately. Statistical significance, determined by two-way ANOVA followed by Holm-Sidak pairwise comparisons, denoted in this graph represents repeatable differences observed between replicates. Boxes around data at 1 and 3 hours of infection indicate significant differences between mouse genotypes: at 1 hour, more *C. jejuni* was recovered from C57BL/6 IL-10$^{-/-}$ than BALB/c IL-10$^{-/-}$ mice, and at 3 hours, significantly more *C. jejuni* 11168 was recovered from C57BL/6 IL-10$^{-/-}$ than BALB/c IL-10$^{-/-}$ mice. Asterisks above the 2, 3, and 23 hour time points indicate a statistically significant difference in *C. jejuni* strain recovery: beginning at 2 hours of infection, significantly more *C. jejuni* 11168 was recovered than *C. jejuni* 260.94. For details including CFU/mL results and statistical analyses including the experiments graphed and other replicates, see **Tables 4.2A-2D**.

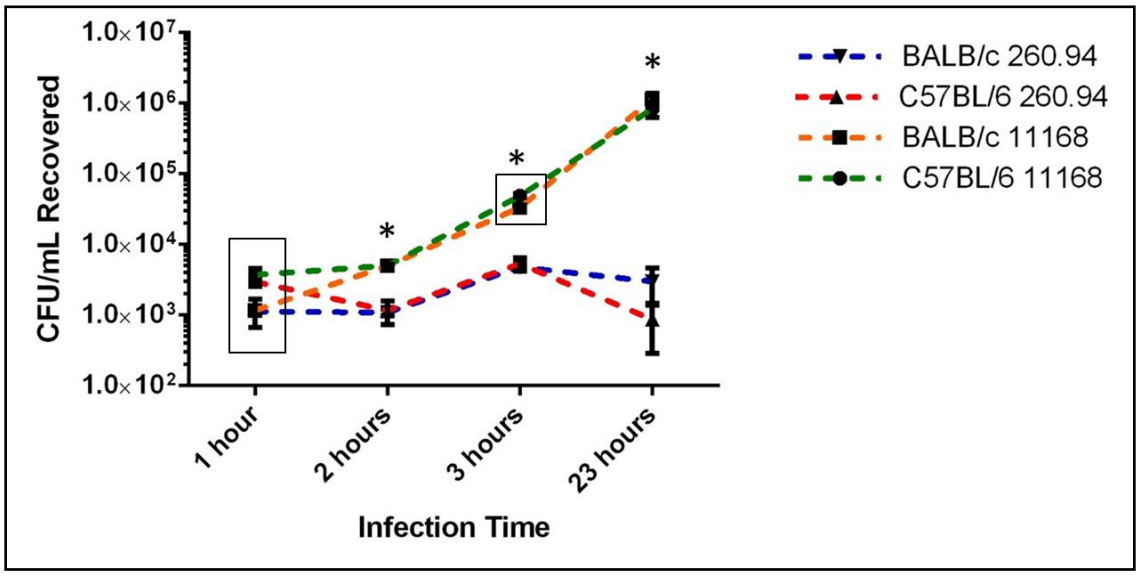


Figure 4.4. **Differences in intracellular survival of two *C. jejuni* strains in bone marrow-derived dendritic cells (BMDCs) from wild-type (WT) and IL-10$^{-/-}$ mice on both C57BL/6 and BALB/c backgrounds as assessed by gentamicin killing assay**. Cells were infected with either *C. jejuni* 11168 or 260.94 strains for 1 hour, treated with gentamicin to kill extracellular bacteria, washed, and incubated until 24 hours post-infection. BMDCs were lysed, and lysates (diluted when needed) were spread and cultured on Bolton agar plates for 72 hours to enumerate viable intracellular *C. jejuni*, presented as colony forming units (CFU)/mL. Data are from three-wells in an experiment, expressed as mean ± SEM. (A) One representative experiment of three, WT mice; (B) One representative experiment of two, IL-10$^{-/-}$ mice. *C. jejuni* 260.94 was not recovered from BALB/c IL-10$^{-/-}$ cells and does not show up on the graph; (C) Experiments from (A) and (B) combined in same graph for comparison purposes. Killing ability of WT mice could not be compared statistically to IL-10$^{-/-}$ mice, as the experiments were performed separately. Asterisks indicate statistically significant differences; 2-way ANOVA, with Holm-Sidak pairwise comparisons. For details including CFU/mL results and statistical analyses including the experiments graphed and other replicates, see **Tables 4.3A-B**.

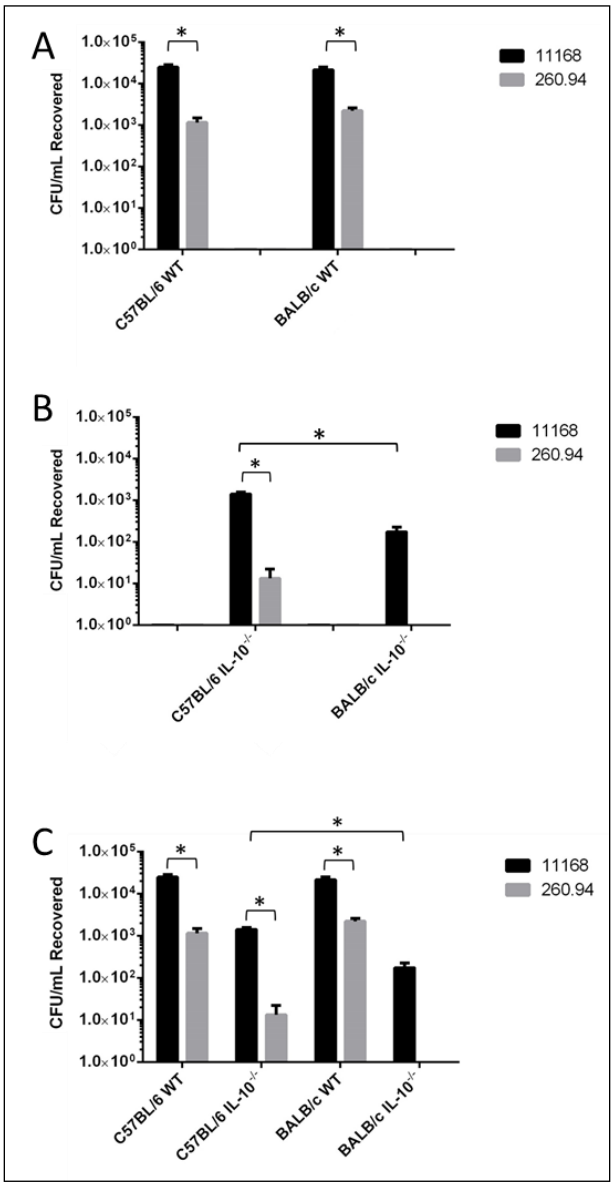
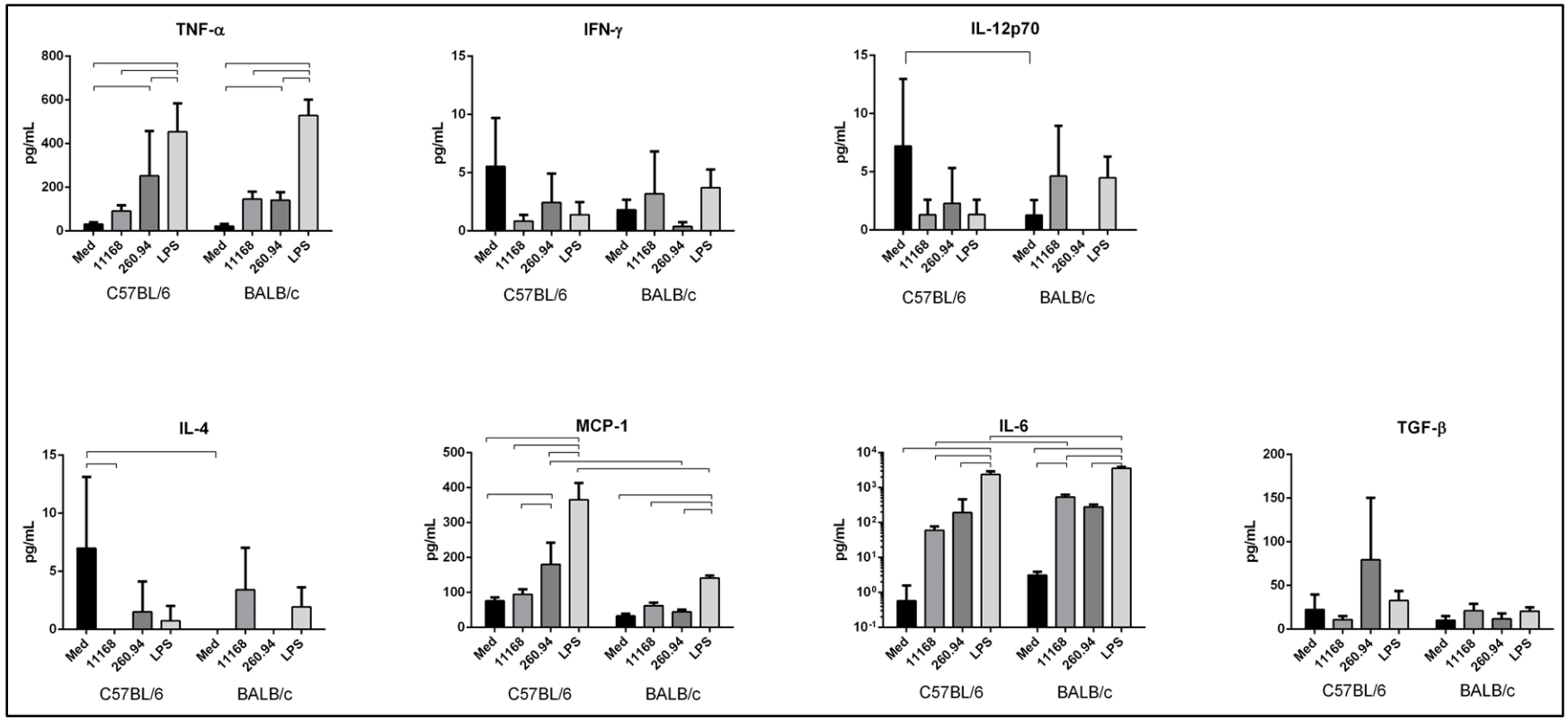


Figure 4.5. Cytokine production in *C. jejuni*-infected bone marrow-derived dendritic cells (BMDCs) derived from C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice. BMDCs were collected on day 10 of differentiation, seeded into 24-well culture plates, and infected with *C. jejuni* 11168 (multiplicity of infection [MOI]: 170), *C. jejuni* 260.94 (MOI: 138), or incubated with lipopolysaccharide (LPS; positive control) or medium only (Med; negative control) for 1 hour. Cells were then treated with gentamicin, washed, and incubated with fresh medium until supernatants were collected at approximately 24 hours p.i. Pro-inflammatory cytokines and those directing Th polarization into Th1, Th2, or Th17 cells were measured using a multiplexed flow cytometry-based bead assay. Data are from 3-wells, and mean ± SD is shown. One experiment representative of three independent experiments is shown. Brackets indicate statistically significant differences between treatment groups (2-way ANOVA, followed by Holm-Sidak pair-wise comparisons; **Table 4.5**). For details regarding statistical analyses of the other two replicates, see **Table 4.4** and **Table 4.6**.



APPENDIX B: SUPPLEMENTAL DATA

This appendix comprises **Table A.1**, showing results of additional gentamicin killing assay (GKA) replicates evaluating intracellular survival. These experiments were performed after completion of those included in the dissertation and are presented as supplemental data.

Table A.1. Results of additional GKAs assessing intracellular survival of *C. jejuni* 11168 and 260.94 strains in cultured dendritic cells derived from the bone marrow of wild-type or interleukin-10$^{-/-}$ mice on C57BL/6 and BALB/c genetic backgrounds. Stem cells isolated from bone marrow were seeded at 2.5 × 10^6 cells/10 mL medium (except 1 mouse in 1 experiment, seeded at 2.3 × 10^6 cells/10 mL) and differentiated for 10 days. Dendritic cells were infected for 1 hr, treated with gentamicin, washed, and lysed at 24 hr to enumerate viable intracellular *C. jejuni*. WT = wild-type; IL-10$^{-/-}$= interleukin-10-deficient; MOI = multiplicity of infection; SD = standard deviation; CFU = colony forming units; C57 = C57BL/6; BALB = BALB/c; ns = non-significant.

Additional Replicate	MOI (Approx. Range Derived from Limiting Dilution)	Mean ± SD, CFU/mL	Statistical Analyses and Results
WT #1	11168: 104-150 260.94: 102-335	C57 11168: 2.1 ± 2.6 × 10^4 BALB 11168: 3.12 ± 1.67 × 10^4 C57 260.94: 3.3 ± 1.5 × 10^3 BALB 260.94: 4.7 ± 3.1 × 10^3	**2-way ANOVA** (failed equal variance): Only ***C. jejuni* strain factor significant (P = 0.031). Holm-Sidak post-test: more *C. jejuni* 11168 than 260.94 recovered.** Kruskal-Wallis (due to failing equal variance): ns (P = 0.168). (NOTE: one of 3 plates from the C57 11168 group was excluded due to too numerous to count colonies.)
WT #2	11168: 30-105 260.94: 60-115	C57 11168: 6.21 ± 3.01 × 10^4 BALB 11168: 6.53 ± 1.16 × 10^4 C57 260.94: 1.7 ± 1.3 × 10^4 BALB 260.94: 1.82 ± 1.70 × 10^3	**2-way ANOVA:** Only ***C. jejuni* strain factor significant (P < 0.001). Holm-Sidak post-test: more *C. jejuni* 11168 than 260.94 recovered.**
IL-10$^{-/-}$ #1	11168: 190-340 260.94: 219-270	C57 11168: 6.14 ± 1.60 × 10^4 BALB 11168: 2.04 ± 1.79 × 10^4 C57 260.94: 2.4 ± 2.0 × 10^3 BALB 260.94: 3.8 ± 4.5 × 10^2	**2-way ANOVA** (failed normality, passed equal variance): both factors significant (***C. jejuni* strain, P <0.001; Genotype, P = 0.015**) and significant **interaction (P = 0.023)** present. Significant pairwise comparisons by **Holm-Sidak post-test: [C57 11168 > C57 260.94]; [C57 11168 > BALB 11168]. 1-way ANOVA: P <0.001.** Significant pairwise comparisons (Holm-Sidak): **[C57 11168 > BALB 260.94]; [C57 11168 > C57 260.94]; [C57 11168 > BALB 11168].** Kruskal-Wallis (due to failing normality): ns (P = 0.078).
IL-10$^{-/-}$ #2	260.94: 144-450	C57 260.94: 4.4 ± 1.9 × 10^3 BALB 260.94: 6 ± 6 × 10^2	**t-test: Significantly more *C. jejuni* 260.94 recovered from C57 than from BALB mice (P = 0.031).**
IL-10$^{-/-}$ #3	11168: 159-250 260.94: 203-215	C57 11168: 1.5 ± 1.9 × 10^4 BALB 11168: 8 ± 11 × 10^2 C57 260.94: 1 ± 2 × 10^3 BALB 260.94: 3 ± 6	**2-way ANOVA** (failed normality): both factors and interaction **all ns. Kruskal-Wallis** (due to failing normality): **ns (P = 0.186).**

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CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

SUMMARY

Campylobacter jejuni is an important cause of bacterial gastroenteritis worldwide and is associated with numerous post-infectious sequelae. *C. jejuni* strains vary widely in factors determining pathogenicity, including invasive potential, ability to stably colonize a host, and expression of ganglioside mimics in the lipooligosaccharide. Similarly, host factors including microbiota and genetic background predisposing to immunological biases are highly variable. In people, *C. jejuni* most typically causes a severe but self-limiting gastroenteritis.[5] Development of post-infectious complications, including the peripheral neuropathy Guillain-Barré syndrome (GBS), occurs in a small proportion of cases.[12] The immunopathogenesis and factors determining susceptibility to GBS are incompletely understood, but both infecting *C. jejuni* strain characteristics and host factors such as immunogenetic background are thought to contribute.

The complex interplay of pathogen and host, beginning with the initial host-microbe interaction, determines innate and adaptive immunity and corresponding disease outcomes. The overarching aim of this study was to determine how these interactions underlie contrasting immune responses to *C. jejuni* in mice. *C. jejuni* strains associated with colitis (*C. jejuni* 11168) and GBS (*C. jejuni* 260.94) have resulted in contrasting adaptive immune responses within C57BL/6 IL-$10^{-/-}$ mice.[15] BALB/c and C57BL/6 are two commonly studied laboratory mouse strains, chosen specifically for this study because of documented immunological biases. In many models, C57BL/6 mice exhibit a pro-inflammatory and Th1 bias. In contrast, BALB/c mice are reportedly Th2-biased, in addition to harboring a greater proportion of immunomodulatory T regulatory (Treg) cells with greater suppressive potential on responding T cells.[6; 9; 18; 20; 25] Thus, this system was designed to evaluate the interplay of infecting *C. jejuni* strain and host

characteristics, beginning with the initial interaction of *C. jejuni* with dendritic cells, and assessing the impact on development of innate and adaptive immunity and disease outcome.

Dendritic cells (DCs) are sentinel antigen presenting cells located throughout the body. These cells continually surveil the local environment and upon interaction with antigen, undergo a maturation process culminating in migration to local lymph nodes and interaction with naïve T cells. This interaction involves presentation of antigen through peptide-MHC II complexes with the T cell receptor, costimulatory molecule expression for T cell activation, and cytokine production. Depending upon the signals generated during this interaction, the Th cell differentiation is polarized toward certain subsets, including Th1, Th2, Th17, or Treg cells; mixed responses are also possible. DCs are thus a vital bridge between innate and adaptive immunity, driving initial polarization of immunity that subsequently determines disease outcomes.

An *in vitro* culture system was employed to evaluate host genetic background, in addition to differences in *C. jejuni* strain invasion efficiency and intracellular survival capability, in the initial host-microbe interaction. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was used to obtain bone marrow-derived DCs (BMDCs) from hematopoietic precursors. BMDCs from wild-type (WT) and IL-$10^{-/-}$ mice on both C57BL/6 and BALB/c backgrounds were used in this study. Confirmation of BMDC phenotype in differentiated cells was performed by flow cytometric analysis of CD11c, MHC II, and F4/80 expression. Interestingly, populations differentiated from stem cells of C57BL/6 IL-$10^{-/-}$ and BALB/c IL-$10^{-/-}$mice have a high percentage of cells expressing CD11c but differ in proportions of immature DCs, mature DCs, and macrophages within final populations. BMDCs from C57BL/6 IL-$10^{-/-}$ mice reflected a more mixed population of immature and mature DCs along with approximately 25% macrophages, while BALB/c IL-$10^{-/-}$ populations comprised a higher proportion of more mature DCs and approximately 16% macrophages. Presence or absence of IL-10 did not appear to impact differentiation

within either C57BL/6 or BALB/c mice, but may affect maturation and function following stimulus with *C. jejuni* or LPS.

Invasion efficiency and intracellular survival of *C. jejuni* strains 11168 and 260.94 in BMDCs derived from C57BL/6 and BALB/c mice were assessed by gentamicin killing assay. In invasion assays using BMDCs derived from IL-$10^{-/-}$ mice, *C. jejuni* 11168 exhibited higher combined invasion efficiency and intracellular survival compared to *C. jejuni* 260.94, with the difference becoming more pronounced with increased infection time. Similarly, *C. jejuni* 11168 exhibited higher intracellular survival in BMDCs derived from both WT and IL-$10^{-/-}$ mice, compared to *C. jejuni* 260.94. The enhanced invasion and survival of *C. jejuni* 11168 compared to *C. jejuni* 260.94 observed *in vitro* correlates with previous comparison of genes associated with invasion, adherence, and acid resistance in these two strains,[1] and with immunohistochemical labeling of the two *C. jejuni* strains in the proximal colon of BALB/c mice (Brudvig, Chapter 3). Thus, BMDCs provide a useful model for studying *C. jejuni* strain characteristics *in vitro*.

In these gentamicin killing assays, differences in *C. jejuni* recovery between mouse genotypes were less consistent than differences identified between *C. jejuni* strains. The most consistent pattern that emerged was higher intracellular viability of *C. jejuni* 11168 in C57BL/6-derived BMDCs. This pattern likely reflects a combination of both enhanced invasion and survival of *C. jejuni* 11168 compared to *C. jejuni* 260.94, and also a relatively higher proportion of immature DCs and macrophages in C57BL/6 compared to BALB/c populations. These findings emphasize the important interplay between host cells and *C. jejuni* strains during the initial interaction.

Cytokine production by DCs is an important signal for Th cell polarization. The final characterization of the host-microbe interaction was assessed by cytokine production of BMDCs from IL-$10^{-/-}$ mice infected with either *C. jejuni* 11168 or *C. jejuni* 260.94. Heightened production of the chemokine MCP-1 in BMDCs from C57BL/6 IL-$10^{-/-}$ mice, especially following infection with *C. jejuni*

260.94, reflects both host cell and *C. jejuni* strain characteristics. Macrophages are primary producers of MCP-1, and C57BL/6 IL-10$^{-/-}$ cells contained a higher proportion of macrophages than BALB/c IL-10$^{-/-}$ cells. MCP-1 was shown to be an important determinant for Th2 polarization,[10] and Th2-mediated immunity was previously reported in C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94.[15] Therefore, early production of MCP-1 by DCs may contribute to Th2 polarization in some models. Similarly, BMDCs from BALB/c IL-10$^{-/-}$ mice exhibited enhanced production of IL-6, especially following infection with *C. jejuni* 11168. As strong Th1/Th17 immunity was seen *in vivo* in BALB/c IL-10$^{-/-}$ mice following infection with *C. jejuni* 11168 (Brudvig, Chapter 3), these results further support the early contributions of DCs in determining adaptive immunity and disease outcome.

In addition to *in vitro* methods to assess how initial host-microbe interactions help determine immunity, *in vivo* mouse models were also used to assess both immune response and pathology mimicking human disease. In the first study, WT and IL-10$^{-/-}$ BALB/c mice were infected with *C. jejuni* 260.94 to investigate the potential for a new GBS model. Considering the previous Th2-mediated responses in C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94[15] and the reported relative Th2-bias in BALB/c compared to C57BL/6 mice, BALB/c mice were expected to mount strong Th2-mediated immunity. Production of anti-ganglioside antibodies, a hallmark of GBS, and development of neurological deficits and accompanying inflammatory lesions in dorsal root ganglia were expected. Instead, both WT and IL-10$^{-/-}$ BALB/c mice infected with *C. jejuni* 260.94 exhibited systemic Th1/Th17 responses, manifested by significant increases in *C. jejuni*-specific IgG2a, IgG2b, and IgG3 plasma antibodies. Infected mice also did not develop anti-ganglioside antibodies or peripheral nerve lesions as induced with *C. jejuni* 260.94 in other mouse strains.[15; 23] Systemic Th1/Th17 responses were exacerbated by IL-10 deficiency, but even in WT mice no Th2 response was seen. Results of this study further support the impact of host genetic background in determining adaptive immunity and susceptibility to GBS. With the results of this study, different immune responses had now been shown

between *C. jejuni* strains 11168 and 260.94 within C57BL/6 IL-10$^{-/-}$ mice,[15] and between BALB/c IL-10$^{-/-}$ and C57BL/6 IL-10$^{-/-}$ mice infected with *C. jejuni* 260.94 (Malik, *et al*. 2014[15] and Brudvig, Chapter 2). Therefore, the final study aimed to further investigate the *in vivo* interplay between host genetic background and *C. jejuni* strain characteristics in a single system.

C57BL/6 IL-10$^{-/-}$ and BALB/c IL-10$^{-/-}$ mice were chosen due to contrasting immune responses to *C. jejuni* strains in our models, and to respective Th1 and Th2 immunological biases reported in the literature. *C. jejuni* strains 11168 and 260.94 are associated with colitis and GBS, respectively, and were shown to vary in colitogenic potential and magnitude of immune response.[1] In the current 4-week study, BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 showed the lowest survivorship, most severe colitis, marked systemic and local Th1/Th17-mediated immunity reflected by *C. jejuni*-specific plasma antibodies and increased IFN-γ, TNF-α, and IL-22 production in the proximal colon, and robust mucosal immunity reflected by *C. jejuni*-specific IgA in fecal supernatants. *C. jejuni* 260.94-infected BALB/c IL-10$^{-/-}$ mice also mounted systemic Th1/Th17-mediated immune responses, but did not develop colitis or marked colonic or mucosal immunity. Interestingly, significant production of anti-GM1 and anti-GD1a ganglioside antibodies was seen only in BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168, a strain associated with enteritis and not GBS, while infection with *C. jejuni* 260.94 did not induce anti-ganglioside antibody production. The anti -GM1 and -GD1a plasma antibodies produced were Th1/Th17-associated IgG2a and IgG2b isotypes, and thus the possibility that the marked systemic and local immunity induced by *C. jejuni* 11168 in BALB/c IL-10$^{-/-}$ mice was necessary for anti-ganglioside antibody production in this model should be considered. For BALB/c IL-10$^{-/-}$ mice, results of the first *in vivo* study were confirmed, in that *C. jejuni* 260.94 induced Th1/Th17 systemic responses, but without colitis or anti-ganglioside antibody production, and no Th2 component was seen. BALB/c IL-10$^{-/-}$ mice infected with *C. jejuni* 11168 exhibited strong Th1/Th17-mediated immunity and colitis, and provide an avenue for studying *C. jejuni* pathogenesis in mice of an additional genetic background.

Contrary to the striking immune response and colitis seen in *C. jejuni* 11168-infected BALB/c IL-$10^{-/-}$ mice, C57BL/6 IL-$10^{-/-}$ mice infected with either *C. jejuni* strain demonstrated atypical results compared to previous studies by our group.[1; 2; 15-17; 21] Despite colonization and an enlarged regional lymph node in many infected mice, virtually no systemic, mucosal, or local colonic immune response was seen following infection with either *C. jejuni* strain, and the mice did not develop the colitis or anti-ganglioside antibodies following *C. jejuni* 11168 or 260.94 infection, respectively, as in other models.[1; 15-17] Interestingly, *Lactobacillus murinus* was cultured from all C57BL/6 IL-$10^{-/-}$ mice, but no BALB/c IL-$10^{-/-}$ mice, at the end of the study. The presence of *L. murinus* was unexpected and the source is unknown, as is why it was cultured only from C57BL/6 IL-$10^{-/-}$ and not BALB/c IL-$10^{-/-}$ mice. Both genotypes of mice received the same *C. jejuni* inocula, which were pure and motile *C. jejuni* cultures based upon wet mount and Gram stain preparations.

Lactobacilli comprise one group of bacteria currently being investigated for potential probiotic effects. Different *Lactobacillus* spp. have demonstrated protective effects against development of spontaneous colitis in IL-$10^{-/-}$ mice.[14; 19] Furthermore, both *in vitro* and *in vivo* studies also have demonstrated antagonism of *Lactobacillus* spp. against *C. jejuni.* [3; 8; 22; 24] Infection of C57BL/6 IL-$10^{-/-}$ mice with *C. jejuni* 11168 has provided a repeatable model of colitis with few exceptions.[1; 15-17; 21] Thus, the relative protection exhibited by C57BL/6 IL-$10^{-/-}$ mice in this study, with concurrent carriage of *L. murinus*, warrants further study.

The current study adds to the relatively limited knowledge of the combination of host factors and infecting *C. jejuni* strain in determining disease outcome. The relative protection of C57BL/6 IL-$10^{-/-}$ mice from *C. jejuni*-induced pathology, compared to BALB/c IL-$10^{-/-}$ mice in the current study and to C57BL/6 IL-$10^{-/-}$ mice in previous studies, indicates a potential probiotic effect of *L. murinus*. Overall, our results indicate that BALB/c mice, with and without IL-10, respond to *C. jejuni* infection with Th1/Th17- but not Th2-mediated responses. The magnitude of the immune response and induction of colitis is

dependent upon infecting strain, with *C. jejuni* 11168 showing higher pathogenicity. *In vitro* studies showed that the colitogenic *C. jejuni* 11168 exhibits enhanced invasion and intracellular survival in dendritic cells compared to GBS-associated *C. jejuni* 260.94, a finding reflected by *in vivo* immunohistochemical labeling of these two *C. jejuni* strains in the ileocecocolic junction. While developing an *in vitro* system for assessment of the initial *C. jejuni*-dendritic cell interaction, important differences were found between C57BL/6 and BALB/c mice in differentiation of hematopoietic stem cells into dendritic cells. Functional differences in cytokine production and ability to kill intracellular *C. jejuni* were consistent with the different BMDC phenotypes. Cytokines produced by infected IL-$10^{-/-}$ BMDCs correlate with *in vivo* responses in the current study (Brudvig, Chapter 3) and reported previously,[15] indicating that DCs play an important role in polarizing the immune response. This study provides critical insight into the complex interplay of *C. jejuni* strain characteristics and host factors, beginning with the contribution of dendritic cells in the initial host-microbe interaction.

FUTURE DIRECTIONS

What is the effect of IL-10 on DC maturation and function between BMDCs derived from C57BL/6 and BALB/c mice?

Presence or absence of IL-10 did not markedly affect the differentiation of hematopoietic stem cells derived from either C57BL/6 or BALB/c mice as assessed by CD11c and MHC II expression. At the end of the 9-11 day differentiation period, cells derived from both WT and IL-10$^{-/-}$ mice on their respective C57BL/6 or BALB/c backgrounds exhibited similar proportions of CD11c(+) cells and did not differ markedly in MHC II expression with or without IL-10. IL-10 has been shown to affect both spontaneous and induced DC maturation, in addition to pro-inflammatory cytokine production and T-cell stimulating ability.[7; 11] Therefore, while IL-10 apparently does not substantially impact differentiation of stem cells into BMDCs, it would be informative to determine if there is a differential effect exerted by IL-10 on DC maturation and function in C57BL/6 compared to BALB/c mice. To test this, BMDCs derived from WT and IL-10$^{-/-}$ on C57BL/6 and BALB/c backgrounds could be cultured as before for 10 days and stimulated with LPS. Expression of MHC II in addition to costimulatory molecules CD80 and CD86 analyzed by flow cytometry would indicate differences in maturation. Differences in pro-inflammatory cytokine production and putative T-cell stimulating ability determined by measurement of cytokines including TNF-α, IFN-γ, IL-6, IL-12, and IL-4 would reflect effects of IL-10 on DC function. These experiments could also include infection with different *C. jejuni* strains, to determine if *C. jejuni*-induced DC maturation and function is variable depending upon presence or absence of IL-10.

Another informative experiment would be to assess the intracellular survival of *C. jejuni* strains 11168 and 260.94 in BMDCs derived from WT and IL-10$^{-/-}$ on the same genetic background. In the current study, BMDCs from WT C57BL/6 and BALB/c mice were compared in single experiments, and BMDCs from IL-10$^{-/-}$ C57BL/6 and BALB/c mice were compared in single experiments. Killing ability of WT versus IL-10$^{-/-}$ cells could not be compared statistically for this reason, but the patterns suggest that IL-

$10^{-/-}$ cells exhibit enhanced killing of *C. jejuni*. This would be consistent with increased maturation and production of pro-inflammatory cytokines induced by neutralization of IL-10 in human DC studies.[7] To further evaluate the effect of IL-10 on ability of DCs to kill different *C. jejuni* strains, BMDCs from WT and IL-$10^{-/-}$ C57BL/6 mice can be compared, and BMDCs from WT and IL-$10^{-/-}$ BALB/c mice can be compared, in separate experiments. It is logistically prohibitive to test four genotypes of mice with two *C. jejuni* strains in a single gentamicin killing assay. Thus, the question of effect of IL-10 within the separate genotypes should be tested independently. Further experiments can then be designed based on these results.

Is increased viable intracellular *C. jejuni* 11168 compared to *C. jejuni* 260.94 in BMDCs, determined by gentamicin killing assay, reflecting enhanced survival more than invasion?

In assays using BMDCs from IL-$10^{-/-}$ mice, viable intracellular *C. jejuni* 11168 was not different from *C. jejuni* 260.94 after 1 hour of invasion, gentamicin treatment, and immediate lysis. However, *C. jejuni* 11168 was recovered in significantly higher numbers than *C. jejuni* 260.94 from C57BL/6 IL-$10^{-/-}$-derived BMDCs with the same infection time of 1 hour followed by gentamicin treatment, but without lysis until 24 hours p.i. This implies that the increased intracellular *C. jejuni* 11168 over time may be more due to intracellular survival and replication than invasion. In order to further delineate the relative contributions of invasion and survival leading to the marked increase in *C. jejuni* 11168 recovery over time, time-course experiments performed with 1 hour of infection, gentamicin treatment, and then lysis of the BMDCs at various time points such as 2, 4, 8, and 12 hours would further characterize the survival and replication over time. It would be logistically prohibitive to test all mouse genotype/*C. jejuni* strain combinations with sufficient replicate wells in a single assay by this method; thus individual assays testing single combinations could be performed, and identification of broad patterns can direct further studies.

What genes are involved in enhanced invasion and survival of *C. jejuni* 11168 compared to *C. jejuni* 260.94?

Multiple genes with putative functions including adherence, colonization, invasion, acid resistance, the LOS, and motility present in *C. jejuni* 11168 are divergent or altogether absent in *C. jejuni* 260.94.[1] Differences between these *C. jejuni* strains in invasion and intracellular survival *in vitro* may be due to any one gene or a combination. To further determine if one of the divergent or absent genes is enhancing *C. jejuni* 11168 survival or invasion compared to *C. jejuni* 260.94, mutant *C. jejuni* 11168 strains lacking one of these genes could be generated and tested *in vitro* for a reduction in invasion or survival; rescue of the phenotype by complementation would further confirm a role for the particular gene.

What effect does colonization with *Lactobacillus murinus* have on development of spontaneous colitis in IL-10$^{-/-}$ mice?

In the second *in vivo* model (Chapter 3), *Lactobacillus murinus* was cultured from all C57BL/6 IL-10$^{-/-}$ mice that also appeared to be protected from *C. jejuni*-induced pathology. *L. reuteri* and *L. salivarius* ssp. *salivarius* have demonstrated protective effects against development of spontaneous colitis in 129 Sv/Ev IL-10$^{-/-}$ and C57BL/6 IL-10$^{-/-}$ mice.[14; 19] IL-10$^{-/-}$ mice can develop spontaneous colitis as early as 3-4 weeks of age, with variable severity depending upon housing, and with more severe lesions in BALB/c than C57BL/6 mice.[4; 13] It is worth noting that in the current study, 3/10 sham-inoculated BALB/c mice exhibited mild spontaneous colitis and 1/10 sham-inoculated C57BL/6 mice had mild colitis. The mice were inoculated at a younger age than in the first *in vivo* experiment (Brudvig, Chapter 2) in order to avoid the confounding spontaneous colitis seen with older age. Mice in the second *in vivo* model (Brudvig, Chapter 3) were euthanized at approximately 10 weeks of age, and the higher rate of spontaneous colitis in BALB/c mice may only reflect the previously reported pattern of increased

severity compared to C57BL/6 mice. However, as the C57BL/6 mice harboring *L. murinus* may have also been protected from *C. jejuni*-induced disease, the possibility that *L. murinus* also conferred protection against spontaneous colitis should be considered. Standardized experiments specifically designed to examine the prevalence of spontaneous colitis in IL-10$^{-/-}$ mice of both BALB/c and C57BL/6 backgrounds with and without *L. murinus* carriage would be necessary to further test the possibility that *L. murinus* abrogated spontaneous colitis in C57BL/6 IL-10$^{-/-}$ mice. For descriptive purposes, C57BL/6 IL-10$^{-/-}$ mice in the colony could be periodically tested for *L. murinus* by culture, monitored over time for clinical signs, and assessed histologically at sacrifice to determine if there was a correlation between *L. murinus* carriage and delayed onset or less severe spontaneous colitis. Controlled studies could also be performed using BALB/c IL-10$^{-/-}$ mice verified to be free of *L. murinus* in the beginning of the study. A placebo-controlled trial could then be conducted[19] to determine if *L. murinus* was associated with reduced spontaneous colitis.

Is there an inhibitory, strain-specific interaction between *L. murinus* and *C. jejuni* strains 11168 and 260.94 *in vitro*?

Prior to *in vivo* experiments, *in vitro* assays should be conducted as further confirmation of an inhibitory effect of *L. murinus* on *C. jejuni* growth and invasiveness. The gentamicin killing assay performed in this study could incorporate pre-treatment with *L. murinus* prior to infection as previously described,[24] followed by assessment of *C. jejuni* invasion and survival in BMDCs. *In vivo* immunohistochemical labeling of *C. jejuni* showed no *C. jejuni* 11168 was present beneath the surface epithelium in C57BL/6 IL-10$^{-/-}$ mice, while infected BALB/c IL-10$^{-/-}$ mice showed intracellular labeling in macrophages/dendritic cells within both the lamina propria and submucosa (Brudvig, Chapter 3). Therefore, using an epithelial line such as young adult mouse colon cells (YAMC) pretreated with *L.*

murinus prior to invasion assays would further characterize the interplay of *C. jejuni* and *L. murinus* at the epithelium.

Testing antimicrobial activity of *L. murinus* against *C. jejuni* strains 11168 and 260.94 by previously reported methods[22] would also be informative. *L. murinus* cultures can be spotted onto agar plates and allowed to grow, with subsequent overlaying of broth and agar inoculated with *C. jejuni*. Zones of inhibition would represent inhibitory effects of *L. murinus* on *C. jejuni* growth.

Is *Lactobacillus murinus* protective against *C. jejuni*-mediated immunopathology *in vivo*?

Antagonism of pathogens by probiotic bacteria can depend upon the pathogen, the probiotic, and the environment. If results of *in vitro* invasion and inhibition assays indicate specificity of *L. murinus* against *C. jejuni, in vivo* experiments specifically designed to determine if *L. murinus* ameliorates *C. jejuni*-induced colitis can be performed. Because BALB/c IL-$10^{-/-}$ mice were shown to be free of *L. murinus* and susceptible to severe *C. jejuni* 11168-induced colitis (Brudvig, Chapter 3), these mice provide an ideal system. Following confirmation of *L. murinus* negativity, BALB/c IL-$10^{-/-}$ mice can be either prophylactically or therapeutically treated with *L. murinus*, with and without *C. jejuni* inoculation. Effects of *L. murinus* on *C. jejuni* colonization and invasiveness and effects on systemic and mucosal immunity and local cytokine production can then be used to further support or refute that *L. murinus* protected C57BL/6 IL-$10^{-/-}$ mice from *C. jejuni*-induced pathology in the current study.

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