COMPOSITION OF GUT MICROBIOTA AFFECTS *C. JEJUNI*-MEDIATED INFLAMMATION AND AUTOIMMUNITY IN MURINE MODELS

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ABSTRACT

COMPOSITION OF GUT MICROBIOTA AFFECTS *C. JEJUNI*-MEDIATED INFLAMMATION AND AUTOIMMUNITY IN MURINE MODELS

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Campylobacter jejuni is the leading antecedent infection to the acute peripheral neuropathy Guillain-Barre Syndrome (GBS). GBS is debilitating, often causes paralysis, and can require several months or more for recovery. Most concerning is that GBS patients are frequently left with long-term neurologic disabilities. Because a vaccine for *Campylobacter* is lacking there are no viable approaches for preventing this form of GBS. Currently, therapeutic approaches for GBS include plasma exchange and intravenous immunoglobulin but they require specialized equipment, pose significant financial burden, and produce mixed results. These strategies lack a strong rationale because GBS is poorly defined mechanistically. While new working mouse models of GBS may lead to alternative therapies, confirmation of *C. jejuni's* specific role in precipitating GBS and the mechanism(s) through which this occurs remain elusive. Thus far, evidence gathered from murine models demonstrates that multiple factors influence *C. jejuni* pathogenesis, including host genetics and *C. jejuni* genetics, particularly the genetic plasticity of this pathogen. Notably, the gut microbiota can modulate *C. jejuni* colonization- and colitis-resistance; however, its role in modulating *C. jejuni*-triggered autoimmunity remains unknown. The overarching goal of this study is to determine if the composition of gut microbiota affects *C. jejuni*-triggered autoimmunity in murine models. The chapters of this thesis present the following data addressing this goal; mice infected with antimicrobial resistant *Campylobacter* strains from Guillain-Barré syndrome patients produced severe colitis and type 2 autoimmune responses when their microbiota were depleted by antibiotics. Furthermore, we demonstrated that transplanted human fecal microbiota alters the immune response to *Campylobacter jejuni* infection in C57BL/6 mice, potentially increasing the risk of autoimmune sequelae. Finally,

comparative genomic analysis of passaged *C. jejuni* populations revealed genetic variation in multidrug transporter genes *cmeB* and *cmeR* in *Campylobacter jejuni* populations from antibiotic treated mice. CmeR regulates expression of *C. jejuni* cell surface molecules, again potentially impacting the risk of autoimmune sequelae. Taken together, our results demonstrate that composition of gut microbiota is a critical determinant of inflammatory and autoimmune outcomes in *C. jejuni* murine models.

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KEY ABBREVIATIONS

ATP	Adenosine Triphosphate
CPZ	Cefoperazone
DNA	Deoxyribonucleic acid
Hu	Humanized
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Mo	Mouse
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
PMA	Phorbol mysirate acetate
rRNA	Ribosomal Ribonucleic acid

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

GUILLAIN-BARRÉ SYNDROME: A POST-INFECTIOUS DISORDER

General disease characteristics. GBS is the leading cause of polyneuropathy worldwide, excluding locations that have failed to eradicate poliomyelitis (Hughes and Rees 1997). While generally accepted as a disease entity, GBS can manifest in many forms, with a wide range of disease severity, neurological deficits, antecedent infections, electrophysiological responses, and antibody responses (van den Berg, Marrink et al. 1992, Hadden, Karch et al. 2001, Yuki and Hartung 2012, Kuwabara and Yuki 2013, van den Berg, Walgaard et al. 2014). Diagnostic criteria for GBS include acute onset of symptoms, symmetry of symptoms, areflexia, delayed nerve conductivity, and elevated protein levels in cerebral spinal fluid (Asbury and Cornblath 1990). At present, GBS can be divided into at least four distinct subtypes: acute inflammatory demyelinating polyradiculoneuropathy (AIDP) that resembles experimental autoimmune nephritis (EAN), which is a T-cell driven disease. In contrast the two axonal forms, acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN) are known to be antibody mediated. Finally, Miller Fisher Syndrome (MFS) is a rare form of GBS that is not well characterized but often involves areflexia, ataxia, opthalmoplegia, and subclinical motor nerve dysfunction (Hughes and Cornblath 2005).

Associated infecting microorganisms can be identified in approximately fifty-percent of GBS cases, thus it has been deemed a post-infectious disorder [2]. Although, at least sixteen microorganisms have been linked to GBS, most cases have antecedent infections of *Cytomegalovirus*, Epstein-Barr virus, *Mycoplasma pneumoniae*, and *Campylobacter jejuni* (Hughes and Cornblath 2005). Infection with *C. jejuni* infection precedes approximately one-third of GBS cases in which an infectious agent can be identified (Mishu and Blaser 1993, van den Berg, Walgaard et al. 2014), and it is most often associated with the AMAN form.

Acute motor axonal neuropathy. The AMAN form of GBS is likely the result of complement-mediated attack on peripheral nerves following the binding of anti-ganglioside

antibodies elicited by infection. Although mortality is rare with GBS, varying between 3–7%, routine histologic analysis of AMAN patients has been performed and informs model development. Taken together, results of these studies show that the AMAN form of GBS is not associated with significant demyelination or lymphocytic inflammation found with other forms but often results in Wallerian-like degeneration and increased macrophage presence (McKhann, Cornblath et al. 1993, Griffin, Li et al. 1995). Ultrastructural analysis of the dorsal root ganglia and peripheral nerves of 7 patients that died following the onset of neurological symptoms showed the following: immunoglobulins bound to the node of Ranvier, complement deposits on the axolemma, and enhanced macrophage numbers in the axon and paranodal space (Griffin, Li et al. 1996). Furthermore, peripheral nerve dysfunction and paralysis correlated in time with peak presence of anti-ganglioside antibodies (Willison, Jacobs et al. 2016). These results are consistent with the hypothesis that anti-ganglioside antibodies bind to peripheral nerves and activate a complement-mediated immune response that leads to peripheral nerve damage, macrophage scavenging of myelin and axonal surfaces in the peripheral nerves with subsequent loss of nerve conduction velocity (Fig. 1.1). Experimental inoculation of mice with *C. jejuni* strains from patients with GBS evoked anti-ganglioside antibodies; however, whether these antibodies lead to neuropathy was not determined (Malik, Sharma et al. 2014).

CAMPYLOBACTER JEJUNI BIOLOGY

General enteric disease characteristics. *C. jejuni* is not only the leading antecedent infection to GBS but it is also a leading cause of enteric disease in both the developed and developing world (Willison, Jacobs et al. 2016). *C. jejuni* primarily colonizes the gastrointestinal tract and initiates inflammation termed gastroenteritis. Experimental evidence shows that *C. jejuni* gastroenteritis is associated with specific strains of *C. jejuni* (Bell, St Charles et al. 2009, Malik, Sharma et al. 2014) and can be enhanced by serial passage (Bell, St Charles et al. 2009), by

depleting the microbiota with antibiotics (O'Loughlin, Samuelson et al. 2015), or by infecting gnotobiotic animals (Chang and Miller 2006). These outcomes raised the question of whether *C. jejuni*-mediated-autoimmunity would also be enhanced under the environmental conditions employed in these models.

Immune responses to *C. jejuni* infection. *C. jejuni* infection evokes both T helper-1 (T_H1) and T helper-2 (T_H2) cellular responses and in some case anti-ganglioside antibodies (Malik, Sharma et al. 2014). The response to infection is determined by a variety of factors including the *C. jejuni* genetic background. This has been observed in humans where *Campylobacter* initiated autoimmunity results in a wide variety of diseases that includes, but is not limited to, the peripheral neuropathies Guillain-Barré syndrome (GBS) and its variant Miller Fisher syndrome (MFS) that causes descending paralysis (Willison and Veitch 1994). *C. jejuni* strains have been isolated from many patients with AMAN and MFS allowing comparisons to strains from patients with enteritis. GBS has been associated with specific strains of *C. jejuni* that possess lipooligosaccharide (LOS) structures on their outer core that are similar to gangliosides found on peripheral nerves near the node of Ranvier (Wim, Bart et al. 2004). Gangliosides are sialylated glycosphingolipids that are located on cell surfaces throughout the nervous system and play a role in cell-to-cell communication. Variation in ganglioside mimics on *C. jejuni* result from genetic variation in *C. jejuni* LOS loci (Parker, Horn et al. 2005, Parker, Gilbert et al. 2008). Synthesis of ganglioside mimics is dependent upon a variable set of genes and enzymes including glycosyltransferases *cstII* and *cstIII* that attach neuraminic acids to galactose on the outer core.

Experimentally, biochemical analysis of *C. jejuni* 11168 demonstrated that ganglioside mimics or the presence of genes required for their biosynthesis are not the only factors contributing to the autoimmune potential of *C. jejuni* strains (Godschalk, Heikema et al. 2004). Moreover, even strains possessing these mimics do not always elicit significant anti-ganglioside antibody responses as was seen when C57BL/6 IL-$10^{-/-}$ mice were infected with *C. jejuni* strain 11168 (Malik, Sharma et al. 2014). In addition, *C. jejuni's* genome contains hypervariable

nucleotide tracts, some of which are found in genes involved in surface structure biosynthesis; these tracts undergo expansion or contraction during passage in the host (Jerome, Bell et al. 2011) which can affect transcript abundance and gene expression further complicating our understanding of *C. jejuni* pathogenesis. This slip strand mutagenesis was demonstrated to affect genes that encode outer surface structures such as lipo-oligosaccharides (LOS), capsule, and O-linked glycosylation of the flagellum important for pathogenesis (Jerome, Bell et al. 2011).

***Campylobacter*: inflammation and autoimmunity in humans**. The following sections summarize the role of the gut microbiota in mediating colonization and disease in regards to *C. jejuni* and some other well-characterized enteric pathogens. Campylobacteriosis is the disease caused by infection with bacteria from the genus *Campylobacter; C. jejuni* is the most common cause of human *Campylobacter* infection (Cody, McCarthy et al. 2013), followed by *C. coli*. *Campylobacter jejuni* is an important zoonotic pathogen that causes 1.3 million cases of gastroenteritis in the US each year, leading to 13,000 hospitalizations and 120 deaths (Scallan, Griffin et al. 2011). Symptoms of campylobacteriosis often include intestinal inflammation, fever, diarrhea, and abdominal pain (Black, Levine et al. 1988, Young, Davis et al. 2007). These symptoms often resolve 4–7 days after initiation but may last up to 10 days (CDC 2014). Human infection occurs via the oral route and most often results from the consumption of raw or undercooked poultry (Young, Davis et al. 2007). Most avian species are asymptomatic carriers, serving as reservoirs for the *Campylobacter,* and infect other birds through common water and feeding sources (CDC 2014). The National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) reported that almost 50% of raw chicken in stores in the United States was contaminated with *Campylobacter* and that most of these cases may be attributed to contamination during slaughter and subsequent processing. Consumption of raw or unpasteurized milk and untreated water also contributes to *Campylobacter* infection (Young,

Davis et al. 2007). Milk may become contaminated when *Campylobacter* infects the udder or when cow fecal matter containing *Campylobacter* contaminates the milk (CDC 2014).

Although most cases resolve on their own, antimicrobials may be prescribed for *C. jejuni* enteritis when symptoms last longer than 7 days. Azithromycin or ciprofloxacin are most often prescribed to treat *Campylobacter* infection (CDC 2014) but rates of antibiotic resistance in *Campylobacter* species including *C. jejuni* and *C. coli* are high (Engberg, Aarestrup et al. 2001, Gibreel and Taylor 2006). *C. jejuni* was recently designated a serious antimicrobial resistant threat by the Centers for Disease Control and Prevention (CDC 2013). Antibiotic resistant (AR) *C. jejuni* have been suggested to cause more severe infections requiring lengthier hospitalizations when compared to susceptible infections and, thus, represent an important public health concern (Moore, Barton et al. 2006). Death following *Campylobacter* infection is rare, with an incidence of approximately 80 people dying of an estimated total of 1–1.5 million cases of infection per year in the United States (CDC 2014). Death usually occurs in those with underdeveloped or declining immune systems, including children under 2, the elderly, and the immune compromised such as those with acquired immune deficiency syndrome (AIDS) (WHO 2011). In rare instances, infected patients may experience long-term consequences including flare-ups of inflammatory bowel disease (Kim, Hans et al. 2009), irritable bowel syndrome (Qin, Wu et al. 2011), Reiter's arthritis (Garg, Pope et al. 2008), and the acute peripheral neuropathy GBS (Yuki, Taki et al. 1993, Hughes, Hadden et al. 1999).

***Campylobacter*: inflammation and autoimmunity in mouse models**. Many animals have been used to study the effects of *C. jejuni* infection, including mice, rats, rabbits, pigs, chickens and ferrets (Mansfield, Bell et al. 2007). Mice provide many advantages including 1) low cost to maintain, 2) relatively small spaces required for housing, allowing for larger experiments, 3) ease of manipulation, and 4) availability of genetic knockouts. Development of murine *C. jejuni* colonization and colitis models has been greatly advanced by manipulation of host genetics and host microbiota (Chang and Miller 2006, Mansfield, Patterson et al. 2008, Bereswill, Fischer et al.

2011, Malik, Sharma et al. 2014, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015). A summary of these advances is provided in the following sections.

Inflammation is associated with persistent *C. jejuni* colonization. Limited Enteric Flora (LF) C3H Severe Combined Immune Deficient (SCID) mice infected with *C. jejuni* displayed high level *C. jejuni* colonization for up 224 days. In contrast, immune competent congenic LF C3H mice began to clear the bacteria at approximately 28 days (Chang and Miller 2006). LF C3H SCID mice but not LF C3H immune competent mice displayed inflammation of the cecum and the colon (Chang and Miller 2006) suggesting that inflammation may allow *C. jejuni* to persist in the gut. This explanation would be consistent with experimental data from other pathogens including *Salmonella enterica serovar Typhimurium* (Winter, Thiennimitr et al. 2010) and some pathogenic *E. coli* (Horwitz and Silverstein 1980) that have evolved mechanisms to exploit inflammation by utilizing tetrathionate and evading compliment fixation, respectively. In general, two hypotheses exist to explain how pathogens may benefit from inflammation: 1) inflammation alters microbiota structure in a way that frees up nutrients that are exploited by pathogens but not the microbiota (i.e. food hypothesis) and 2) changes in antimicrobial compounds produced by the inflamed tissue may be detrimental to the microbiota but not the pathogen (i.e. differential killing hypothesis) (Stecher and Hardt 2008).

IL-10$^{-/-}$ but not IL-10$^{+/+}$ mice develop colitis after *C. jejuni* infection. Interleukin-10 (IL-10) is a regulatory cytokine produced by T cells, B cells and some monocytes that tends to suppress lymphocyte responses (Couper, Blount et al. 2008). Generally, IL-10 is classified as an anti-inflammatory cytokine that downregulates the host response to invasion by intracellular pathogens by inhibiting several key inflammatory regulators, including major histocompatibility complex II and T-cell co-stimulatory factors B7-1 and B7-2, and expression of interferon (IFNγ) (Moore, Malefyt et al. 2001, Ouyang, Rutz et al. 2011). Congenic C57BL/6 IL-10 deficient mice (C57BL/6 IL-10$^{-/-}$) but not their IL-10$^{+/+}$ counterparts are susceptible to colitis when infected by *C. jejuni* 11168 (Mansfield, Bell et al. 2007). *C. jejuni* 11168 successfully colonized the GI tract of

C57BL/6 wild type and IL-10$^{-/-}$ mice; however, only IL-10$^{-/-}$ mice developed inflammation of the colon and cecum. The cecum was the GI site with the highest level of colonization; and *C. jejuni* was isolated from most GI compartments (i.e. cecum, stomach, colon, and jejunum) or detected by *C. jejuni* specific (*gyr*A) PCR of tissue homogenates. All mice were colonized at comparable levels and colonization was required but not sufficient for GI lesions as only IL-10$^{-/-}$ mice developed disease and lesions (Mansfield, Bell et al. 2007).

Colitis in IL-10$^{-/-}$ mice is *C. jejuni* strain dependent (Bell, Jerome et al. 2013, Malik, Sharma et al. 2014), and genomic composition of the *C. jejuni* strain is an important factor (Bell, Jerome et al. 2013). To date the entire suite of genes required for *C. jejuni* colitis remains unknown; however, comparative genomics of available *C. jejuni* genomes and gene expression analysis of *C. jejuni* strains that caused colitis in C57BL/6 IL-10$^{-/-}$ mice compared to those that did not yielded 201 potential virulence genes, collectively called the *C. jejuni* virulome (Bell, Jerome et al. 2013). In addition, motility is likely a major determinant of *C. jejuni* pathogenesis. *C. jejuni* diminished motility and non-motile mutants colonize at rates 100 to 1000 fold less than the wild-type (Wassenaar, Zeijst et al. 1993) thus variation in motility amongst strains play a role in infection outcomes. Further, an experiment in *C. jejuni* 11168-infected germ-free C57BL/6 mice showed that expression levels of ninety open reading frames (ORFs) were significantly up- or down-regulated in the mouse cecum at least two-fold compared to *in vitro* growth (Bell, Jerome et al. 2013). Genomic content of these ninety *C. jejuni* 11168 ORFs was significantly correlated with the capacity to colonize and cause enteritis in mice. Differences in gene expression levels and patterns are thus an important determinant of pathotype in *C. jejuni* strains in this mouse model.

Antibiotic treated *Campylobacter* mouse models. Results from experimental murine *C. jejuni* inoculation suggest that competition from members of the resident gut microbiota play a role in *C. jejuni* colonization resistance (Chang and Miller 2006, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015). Two studies have shown that antibiotic depleted gut microbiota is sufficient to overcome *C. jejuni* colonization resistance in wild-type mice. Consistent

with results in LF C3H mice, antibiotic depletion of gut microbiota with ampicillin enhanced susceptibility to *C. jejuni* colonization and mild enteric disease in CBA/J mice. Mice received two doses of ampicillin at 24 and 48 hours prior to *C. jejuni* infection. 16S rRNA gene amplicon analysis revealed decreased overall diversity in the fecal microbiota of antibiotic treated mice. Notably, antibiotic treated mice that were supplemented with *Enterococcus faecalis or Lactobacillus acidophilus* showed diminished *C. jejuni* loads at 7-days post-inoculation indicating a role for these microorganisms in *C. jejuni* colonization resistance (O'Loughlin, Samuelson et al. 2015).

Similarly, vancomycin treatment enhanced susceptibility to *C. jejuni* colonization and gastrointestinal lesions in C57BL/6 wild-type mice. Mice were pretreated with vancomycin and subsequently infected with *C. jejuni* by oral gavage and maintained for 7-days post-inoculation. In line with previous results in LF microbiota C3H mice (Chang and Miller 2006), enteric disease seen in depleted microbiota wild-type mice was exacerbated in single immunoglobulin interleukin-1 receptor-related protein (SIGIRR) deficient mice. These C57BL/6 Sigrr$^{-/-}$ mice are immune deficient due to this gene knockout, which is a negative regulator of MyD88 signaling. Despite enhanced enteric disease in Siggr$^{-/-}$ mutants, *C. jejuni* pathogen loads were very similar compared to wild-type mice; yet, differences in *C. jejuni* localization in the gut were observed. In wild type mice, *C. jejuni* were primarily found in the lumen and luminal surface of the mucus layer. In stark contrast, *C. jejuni* penetrated the mucus and was often found in the intestinal crypts of Sigrr$^{-/-}$ mice. Further investigation demonstrated that Toll like receptors 2 (TLR2) and 4 (TLR4) were required for enteric disease in Sigrr$^{-/-}$ mice (Stahl, Ries et al. 2014). These results are consistent with several previous reports showing the *C. jejuni* activates TLR2 and TLR4 during inflammatory activation of dendritic cells (Rathinam, Appledorn et al. 2009). Together these studies demonstrate that the gut microbiota plays a significant role in mediating *C. jejuni*-mediated inflammation, which is exacerbated in immunocompromised hosts; however, neither study evaluated autoimmune responses in *C. jejuni* infected mice.

Humanized microbiota *Campylobacter* mouse model. Inoculation of mice with human fecal material has been used to generate humanized microbiota mice (^{Hu}microbiota). In one model, ^{Hu}microbiota mice were generated by using a five-antibiotic cocktail (ampicillin, vancomycin, ciprofloxacin, imipenem, and metronidazole) to deplete the microbiota, followed by inoculation with either murine or human feces. Peroral *C. jejuni* infection of these mice resulted in clearance of *Campylobacter jejuni* in 2 days by murine microbiota mice. In contrast, human microbiota mice remained colonized for 6 weeks and displayed exacerbated T cell, B cell, and pro-inflammatory cytokine responses in the colonic mucosa (Bereswill, Fischer et al. 2011). Notably, murine microbiota controls were also pre-treated with antibiotics thus raising the question of whether this affected their immune responses.

CAMPYLOBACTER JEJUNI WITHIN-HOST ADAPTATION

C. jejuni's genome is not static during *in vivo* passage (Wassenaar, Geilhausen et al. 1998, Nuijten, Berg et al. 2000, de Boer, Wagenaar et al. 2002, Jerome, Bell et al. 2011, Kim, Artymovich et al. 2012, Kivistö, Kovanen et al. 2014). A significant proportion of this genomic variation occurs in virulence associated genes that are involved in the synthesis of antigenic structures including the LOS, flagella, and capsule that are involved in triggering immune responses and potentially aiding in immune evasion (Jerome, Bell et al. 2011, Kivistö, Kovanen et al. 2014). Some genomic variants have direct links to biological outcomes, such as increased motility; thus *C. jejuni* adaptation may influence infection outcomes.

The first evidence for *C. jejuni* adaptation *in vivo* came from variability in pulse-field gel electrophoresis banding patterns following passage in chickens, where analysis of initially clonal isolates of *C. jejuni* revealed multiple banding patterns in recovered isolates, providing evidence that large-scale genomic rearrangements occurred during *in vivo* passage (Wassenaar, Geilhausen et al. 1998). Concurrently, Parkhill *et al.,* (2000) identified hypervariable regions in

the *C. jejuni* 11168 genome consisting of homopolymeric tracts of nucleotides. Since this discovery our laboratory has shown that insertions or deletions in homopolymeric tracts of nucleotides allow *C. jejuni* 11168 to rapidly adapt during passage in mice (Jerome, Bell et al. 2011). *C. jejuni* farm isolates also contained variants in homopolymeric tracts (Kivistö, Kovanen et al. 2014). In both cases, the majority of variants in homopolymeric tracts were found in the LOS, capsular, and flagellar genes. Collectively, these homopolymeric tracts are called contingency loci as they have higher rates of mutation than the rest of the genome. Mutations in contingency loci contribute to phase variation: the ability to turn gene expression on or off (Moxon, Paul et al. 1994); phase variation may directly impact pathogenesis by altering the expression of virulence factors including LOS, capsule, and flagella (Jerome, Bell et al. 2011).

Variation in homopolymeric tract length can result in observable biological outcomes. Notably, passage of *C. jejuni in vivo* led to presence of antigenic ganglioside mimics on the LOS of *C. jejuni* 81-176 that initially lacked any ganglioside mimics (Prendergast, Tribble et al. 2004). Site directed mutagenesis of homopolymeric tracts in the *cgt*A gene (N-acetylgalactosaminyltransferase) in *C. jejuni* 81-176 shifted the ratio of GM2 and GM3 ganglioside mimics and enhanced the invasiveness of the *C. jejuni cgt*A mutant compared to the wild-type strain (Guerry, Szymanski et al. 2002). The host environmental cues that drive evolutionary selection for phase variation are unknown, but it is known that this process allows for rapid adaptation to novel environments, increased diversity, and evasion of the host immune system (van der Woude and Baumler 2004, Jerome, Bell et al. 2011). Slipped strand mutagenesis (Moxon, Paul et al. 1994, Zhou, Aertsen et al. 2014) and the absence of several homologues of *E. coli* DNA repair genes contribute to the high incidence of phase variation in *C. jejuni* (van der Woude and Baumler 2004).

Other mechanisms of *C. jejuni* adaptation. *C. jejuni* has other mechanisms of adaptation to novel environments in addition to variation in homopolymeric tracts. Several recent studies have demonstrated that single nucleotide variants outside of homopolymeric tracts

contribute genetic diversity to *C. jejuni* strains (Cagliero, Cloix et al. 2006, Mohawk, Poly et al. 2014, Thomas, Lone et al. 2014). In addition, phenotypic adaptation in *C. jejuni* has also been observed. *In vivo* passage conferred increased motility in mice and rabbits (Caldwell, Guerry et al. 1985, Jones, Marston et al. 2004), although this is not surprising since motility is a major factor in *C. jejuni* colonization (Nachamkin, Yang et al. 1993, Wassenaar, Zeijst et al. 1993, Yuki, Taki et al. 1993). It has also been shown that *C. jejuni* virulence increases following passage both in similar (Bell, St Charles et al. 2009) or divergent hosts (Kim, Artymovich et al. 2012), which may result from enhanced colonization potential (up to 10,000 fold increase) (Cawthraw, Wassenaar et al. 1996), alterations in virulence associated gene expression (Bell, Jerome et al. 2013), or the presence of antigenic stimuli based on modifications of surface structures of *C. jejuni* (Prendergast, Tribble et al. 2004). Intragenomic recombination can also occur during passage and even restore motility in non-motile *flaA* mutants (Nuijten, Berg et al. 2000) or enhance phage resistance (Scott, Timms et al. 2007). Intergenomic recombination is possible but current data are limited to recombination between *C. jejuni* strains with highly similar gene content (de Boer, Wagenaar et al. 2002). Together, these results confirm the plasticity of the *C. jejuni* genome while establishing that adaptation of *C. jejuni* often affects loci with potential to modulate host immune responses. Because microbiota have been shown to modulate *C. jejuni* infection outcomes, determining whether alterations in the microbiota affect genes encoding antigenic determinants will further our understanding of mechanisms governing *C. jejuni* pathogenesis.

MICROBIOTA MEDIATED PATHOGEN COLONIZATION RESISTANCE

The gut microbiota participates in many critical functions for the host, including shaping the immune response, regulating metabolic processes, preventing pathogen colonization, and preventing the overgrowth of indigenous bacteria with pathogenic potential termed pathobionts (Artis 2008, Kinross, Darzi et al. 2011, Buffie and Pamer 2013). Current estimates from culture-

and non-culture-based enumeration techniques estimate that there are at least 52 (Rappé and Giovannoni 2003) and as many as 1000 (Yarza, Yilmaz et al. 2014) bacterial phyla on earth, but the human gut microbiota is dominated by just four; *Bacteroidetes, Proteobacteria, Actinobacteria, and Firmicutes* (Eckburg, Bik et al. 2005). In addition, the advent of non-culture based methods revealed that at least eighteen-hundred genera and at least fifteen-thousand species are present in the human gut (Frank, St Amand et al. 2007). In healthy adult humans, gram-positive *Firmicutes*, such as *Clostridiales* and *Lactobacillales*, and gram-negative *Bacteroidetes* represent the major phyla in the gut microbiota (Eckburg, Bik et al. 2005, Ley, Peterson et al. 2006). In all, there are at least as many bacterial cells as human cells in and on the human body (Sender, Fuchs et al. 2016) and potentially 10 times more (Ley, Peterson et al. 2006).

It is well documented that there are differences in the ecological community structure of the microbiota between healthy individuals and patients with disease, including those with Inflammatory Bowel Disease (IBD) (Frank, St Amand et al. 2007), the obese (Ley, Turnbaugh et al. 2006, Zhang, DiBaise et al. 2009), and those with diabetes (Qin, Li et al. 2012). Notably, the factors that play a role in determining the structure of the microbiota are numerous and difficult to delineate; however, it is clear that the microbial community structure is a reflection of natural selection and of a multitude of interactions including microbe-microbe (Buffie and Pamer 2013), host-microbe (Dominguez-Bello, Costello et al. 2010, Koren, Goodrich et al. 2012, Yatsunenko, Rey et al. 2012, Kostic, Howitt et al. 2013), and microbe-environment interactions (Costello, Lauber et al. 2009, Org, Parks et al. 2015). However, it is not clear whether differences in microbiota structure are the cause of disease or a reflection of the disease itself.

Both epidemiological data and data from animal models make it clear that a diverse microbiota with an abundance of beneficial microorganisms is critical for prevention of pathogen colonization in human (Qin, Li et al. 2012, Le Chatelier, Nielsen et al. 2013) and non-human animals (Hildebrand, Nguyen et al. 2013). Mouse models with defined microbiota (gnotobiotic) and without microbiota (germfree) have played a critical role in expanding our knowledge about pathogen-

microbiota interactions because mice are easily bred, maintained, and experimentally manipulated. Infection in in germ-free models results in varied responses ranging from increased (Nardi, Silva et al. 1989, Inagaki, Suzuki et al. 1996) to decreased susceptibility to pathogen colonization and inflammation. In summary, the microbiota is critical in mediating pathogen colonization, and animal models have been critical in understanding how the microbiota prevents pathogens from colonizing.

Dysbiosis and susceptibility to pathogens. Dysbiosis is a term used to describe microbial communities that are depleted of beneficial bacteria; such depletion is associated with increased susceptibility to both pathogen-mediated and non-pathogen associated diseases. Dysbiosis may result from immune deficiencies, changes in diet, antibiotic treatment, and acute inflammation (Honda and Littman 2012). One consequence of dysbiosis in human and non-human animals includes diminished pathogen colonization resistance (Buffie and Pamer 2013). In addition, when compared to healthy individuals dysbiosis has been found in patients with various chronic inflammatory and autoimmune diseases including IBD, Multiple Sclerosis, and Type 1 diabetes (Ercolini and Miller 2009). In some cases, these diseases have direct links to pathogenic organisms, however, others are associated with fluctuations in the abundance of particular commensal microorganisms (Shimon 2000, Ercolini and Miller 2009, Chervonsky 2013). There are indications that obligate anaerobes play a critical role in colonization resistance to pathogens. Depletion of these organisms may free up nutrients for fast growing organisms including *Proteobacteria* (van der Waaij, Berghuis-de Vries et al. 1971, van der Waaij, Berghuis-de Vries et al. 1972, Wells, Maddaus et al. 1988, Shin, Suzuki et al. 2002). To evaluate the role of the microbiota in pathogen-associated diseases, germfree, gnotobiotic, and antibiotic depleted microbiota mice have been experimentally infected and some are described in the following sections.

Microbiota mouse models. Germfree mice provide the best model for examining host-microbiota relationships because no competing microbiota exists. In particular, selective

colonization of germfree mice has allowed us to probe the function of specific components of the microbiota. In general, germfree mice have been shown to be more susceptible to colonization with pathogens including *Shigella flexneri*, *Salmonella spp*., and *Clostridium difficile* (Bohnhoff, Drake et al. 1954, Bammann, Clark et al. 1978). However, the immune response to pathogen colonization to these pathogens was variable, with reports of both diminished and exacerbated inflammatory responses (Yrios and Balish 1986, Silva, Evangelista et al. 1987, Inagaki, Suzuki et al. 1996). Notably, germfree mice do not make good models for immunophenotyping due to stunted digestive tracts and immune system development, including altered mucus secretion and enlarged ceca (Gordon and Pesti 1971, O'Hara and Shanahan 2006).

Antibiotic treatment diminishes *Salmonella* and *Clostridium* colonization resistance in mice. *S. enterica serovar* Typhimurium colonizes poorly in the mouse gastrointestinal tract; however, disruption of the microbiota with antibiotics (Barthel, Hapfelmeier et al. 2003, Sekirov, Tam et al. 2008, Croswell, Amir et al. 2009, Garner, Antonopoulos et al. 2009, Ferreira, Gill et al. 2011, Fernández-Santoscoy, Wenzel et al. 2015) or presence of a low-complexity microbiota (Garner, Antonopoulos et al. 2009) can result in enhanced *S. enterica* colonization and inflammation. *C. difficile* is another opportunistic pathogen that may infect and dominate the gut microbiota following antibiotic treatment. In mice antibiotic treatment abolishes *C. difficile* colonization resistance (Theriot, Koenigsknecht et al. 2014). Disruption of the microbiota with broad-spectrum antibiotic treatment alters the metabolic profile of the gut, which *C. difficile* exploits for growth (Theriot, Koenigsknecht et al. 2014), and results in enhanced GI pathology (Theriot, Koumpouras et al. 2011).

Early attempts to develop a murine model of *C. jejuni* colonization and colitis were retarded by an inability to colonize wild-type mice with *C. jejuni* or low-level *C. jejuni* colonization with subclinical disease (Chang and Miller 2006, O'Loughlin, Samuelson et al. 2015). *C. jejuni* colonization resistance was abolished by infecting gnotobiotic mice; and persistent, high level *C. jejuni* colonization was achieved by infecting immune deficient gnotobiotic mice (Chang and Miller

2006). Along with persistent *C. jejuni* colonization, immune deficient (i.e. SCID) gnotobiotic mice had marked inflammation of the cecum, colon, and stomach (Chang and Miller 2006). Other reports show that both specific pathogen-free (SPF) C57BL/6 wild-type and congenic C57BL/6 interleukin-10 deficient (IL-$10^{-/-}$) mice were colonized by *C. jejuni,* but only IL-$10^{-/-}$ mice were susceptible to colitis after infection with *C. jejuni* 11168 (Mansfield, Bell et al. 2007, Mansfield, Patterson et al. 2008). In this work, it was also shown that presence of *Helicobacter hepaticus* or related mouse pathogens conferred immunological resistance to colonization with *C. jejuni* strains. This dissertation builds upon the C57BL/6 IL-$10^{-/-}$ mouse model to determine if the microbiota plays a critical role in the inflammation seen in *C. jejuni* infected C57BL/6 IL-$10^{-/-}$ mice.

Dysbiosis and microbiota mediated autoimmunity. The gut microbiota plays a role in both intestinal and extra-intestinal autoimmune diseases. Inflammatory bowel disease (IBD) is an intestinal autoimmune disease that manifests as long- lasting and often debilitating intestinal inflammation. There are two main lines of evidence that suggest that IBD is affected by microbiota structure: 1) antibiotic administration is beneficial for many IBD patients (Khan, Ullman et al. 2011), and 2) there are distinct structural differences, specifically reduced diversity in the microbiota of IBD patients (Manichanh, Rigottier-Gois et al. 2006). It is not yet clear why antibiotics are beneficial for IBD patients; however, there are indications that bacteria, fungi, and even viruses may play a role in inducing IBD. Experimentally, some female non-obese diabetic (NOD) mice, an experimental model of Type I diabetes, are protected from disease when supplemented with segmented filamentous bacteria. This was associated with the development of a robust T_H17 population (Kriegel, Sefik et al. 2011). Other diseases with links to ecological shifts in microbiota structure include Irritable Bowel Syndrome, Celiac disease, and multiple sclerosis (Round and Mazmanian 2009, Mathis and Benoist 2011). In summary, this evidence suggests a role for the microbiota in autoimmune disease; however, no particular microbiota structure is indicative of susceptibility to autoimmunity when pathogens are involved. In lieu of this, much work has been focused on determining the mechanism(s) of pathogen-initiated autoimmunity, and it is

hypothesized that when pathogens are involved, autoimmunity may be the result of molecular mimicry of host antigens resulting in cross reactivity.

SUMMARY

The enteric microbiota is a critical factor in the *C. jejuni* enteric disease; however, its role in *C. jejuni* autoimmunity including GBS has not been determined. It is well established that dysbiosis can enhance susceptibility to pathogens increasing the likelihood of disease in the host following infection with *S. typhimurium, E. coli,* and *C. jejuni.* Because these pathogens pose significant health burdens worldwide, further investigation of the microbiota as a regulator of infection outcomes is essential. Elucidating the mechanism of *C. jejuni* pathogenesis is of critical importance because *C. jejuni* is a significant health burden in both developed and developing countries as a leading cause of gastroenteritis with potential long-term consequences including GBS. In summary, evidence gained from murine infection models has established that *C. jejuni* infection is a multifactorial disease process modulated by host genetics, host microbiota, and *C. jejuni* genetics. Currently, C57BL/6 IL-$10^{-/-}$ mice serve as an established model of *Campylobacter* colitis. Furthermore, anti-ganglioside antibodies found in the plasma of these mice when infected with GBS-associated *C. jejuni* strains confirm *Campylobacter* autoimmunity (Malik, Sharma et al. 2014); however, a link to peripheral neuropathy is lacking. In conclusion, with the knowledge that the microbiota modulates *C. jejuni* pathogenesis, we sought to determine the role of gut microbiota composition in *C. jejuni*-mediated autoimmunity.

RATIONALE FOR THIS STUDY

Our understanding of the interaction between *C. jejuni* and the gastrointestinal microbiota, a key regulator of the host immune response and pathogen colonization, is limited. Depletion of

beneficial microorganisms in the gut, termed dysbiosis, is sufficient for the host to develop overt disease after infection with intestinal pathogens including *S. Typhimurium, C. difficile,* and *C. jejuni.* Limited flora gnotobiotic mice, ampicillin treated, and vancomycin treated mice are more susceptible to *C. jejuni* colonization than conventional murine microbiota mice (Chang and Miller 2006, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015). Furthermore, a combination of immune deficiency and depleted microbiota exacerbated *C. jejuni* colonization and enteric disease (Chang and Miller 2006, Stahl, Ries et al. 2014). However, the effects of depleted microbiota on *C. jejuni*-mediated autoimmunity including anti-ganglioside antibody elicitation were not investigated. Moreover, the involvement of *C. jejuni* and the mechanism through which *C. jejuni* initiates the acute peripheral neuropathy GBS in humans and non-human animals has not been confirmed. Finally, the possibility that *C. jejuni*-host-microbiota interactions influence variation in the highly mutable *C. jejuni* genome and affect infection outcomes has not been determined. The main objective of this study was to address these knowledge gaps through the following specific aims:

Aim 1: Determine if the conventional microbiota plays a protective role in the host response to infection with *C. jejuni* GBS patient strains in C57BL/6 IL-10$^{-/-}$ mice.

Hypothesis 1: Altering the gut microbiota with cefoperazone, a broad-spectrum antibiotic, will 1) enhance *C. jejuni* colonization, 2) enhance gastrointestinal lesions, and 3) exacerbate levels of anti-ganglioside antibodies compared to infected, untreated controls.

Aim 2: Determine if a humanized microbiota is sufficient to alter the host inflammatory and autoimmune response to infection with *C. jejuni* colitis and GBS strains in C57BL/6 wild-type mice.

Hypothesis 2: Humanized mice will exhibit 1) enhanced colonization by *C. jejuni*, 2) higher levels of anti-ganglioside antibodies, and 3) increased lesions in both the GI tract and

peripheral nerves compared to mice with conventional murine microbiota.

Aim 3: Determine if distinct patterns of genomic variation occur in *C. jejuni* isolates recovered from conventional, antibiotic-treated, and humanized mice (Fig. 1.2).

Hypothesis 3: *C. jejuni* isolates will have distinct patterns of genomic variation that are unique to their microbial environment during passage.

The findings for specific aims 1, 2 and 3 can be found in chapters 2, 3, and 4 respectively. Finally, chapter 5 summarizes the findings reported in chapters 2-4 and provides a new set of specific aims for expanding upon the results of this study.

APPENDIX

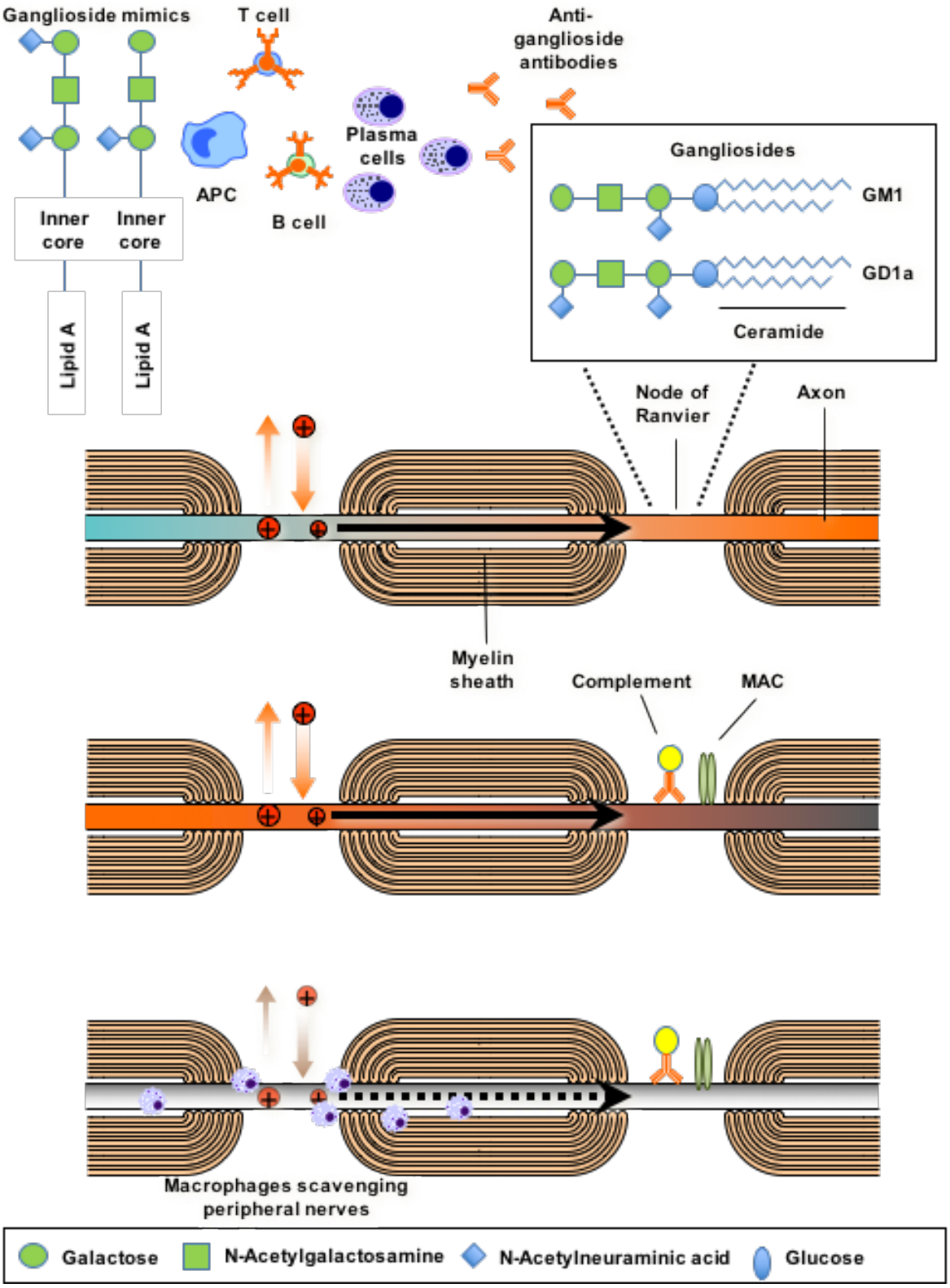

Figure 1.1. Hypothesized mechanism of GBS preceded by *C. jejuni* infection. *Campylobacter jejuni* infection in the host evokes anti-ganglioside antibodies in response to ganglioside mimics

Figure 1.1. (cont'd). on its LOS. Anti-ganglioside antibodies bind to ganglisodes (GM1 and GD1a) at the node of ranvier activating complement and formation of the membrane attack complex which lead to nerve injury and diminished nerve signal transmission. Finally, macrophages scavenge damaged nerves.

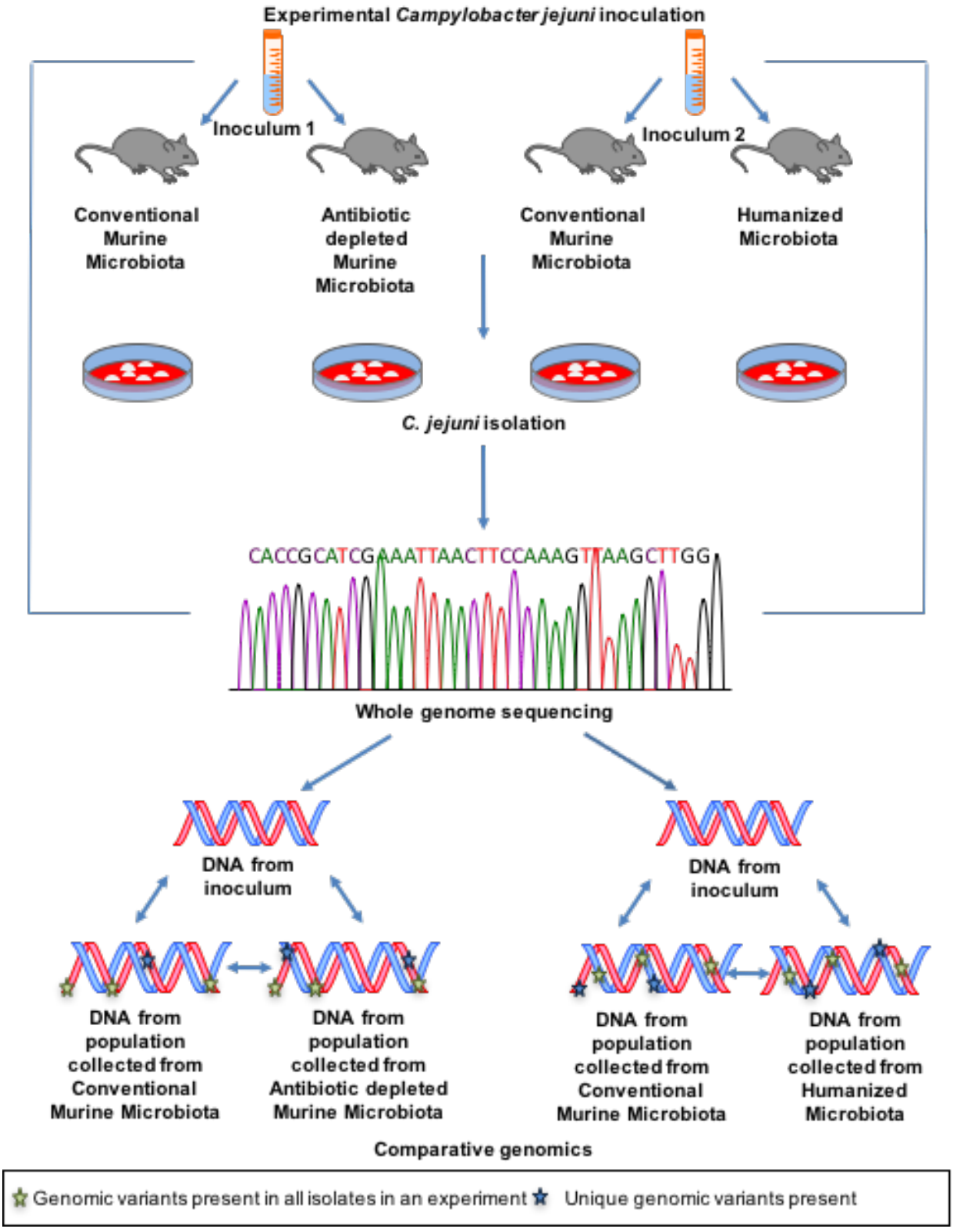

Figure 1.2. Experimental design for comparative genomic analysis of passaged *C. jejuni*.

Mice with conventional, antibiotic depleted, and humanized microbiota were experimentally

Figure 1.2 (cont'd). inoculated with *C. jejuni* in one of two experiments. *C. jejuni* were isolated from fecal or cecal samples collected at necropsy and streaked for growth on selective media. The entirety of the *C. jejuni* population was collected and DNA were extracted. DNA from both passaged populations and inocula (i.e. ancestral isolates) were sequenced. Comparative genomic analysis was conducted to determine if genomic variants were specific to treatment or microbiota.

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CHAPTER 2

ANTIBIOTIC DEPLETION DRIVES SEVERE *CAMPYLOBACTER*-MEDIATED TYPE 1/17 COLITIS AND TYPE 2 AUTOIMMUNITY

ABSTRACT

Infection with antimicrobial resistant *Campylobacter jejuni* strains from GBS patients produced both severe Type 1/17 colitis and Type 2 autoimmune responses when C57BL/6 IL-10$^{-/-}$ mice were treated with a broad spectrum antibiotic that decreased complexity and abundance of the gut microbiota. Antibiotic depletion of microbiota was the main factor in inducing enhanced enteric disease and GBS associated phenotypes although the degree of severity was also dependent on the *C. jejuni* strain. Notably, antibiotic treatment drove severe colitis by *C. jejuni* strains isolated from GBS patients that had heretofore produced no or only mild colitis in this model. Antibiotic treated infected mice had high numbers of *C. jejuni* in the apical, basolateral and paracellular junctions of gut epithelium and within cells of the lamina propria, submucosa and lymph nodes indicating increased invasion and translocation from the gut. These results indicate that antibiotic depletion of gut microbiota alters immune responses to *C. jejuni* in a manner that exacerbates colitis and promotes GBS associated autoantibodies.

INTRODUCTION

Campylobacter spp. are a leading cause of bacterial diarrheal illness (Young, Davis et al. 2007, Scallan, Griffin et al. 2011). Two species; *C. jejuni* and *C. coli*, are responsible for the majority of *Campylobacter* infections. *C. jejuni*—a gram-negative, spiral rod-shaped, bacterial pathogen—is responsible for approximately 1.3 million infections in the USA annually. Human infections result from spread of the organism from animals through contaminated meat, milk or water (CDC 2014). Undercooked poultry and unpasteurized milk are the most important sources. *C. jejuni* causes fever, vomiting and diarrhea in the majority of cases. These symptoms can be debilitating but are usually self-limiting within 7–10 days (Young, Davis et al. 2007). Occasionally,

autoimmune diseases arise following exposure to *C. jejuni*, including inflammatory bowel disease (Kim, Hans et al. 2009), Reiter's arthritis (Garg, Pope et al. 2008), and Guillain-Barré or Miller-Fisher syndromes (GBS) (Hughes and Rees 1997, Yuki 2012).

GBS is an acute peripheral neuropathy and the leading cause of acute flaccid paralysis (Hughes and Rees 1997, Yuki and Hartung 2012). *C. jejuni* is the leading antecedent infection for GBS with 3000–6000 cases reported annually. At present, GBS can be divided into at least four subtypes: two axonal forms; acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN), a demyelinating form; acute inflammatory demyelinating polyradiculoneuropathy (AIDP), acute inflammatory demyelinating polyradiculoneuropathy (AIDP), and a distinct variant; Miller Fisher Syndrome (MFS) that is characterized by opthalmoplegia, muscle weakness, and areflexia (Hughes and Rees 1997, Hughes, Hadden et al. 1999). Antecedent *C. jejuni* infection is most often associated with the AMAN subtype and production of GM1 and GD1a anti-ganglioside antibodies (Yuki, Susuki et al. 2004). In humans AMAN is associated with IgG binding at the node of Ranvier, complement deposition on nerves, and enhanced macrophage numbers in axons (Griffin, Li et al. 1996, van den Berg, Walgaard et al. 2014). It is suspected that when GBS is preceded by *C. jejuni* infection, bacterial lipo-oligosaccharides resembling host gangliosides activate the immune system to produce antibodies that are cross-reactive with gangliosides on peripheral nerves (Yuki, Susuki et al. 2004). Binding of anti-ganglioside antibodies to myelin or the axon at the node of Ranvier is hypothesized to lead to complement activation, macrophage recruitment, and damage to the axon that impairs function (Hughes, Hadden et al. 1999, Keiichiro, Matthew et al. 2007). Although infection with *C. jejuni* GBS patient strains has been shown to elicit anti-ganglioside antibodies in mice (Malik, Sharma et al. 2014), it is unknown whether this antibody production leads to peripheral neuropathy consistent with human GBS. Despite its potential benefit to human health, a working model for the study of GBS in which *C. jejuni* infection initiates autoimmunity that mimics the AMAN form of the human disease remains elusive. Mice have often been used to study *C. jejuni* pathogenesis,

and depleted microbiota have been shown to enhance susceptibility to *C. jejuni* colonization and enteric inflammation, raising the question of whether depleted microbiota would enhance susceptibility to *C. jejuni*-mediated-autoimmunity.

It is well known that manipulation of the resident gut microbiota can significantly impact host-susceptibility to pathogen colonization (Kamada, Chen et al. 2013). Disruption of the resident gut microbiota with antibiotics increased susceptibility to *C. jejuni* colonization and enteric disease (Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015). Indeed, such manipulation has been critical to the development of several pathogen infection models including *Clostridium difficile* (Antonopoulos, Huse et al. 2009, Theriot, Koenigsknecht et al. 2014) and *Salmonella enterica* Typhimurium (Sekirov, Tam et al. 2008) murine models. Recently, two studies have shown that treatment with ampicillin (O'Loughlin, Samuelson et al. 2015) and vancomycin (Stahl, Ries et al. 2014) enhanced susceptibility to *C. jejuni* in CBA/J mice. Presumably, depletion of microorganisms that provide *C. jejuni* colonization resistance were eliminated by antibiotic treatment. Host genetics also play a role in *C. jejuni*-mediated inflammation. As Stahl et al (2014) demonstrated, antibiotic depletion of gut microbiota enhanced susceptibility to *C. jejuni* colonization in C57BL/6 mice, but genetic manipulation— depletion of the single immunoglobulin interleukin-1 receptor-related protein (SIGIRR$^{-/-}$) in addition to antibiotic treatment—was required for severe enteric disease. Similarly, decreased diversity of the intestinal microbiota present in limited flora (LF) mice diminished *C. jejuni* colonization resistance while only *C. jejuni* infected LF severe combined immune deficient mice (SCID) were susceptible to enteric disease (Chang and Miller 2006). Notably, we have shown that untreated C57BL/6 IL-10$^{-/-}$ mice infected with GBS patient strains are susceptible to mild T cell mediated colitis and develop anti-ganglioside antibodies confirming autoimmunity. Thus, we hypothesized that altering the gut microbiota of C57BL/6 IL-10$^{-/-}$ mice with the broad spectrum antibiotic cefoperazone and infecting them with *C. jejuni* GBS-associated isolates would (1) increase *C. jejuni* colonization, (2) increase the severity

of host gastrointestinal lesions, and (3) increase levels of antibodies cross-reactive with neuronal gangliosides compared to infected and uninfected, untreated controls.

To investigate how antibiotic depleted gut microbiota affects *C. jejuni-mediated-* autoimmunity, we used an established mouse model of *C. jejuni* colitis (i.e. C57BL/6 IL-10$^{-/-}$) (Mansfield, Bell et al. 2007, Malik, Sharma et al. 2014) and treated the mice with cefoperazone (CPZ), a broad-spectrum antibiotic that has been shown to significantly deplete the murine microbiota (Antonopoulos, Huse et al. 2009, Nagalingam, Robinson et al. 2013, Theriot, Koenigsknecht et al. 2014, Yin, M et al. 2015). *C. jejuni* resistance to cefoperazone results from the multidrug efflux pump *cmeABC* (Lin, Michel et al. 2002, Guo, Lin et al. 2010). Mice received either sterile drinking water or 0.5 mg/mL CPZ in sterile drinking water followed by oral gavage with one of three *C. jejuni* GBS patient strains or tryptone soy broth (the vehicle); antibiotic treatment was continued for the entire duration of the experiment. Using established assays, we measured anti-ganglioside antibodies in plasma collected from all experimental mice following sacrifice and compared them to controls. To determine whether the presence of anti-ganglioside antibodies correlated with enhanced macrophage infiltration consistent with GBS we quantified immunohistochemically-labeled macrophages in peripheral nerves and dorsal root ganglia. Next, because *Campylobacter* is a leading cause of bacterial gastroenteritis and C57BL/6 IL-10$^{-/-}$ mice are prone to *C. jejuni* induced T cell mediated colitis (Malik, Sharma et al. 2014) we characterized colonic T cell populations and evaluated enteric lesions. In this study, we have identified antibiotic depleted gut microbiota as a factor in *C. jejuni* mediated-autoimmunity. We show that antibiotic treatment enhanced *Campylobacter* mediated inflammation and autoimmunity, manifesting as exacerbated enteric lesions and anti-ganglioside antibodies, respectively. Finally, we show that in addition to the microbiota *C. jejuni* genetics modulate host immune responses. Together, these data identify a novel model of *C. jejuni* inflammation and autoimmunity initiated by infection with a single strain from a patient with GBS.

RESULTS

Results are from three separate experiments and are organized by the condition assessed or the assay performed. Experimental study designs are shown in Table 1.

Enteric Pathogens. To determine if mice were positive for enteric pathogens that could contribute to inflammatory responses we screened fecal samples from all experimental mice for *Campylobacter* spp.(Linton, Owen et al. 1996), *Helicobacter* spp.(Riley, Franklin et al. 1996), *Enterococcus faecalis* (Dutka-Malen, Evers et al. 1995), and *Citrobacter rodentium* (McKeel, Douris et al. 2002). Mice were negative in all cases with the exception of *Campylobacter spp*. in experimentally inoculated mice.

Survivorship. To compare the time to humane endpoint in all experimental mice we recorded the number of days post-inoculation until mice required sacrifice due to humane endpoints. In all cases mice were sacrificed if severe disease was detected using a standardized scoring sheet (see Methods). In the pilot experiment, one mouse in the 260.94 + CPZ group reached its humane endpoint prior to 5 weeks (Fig. 2.1 A). In experiment 1, 0% of uninfected + H_2O, 0% of uninfected + CPZ, 40% of 260.94 + H_2O, 40% of 260.94 + CPZ, 40% of D8942 + H_2O, and 60% of D8942 + CPZ mice were sacrificed prior to 5-weeks because they reached the humane endpoint (Fig. 2.1 B). In experiment 2 a single HB93-13 + H_2O mouse compared to 4 HB93-13 + CPZ mice reached the humane endpoint prior to 7 weeks (Fig. 2.1 C).

Weight. Mice were weighed at the time of sacrifice to investigate if *C. jejuni* inoculation resulted in disparity in final weight compared to controls**.** In the pilot experiment, no differences in weight were detected (Data not shown). Next, to determine if a disparity in weight gain occurred between experimentally infected and controls we compared weight prior to inoculation and at the time of sacrifice in experiment 1 and 2. Antibiotic treatment did not result in a disparity in weight gain in 260.94 or D8942 infected mice (Fig. 2.1 E), however, HB93-13 infected, CPZ treated mice gained significantly less weight than uninfected-CPZ mice (Fig. 2.1 F).

Gross pathology. During the course of necropsy we evaluated gross pathologic changes in the cecum, colon and lymph nodes to determine if antibiotic treatment exacerbated pathology in infected mice. Inflammation of the cecum or colon; enlargement of lymph nodes or bloody feces were observed in at least one mouse at necropsy and assigned to a ranked scoring system (see Methods). In the pilot experiment, 3 of 4 260.94 + CPZ mice had gastrointestinal gross pathology while 1 of 4 260.94 + H_2O mice had GI gross pathology (Fig 2.1 C). To determine if enhanced inflammatory and autoimmune responses (see below) in antibiotic treated mice were strain dependent, C57BL/6 IL-$10^{-/-}$ mice were inoculated with *C. jejuni* 260.94, D8942 or HB93-13 in one of two subsequent experiments. Gastrointestinal gross pathological changes could be detected in all but one infected + CPZ mice, infected with any strain in experiment 1 and 2 (Fig. 2.1 H and I). Three of four 260.94 + CPZ mice had grade 2 pathology and one had grade 3 (Fig. 2.1 H). In contrast, only one 260.94 + H_2O mouse had grade 2 pathology and none had grade 3 (Fig. 2.1 H). Three of four D8942 + CPZ and 3 of 4 D8942 + H_2O mice had grade 2 pathology (Fig. 2.2 C). In experiment 2, a single mouse in both the uninfected + H_2O mouse and uninfected + CPZ showed gross pathological changes (Fig. 2.1 J). Gross pathological changes were similar in HB93-13 + H_2O and HB93-13 + CPZ mice (Fig. 2.1 I).

Antibiotic treatment depleted the microbiota and enhanced *C. jejuni* colonization in cecal and colon contents. To determine if cefoperazone depleted the gut microbiota we used PCR to amplify the V3 region of the 16S rRNA gene in DNA isolated from the feces of experimental mice. The V3 region could be amplified from uninfected + H_2O but not uninfected + CPZ mice confirming depletion of gut microbiota. To compare the level of *C. jejuni* colonization in the colon and cecum of experimentally inoculated mice we streaked portions of both organs on TSA-CVA plates and analyzed *C. jejuni* levels using a semi-quantitative scoring system. In the pilot experiment, 3 of 4 *C. jejuni* 260.94 + CPZ mice were culture positive in either the cecum or the colon or both compared to 1 of 5 *C. jejuni* 260.94 + H_2O mice, indicating increased colonization of CPZ-treated mice (Fig. 2. 1 J). In experiment 1, 2 of 4 260.94 + H_2O and 4 of 5 260.94 + CPZ

mice were culture positive at necropsy (Fig. 2.1 K). All mice infected with *C. jejuni* D8942 were culture positive at necropsy (Fig. 2.1 K). Semi-quantitative analysis of *C. jejuni* by culture did not reveal differences in the number of CFUs between antibiotic treated and untreated mice (data not shown). In experiment 2, 1 of 8 HB93-13 + H_2O mice compared to 7 of 7 HB93-13 + CPZ mice were culture positive (Fig. 2.1 1L); all control mice were negative by culture.

Antibiotic treatment of *C. jejuni* infected mice was associated with increased amounts of immunohistochemical (IHC) staining and shifting of location of staining to deeper gut tissues and draining lymph nodes. To determine whether the *C. jejuni* GBS patient strains might be capable of invading tissues of the ileocecocolic junction and to determine the location of the organism in these tissues, IHC staining of paraffin embedded tissue was performed using a commercial *C. jejuni* specific antiserum. Results appear in Figure 2. Sections from mice in the TSB sham inoculated controls and cefoperazone alone treatment groups had no evidence of *C. jejuni* specific staining in any tissues, while mice in the *C. jejuni* infected groups all had staining associated with the contents and intestinal crypts. However, the degree of staining and location in tissues varied depending upon the infecting strain and whether antibiotic treatment was administered. Mice given all three *C. jejuni* strains had organism in the contents, mucus, crypts and adherent to the apical epithelium. Infected mice given antibiotics had organisms in these sites as well as within the paracellular junctions of the epithelium, at the basolateral surface of the epithelium, intracellular within the lamina propria and submucosa, and within cells of the draining lymph nodes. We found that the higher the IHC score the greater the amount of staining and the greater the number of deeper tissue sites demonstrated *C. jejuni* specific staining. Thus, deep gut and lymphoid tissues from mice infected with *C. jejuni* 260.94 had the highest scores indicating greater tissue invasion and dispersal to draining lymphoid tissue. These mice also had significant staining associated with crypt abscesses and effacing lesions of the GI tract, while antibiotic treated infected mice given HB93-13 and D8942 had less staining in these sites and fewer lesions of this type.

Antibiotic treatment elicited a *C. jejuni* strain independent increase in typhlocolitis at the ileocecocolic junction. We examined hematoxylin and eosin stained sections of the ICCJ of experimentally inoculated mice for inflammation and other pathologic changes. Tissues represent all experimentally inoculated mice colonized for 5 weeks (pilot and experiment 1), 7 weeks (experiment 2) or humane endpoint with *C. jejuni* 260.94, D8942, and HB93-13, and from sham-inoculated controls. Histologic scores were assigned using a standardized scoring system with a range of 0 to 40 (Mansfield, Bell et al. 2007). In all, two control mice showed inflammation in the ICCJ (Fig. 2.3 A and C) consistent with previous reports of spontaneous colitis in C57BL/6 IL-10$^{-/-}$ mice in response to stimulation by microbiota (Bristol, Farmer et al. 2000). Mice with spontaneous colitis were negative by PCR for enteric pathogens (see Methods) including *C. jejuni* culture and *gyr*A PCR.

In the pilot or experiment 1, there was a strong trend to high histologic scores in the ICCJ in *C. jejuni* infected mice given antibiotics compared to the uninfected control group, but statistically significant differences were not detected. In the pilot experiment, 3 of 4 260.94 + CPZ mice had histologic scores of greater than 30 indicating severe tissue damage (Fig. 2.3 A). In experiment 1, these high histologic scores in *C. jejuni* infected mice given CPZ were also severe with all mice achieving the highest grade of 3 compared to uninfected controls that were all grade 1, which are considered normal. In experiment 1, ileocecocolic (ICJ) histopathology the median scores of the infected groups were as follows: 3 for 260.94 + H_2O, 36 for 260.94 + CPZ, 12 for D8942 + H_2O, and 35.5 for D8942 + CPZ (Fig. 2.3 A–B). One mouse in the 260.94 group had a score of 36; all other mice had a score of 3. In experiment 2, histopathology scores were significantly increased in HB93-13 + CPZ mice compared to uninfected, antibiotic treated and untreated controls (Fig. 2.3 C). A single mouse in both the uninfected + H_2O and HB93-13 + H_2O groups in experiment 2 had a score 27 and 36 respectively (Fig. 2.3 C).

When present, inflammation and tissue damage affected the colon and cecum but not the ileum. In general, with the exception of 2 mice with spontaneous colitis, uninfected + H_2O mice

did not show inflammation (Fig. 2.3 D–F, 2X, 20X, 40X) and antibiotic treatment did not elicit a pathological changes in the absence of *C. jejuni* (Fig. 2.3 G-I, 2X, 20X, 40X). Infection with *C. jejuni* 260.94 alone did not elicit significant inflammation yet generally abnormal responses consisted of neutrophils and mononuclear cells in the lumen and epithelium of the cecum and colon (Fig. 2.3 J–L 2X, 20X, 40X). In stark contrast, antibiotic treatment instigated severe inflammation in the cecum and colon of mice infected with 260.94 presenting as increased neutrophils and mononuclear cells in the lumen, epithelium, lamina propria, and submucosa; excess mucus in the lumen; epithelial effacing lesions and ulcerations; marked crypt hyperplasia; increased neutrophils in crypts; marked increases in lamina propria cellularity consisting primarily of diffusely distributed mononuclear cells; inflamed myenteric plexus; diffuse mononuclear submucosal inflammation; edema; and vasculitis (Fig. 2.3 M–O). Infection with D8942 elicited mild inflammation associated primarily with neutrophilic and mononuclear cells in the lumen and epithelium (Fig. 2.3 P, 20X) which was exacerbated by antibiotic treatment (Fig. 2.3 Q, 20X) in a manner consistent with that seen in mice given 260.94 + CPZ and HB93-13 + CPZ mice (Fig. 2.3 R, 20X).

Antibiotic treatment enhanced T cell responses in infected, antibiotic treated mice. Consistent with our previous report showing that T-cells are required for colitis in C57BL/6 IL-$10^{-/-}$ mice (Malik, Sharma et al. 2014) we isolated colonic lamina propria leukocytes and sorted them by flow cytometry to determine if expected T cell mediated inflammation in *C. jejuni* infected mice was exacerbated by antibiotic treatment. In the pilot experiment, 260.94 + CPZ mice exhibited significant increases in total T cells ($CD19^- CD3^+$) compared to uninfected mice and infected mice not given antibiotics (Fig. 2.4 A), T_H cells ($CD3^+CD19^-CD4^+$)(Fig. 2.4 D) T_H1 cells ($CD3^+CD19^- CD4^+IFN\gamma^+$)(Fig. 2.4 G), and T_H2 cells ($CD3^+CD19^-CD4^+IL4^+$)(Fig. 2.4 J) compared to uninfected controls. **I**n experiment 1, 260.94 + CPZ mice had a significant increase in total T cells ($CD3^+ CD19^-$)(Fig. 2.4 B), total T helper cells ($CD3^+ CD19^- CD4^+$) (Fig. 2.4 E), and T_H1 cells ($CD3^+ CD19^-$

$CD4^+$ $IFN\gamma^+$) (Fig. 2.4 H). This enhanced T_H1 response was not associated with a decrease in T_H2 cells ($CD3^+$ $CD4^+$ $IL4^+$)(Fig. 2.4 K), indicating that antibiotic administration was associated with a mixed T_H1/T_H2 response in *C. jejuni* 260.94 infected mice (Fig. 2.4 E and H). These results were strain specific. In experiment 2, there was a significant increase in total T-cells (Fig. 4C) and T helper cells (Fig. 2.4 F) in HB93-13 + CPZ mice however, T_H1 and T_H2 cells did not achieve statistical significance in HB93-13 + H_2O or HB93-13 + CPZ compared to controls (Fig. 2.4 I and L). Next, we evaluated the frequency of T_H cells and T_H1 and T_H2 to determine if a shift in in T_H1 and T_H2 cells was apparent. In the pilot experiment, T_H1 cells were elevated compared to controls (i.e. uninfected + H_2O) and T_H2 cells diminished compared to 260.94 + H_2O (Fig. 2.4 M and P). Similarly, the frequency of T_H1 cells was elevated in 260.94 + CPZ and D8942 + CPZ mice compared to uninfected + CPZ mice (Fig. 2.4 N). In addition, T_H2 cells were diminished in 260.94 + CPZ mice but not D8942 + CPZ mice (Fig. 2.4 R). No differences were detected in the frequency of T_H cells in experiment 3 (Fig. 2.4 O and R).

T_H1 associated antibody responses. Type 1, 2, and 17 cytokines modulate B cell class switching; IFNγ is associated with IgG2c and IgG3 responses, IL-4 is associated with IgE and IgG1 responses, and IL-17 is associated with IgG2b responses (Germann, Bongartz et al. 1995, Bai, Liu et al. 2008, Zhang, Li et al. 2013). To determine if T_H1 associated antibody responses were elicited by *C. jejuni* infection and exacerbated by antibiotic treatment we measured IgG2c type antibodies in the plasma of experimental mice by indirect ELISA**.** In the pilot experiment, a significant increase in anti-*Campylobacter* IgG2c antibodies was detected between untreated + H_2O and 260.94 + CPZ groups (p= 0.171) (data not shown). In experiment 1, antibiotic treatment again elicited an anti-*Campylobacter* IgG2c response in 260.94 infected but not in D8942 infected mice (Fig. 2.5 A). In experiment 2, anti-*Campylobacter* IgG2c responses were modest but elevated in the HB93-13 + H_2O and HB93-13 + CPZ groups (Fig. 2.5 B). Next, to determine if T_H1 associated antibodies were cross-reactive with peripheral nerve gangliosides, we performed indirect IgG2c anti-ganglioside ELISAs. Although anti-*C. jejuni* IgG2c antibodies were only

elevated in 260.94+ CPZ mice in experiment 1, IgG2c anti-ganglioside antibodies cross reactive with both GM1 (Fig. 2.5 C) and GD1a (Fig. 2.5 E) were increased in both 260.94 + CPZ and D8942 + CPZ groups compared to uninfected + CPZ mice. When comparing this response in mice given another *C. jejuni* strain, GM1 and GD1a antibodies were elevated in HB93-13+ CPZ compared to both untreated + CPZ and untreated + H_2O mice (Fig. 2.5 D and F).

T_H17 associated antibody responses. We next analyzed the levels of T_H17 associated antibodies reactive with *C. jejuni* and gangliosides GM1 and GD1a. In the pilot experiment, antibiotic treatment resulted in significant increases in anti-*C. jejuni* IgG2b in 260.94 + CPZ compared to uninfected + H_2O ($p = 0.0040$) (data not shown). In experiment 1, significant differences were detected in anti-*C. jejuni* IgG2b antibody levels between uninfected + CPZ and 260.94 + CPZ but not D8942 + CPZ groups (Fig. 2.6 A). In contrast, in experiment 2, anti-*C. jejuni* Ig2b antibodies were elicited by infection alone and exacerbated by antibiotic treatment (Fig. 2.6 B). Interestingly, neither *C. jejuni* 260.94 nor D8942 alone elicited significant IgG2b anti-ganglioside antibodies; however, consistent with the anti-*C. jejuni* IgG2b responses produced by these two *C. jejuni* strains, infection with HB93-13 also elicited anti-GD1a IgG2b antibodies which were exacerbated by antibiotic treatment compared to uninfected + H_2O group (Fig. 2.6 F).

T_H2 associated antibody responses. Finally, we assessed the abundance of T_H2 associated IgG1 anti-ganglioside antibodies in the same manner described for other antibody types**.** We did not detect a significant increase in anti-*Campylobacter* or anti-ganglioside antibodies in either the pilot experiment or the experiment 1. In contrast, infection with *C. jejuni* HB93-13 elicited both anti-*Campylobacter* and anti-ganglioside antibodies compared to controls. As shown in Fig. 2.7 C, anti-*Campylobacter* IgG1 antibodies were elevated in the HB93-13 + CPZ group compared to the uninfected + H_2O group. Furthermore, both the HB93-13 + H_2O and HB93-13 + CPZ groups had enhanced anti-GM1 IgG1 (Fig. 2.7 F) but not anti-GD1a (Fig. 2.7 I) antibody responses compared to the uninfected + H_2O group.

Sciatic nerve and dorsal root ganglia anti-F4/80 immunohistochemical staining. Finally, having detected anti-ganglioside antibodies in *C. jejuni* infected mice we hypothesized that infected + H_2O groups would display enhanced macrophage numbers in peripheral nerves and dorsal root ganglia compared to controls, and these lesions would be exacerbated in infected + CPZ groups. Significant increases in macrophage number were not detected in any group compared to the uninfected + H_2O groups (Fig. 2.8 A-D). Thereafter, antibody responses divided by class and ganglioside cross reactivity (i.e. GM1 or GD1a), corresponding to individual mice, were compared to macrophage counts in both the DRG and the SN, to determine if the presence of anti-ganglioside antibodies or of particular types of anti-ganglioside antibodies was correlated with increased macrophage count (Table 2). This analysis revealed (1) that many of uninfected + CPZ mice had increased macrophage numbers compared to uninfected + H_2O group controls; (2) that macrophages were more prevalent in experiment 1 and were generally located in the DRG; and (3) that increased macrophage number was not associated with the presence of any particular class of anti-ganglioside antibody. Notably, 1 of 4, 260.94 + H_2O group mice had increased macrophage numbers versus 5 of 5 *C. jejuni* 260.94 + CPZ group mice. In contrast, 4 of 5 D8942 + H_2O group mice had increased macrophages numbers in their DRG compared to only 1 of 4 D8942 + CPZ group mice. No HB93-13 infected mice displayed increased macrophage numbers in the DRG and exacerbated macrophage numbers were infrequent in SN nerves of both treated and untreated HB93-13 infected mice.

DISCUSSION

Previously we determined that contrasting immune responses mediate *Campylobacter jejuni* induced colitis and autoimmunity in interleukin-10 (IL-10) deficient mice, dependent upon the infecting strain (Malik, Sharma et al. 2014). *C. jejuni* Guillain-Barré Syndrome (GBS) patient strains induced mild colitis associated with blunted Type1/17 but enhanced Type2 responses;

Type2 but not Type1/17 antibodies cross-reacted with peripheral nerve gangliosides demonstrating autoimmunity. In this study we tested whether colonization resistance afforded by commensal gut microbiota play an important role in susceptibility to GBS. We found that antimicrobial resistant *C. jejuni* strains from GBS patients produced both severe Type 1/17 colitis and Type 2 autoimmune responses when C57BL/6 IL-10$^{-/-}$ mice were treated with a broad spectrum antibiotic that decreased complexity and abundance of the gut microbiota. Antibiotic depletion of gut microbiota was the main factor in the manifestation of enhanced enteric disease and GBS associated phenotypes although the degree of severity was also dependent on the infecting strain of *C. jejuni*. Interestingly, antibiotic treatment drove severe colitis by *C. jejuni* strains isolated from GBS patients that had heretofore produced no or only mild colitis in this model. Antibiotic treated infected mice had high numbers of *C. jejuni* in the apical, basolateral and paracellular junctions of the epithelium and within cells of the lamina propria, submucosa and lymph nodes indicating increased invasion and translocation from the gut. Our results indicate that antibiotic depletion of the gut microbiota alters immune responses to *C. jejuni* in a way that exacerbates colitis and promotes GBS associated antibody responses.

Our finding that *C. jejuni*-mediated colonic inflammation is enhanced in mice with depleted microbiota is consistent with findings in three previous studies in which limited flora mice (Chang and Miller 2006), vancomycin treated mice (Stahl, Ries et al. 2014), and ampicillin treated mice (O'Loughlin, Samuelson et al. 2015) were shown to be more susceptible to *C. jejuni* colonization and enteritis. Furthermore, exacerbated inflammatory responses in limited-flora SCID mice (Chang and Miller 2006) and vancomycin treated Sigrr$^{-/-}$ mice (Stahl, Ries et al. 2014), taken together with our results in cefoperazone treated IL-10$^{-/-}$ mice, indicate that *C. jejuni*-mediated gastrointestinal inflammation is a multifactorial disease process mediated by both host microbiota and host genetics. In our experimental *C. jejuni* oral inoculation model, antibiotic depletion of gut microbiota was sufficient to enhance gastrointestinal lesions in the ICCJ of *C. jejuni* infected mice consistent with our hypothesis. Notably, semi-quantitative culture assessment of *C. jejuni*

colonization in cecum and colon did not indicate significant increases in *C. jejuni* colonization levels in the infected and infected/antibiotic treated groups because even without antibiotics colonization levels are high (data not shown); however, immunohistochemical analysis showed darker staining, a greater area of staining and a greater depth of tissue staining in infected mice given antibiotics. Regardless of the *C. jejuni* load in infected mice, there was a dramatic increase in the number of animals colonized with HB93-13 in the cecum or colon when antibiotic treatment was given compared to untreated mice (Fig. 2.1 L). Antibiotic treatment also caused higher numbers of invasive *C. jejuni* that were found associated with the apical, basolateral and paracellular junctions of the epithelium and within cells of the lamina propria, submucosa and lymph nodes indicating increased invasion and translocation from the gut. Collectively these results indicate immune deficient hosts are made more vulnerable to *C. jejuni*-mediated-inflammation when microbiota are depleted as shown by antibiotic treatment. These results also strongly suggest that the growing prevalence of antibiotic resistant *C. jejuni* will be accompanied by an increase in severe gastroenteritis and higher rates of GBS in human populations.

Our work provides the first evidence that antibiotic depletion of gut microbiota results in enhanced anti-ganglioside antibody responses (Fig. 2.5–7). Although depletion of the microbiota has been shown to increase *C. jejuni* intestinal colonization (Chang and Miller 2006, O'Loughlin, Samuelson et al. 2015) no one has explored whether increased colonization results in increased anti-ganglioside antibodies. Our data demonstrate that exacerbated anti-ganglioside antibodies were correlated with an increase in the levels of *C. jejuni* and in the number of mice with *C. jejuni* 260.94 and HB93-13 colon and cecum positive cultures at sacrifice (Fig. 2.1 J–K). Clearly, elimination of microbiota with antibiotic treatment removed commensal organisms responsible for *C. jejuni* colonization resistance. *C. jejuni* persisted in the gut despite antibiotic treatment due to resistance mediated by a highly conserved multidrug efflux system, CmeABC (Lin, Michel et al. 2002, Guo, Lin et al. 2010). Because macrophage infiltration of nerve tissue was not exacerbated

by *C. jejuni* infection we cannot confirm that anti-ganglioside antibodies instigated peripheral neuropathy consistent with the hypothesized mechanism of GBS (van den Berg, Marrink et al. 1992). However, the presence of antibodies cross-reactive with *C. jejuni* and peripheral nerve gangliosides confirms *C. jejuni*-mediated-autoimmunity which is exacerbated by antibiotic treatment in our mouse model. If in fact these antibodies initiate the immune attack on nerve tissue that is hypothesized to cause GBS, our results suggest that depleted microbiota may not only modulate *C. jejuni*-meditated-autoimmunity but may also enhance host susceptibility to GBS. Future work will seek to optimize the time after infection at which nerve lesions peak.

Finally, the microbiota is not the only factor mediating the host immune response to *C. jejuni* infection. Our results show that anti-ganglioside antibody responses varied by the infecting *C. jejuni* strain, indicating *C. jejuni* genetics plays a role in *C. jejuni*-mediated-autoimmunity. Anti-ganglioside antibodies are elicited following *C. jejuni* infection in response to carbohydrate structures resembling gangliosides found on the outer core of the *C. jejuni* LOS (Yuki, Taki et al. 1993, Sheikh, Ho et al. 1998). Variation in type and degree of anti-ganglioside antibody elicitation in response to *C. jejuni* infection has been shown by our lab previously (Malik, Sharma et al. 2014). This response is affected by differences in ganglioside mimicry and is correlated with genetic variation in LOS loci (Gilbert, Karwaski et al. 2002, Parker, Horn et al. 2005, Houliston, Vinogradov et al. 2011). Strain D8942 was from the US GBS outbreak and has not been used in laboratory experiments prior to our experimental inoculation, thus comparative analysis was not possible. However, consistent with our previous work (Malik, Sharma et al. 2014), *C. jejuni* 260.94 elicited both GM1 and GD1a IgG1 anti-ganglioside antibodies compared to sham inoculated mice (Table 2), although individual mouse responses varied in our experiment and the group did not achieve statistical significance (Fig. 2.7). Notably, antibiotic depletion of gut microbiota exacerbated anti-ganglioside responses resulting in significant Ig2b and IgG2c anti-ganglioside antibodies (Fig. 2.5 and 2.6), consistent with enhanced inflammation associated with antibiotic treatment. IgG1 antibodies are of particular interest because previous reports on GBS patients

have identified GM1 IgG1 antibodies as predictors of severe outcomes and prolonged recovery in humans (Koga, Yuki et al. 2003). Because HB93-13 elicited significant IgG1 anti-ganglioside antibody responses in previous work, we hypothesized that these responses would be repeated here and would be exacerbated by antibiotic treatment. Consistent with our previous report (Malik, Sharma et al. 2014), HB93-13 elicited IgG1 anti-ganglioside antibodies which were exacerbated by antibiotic treatment in our experiment. Thus, we accepted our hypothesis.

To the best of our knowledge, no link between antibiotic treatment and enhanced susceptibility to *C. jejuni* infection or *C. jejuni*–mediated-inflammation or autoimmunity in humans has been made; however, antibiotics are often used to treat severe cases of campylobacteriosis. Yet, our findings in this study show that the host microbiota is a key modulator of *C. jejuni* pathogenesis in mice. Assuming similar affects in humans and taking our results into consideration along with evidence of widespread antibiotic resistance in *Campylobacter*, it may be advisable to exercise caution when treating *C. jejuni* infections with antibiotics, especially in cases where antibiotic resistance elements of the infecting strain is unknown. Furthermore, these results provide a novel model for the study of *C. jejuni* enteric disease as well as autoimmunity allowing researchers to explore factors that may tip the balance from one disease manifestation to the other.

Finally, this work suggests that probiotics may be a fruitful path for decreasing autoimmune disease following *C. jejuni* infection. Future studies should explore the role of specific components of the microbiota in the regulation of *C. jejuni*-mediated autoimmunity. This study and other mouse experimental inoculation studies (O'Loughlin, Samuelson et al. 2015) and food animal studies provide evidence that probiotics can reduce the growth of *C. jejuni in vivo* (Ghareeb, Awad et al. 2012). Thus evaluation of probiotics as disease modulators in this *C. jejuni*-mediated inflammation and autoimmunity mouse model is warranted. Furthermore, analysis of the mechanisms governing the strain to strain variation in *C. jejuni* pathogenesis is required and will be critical in predicting and preventing human disease following infection.

MATERIALS AND METHODS

***Campylobacter jejuni* strains and inoculum preparation**. *C. jejuni* strains 260.94 (ATCC BAA-1234) and HB93-13 (ATCC 700297) were obtained from the American Type Culture collection (Manassas, VA). *C. jejuni* D8942 was obtained from the Centers for Disease Control (Atlanta, GA). All three strains used in these experiments were isolated from patients with Guillain-Barré syndrome (Prendergast, Lastovica et al. 1998, Sheikh, Nachamkin et al. 1998, Jackson, Zegarra et al. 2013). Strain HB93-13 was originally isolated from feces of an 8-year-old boy in China diagnosed with the acute motor axonal neuropathy (AMAN) form of GBS, while strain 260.94 was isolated from a patient of the Red Cross Children's hospital of Cape Town, South Africa, diagnosed with the acute inflammatory demyelinating polyneuropathy (AIDP) form of GBS. Strains HB93-13 and 260.94 both possess GM1 ganglioside mimics, while HB93-13 also has a GD1a mimic; D8942 ganglioside mimicry is unknown. Inocula were prepared as described previously and checked for purity by Gram stain and >90% darting motility by dark field microscopy (Mansfield, Bell et al. 2007).

Mice. All procedures involving animals were performed in accordance with the recommendations described in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under protocols approved by the Michigan State University Institutional Animal Use and Care Committee (approval numbers 06/12-107-00 and 06/15-101-00). B6.129P2-IL-10^{tm1Cgn}/J (referred to here as C57BL/6 IL-10$^{-/-}$) mice were obtained from Jackson Laboratories (Bar Harbor, ME), a breeding colony was established in a *Campylobacter/Helicobacter*-free facility, and offspring were used for all experiments described here. Mice were housed in specific-pathogen-free conditions, fed an irradiated mouse diet (mouse breeder diet 7904; Harlan Teklad, Indianapolis, IN), kept on autoclaved bedding, and given filter sterilized water (autoclaved water in bottles for weanlings) in an MSU limited-access room. PCR assays specified by Jackson Laboratories were used to confirm mouse genotypes both before

and after experiments (https://www2.jax.org/protocolsdb/f?p=116:2:27254394395538::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:2631,002251). Experimental mice were screened for colitogenic bacteria including *Campylobacter* spp. (Linton, Owen et al. 1996), *Helicobacter* spp. (Riley, Franklin et al. 1996), *Enterococcus faecalis* (Dutka-Malen, Evers et al. 1995), and *Citrobacter rodentium* (McKeel, Douris et al. 2002) using DNA isolated from fecal samples both prior to and at the conclusion of the experiment. The screening assays used were 16S rRNA gene PCR assay for *Campylobacter* spp. and *Helicobacter* spp., *espB* gene-specific PCR for *C. rodentium*, and *ddl* gene-specific PCR for *E. faecalis*. Dedicated sentinel mice were used to assess extraneous infection with bacterial, protozoan and viral agents (Charles River Laboratories, Wilmington, MA) and were monitored by the MSU Campus Animal Resources (CAR). Once mice reached 7 weeks of age they were transferred to the University Research Containment Facility for the following experiments. All experimental mice were housed individually in Innovive filter-top cages on sterile bedding, fed irradiated diet 7904 (Harlan-Teklad, US), given autoclaved water and autoclaved cotton nestlets and randomly assigned to cage slot locations on the rack without respect to their treatment group.

Experimental designs. Ten to 12-week-old C57BL/6 IL-$10^{-/-}$ mice received either sterile drinking water or 0.5 mg/mL cefoperazone (CPZ) in sterile drinking water for the duration of the experiment. Seven days after initiating CPZ treatment, mice receiving CPZ or normal drinking water were inoculated by oral gavage with 0.2 mL of either tryptone soya broth (TSB) or approximately 1 x 10^{10} cfu *C. jejuni* suspended in TSB Administration of CPZ in sterile drinking water or antibiotic-free drinking water continued until euthanasia. Three separate experiments were performed: Pilot and experiment 1 concluded 5 weeks PI; experiment 2 concluded 7 weeks PI (Table 1). Trained animal handlers monitored food, water, and general animal welfare daily. In order to limit avoidable discomfort, distress, pain, and injury all animals were monitored for clinical signs of disease twice daily after clinical signs appeared based on a scoring sheet developed for this purpose (Mansfield, Bell et al. 2007). Each clinical sign observed had a point designation.

Upon each scoring all points were added and if the sum was 9 or above animals were humanely euthanized to prevent suffering. Thus a standardized humane endpoint was established. At 5 weeks post-infection (PI)(Pilot and experiment 1), 7 weeks PI (experiment 2), or at the humane endpoint, mice were administered an overdose of CO_2 in a sealed chamber according to guidelines of the AVMA (Leary, Underwood et al. 2013). After euthanasia we also ruptured the diaphragm or thoracic wall to ensure that the mice would not revive.

Necropsy and sample collection. A fecal sample was collected prior to euthanasia. Mice were euthanized, weighed and quickly prepared for necropsy. Blood was obtained by cardiac puncture and immediately mixed with 0.68% sodium citrate. Any instance of gross pathological change in the GI tract was recorded. Thickened (TW) or enlarged (ENL) colon or cecum walls and bloody intestinal contents were observed and recorded at necropsy. Gross pathological findings were graded as follows: Grade 0 = no gross pathology detected, Grade 1 = thickened wall (TW) or enlarged (ENL) colon or cecum, Grade 2 = TW or ENL colon and cecum, Grade 3 = TW or ENL colon and cecum and bloody feces or luminal contents. Plasma was collected after centrifugation and quickly stored at -80°C until further analysis could be performed. The colon and cecum were divided into 3 sections; one section of each organ was stored in formalin, flash frozen, or streaked on tryptone soya agar plates supplemented with 20 µg CPZ per mL, 10 µg vancomycin per mL, and 2 µg amphotericin B per mL (TSA-CVA) (all antibiotics from Sigma-Aldrich, St. Louis, MO) and incubated in a sealed container with a Campy*Gen* sachet for 48 hours at 37°C.

Quantification of *C. jejuni* in the cecum and colon. Small snips of cecum and colon tissue were taken and streaked on TSA-CVA plates. Semi-quantitative analysis of *C. jejuni* growth on these plates were judged using a standardized scoring system: 0 (no growth), 1 (1-20 CFU), 2 (20-200 CFU), 3 (200-400) and 4 (confluent growth) (Mansfield, Bell et al. 2007).

Preparation of ileocecocolic junction, histopathological scoring and *C. jejuni* specific immunohistochemistry. Tissue samples previously fixed in 10% neutral buffered

formalin were processed and vacuum infiltrated with paraffin on the Sakura VIP 2000 tissue processor; followed by embedding with the ThermoFisher HistoCentre III embedding station. Once blocks were cooled, excess paraffin was removed from the edges; placed on a Reichert Jung 2030 rotary microtome and faced to expose tissue sample and sectioned at 4-5 microns. Sections were dried at 56°C slide incubator to for 2 – 24 hours. Slides were removed from the incubator and stained with a routine Hematoxylin and Eosin (H & E) method as follows: two changes of xylene – 5 minutes each, two changes of 100% ethanol – 2 minutes each, two changes of 95% ethanol – 2 minutes each, running tap water rinse for 2 minutes, Hematoxylin (Cancer Diagnostics – Durham, NC) for 1 ½ minutes followed directly by a 10–15 second differentiation in 1% aqueous glacial acetic acid and running tap water for 2 minutes to enhance nuclear detail. Upon completion of running tap water slides were placed in one change of 95% ethanol – 2 minutes, 1% Alcoholic Eosin-Phloxine B – 2 minutes to stain cytoplasm, one change of 95% ethanol for 2 minutes, four changes of 100% ethanol – 2 minutes each, four changes of xylene – 2 minutes each followed by coverslipping with synthetic mounting media for permanent retention and visualization with light microscopy. Scoring of the distal ileum, cecum and proximal colon was conducted as described in Mansfield et al., 2007. Briefly, the lumen, epithelium, lamina propria and submucosa of the ileocecocolic junction of each mouse were observed for histopathological changes by an investigator (LSM) blinded to sample identity and a score from 1-41 was assigned based on the lesions (Mansfield, Bell et al. 2007). Another section of the ICCJ was sectioned and prepared for *C. jejuni* specific immunohistochemistry using a rabbit polyclonal antibody against *C. jejuni* (US Biologicals, Swampscott, MA) diluted 1:500 with normal antibody diluent (Scytek, Logan, UT) according to a previously published protocol (Mansfield, Bell et al. 2007).

Assessment of anti-*C. jejuni* and anti-ganglioside antibodies by enzyme-linked immunosorbent assay. Antibodies reactive with GM1 (Sigma-Aldrich), GD1a (USBio, Salem, MA) and a crude *C. jejuni* protein preparation were measured via indirect enzyme-linked immunosorbent assay (ELISA). Biotinylated goat anti-mouse secondary antibodies (Southern

Biotech Birmingham, AL) were used to determine IgG subclasses (IgG1, IgG2b or IgG2c).

Collection of nerves and dorsal root ganglia and Immunohistochemistry. We collected the sciatic nerve and 1 to 3 dorsal root ganglia from experimental mice for histological and morphological analysis. These structures were exposed by dissection at necropsy, the mouse carcass was fixed in formalin for 24 hours and switched to 60% ethanol thereafter, and the structures further dissected and embedded en bloc in order to assess the segmental nature of any GBS lesions (Gadsden et al. unpublished). Slides were prepared by the Michigan State University Investigative Histopathology Laboratory. Four micron sections were placed on charged slides and dried at 56°C overnight. The slides were subsequently deparaffinized in xylene and hydrated through descending grades of ethyl alcohol to distilled water. Slides were placed in Tris Buffered Saline (TBS) pH 7.4 (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 and 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. One section was stained with Hematoxylin and Eosin for assessment of inflammation and general tissue damage. F4/80 stained cells were counted and normalized for tissue area using ImageJ version 2.0.0-rc43/1.50e (Caroline, Wayne et al. 2012).

Preparation of lamina propria leukocytes and characterization by flow cytometry. An approximately 3 cm segment of proximal colon tissue was collected from each mouse and held in RPMI 1640 containing 10% fetal bovine serum (FBS) until processing (30 mins). Epithelial cells were isolated by washing and cutting the colon into 1 cm pieces and incubating the pieces in calcium- and magnesium-free Hanks Buffered Saline Solution (HBSS) supplemented with 5% FBS and 5 mM EDTA while shaking at 150 rpm and 25°C for 30 min. The tissues were then incubated in 1640 RPMI containing 10% FBS and 0.5 mg/mL collagenase type IV and 0.5 mg/mL DNase I (Sigma-Aldrich) while shaking at 150 rpm and 37°C for 1 hour. Liberated cells were filtered through a 100 µm nylon cell strainer (Falcon). Isolated cells were separated by a 40/80% discontinuous Percoll (GE Healthcare Life Sciences) gradient. Prior to immunostaining, the cells were re-stimulated with phorbol 12-myrisate 13-acetate (Sigma Aldrich) and ionomycin (BD Biosciences). Cytokine secretion was inhibited with brefeldin A (BD Biosciences) and BD GolgiStop™ (monensin) (BD Biosciences). Thereafter, cells were stained with anti-mouse CD19 PerCP-Cyanine5.5 (eBioscience) for B-cells, anti-mouse CD3e PerCP-Cyanine5.5 (eBiosciences) for T-cells, anti-mouse CD4 FITC (eBiosciences) for T-helper cells, interferon gamma (PE/Cy7) (eBioscience), and interleukin-4 (PE) (BD Biosicences). All cells were gated on $CD^{-}19$ $CD3^{+}$ gate. Cell viability was assessed via trypan blue staining and forward and side scatter. At least 90% cell viability was required for inclusion.

Statistical Analyses. Pilot study data analyses were conducted using the Kruskal-Wallis non-parametric one-way analysis of variance and Dunn's post-test when appropriate. Experiment 1 and 2 data analyses were conducted using a one-way analysis of variance (ANOVA) and Tukey's post-test when appropriate. Histopathology data from the ileocecocolic junction were

analyzed with the Kruskal-Wallis test on ranks regardless of the experiment. If statistical significance was achieved, pairwise comparisons were made with Fishers Exact Test (http://vassarstats.net/fisher2x3.html) and corrected for multiple comparisons by implementing Holmes step-down method (Ludbrook 1998). Scores were grouped in a 2-way table assigned a grade 0 (≤9), 1 (10-20) or 2 (>20). Unless otherwise noted statistical analyses were performed using GraphPad Prism 6.0h for Mac OS X, GraphPad Software, La Jolla California USA. Standard deviations for anti-ganglioside antibody and F4/80 comparative analysis were calculated with the STDEV.P function in Microsoft Excel for Mac version 15.15. Plasma samples were considered positive for *Campylobacter,* GM1, or GD1a antibodies an OD_{450} value two or more standard deviations above the mean value for plasma from uninfected, untreated controls was obtained.

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APPENDIX

GROUP	NO. OF MICE	WEEKS PI
Pilot		
TSB	**5**	**5**
260.94	**5**	
260.94 + CPZ	**4**	
Experiment 1		
TSB	**4**	**5**
CPZ	**5**	
260.94	**4**	
260.94 + CPZ	**5**	
D8942	**5**	
D8942 + CPZ	**4**	
Experiment 2		
TSB	**8**	**7**
CPZ	**8**	
HB93-13	**8**	
HB93-13 + CPZ	**7**	

Table 2.1. Distribution of mice in pilot, experiment 1, and experiment 2. Table represents the treatment groups and number of animals per treatment group for all experiments at the time of inoculation. n = number1. of animals

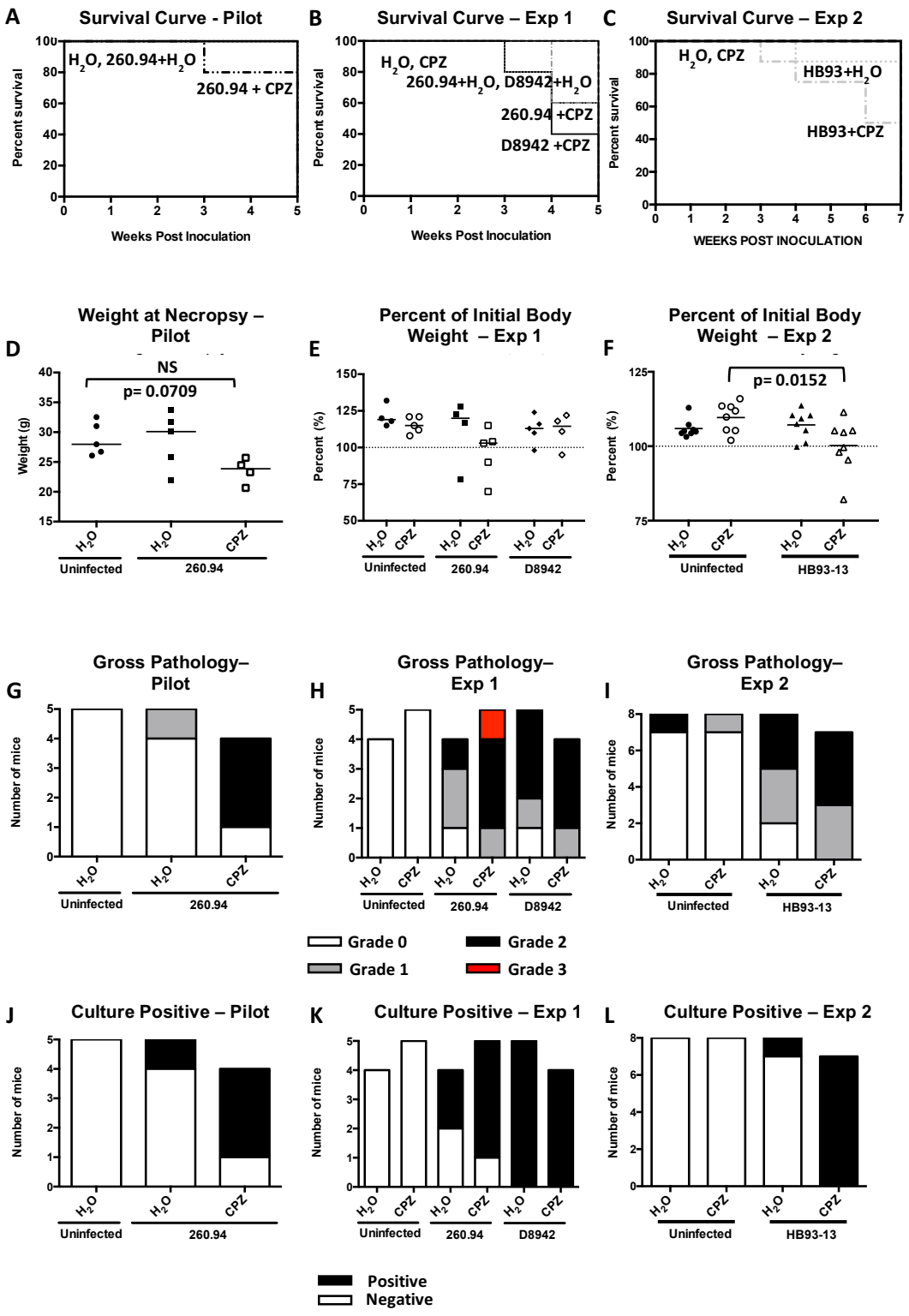

Figure 2.1. Survival curve, endpoint body weight, gross pathology and culturable *C. jejuni.*

C57BL/6 IL-10$^{-/-}$ mice were mice were inoculated with TSB or *C. jejuni* 260.94, D8942, or HB93-

Figure 2.1 (cont'd). 13 in three separate experiments and euthanized at 5-weeks (pilot and experiment 1), 7 weeks (experiment 2) or at the humane endpoint. Mice received sterile drinking water or 0.5 mg/mL CPZ in sterile drinking water for 7 days prior to infection; antibiotic treatment continued for the duration of the experiment. Panels A, B, and C) Survival curves, D) Body weight at necropsy, E and F) percent of initial body weight, G, H and I) gross pathological changes in the colon, cecum and lymph nodes (Grades 0 though 3 defined in Materials and Methods), J, K and L) number of culture positive mice at necropsy. Data represent 4–5 mice per group (pilot and experiment 1) or 7-8 mice per group (experiment 2) and were analyzed by the Kruskal-Wallis test on ranks and Dunn's Post test. In panel D, E, and F each symbol represents a single animal; bars represent group medians. NS, not significant.

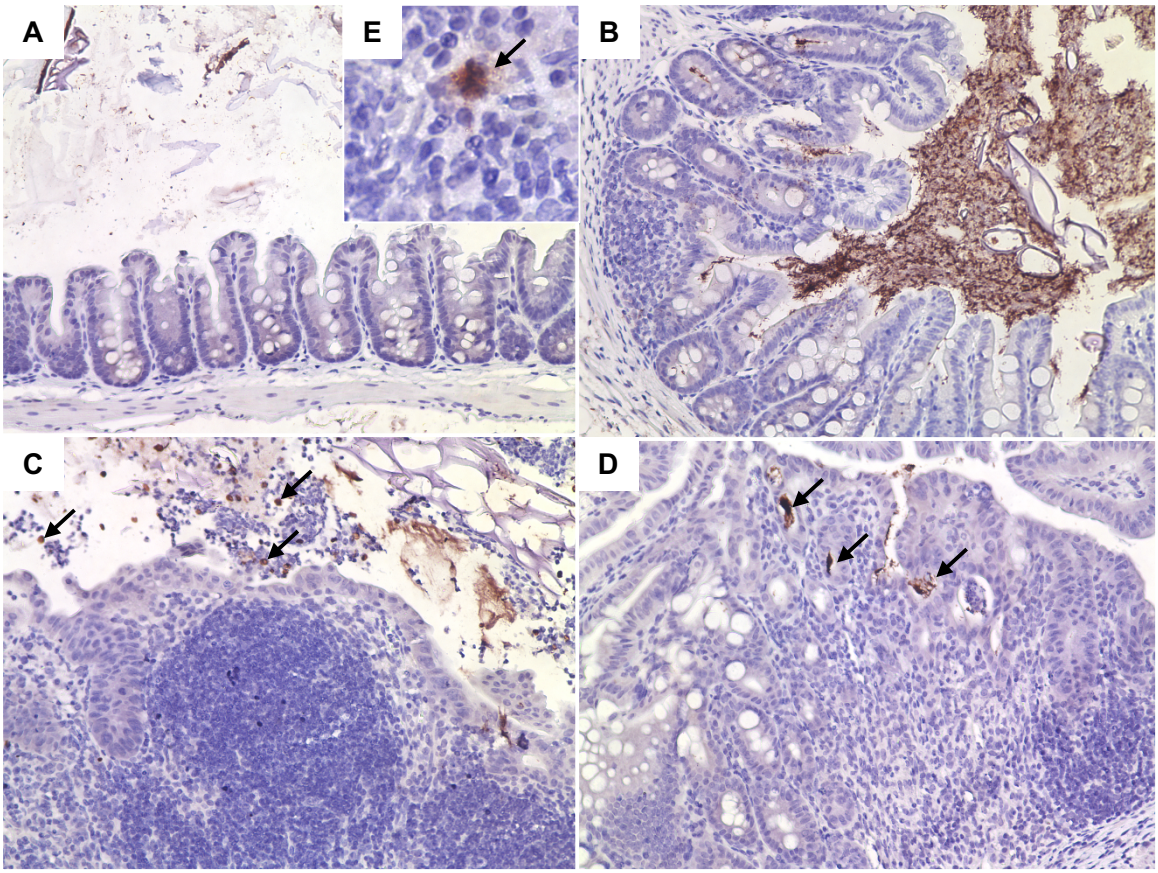

Figure 2.2. Shows immunohistochemical staining for *Campylobacter jejuni* in mouse ileocecocolic tissues. Panel A is from a mouse given tryptone soya broth alone. Panel B was from a mouse given *C. jejuni* HB-93-13 with no antibiotic that shows staining associated with the contents, crypts and epithelial surface. Panel C is from a mouse given *C. jejuni* 260.94 and cefoperazone that show severe inflammation and staining associated with neutrophils in the exudate at the villus tip (arrows). Panel D is from the same mouse as C but shows staining associated with crypt abscesses (arrows). Inset E is from a mouse given *C. jejuni* D8942 and cefoperazone and shows the ileocecocolic lymph node draining the proximal colon. The arrow indicates a phagocytic cell with intracytoplasmic staining.

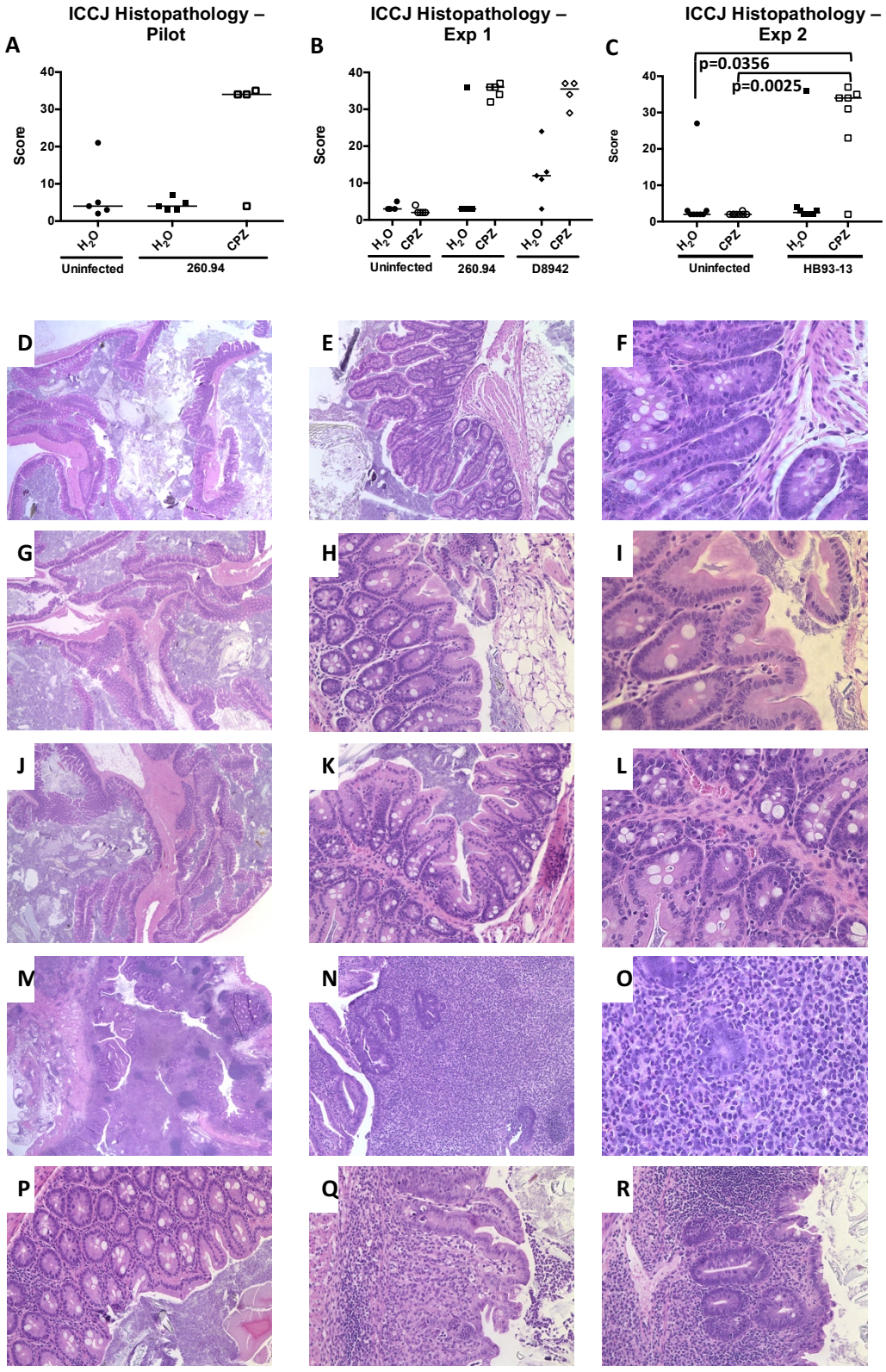

Figure 2.3. CPZ treatment enhances ICCJ histopathology. Figure 4. CPZ treatment enhances ICCJ histopathology. Ileocecocolic junctions (ICCJ) were collected at necropsy and

Figure 2.3 (cont'd). fixed in 10% formalin. Sections were stained with hematoxylin and eosin (H and E), and received a score ranging from 0 to 41. A) pilot, B) experiment 1, and C) experiment 2. Symbols represent the score of 4–8 animals per group and bars represent the median. Data were considered statistically significant if $p<0.05$ after correction for multiple comparisons. D) uninfected + H_2O, 2X, E) uninfected + H_2O, 20X, F) uninfected + H_2O, 40X, G) uninfected + CPZ, 2X, H) uninfected + CPZ, 20X, I) uninfected + CPZ, 40X, J) 260.94 + H_2O, 2X, K) 260.94 + H_2O, 20X, L) 260.94 + H_2O, 40X, M) 260.94 + CPZ, 2X, N) 260.94 + CPZ, 20X, O) 260.94 + CPZ, 40X, P) D8942 + H_2O 20X, Q) D8942 + CPZ 20X, and R) HB93-13 + CPZ ,20X are H and E stained ICCJ sections at various magnifications.

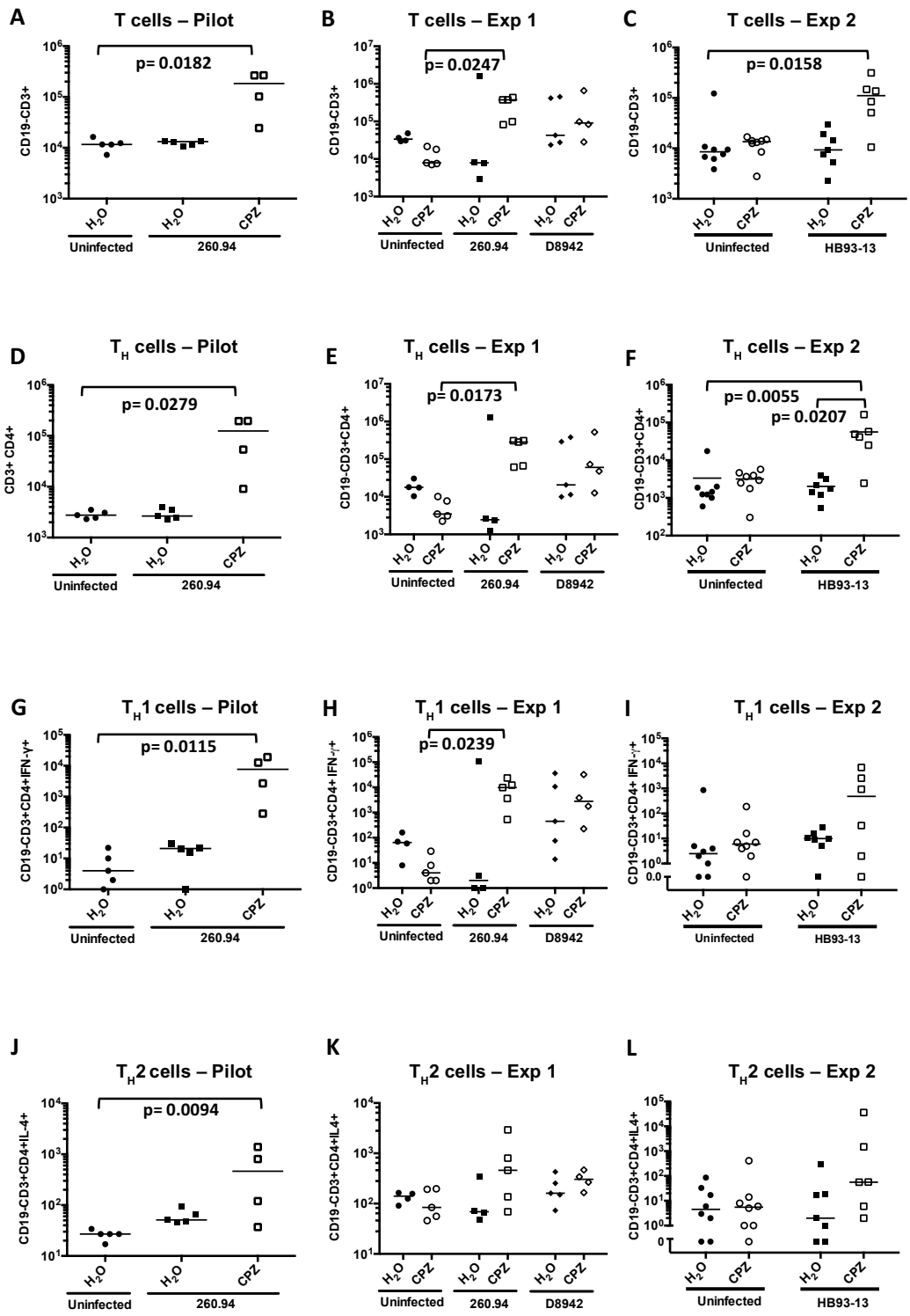

Figure 2.4. Endpoint lamina propria leukocyte analysis via flow cytometry: Pilot experiment. Colon leukocytes were isolated from C57BL/6 IL-10$^{-/-}$ mice at 5 weeks (pilot and experiment 1), 7 weeks (experiment 2), or humane endpoint and sorted by flow cytometry. Dead

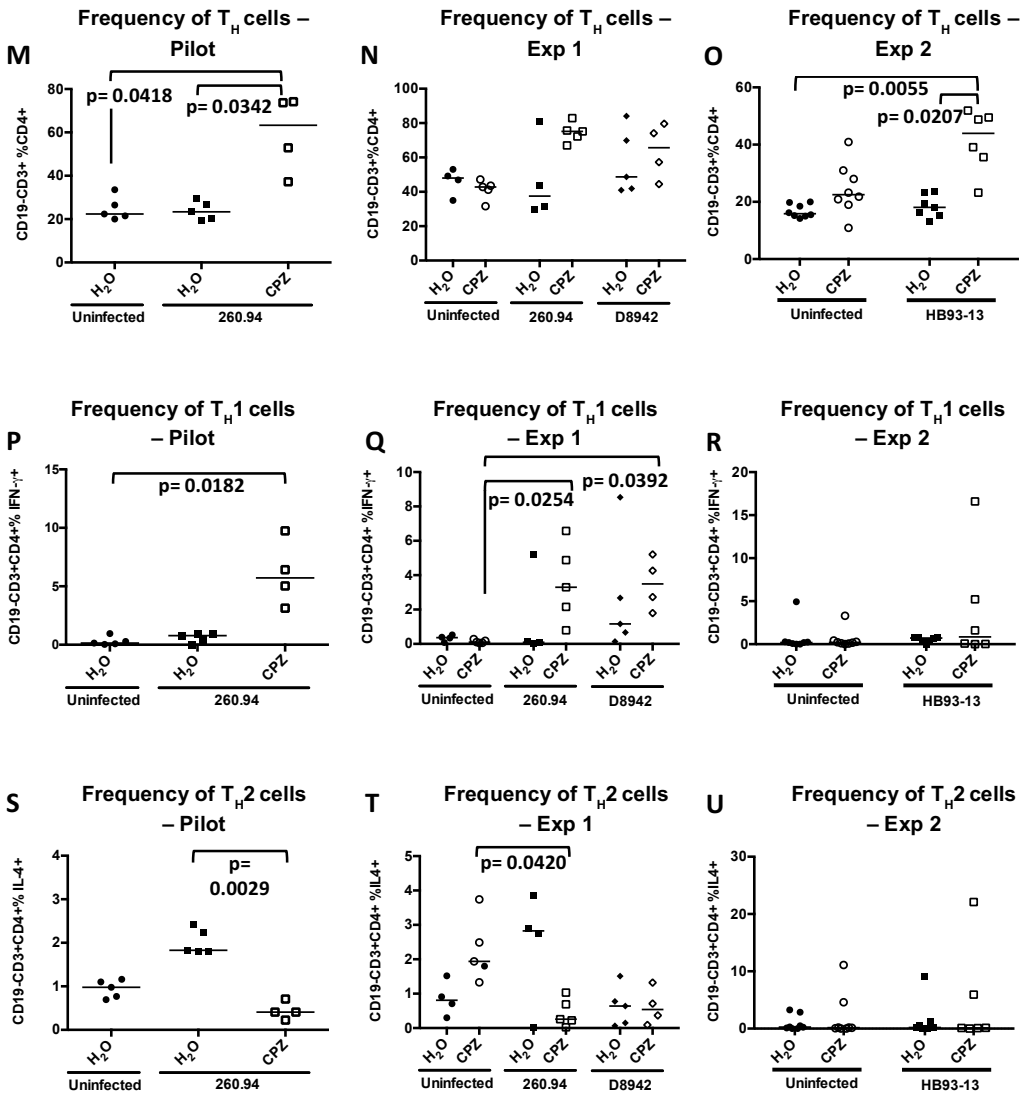

Figure 2.4 (cont'd). and dying cells were excluded based on forward and side scatter. All cells were gated on $CD19^{-}$ $CD3^{+}$ gate. Each symbol represents a single animal; bars represent medians with 4–5 mice per group (Pilot and experiment 1) or 6–8 mice per group (experiment 2); data were analyzed by the Kruskal-Wallis test on ranks with Dunn's post test and $p<0.05$ was considered statistically significant.

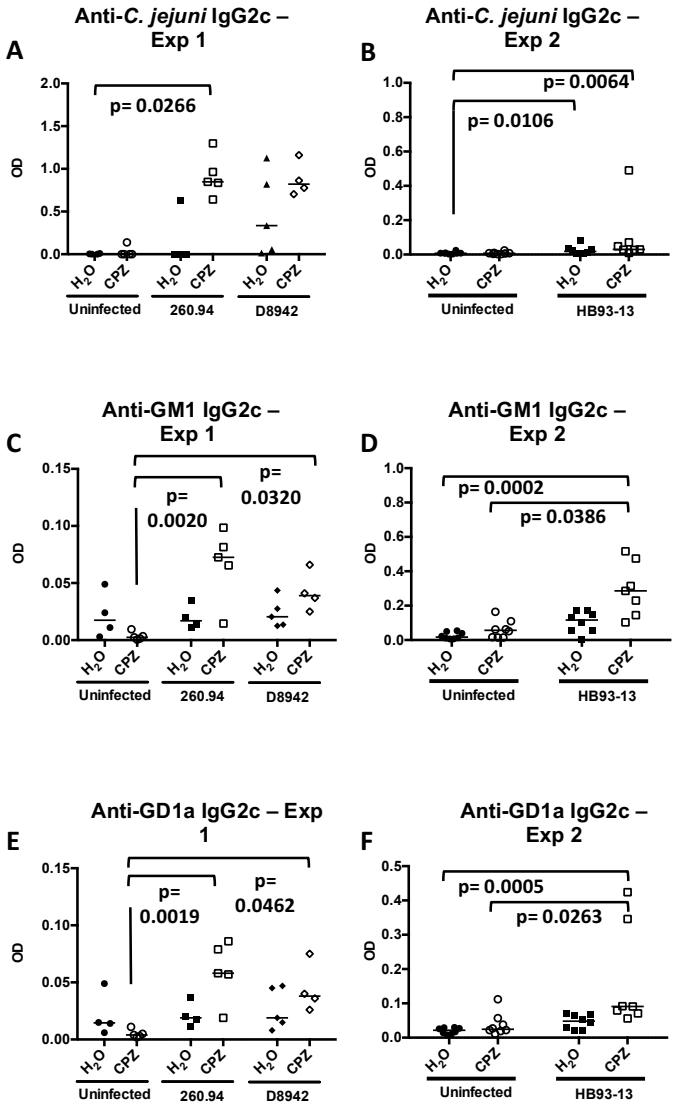

Figure 2.5. Type 1 antibody responses were exacerbated by antibiotic treatment. Plasma antibodies reactive with gangliosides (GM1 and GD1a) and *C. jejuni* antigen were measured via indirect ELISA. Biotinylated goat anti-mouse secondary antibodies were used to determine IgG subclass. Each symbol represents a single animal; bars represent group means. Data represent 4–5 mice per group (Pilot and Experiment 1) or 6–8 mice (Experiment 2); and the means of 3 replicate wells per sample were analyzed by Kruskal-Wallis test on ranks with Dunn's post test and $p<0.05$ was considered statistically significant.

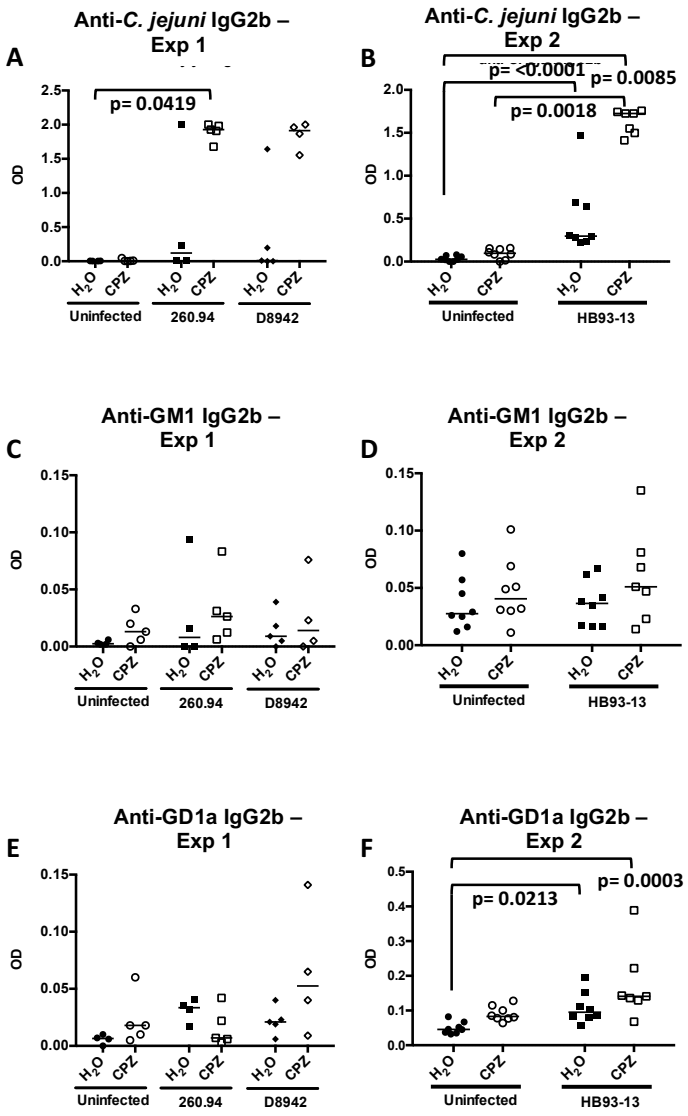

Figure 2.6. Type 17 antibody responses were enhanced by antibiotic treatment in a strain specific manner. Plasma antibodies reactive with gangliosides (GM1 and GD1a) and *C. jejuni* antigen were measured via indirect ELISA. Biotinylated goat anti-mouse secondary antibodies were used to determine IgG subclass. Each symbol represents a single animal; bars represent group means. Data represent 4-5 mice per group (pilot and experiment 1) or 6-8 mice (experiment 2); and the means of 3 replicate wells per sample were analyzed by Kruskal-Wallis test on ranks with Dunn's post test and $p<0.05$ was considered statistically significant.

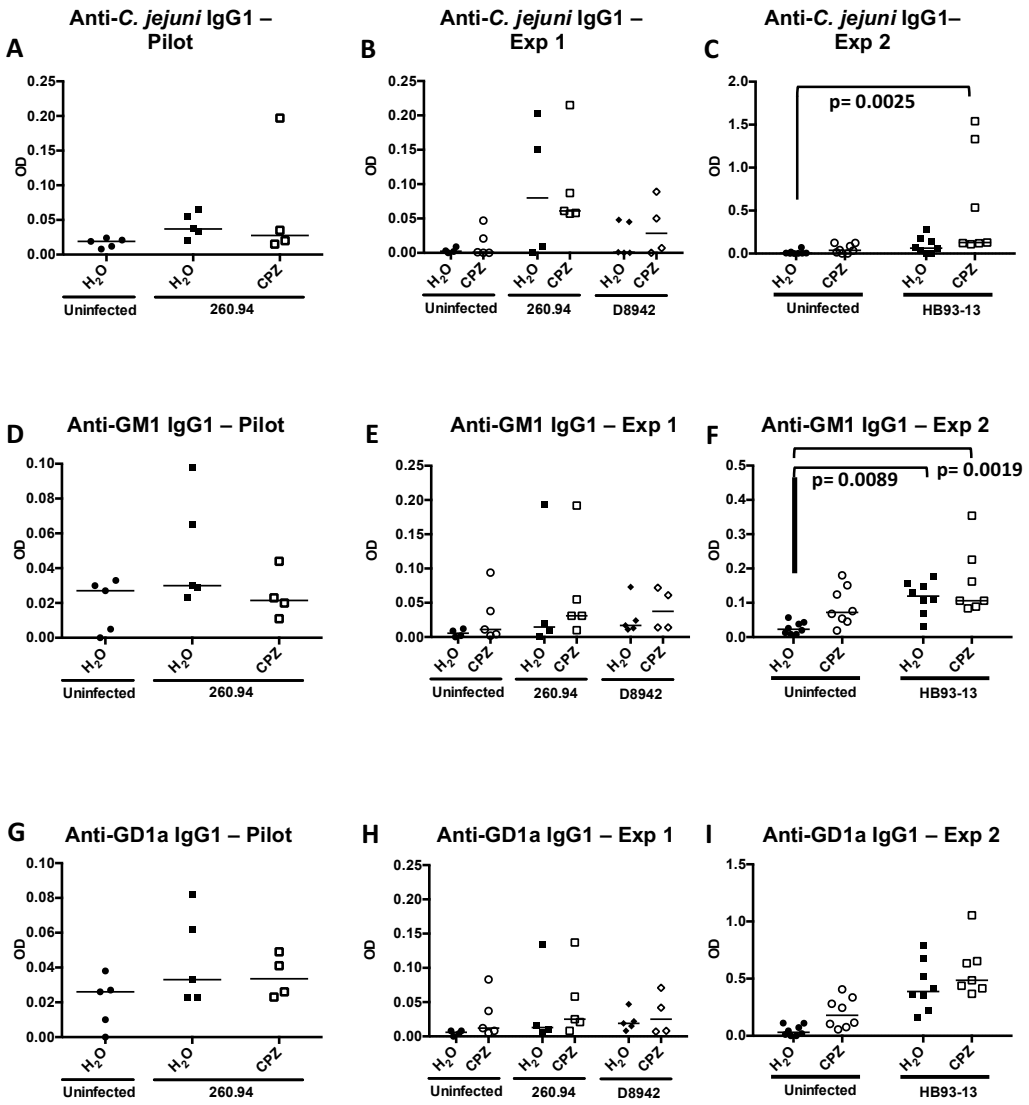

Figure 2.7. *C. jejuni* HB93-13 but not 260.94 or D8942 elicited Type 2 anti-*C. jejuni* and anti-ganglioside antibody responses. Plasma antibodies reactive with gangliosides (GM1 and GD1a) and *C. jejuni* antigen were measured via indirect ELISA. Biotinylated goat anti-mouse secondary antibodies were used to determine IgG subclass. Each symbol represents a single animal; bars represent group medians. Data represent 4–5 mice per group (pilot and experiment 1) or 6–8 mice (experiment 2); and the means of 3 replicate wells per sample were analyzed by Kruskal-Wallis test on ranks with Dunn's post test and $p<0.05$ was considered statistically significant.

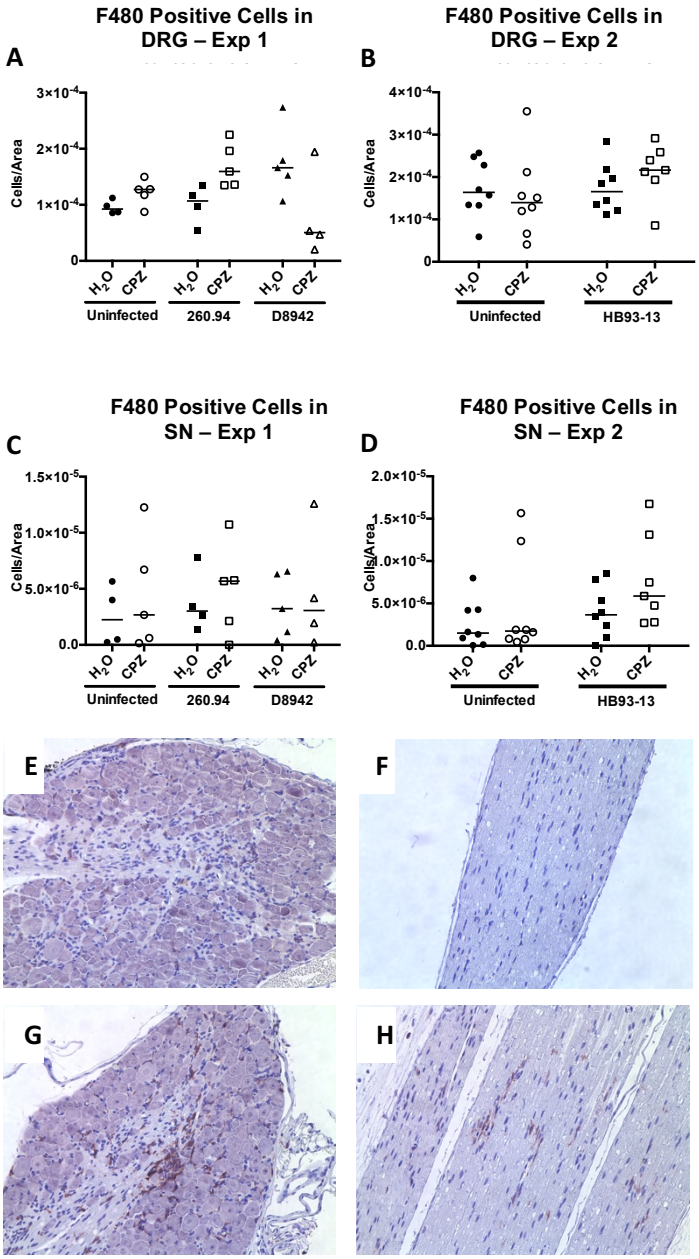

Figure 2.8. Anti-F480 immunohistochemical labeling of sciatic nerves and dorsal root ganglia. Panels A and B) counts of F480 positive cells in dorsal root ganglia, C and D) counts of F480 cells in sciatic nerves, E and F) anti-F480 labeled sciatic nerves, G and H) anti-F480 labeled dorsal root ganglia. Data represent 4–8 animals per group with the median values. No significant differences were detected. NS= Not significant.

	ANTI-GANGLISIDE ANTIBODIES						MACROPHAGES	
	IgG1		IgG2b		IgG2c			
TREATMENT	GM1	GD1a	GM1	GD1a	GM1	GD1a	DRG	SN
EXPERIMENT 1								
TSB								
TSB								
TSB								
TSB								
CPZ								
CPZ								
CPZ								
CPZ								
CPZ								
260.94								
260.94								
260.94								
260.94								
260.94 + CPZ								
260.94 + CPZ								
260.94 + CPZ								
260.94 + CPZ								
260.94 + CPZ								
D8942								
D8942								
D8942								
D8942								
D8942								
D8942 + CPZ								
D8942 + CPZ								
D8942 + CPZ								
D8942 + CPZ								
EXPERIMENT 2								
TSB								
TSB								
TSB								
TSB								
TSB								
TSB								
TSB								
TSB								
CPZ								
CPZ								
CPZ								
CPZ								
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HB93-13 + CPZ								
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HB93-13 + CPZ								
HB93-13 + CPZ								
HB93-13 + CPZ								
HB93-13 + CPZ								
HB93-13 + CPZ								

Table 2.2. Anti-ganglioside antibody and F4/80 comparative analysis. Represents individual positive antibody and F4/80 responses. A score two or more standard deviations from the mean

Table 2.2. (cont'd). of the uninfected + H_2O was deemed a positive response. Blue color indicates significantly elevated antiganglioside antibodies of that specificity and isotype.

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CHAPTER 3

TRANSPLANTED HUMAN FECAL MICROBIOTA ALTERS ADAPTIVE IMMUNE RESPONSES TO *CAMPYLOBACTER JEJUNI* INFECTION IN C57BL/6 MICE

ABSTRACT

Campylobacter jejuni is the leading antecedent infection to the acute peripheral neuropathy Guillain-Barré Syndrome (GBS), which is associated with development of antiganglioside antibodies. Previously we determined that contrasting immune responses mediate *C. jejuni* induced colitis and autoimmunity in interleukin-10 (IL-10) deficient mice, dependent upon the infecting strain. Colitis-associated strains elicited T helper 1 (T_H1)-dependent inflammatory responses while GBS-associated strains elicited T_H2-dependent antibody production. We also found that both responses were exacerbated by antibiotic depletion of the microbiota. Here, we examined whether the structure of the gut microbial community alters the gastrointestinal inflammatory response or the host's anti-ganglioside antibody response to infection with *C. jejuni* strains from colitis or GBS patients. We aimed to determine if presence of human gut microbiota alters host inflammatory and autoimmune responses to *C. jejuni* infection when compared to congenic mice with conventional mouse microbiota. ^{Hu}microbiota and conventional microbiota mice had statistically significant differences between their microbial communities, although alpha diversity indices showed no differences in diversity between experimental groups. C57BL/6 mice carrying a stable humanized gut microbiota (^{Hu}microbiota) and experimentally infected with *C. jejuni* strains from a colitis patient and a GBS patient had higher *C. jejuni* colonization levels and colonic inflammation scores than congenic mice with conventional murine microbiota. *C. jejuni* 11168 but not 260.94 elicited T_H1 and T_H17 associated anti-*C. jejuni* antibody responses. Notably, ^{Hu}microbiota mice displayed a T_H2 biased anti-*C. jejuni* antibody response independent of inoculation status. Finally, ^{Hu}microbiota mice infected with both *C. jejuni* patient strains had elevated GM1 anti-ganglioside antibody responses, but only those given strain 260.94 were significantly higher than conventional microbiota mice given the same strain. These data demonstrate that human microbiota alters host-pathogen interactions in infected mice increasing colonization and autoimmune responses in a *C. jejuni* strain-dependent

manner. This suggests that particular human microbiota compositions likely enhance host susceptibility to GBS following *C. jejuni* infection.

INTRODUCTION

Dysbiosis, the depletion of beneficial organisms in the gut microbiota, has been implicated in the manifestation of several autoimmune and chronic inflammatory diseases. Both autoimmune diseases and chronic inflammatory diseases are characterized by destruction of tissues and functional impairment modulated by immune mechanisms (Mathis and Benoist 2011, Chervonsky 2013, Rosenblum, Remedios et al. 2015). The microbiota modulates the host immune response and affects the production of cytokines, antibodies, and antimicrobial peptides that target pathogens for removal (Buffie and Pamer 2013, Kamada, Chen et al. 2013). Microbial regulation of these immune responses highlights the importance of host-microbiota mutualism. Thus, several autoimmune diseases likely have origins in dysbiotic microbiota or abnormal mucosal barrier function including Inflammatory Bowel Disease (IBD) (Mathew 2008), Type 1 diabetes (Wen, Ley et al. 2008), multiple sclerosis (Westall 2006, Pender 2009), and Reiter's arthritis (Toivanen 2003, Rodríguez-Reyna, Martínez-Reyes et al. 2009). A substantial number of autoimmune diseases including Guillain-Barré Syndrome (van den Berg, Walgaard et al. 2014), Miller-Fisher syndrome (Koga, Gilbert et al. 2005), and Lyme arthritis (Nardelli, Callister et al. 2008) have been linked to previous infection with pathogenic organisms (Ercolini and Miller 2009). Considerable effort has been made to understand how infection with pathogenic microorganisms results in a loss of tolerance and initiates autoimmunity (Ercolini and Miller 2009, Sfriso, Ghirardello et al. 2010). One leading hypothesis is that molecular mimicry, a similarity between molecular structures on the infectious agent and host tissues, results in cross reactivity (Blank, Barzilai et al. 2007, Cusick, Libbey et al. 2012), which in turn leads to autoimmune attack.

Campylobacter jejuni, a leading cause of bacterial gastroenteritis worldwide, precedes at least one-fourth of all cases of the acute peripheral neuropathy Guillain-Barré Syndrome (Rees, Soudain et al. 1995, Islam, Jacobs et al. 2010, Willison, Jacobs et al. 2016). It is hypothesized that molecular mimicry of host nerve gangliosides by lipooligosaccharides on the surface of *C. jejuni* initiate cross-reactive antibody responses resulting in complement-mediated nerve damage (Yuki 2012). Other factors may also contribute to GBS disease. When enteric disease is severe, *C. jejuni* infection may be treated with fluoroquinolone or macrolide antibiotics, however, increasing resistance to these drugs has been reported (Pamela, Curt et al. 2006). Notably, antibiotic treated and gnotobiotic mice display increased susceptibility to *C. jejuni* colonization and enhanced incidence of gastrointestinal inflammation (Chang and Miller 2006, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015), leading to the hypothesis that components of the resident mouse gut microbiota protect against *Campylobacter*-mediated disease (O'Loughlin, Samuelson et al. 2015). Moreover, approximately two-thirds of GBS patients report gastrointestinal or respiratory inflammation in the weeks preceding neurological symptoms (Jacobs, Rothbarth et al. 1998, Hughes and Cornblath 2005, Koga, Gilbert et al. 2005, Shui, Rett et al. 2012); thus, host determinants of inflammation including gut microbiota may play a critical role in GBS development. Experimentally, normal flora of mice play a critical in preventing *C. jejuni*-mediated inflammation thus raising the question of whether murine models carrying certain human microbiota would show similar susceptibility to enteric disease shown in depleted microbiota models (Chang and Miller 2006, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015).

To determine if a humanized microbial community is sufficient to alter the host inflammatory and autoimmune response to infection with *C. jejuni,* we infected C57BL/6 humanized (^{Hu}microbiota) and conventional microbiota mice with a *C. jejuni* enteric disease patient strain (11168) or GBS patient strain (260.94). Using an established and robust experimental inoculation system and a defined set of disease indicators (Mansfield, Bell et al.

2007, Bell, St Charles et al. 2009, Malik, Sharma et al. 2014) we measured both inflammatory and autoimmune endpoints. We hypothesized that ^{Hu}microbiota mice would exhibit 1) enhanced colonization by *C. jejuni*, 2) higher levels of anti-ganglioside antibodies, and 3) increased lesions in both the GI tract and peripheral nerves compared to mice with conventional murine microbiota. To compare the microbiota of ^{Hu}microbiota and conventional microbiota mice, we characterized the fecal microbiota using 16S rRNA gene amplicon analysis. To examine whether the expected microbiota dependent immune responses resulted in inflammatory changes in the gut (assessed by gross pathology, histopathology, and colon homogenate IFNγ levels), or elevated autoantibody levels (assessed by plasma antibody ELISA), we infected both ^{Hu}microbiota and conventional microbiota mice with *C. jejuni* 11168 and *C. jejuni* 260.94. Here we show that ^{Hu}microbiota mice 1) have higher levels of *C. jejuni* colonization, 2) are more susceptible to *C. jejuni* gastroenteritis, 3) display diminished activity in the open field test that is not the result of infection, and 4) have a type 2 biased antibody response. Consistent with previous results, IL-10 mice were more susceptible to colitis than genetically wild type mice and immune responses were affected by the characteristics of the infecting *C. jejuni* strain as well as by the composition of the gut microbiota.

RESULTS

Fecal Microbiota. To determine if handling (SPF v sterile) altered the microbiota of ^{Hu}microbiota mice we compared the fecal microbiota of ^{Hu}microbiota and conventional mice by qPCR. For this analysis we amplified fecal DNA using 16S rRNA gene primers specific for *Clostridium* group 14*, Clostridium* group 1*, Bacteroidetes, and Enterobacteriaceae.* No statistically significant differences were detected in these four bacterial groups between Hu-TSB (SPF) and Hu-TSB (Ster.)(data not shown).

Disease indicators. To determine if ^{Hu}microbiota mice are 1) susceptible to *C. jejuni* gastroenteritis and 2) display *C. jejuni* strain specific antibody responses to GBS patient strains,

we infected mice with one of two *C. jejuni* strains. Veterinarians and trained animal handlers monitored clinical signs daily. A significant difference in body weight was detected between Hu-260.94 and Conv-11168 mice (Fig. 3.1 A). A single IL-10-deficient infected mouse (Conv-IL-$10^{-/-}$-11168 (SPF)) displayed clinical signs (Fig. 3.1 B). Gross pathology was mild in all cases, infrequent except for Conv-IL-$10^{-/-}$-11168 mice, and restricted to infected mice in all cases (Fig. 3.1 C). Interestingly, other than the IL-10 deficient mice, only infected ^{Hu}microbiota mice showed gross pathology. C57BL/6 genetically wild-type and IL-$10^{-/-}$ were used as gastroenteritis controls due to their well characterized reputation as colitis resistant (wild-type) and colitis susceptible (IL-$10^{-/-}$)(Mansfield, Bell et al. 2007, Malik, Sharma et al. 2014) controls. *C. jejuni* could be cultured from mice in all infected groups (Fig. 3.1 D); however, *C. jejuni* could be only be cultured from 6 of 10 IL-$10^{-/-}$ mice. In comparison, one-hundred percent colonization was achieved in *C. jejuni* infected ^{Hu}microbiota and conventional genetically wild-type mice although the WT and IL-$10^{-/-}$ mice were both derived from the same stock (Jackson Laboratories, Bar Harbor, MA). To assess cross reactive antibody responses, we measured anti-*C. jejuni* and anti-ganglioside antibody responses in infected mice by indirect ELISA. Notably, inoculation with *C. jejuni* 11168 but not 260.94 was sufficient to elicit significant T_H1 (Ig2c and IgG3 isotypes) and T_H17 (IgG2b isotype) associated antibody responses compared to controls (Hu-TSB (Ster.)) (Fig. 3.1 G-I). T_H2 associated antibody responses were elevated in Hu-11168 mice compared to ^{Hu}microbiota-TSB (SPF) but not ^{Hu}microbiota-TSB (Ster.). No other T_H2 associated anti-*Campylobacter* or anti-ganglioside antibody responses were detected compared to controls (Fig. 3.1 G-L).

^{Hu}microbiota mice have a distinct microbiota compared to conventional mice. In experiment 1 (Table 2), to compare microbiota structure in ^{Hu}microbiota and conventional microbiota, infected and sham inoculated mice, we analyzed their fecal microbiota with 16S rRNA amplicon analysis. Analysis revealed that 4 of 5 phyla, 9 of 11 classes and 32 of 52 genera detected in the human fecal slurry used to produce the ^{Hu}microbiota mice could be detected in the ^{Hu}microbiota mice used in this study. The phylum *Verrucomicrobia* constituted less than 2% of the

reads from the original inoculum and could not be found in ^{Hu}microbiota mice used in this experiment. Noteworthy was that clustering of groups based on Bray-Curtis dissimilarity statistic resulted in separation by microbiota but not group assignments according to *C. jejuni* or TSB inocula (Fig. 3.2).

Increased abundance of *Lactobacillus* in conventional microbiota mice. Five bacterial orders had an average abundance of 5% or greater in one or more groups of mice; *Bacteriodales, Bifidobacteriodales, Clostridiales, Lactobacillales* and *Erysipelotrichales* (Fig. 3.3 A-E). *Bacteriodales* was a minor component of the inoculum, constituting approximately 3.3% of the sequences, but a major component of ^{Hu}microbiota (~57–60%) and conventional murine (28–43%) microbiota (Fig. 3.3 A). Within the order *Bacteriodales,* the ^{Hu}microbiota was dominated by *Bacteroidaceae* (~58–63% of *Bacteriodales*) yet it was only a minor component of the conventional murine microbiota (~0.02–0.07% of *Bacteriodales*). In contrast, within the order *Bacteriodales* the conventional murine microbiota was dominated by *Porphyromonadaceae* (~98% of *Bacteriodales*). *Bifidobacteriodales* constituted ~7% of the inoculum, was completely absent in the ^{Hu}microbiota mouse samples, and was a minor component of the conventional murine microbiota (~0.6–1.6%) (Fig. 3.3 B). All reads from the order *Bifidobacteriodales* were assigned to the family *Bifidobacteriaceae.* The inoculum was dominated by the order *Clostridiales* (~70%) which was less abundant in all of the mouse fecal samples but present in similar abundances in ^{Hu}microbiota (~28–38%) and conventional microbiota (22–42%) samples (Fig. 3.3 C). Within the order *Clostridiales, Lachnospiriaceae* was the dominate family in all groups. *Erysipelotrichales* was also present in all mice and more abundant in conventional mice than the inoculum or conventional samples; inoculum (7.2%), ^{Hu}microbiota (~3.6–4.5%) and conventional (~10.4–15.1%) (Fig. 3.3 D). *Lactobacillales* was present in all groups but more abundant in the conventional mice than the inoculum or ^{Hu}microbiota mice; inoculum (0.68%), ^{Hu}microbiota (~0.002–1.7), and conventional (~2.9–6.3%) (Fig. 3.3 E). Within the order *Lactobacillales* ^{Hu}microbiota mice had no or 6000-fold less *Lactobacillaceae* than conventional microbiota

samples. In all cases greater than 97% percent of the reads in the family *Lactobacillaceae* were assigned to the genus *Lactobacillus* (Fig. 3.3 F). At the level of order, unclassified reads could be found in all groups; inoculum (3.7%), ^{Hu}microbiota (~1.9–2.4%) and conventional (~9.2–10.4%) (data not shown).

Diversity statistics: Groups can be distinguished by microbiota but not infection status. To determine if OTUs could be separated into groups based on microbiota or inoculation status we assessed OTUs by alpha and beta diversity metrics. Principle Components Analysis showed that there was clear separation between ^{Hu}microbiota and conventional fecal samples (Fig. 3.5 A) but that microbiota composition was not affected by inoculation status (Fig. 3.5 B and C). These results are supported by two-way ANOSIM, PERMANOVA (Fig. 3.4 D). Comparison of alpha metrics revealed a disparity in OTUs in Hu-11168 compared to Conv-11168 (Fig. 3.6 A). No other disparity in OTUs existed, and this finding was not reflected in species diversity or evenness (Fig. 3.6 B-D).

Majority of the variance between Hu-TSB and Conv-TSB can be attributed to the abundance of Porphyromonadaceae, Bacteroidaceae, and Lachnospiraceae. To determine 1) which OTUs varied in abundance between groups and, 2) the contribution of specific OTUs to the variance between groups we performed a paired T-test with a Benjamin-Hochberg correction for false discovery (http://www.biostathandbook.com/multiplecomparisons.html) and similarity percentages analysis. In summary, ^{Hu}microbiota and conventional samples could be distinguished by OTUs assigned to the *Bacteroidetes* and *Firmicutes* phyla and these phyla contributed to 57.19 and 26.6 percent of the variance between Hu-TSB and Conv-TSB, respectively (Table 3). The most abundant OTUs contributing to the difference between the groups are OTUs 002, the dominant OTU in the conventional samples, and OTUs 001 and 003, which are the dominant OTUs in the ^{Hu}microbiota samples. Collectively OTUs 001, 002, 003 contribute 57.93% of the variance between Hu-TSB and Conv-TSB.

Disease indicators: ^{Hu}microbiota mice displayed increased susceptibility to intestinal inflammation. To compare the progression and severity of disease that manifested in experimental mice we monitored all mice for 1) clinical signs, 2) gross pathology and disparity in body weight. All mice were monitored closely by veterinarians to determine if euthanasia was required prior to the scheduled 7-week endpoint. These determinations were based on hunching, lethargy, and watery or bloody diarrhea in accordance with a standardized scoring system (Mansfield, Bell et al. 2007) available from the Michigan State University Microbiology Research Unit Food and Waterborne Diseases Integrated Research Network-sponsored Animal Model Phenome Database. No significant disease indicators were detected during the experimental course, thus all of the mice were maintained for the entirety of the experiment. No disparity in body weight was detected (Fig. 3.6 A). Clinical signs, predominantly soft feces were detected in several animals, including controls, and were generally more prevalent in ^{Hu}microbiota infected mice (Fig. 3.6 B). Four of 10 of *C. jejuni* 260.94 infected and 5 of 10 *C. jejuni* 11168 infected ^{Hu}microbiota mice had severe gross pathological changes in the GI tract (Fig. 3.6 C). In many of these cases, the ileocecocolic lymph node, spleen, and sometimes the mesenteric lymph nodes were enlarged. One *C. jejuni* 11168 infected ^{Hu}microbiota mouse had a slightly thickened colon wall. To determine if the level *C. jejuni* differed between ^{Hu}microbiota and conventional mice, a potential cause of enhanced GI gross pathology, we quantified *C. jejuni* in both the cecum and colon. Colonization was higher in ^{Hu}microbiota than conventional mice (Fig. 3.6 D and E). These data were supported by a 10-fold and-100 fold increase in *C. jejuni* 11168 and 260.94 in ^{Hu}microbiota fecal microbiota analysis compared to conventional fecal microbiota samples (Fig. 3.6 F). Infection with *C. jejuni* 11168 but not 260.94 was sufficient to elicit a T_H1 (IgG2c and IgG3) and T_H17 (IgG2b) associated antibody response compared to uninfected controls (Fig. 3.6 G-I). T_H2 associated anti-*Campylobacter* (Fig. 3.6 J) and anti-ganglioside (Fig. 3.6 K) antibody responses were elevated in ^{Hu}microbiota mice compared to conventional mice independent of inoculation status, indicating a T_H2 associated antibody bias in ^{Hu}microbiota mice.

IFNγ responses and enteric lesions were not exacerbated in ^{Hu}microbiota mice. We next assessed the ICCJ for histopathology and measured classical inflammatory (IFNγ) and anti-inflammatory (IL-4) cytokine levels in the colon. Histological analyses of the ICCJs was performed as described previously (Mansfield, Bell et al. 2007). No significant increases were detected in enteric lesions (Fig. 3.7 A) or mRNA expression of IFNγ (Fig. 3.7 B) or IL-4 (Fig. 3.7 C) in colon homogenates. A single high reactor mouse was detected in each of the following groups Hu-260.94 (9.4-fold increase/HPRT compared to group mean of 1.7) (Fig. 3.7 B), and Hu-11168 (17.8-fold increase/HPRT compared to group mean of 2.8) (Fig. 3.7 C), and Hu 260.94 (38.3-fold increase/HPRT compared to group mean of 5.244) (Fig. 3.7 C). Add data from pilot experiment.

Assessment of macrophages in peripheral nerves and dorsal root ganglia. To determine if macrophage numbers were increased in *C. jejuni* infected mice compared to controls we immunohistochemically labeled these tissues with F4/80 and counted positive cells. No differences in peripheral nerve lesions were detected.

^{Hu}microbiota mice display hypoactivity in the open-field test and infection alters activity in mice with conventional microbiota. To determine if *C. jejuni* infection was associated with behavioral phenotypes indicative of enteric disease and potential neuropathy we recorded the activity of the experimental mice in the open-field test. In general, mice with ^{Hu}microbiota displayed diminished activity levels compared to conventional mice regardless of infection status (Fig. 3.8 A and 3.8 B), and both groups trended towards a decrease in activity as time progressed. Significant decreases in the number of quadrants crossed were primarily dependent upon having ^{Hu}microbiota; no differences were detected within the ^{Hu}microbiota mice during the seven-weeks, and they generally displayed low levels of activity. Significant differences detected in quadrants-crossed by conventional mice are as follows: Baseline) None; week 1: Conv-TSB vs. Conv-260.94 ($p=0.0031$), and Conv-TSB vs. Conv-11168 ($p= < 0.0001$); week 2: Conv-TSB vs. Conv-11168 ($p= 0.0023$); week 3: Conv-TSB vs. Conv-260.94 ($p= 0.0183$) and

Conv-TSB vs. Conv-11168 (p=0.0105) week 4: Conv-TSB vs. Conv-260.94 (p= 0.0139) and Conv-TSB vs. Conv-11168 (p = 0.0078); week 5: Conv-TSB vs. Conv-11168 (p=0.0183); week 6: none, and week 7: Conv-TSB vs. Conv-260.94 (p= 0.0043) and Conv-TSB vs. Conv-11168 (p=0.0023). ^{Hu}microbiota mice crossed fewer quadrants than ^{Hu}microbiota mice for all comparisons starting from the baseline and continuing through week 4. After week four, cross microbiota comparisons were only significant for comparisons between Conv-TSB and all ^{Hu}microbiota groups (p always less than 0.0078). Similarly, no statistically significant differences in rearing behavior were detected within ^{Hu}microbiota group, and Hu-TSB and Conv-TSB groups differed in rearing activity at prior to inoculation (Fig 3.8 B). Significant differences detected in conventional mice are as follows: Baseline: Conv-TSB vs Conv-260.94 (p= 0.0033), Conv-TSB and Conv-11168 (p=0.0011); Week 1: Conv-TSB vs. Conv-11168 (p= < 0.000). After week 3, no differences in rearing behavior were detected between any groups as mice were generally inactive.

DISCUSSION

C. jejuni is a leading cause of bacterial diarrheal illness and the leading antecedent infection to the acute peripheral neuropathy GBS (Young, Davis et al. 2007, Willison, Jacobs et al. 2016). After six generations of breeding, individually housed mice with a transplanted human microbiota were infected with *C. jejuni* enteric disease or GBS patient strains. These mice retained a microbiota distinct from that of their conventional counterparts that could be primarily distinguished by bacteria belonging to the Phyla *Bacteroidetes* and *Firmicutes*. *C. jejuni* infection did not appear to alter the microbiome composition in either group (Fig. 3.4 B and 4). Moreover, the abundance of *Lactobacillus,* which has been sown to prevent *C. jejuni* colonization in *in vivo* and *in vitro* models (Neal-McKinney, Lu et al. 2012, O'Loughlin, Samuelson et al. 2015), was at least 6000-fold greater in conventional than ^{Hu}microbiota mice. The value of this model is further

validated by the occurrence of distinct disease manifestation in the GI tract and the presence of antiganglioside antibodies in infected ^{Hu}microbiota mice as compared to the conventional controls. Overall, our results support the conclusion that components of the murine microbiota play a role in colonization resistance that is overcome in our ^{Hu}microbiota model, resulting in increased susceptibility to both inflammation and autoimmunity.

We analyzed *C. jejuni* infected mice with ^{Hu}microbiota and conventional microbiota for enteric inflammation and found that ^{Hu}microbiota mice are more susceptible to *C. jejuni*-mediated inflammation determined by having more severe gross pathology especially enlarged lymph nodes. These results are consistent with previous reports showing that the microbiota is a key regulator of enteric disease in *C. jejuni* infected mice (Chang and Miller 2006, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015). Despite this finding, significant enteric histologic lesions were not found and inflammatory cytokine levels were not significantly elevated in the proximal colon of ^{Hu}microbiota mice. These results suggest that most of the immune reactivity was occurring in the draining lymph nodes and not in the colon wall in most infected mice. However, semi-quantitative and 16S amplicon analysis showed that microbiota source affected *C. jejuni* load. 10-fold higher *C. jejuni* 11168 and 100-fold higher *C. jejuni* 260.94 loads were detected in the fecal microbiota of infected ^{Hu}microbiota mice than their conventional microbiota matched counterparts (Fig. 3.7 F). We previously reported that interleukin-10 deficiency significantly increased host-inflammatory responses to *C. jejuni*. Now these results indicate that ecological shifts in the microbiota are another factor sufficient to enhance host-susceptibility to *C. jejuni* resulting in mild enteric disease.

Infection with *C. jejuni* GBS patient strains is associated with T_H2 immune responses and anti-ganglioside antibodies in a C57BL/6 IL-10 deficient mouse model (Malik, Sharma et al. 2014). In our experiment, ^{Hu}microbiota mice showed a biased type 2 antibody titer with or without infection compared to conventional microbiota mice. Analysis of anti-ganglioside antibodies showed exacerbated anti-GM1 antibody levels following infection with *C. jejuni* 260.94 but not

enteric strain 11168 compared to conventional microbiota mice infected with the same *C. jejuni* strain. We have demonstrated microbiota mediated autoimmunity in *C. jejuni* infected mice with depleted microbiota previously (Brooks et al. unpublished); however, this is the first study to show that ecological shifts in a diverse microbial community are sufficient to alter *C. jejuni*-mediated autoimmunity. In fact, while T_H1/T_H17 responses (IgG2c, IgG2b) to the *C. jejuni* 11168 strain in ^{Hu}microbiota mice are similar to IL-10 deficient mice, ^{Hu}microbiota have more pronounced T_H2 responses than IL-10 deficient mice and yet no demonstrable GBS. This suggests that anti-ganglioside antibodies alone cannot produce GBS in the ^{Hu}microbiota C57BL/6 mice were IL-10 is present. These results, in conjunction with anti-*Campylobacter* results show that differences in the antigens presented on the two strains are not the only factor driving enteric and GBS phenotypes. In addition, *C. jejuni* 11168 IgG1 antibody responses also suggest that the 11168 strain is not strictly an enteritis-causing strain. Although it was taken from a human patient with acute gastroenteritis, the possibility that a single strain could cause different manifestations of disease in different individuals, perhaps even depending on the microbiota composition of those individuals, should not be discounted.

A significant decrease in activity in the behavioral open field test was detected in all ^{Hu}microbiota mice, while no overt neurological signs were detected. The open field test has been used as a non-invasive longitudinal measure of quality of life that allows the investigator to make inferences about anxiety and normal exploratory behavior (Walsh and Cummins 1976, Prut and Belzung 2003, Tatem, Quinn et al. 2014). This decrease in activity was not due to infection status, nor were there any obvious signs of neurologic or enteric disease detected in the ^{Hu}microbiota mice; thus we conclude that this decreased activity is not an indicator of nervous system disease in response to infection. Furthermore, sciatic nerves and lumbar dorsal root ganglia were dissected and immunohistochemically labeled and macrophages quantified to determine whether an increase in macrophages could be detected as a potential mechanism of peripheral nerve damage consistent with that seen in patients with GBS (Griffin, Li et al. 1996, van den Berg,

Walgaard et al. 2014, Willison, Jacobs et al. 2016). An increase in macrophages in or around the nerve is indicative of complement dependent injury; however, no differences in macrophage numbers were detected in our study. Our results suggest the possibility of an influence of the microbiota on brain and behavior, which has been shown previously (Cryan and Dinan 2012, Dinan and Cryan 2012, Cryan and Dinan 2015). Taken together these findings show that microbiota can influence pain, cognition, and stress responses.

In summary, we found that the microbiota is a key factor in the regulation of *Campylobacter* inflammation in the intestine and the elicitation of anti-ganglioside antibodies. These data support recent findings that the microbiota is a critical component in *Campylobacter* gastroenteritis and to our knowledge, this is the first report to suggest that the microbiota may in fact be a determinant of host susceptibility to Guillain-Barré Syndrome. *C. jejuni* microbiota mediated colonization resistance in mice is overcome by perturbation of the microbiota; thus factors that mediate the host microbiota including age, diet, antibiotic treatment, prior pathogen exposure etc. may be determinants of susceptibility to Guillain-Barré Syndrome. Because no single human microbiota exists, it is only reasonable to speculate that OTUs distinguishing the human and murine microbiota in our experimental mice play a role in regulating *C. jejuni* loads. Finally, therapeutic approaches that avoid depletion of healthy microbiota and enhance populations of beneficial microorganisms may be appropriate. Recently, probiotics including *Lactobacillus* have been shown to inhibit *C. jejuni* growth in mice (O'Loughlin, Samuelson et al. 2015); thus probiotic supplementation during the early stages of infection may facilitate clearance of *C. jejuni* (Mohan 2015).

MATERIALS AND METHODS

Laboratory 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 (Leary, Underwood et al. 2013). Protocols were reviewed and approved by the Michigan State University Institutional Animal Use and Care Committee. A portion of the mice in each experiment possessed humanized microbiota generated as described previously (Collins, Auchtung et al. 2015) and are indicated with the prefix Hu (^{Hu}microbiota). Briefly, germ-free mice were inoculated by gavage with a human fecal slurry, bred, and the microbiota allowed to pass from mother to offspring without intervention within the germ-free incubator. After initial characterization in founder mice described in Collins et al. 2015, the ^{Hu}microbiota mice were separated into two groups and a new colony was established (Hu-C57BL/6) by LSMansfield by transferring mice in filter top cages within sterile dog crates to Michigan State University. ^{Hu}microbiota mice were housed under specific pathogen free conditions (SPF), and bred for six generations in closed cages on an Innovive (San Diego, CA, USA) mouse rack with filtered air flow and sterile food and water. All cage changes and other manipulations were performed in a laminar flow hood to avoid introduction of microorganisms from the environment.

In the pilot experiment, age matched 10-12 week-old ^{Hu}microbiota C57BL/6 genetically wild-type (Hu), conventional microbiota genetically wild-type (Conv), and conventional microbiota congenic IL-10 deficient mice (Conv-IL-10$^{-/-}$) were used. Mice were inoculated with either tryptone soy broth (TSB; vehicle control), *C. jejuni* 260.94, or *C. jejuni* 11168 and handled with sterile or specific pathogen free (SPF) technique resulting in 6 groups (Table 1). For SPF technique all personnel that were handling animals wore Tyvek coveralls, impermeable plastic booties, face mask, hair bonnet, and gloves. All cage changes were performed at the bench. For sterile technique, all personnel that were handling animals wore impermeable plastic booties, face mask, hair bonnet, sterile surgical gown, and sterile surgical gloves. All breeding mice were and continue to be handled using sterile technique to avoid introducing extraneous organisms to the microbiota. In contrast, all cage changes were performed in a sterile laminar flow hood that was disinfected after each use. To determine if handing would alter outcomes in sham inoculated ^{Hu}microbiota mice we inoculated 20 ^{Hu}microbiota mice with TSB and handled them with either sterile technique

(Hu-TSB (Ster.)) or SPF technique (Hu-TSB (SPF)). Two other ^{Hu}microbiota groups were generated by inoculating ^{Hu}microbiota mice with either *C. jejuni* 260.94 (Hu-260.94 (Ster.)) or *C. jejuni* 11168 (Hu-11168 (Ster.)) and handling them with sterile technique. As a positive control for gastroenteritis, we inoculated and compared outcomes in conventional microbiota wild-type C57BL/6 and C57BL/6 IL-10$^{-/-}$ mice inoculated with *C. jejuni* 11168 and handled with SPF technique. Mice were sacrificed at 5 weeks post-inoculation.

In experiment 1, age matched C57BL/6 ^{Hu}microbiota and conventional microbiota mice were inoculated with tryptone soy broth (TSB), *C. jejuni* 260.94, or *C. jejuni* 11168 (Table 2). In experiment 1, all mice were handled with SPF technique, observed for 7 weeks post-inoculation, and then sacrificed. In all, six experimental groups were generated in experiment 1; conventional TSB inoculated (Conv-TSB), conventional *C. jejuni* 260.94 infected (Conv-260.94), conventional *C. jejuni* 11168 infected (Conv-11168), ^{Hu}microbiota TSB inoculated (Hu-TSB), ^{Hu}microbiota 260.94 infected (Hu-260.94), and ^{Hu}microbiota 11168 infected (Hu-11168).

Enteric pathogen screening. DNA was extracted from feces collected from all mice prior to experimental inoculation and at necropsy for enteric pathogen screening as described in Mansfield et al. (2007). In all cases, no control mice were positive for *C. jejuni* PCR using *gyr*A specific primers (Wilson, Abner et al. 2000). In addition, we screened all samples for *Campylobacter* spp. (16S rRNA gene), *Helicobacter* spp. (16S rRNA gene), *C. rodentium* (*espB* gene), and *E. faecalis* (*ddl* gene). Dedicated sentinel mice were used to assess extraneous infection with bacteria, protozoa and viral agents (Charles River Laboratories, Wilmington, MA) and were monitored by the MSU Campus Animal Resources (CAR).

***Campylobacter jejuni* strains and inoculum preparation**. *C. jejuni strains* 260.94 (ATCC BAA-1234) and NCTC 11168 (ATCC 700819) were obtained from the American Type Culture Collection (Manassas, VA). *C. jejuni* 260.94 is a Guillain-Barré Syndrome patient strain that elicits GM1 and GD1a anti-ganglioside antibody responses in C57BL/6 IL-10$^{-/-}$ mice (Malik, Sharma et al. 2014). *C. jejuni* 11168 is an enteric disease patient strain isolated from a patient

with severe gastroenteritis. *C. jejuni* 11168 has a GM1 ganglioside mimic on its surface (Linton, Karlyshev et al. 2000) but is not associated with GBS and has not been shown to elicit significant anti-ganglioside antibody responses in mice C57BL/6 IL-$10^{-/-}$ mice (Malik, Sharma et al. 2014). Inocula were prepared in same manner for both experiments. Inocula of both *C. jejuni* strains were prepared by streaking frozen stocks onto tryptone soy agar (TSA) (Accumedia) supplemented with 5% defibrinated sheep blood (Cleveland Scientific, Bath Ohio) (TSAB). (Plates were incubated at 37°C in anaerobic jars equilibrated to 10% CO_2, 10% H_2, and 80% N_2 for 48 hours and a portion of the growth re-suspended in tryptone soya broth (TSB) to give an A_{600} of 0.2 to 0.3. One-hundred microliters of this suspension was spread on 2 plates per mouse and the plates incubated for 16 hours in the 10% CO_2, 10% H_2, and 80% N_2 gas mixture. The resulting cells were collected and suspended in TSB; the suspension was adjusted to give an A_{600} of approximately 1.0 when diluted 1:10 (approximately 1×10^{10} CFU/mL final concentration). Purity, morphology, and motility were verified by microscopy and gram straining. Finally, 0.2 mL of the resulting inoculum or the vehicle (i.e. TSB) was carried to the containment facility on ice and delivered to infected and control mice, respectively, by oral gavage, resulting in six groups (Table 1 and 2). Limiting dilution analysis was used to determine the actual inoculum delivered to the mice.

Experimental design. Following infection all mice were observed at least once daily (twice daily after clinical signs were noted) by trained individuals for a period of 5 (Pilot) or 7 (Experiment 1) weeks to ensure mice were euthanized at a humane endpoint. In experiment 1, 1 week prior to infection (i.e. Baseline) and each week for 7 weeks post-inoculation mice underwent behavioral phenotyping in an open field test in a rat cage (18" x 8") divided into 4 quadrants. At 5- (Pilot) or 7 weeks (Experiment 1) the mice were sacrificed and tissues were collected and stored for further analysis. Prior to humane euthanasia by CO_2 overdose, fecal samples were collected, placed in TSB, frozen on dry ice and quickly moved to a -80°C freezer until thawing for

DNA extraction. After euthanasia, mice were weighed and blood was collected by cardiac puncture, immediately mixed with 0.1 mL of 3.8% citrate, spun down and plasma stored at -80°C for analysis of plasma antibodies. During necropsy two veterinarians (a pathologist and a gastroenterologist) observed and recorded any gross pathology prior to the removal of the GI tract. For the pilot and experiment 1, the cecum and colon were harvested, cut in half, and the halves flash frozen or streaked on TSAB-CVA plates for cytokine analysis and quantification of *C. jejuni* in these compartments, respectively. In experiment 1, the ileocecocolic junction was harvested, infiltrated with 10% Neutral Buffered Formalin (NBF) placed in a cassette, and further fixed in NBF for 48 hours and stored in 60% ethanol until processed for histological analysis.

Bacterial DNA isolation from feces and 16S ribosomal RNA gene analysis. In the pilot, DNA was extracted from fecal samples using the QIAamp DNA stool kit (Qiagen). DNA concentrations determined using a NanoDrop ND-1000 Spectrophotometer and concentrations normalized. The quantity of *Clostridium* group 1, *Clostridium* group 1, *Bacteroidetes*, and *Enterobacteriaceae* were measured using an IQ™5 Multicolor Real-Time PCR Detection System. In experiment 1, DNA was extracted from fecal samples using bead beating and the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC) according to manufacturer's instructions. The resulting DNA samples were delivered to the Michigan State University Research Technology Support Facility for library preparation and 16S amplicon analysis. In all, 62 samples were submitted for sequencing, including 60 mouse samples, the original fecal slurry used for inoculation of founder mice, and a mock community (HM-782D, BEI) for estimation of sequencing error. The V4 region of the 16S rRNA gene was amplified using dual indexed primers (Kozich, Westcott et al. 2013). PCR products were normalized using an Invitrogen SequalPrep DNA Normalization plate and the normalized products pooled. After quality control and quantitation, the pool was loaded on a standard MiSeq v2 flow cell and sequenced with a 500 cycle MiSeq v2 reagent kit (paired-end 250 base pair reads). Base calling was performed by Illumina Real Time Analysis (RTA) v1.18.54

and output of RTA was de-multiplexed and converted to FastQ format files with Illumina Bcl2fastq v1.8.4.

Amplicon analysis was performed using mothur (v. 1.35) and protocols available at http://www.mothur.org/wiki/MiSeq_SOP accessed December, 2015. Alignment was achieved using the Silva 16S ribosomal gene database (Quast, Pruesse et al. 2013). Chimeric sequences and any sequences classified as chloroplast, mitochondria, Archaea, or Eukaryota, were removed from the data set using uchime and the mothur formatted version of the Ribosomal Database Project (RDP) training set version 9, respectively, per the mothur protocol. Sequences were clustered in Operational Taxonomic Units (OTUs) of ≥97% sequence identity yielding 128 OTUs. Analyses were performed in mothur and PAST 3.07 (Hammer, Harper et al. 2001). Sequence read data will be made available via NCBI upon publication.

Clinical signs. Mice were observed twice daily by trained animal handlers and upon the appearance of clinical signs they were observed twice daily until reaching their humane endpoint or resolution of clinical signs. Mice were assigned scores for a battery of clinical signs according to a standardized scoring system; 1) Eating/Drinking (0 = yes, 1 =No), 2) Respiration (0 = normal, 1 = abnormal (increased), 10 = labored), 3) Rough hair coat (0 = no, 2 = yes), Hunched posture (0 = no, 9 = yes), Tremors (0 = no, 10 = yes), Movement (0 = normal, 1 = subdued (moves with stimulation), 2 = unresponsive to handling), Crusty eyes (0 = no, 1 = one eye, 2 = 2 eyes), Diarrhea on fur (0= no, 1 = yes), Cool to the touch (0 = no, 10 = yes), and Body weight (0 = 0 – 1% weight loss, 1 = 1 – 5% weight loss). A score greater than 9 required immediate euthanasia. Endpoints resulting in score greater than 9 include loss of body weight greater 5%, cool to touch, blue extremities or points adding up to greater than 9 in other criteria.

Quantification of *C. jejuni* in the cecum and colon. *Campylobacter jejuni* in the colon and cecum were quantified using a standardized semi-quantitative scoring system (Mansfield, Bell et al. 2007). Briefly, colon and cecum tissue snips of the same size were collected at necropsy and were streaked on TSAB containing cefoperazone (2 μg/mL), vancomycin (10 μg/mL), and

amphotericin B (2 μg/mL) (all antibiotics were obtained from Sigma Aldrich, St. Louis MO) agar plates and grown in anaerobic jars equilibrated with CampyGen satchets (Oxoid) at 37°C for 48-72 hours. The resulting growth was assigned a score on a scale of 0-4 based on the density of growth; 0 (no growth), 1 (1-20 CFU), 2 (20-200 CFU), 3 (200-400 CFU) and 4 (confluent growth).

Behavioral phenotyping. Open field testing was performed to detect neurological signs and changes in behavior due to inoculation with either GBS associated or enteric associated strains of *C. jejuni*. All sham inoculated control mice served as controls for phenotyping. Activity of *e*xperimental mice was video recorded once per week for one week prior to inoculation and once per week for 7 weeks post-inoculation. Briefly, mice were placed in the center of an 18" x 8" sterile rat cage divided into 4 marked quadrants and allowed to move freely for 90 seconds. At the completion of the experiment a single investigator (PTB), who was blinded to sample identity, recorded the number of quadrants crossed and the number of rears for each mouse. Quadrants crossed were counted starting with the first line crossed after establishing all four limbs in single quadrant. Rears were counted as extension of hind limbs and placement of both front limbs of the side of the cage.

Scoring of ileocecocolic junction (ICCJ) histopathology. Tissue samples were collected at necropsy, placed in cassettes, fixed in 10% NBF (Fisher Scientific) for 48 hours, and stored in 60% ethanol until final processing. Samples were submitted to the Michigan State University Investigative Histopathology Laboratory where they were processed in the following manner: Fixed samples were vacuum infiltrated with paraffin on the Sakura VIP 2000 tissue processor; followed by embedding with a ThermoFisher HistoCentre III embedding station. Paraffin embedded blocks were sectioned at 4–5 microns with a rotary microtome, dried at 56°C in a slide incubator for 2–24 hours and stained with Hematoxylin and Eosin (H & E). Scoring of the distal ileum, cecum and proximal colon was performed as described in Mansfield et al. (2007). Briefly, the lumen, epithelium, lamina propria and submucosa of the ICCJ of each mouse were

observed for histopathological changes by a single investigator (LSM) blinded to sample identity, and a score from 1-41 was assigned based on lesions using a standardized scoring system (Mansfield, Bell et al. 2007). Specific features evaluated were as follows: 1) excess mucus and inflammatory exudates in the lumen, 2) surface integrity, intraepithelial lymphocyte number, goblet cell hypertrophy, goblet cell depletion, crypt hyperplasia, crypt atrophy, crypt adenomatous changes, and crypt inflammation in the epithelium, 3) increased immune cells in the lamina propria, 4) and fibrosis in the submucosa.

Cytokine analysis. RNA was extracted from proximal colon samples that were flash frozen at the time of necropsy. Equal sized 5 mm cubed tissue snips were homogenized using micropestles, and RNA was extracted following the RNeasy Plus Mini Kit protocol (Qiagen). RNA concentrations were measured using the Nanodrop ND-1000 spectrophotometer and standardized to a concentration of 50 ng/μl. cDNA was obtained by PCR with random primers. A master mix was assembled using reagents from Promega GoTaq qPCR kit and added to the samples. This reaction was run using the following thermal cycler conditions (Step 1) 5 minutes 25°C, Step 2) 20 minutes 42°C, Step 3) 70°C, Step 4) 4°C min – Hold). Interleukin 4 (IL-4) and Interferon gamma (IFNγ) cytokine levels were measured using Q-PCR on a iQ5 (BioRad) with standardization. ANOVAs were performed on $2^{-\Delta\Delta ct}$ data to find the linear fold change in gene expression and are presented as mean fold change of three replicates over levels of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Enzyme-Linked Immunosorbent Assays (ELISAs). Indirect enzyme-linked immunosorbent assays (ELISA) were performed to test for the presence of antibodies reactive with bulk *C. jejuni* antigen and/or gangliosides GM1, GD1a, and GQb1 in the plasma of experimental mice, referred to as anti-*Campylobacter* and anti-ganglioside antibodies, respectively. Preparation of the bulk *C. jejuni* antigen was performed as previously described (Mansfield, Bell et al. 2007). Positive controls (highly reactive plasma samples that tested strongly

for the presence of the antigen in previous experiments) and negative controls (monoclonal mouse anti-Toxoplasma *gondii*, ViroStat) were used in all cases. All samples were run in triplicate and the mean values used for statistical analysis. We tested for antibodies to gangliosides GM1 (Sigma), GD1a (USBio), and mixed GM1-GQ1b (Sigma, Calbiochem, respectively). Immunoglobulin (IgG) subtypes were determined using biotinylated goat anti-mouse-IgG1, IgG2b, IgG2c, and IgG3 (Jackson ImmunoResearch, West Grove, PA) secondary antibodies. Methods for *C. jejuni*-specific antibody ELISAs were described previously (Mansfield, Bell et al. 2007) and ganglioside ELISAs were conducted similarly (reference Malik et al).

Quantification of F4/80 positive cells in sciatic nerves and dorsal root ganglia. Sciatic nerves and 2–3 lumbar dorsal root ganglia (DRG) from L3, L4 and L5 were dissected, isolated and fixed in 10% formalin pH 7.0 fixed. Thereafter, tissues were embedded en bloc in order to assess the segmental nature of any GBS lesions (Referenced St. Charles et al). Slides were prepared by the Michigan State University Investigative Histopathology Laboratory. Briefly, 3-5 micron sections were placed on charged slides, dried at 56°C for approximately 12 hours, and subsequently deparaffinized in xylene and hydrated through descending grades of ethyl alcohol to distilled water. Slides were placed in Tris Buffered Saline (TBS) pH 7.4 (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 and Tween 20 (Scytek). After blocking 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). Reaction development utilized Vector Nova Red Kit

peroxidase chromogen incubation of 15 minutes followed by counterstaining in Gill's Hematoxylin (Cancer Diagnostics – Durham, NC) for 30 seconds, differentiated with 1% acetic acid, dehydrated, and mounted with Permount (Sigma). F4/80 stained cells were counted and normalized for tissue area using ImageJ version 2.0.0-rc43/1.50e (Caroline, Wayne et al. 2012).

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 6.0h for Mac OS X (GraphPad Software, La Jolla, California USA) with the exception of 16S amplicon analysis. Data were entered and then checked for normality and equal variance. If they passed both tests, one-way ANOVA was performed. If they failed either test, a Kruskal-Wallis test was performed instead, followed by Dunn's post-test, with $p<0.05$ constituting significance. Statistical analysis of histopathological scoring of ICCJ was performed using a Kruskal-Wallis test followed by Dunn's post-test. Statistically significant comparisons in histopathology were further analyzed using Fisher's Exact Test (http://vassarstats.net/fisher2x3.html) and corrected for multiple comparisons with the Holm step-down procedure (Aickin and Gensler 1996). Two-way repeated measures ANOVA and Tukey's post-test were used for analysis of open-field and rearing behavior. Analysis of 16S amplicon data was performed using PAST 3 (Hammer, Harper et al. 2001) and statistical procedure indicated in figure legends.

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APPENDIX

GROUP	HANDLING	GENOTYPE	MICROBIOTA	INOCULUM	# OF MICE
Hu-TSB (Ster.)	Sterile	C57BL/6	Humanized	TSB	10
Hu-TSB (SPF)	SPF	C57BL/6	Humanized	TSB	10
Hu-260.94	Sterile	C57BL/6	Humanized	260.94	10
Hu-11168	Sterile	C57BL/6	Humanized	11168	10
Conv-11168	SPF	C57BL/6	Conventional	11168	10
Conv- IL-10$^{-/-}$ 11168	SPF	C57BL/6 IL-10$^{-/-}$	Conventional	11168	10

Table 3.1. Experimental design: Pilot experiment. C57BL/6 wild type (C57BL/6) or congenic C57BL/6 IL-11611611610 deficient (C57BL/6 IL-10$^{-/-}$) mice with Humanized (Hu) or conventional (conv) microbiota were inoculated with TSB, *C. jejuni* 260.94, or *C. jejuni* 11168 and subject to sterile (Ster.) or specific pathogen free (SPF) handling for the duration of the experiment; 5 weeks post-inoculation.

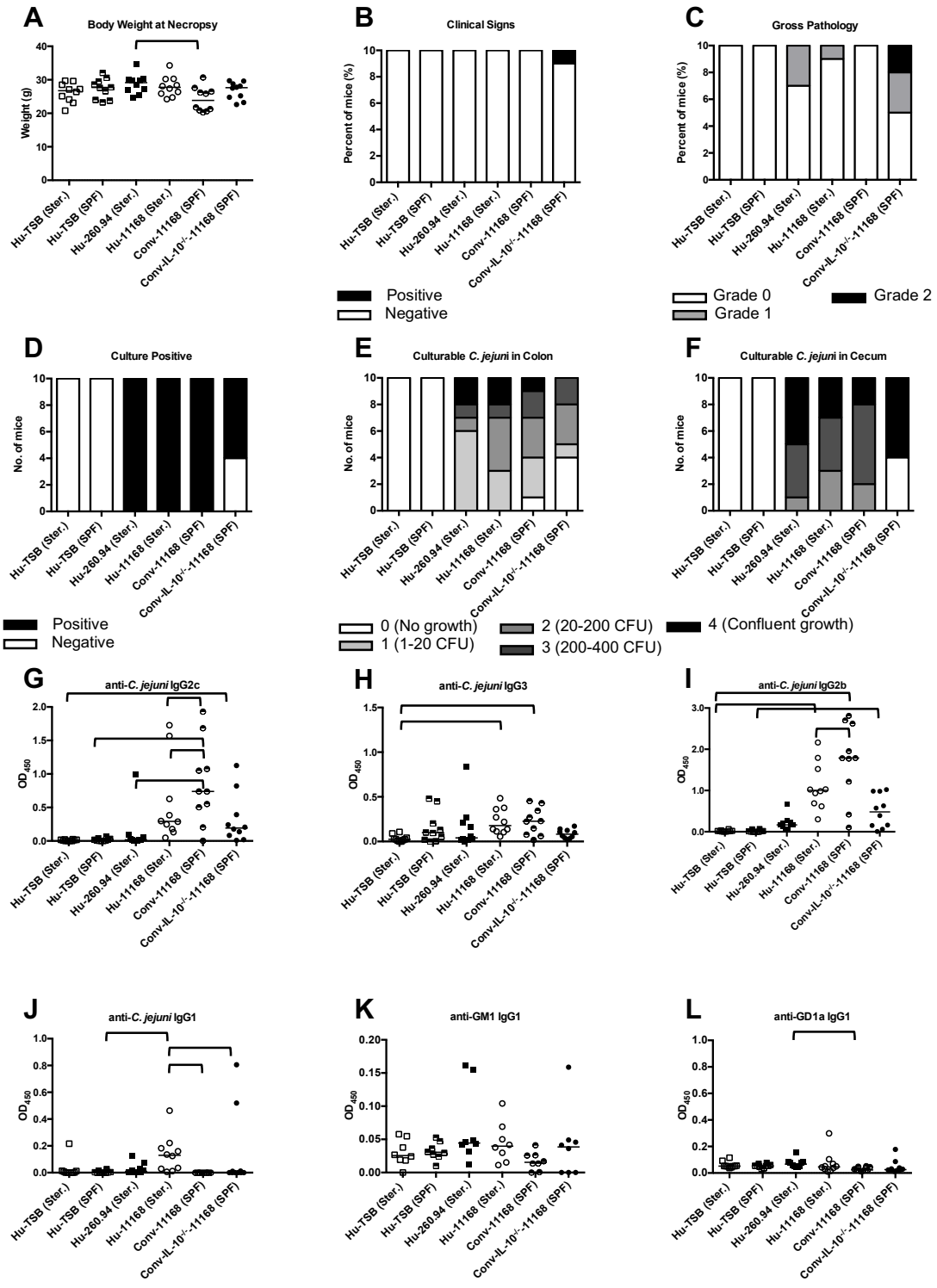


Figure 3.1. Disease indicators: Pilot experiment. Data represent A) body weight at necropsy, B) clinical signs, C) gross pathology at necropsy, D) number of mice that are culture positive for *C. jejuni* in cecum or colon, and semi-quantitative representation of culturable *C. jejuni* in E) colon and F) cecum at necropsy. Panels G-L represent anti-*Campylobacter* (G-J) and anti- ganglioside

Figure 3.1 (cont'd). antibodies (K-L) detected by indirect ELISA. Bars indicate statistical significance.

GROUP	GENOTYPE	MICROBIOTA	INOCULUM	# OF MICE
Hu-TSB	C57BL/6	Humanized	TSB	10
Hu-260.94	C57BL/6	Humanized	*C. jejuni* 260.94	10
Hu-11168	C57BL/6	Humanized	*C. jejuni* 11168	10
Conv-TSB	C57BL/6	Murine	TSB	10
Conv-260.94	C57BL/6	Murine	*C. jejuni* 260.94	10
Conv-11168	C57BL/6	Murine	*C. jejuni* 11168	10

Table 3.2. Experimental Design: Experiment 1. C57BL/6 genetically wild-type mice with Humanized (Hu) or conventional (conv) microbiota were inoculated with TSB, *C. jejuni* 260.94, or *C. jejuni* 11168 and subject to specific pathogen free (SPF) handling for the duration of the experiment; 7 weeks post-inoculation.

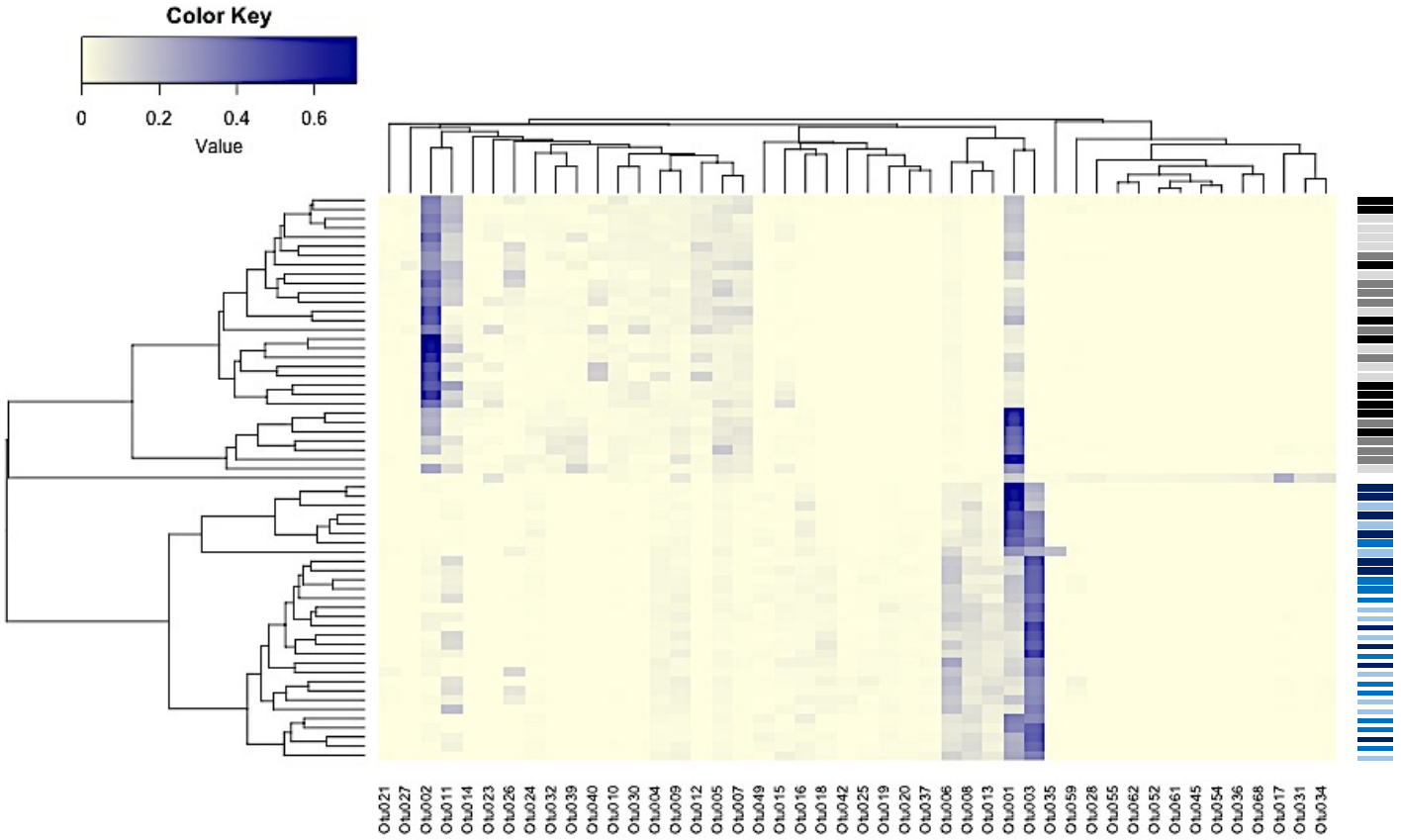


Figure 3.2. Heatmap of relative OTU abundance across samples. Abundances were measured as proportions of samples and only OTUs that constituted at least 1% of 1 sample were included. Samples and OTUs were clustered hierarchically based on Bray-Curtis dissimilarity index of relative abundance profiles. On the right y-axis bars indicate; black = Conv-11168, dark gray = Conv-260.94, light gray = Conv_TSB, dark blue = Hu_11168, blue = Conv_260.94, light blue = Conv_TSB, and white = Inoculum.

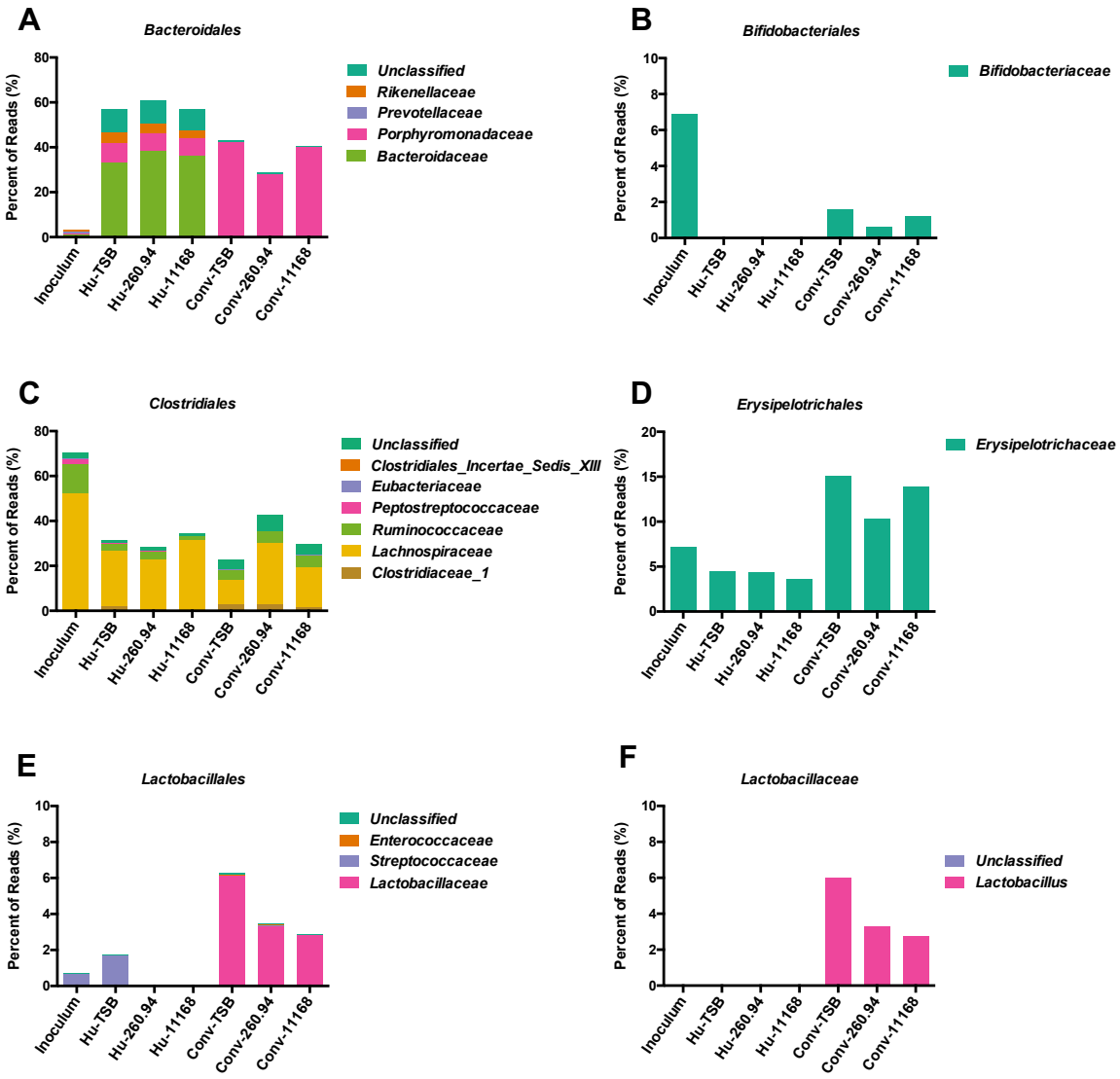
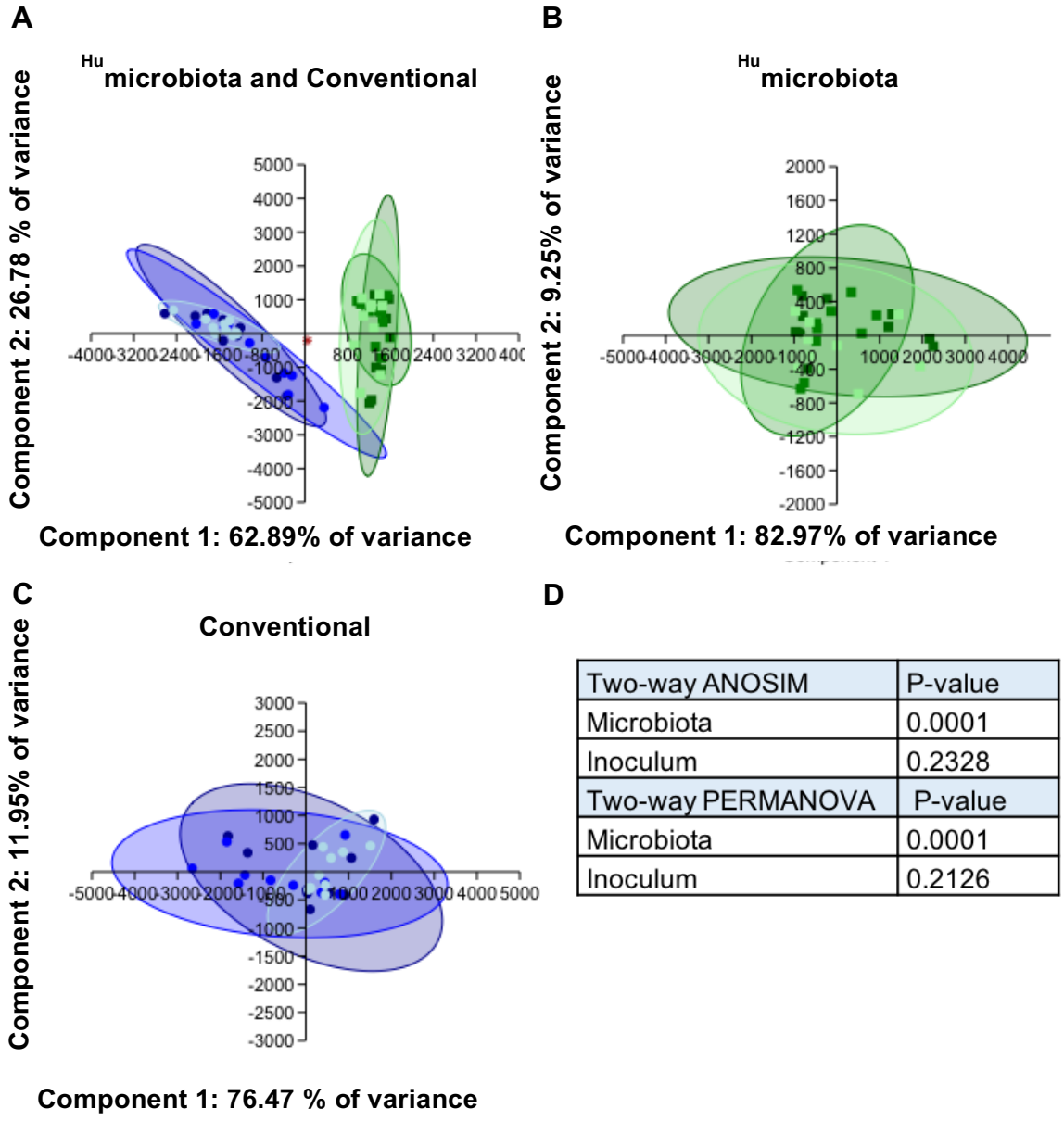


Figure 3.3. Relative abundance of major bacterial orders in fecal microbiota. Data represent relative abundances of OTUs assigned at the Order level with the exception of *Lactobacillaceae*. Orders constituting ≥5% of the average abundance for a single group were included. The average percentage of reads within each order that were assigned to families (Order for panel F) are represented as proportions of the orders (Genus for panel F) bar.



Two-way ANOSIM	P-value
Microbiota	0.0001
Inoculum	0.2328
Two-way PERMANOVA	P-value
Microbiota	0.0001
Inoculum	0.2126

Figure 3.4. Principle Component Analysis (PCA) and multivariate statistics of 16S rRNA taxonomy. PCA modeling was performed using OTU assignments. Resulting plots show separation by microbiota (A) but not inoculum (B and C). Dots represent; dark blue = Conv-11168, blue = Conv-260.94, light blue = Conv-TSB, dark green = Hu-11168, green = Hu-260.94, light green = Hu-TSB, and red = Inoculum. Two-way ANOSIM and two-way PERMANOVA indicate statistically significant differences between microbiota but not inoculum.

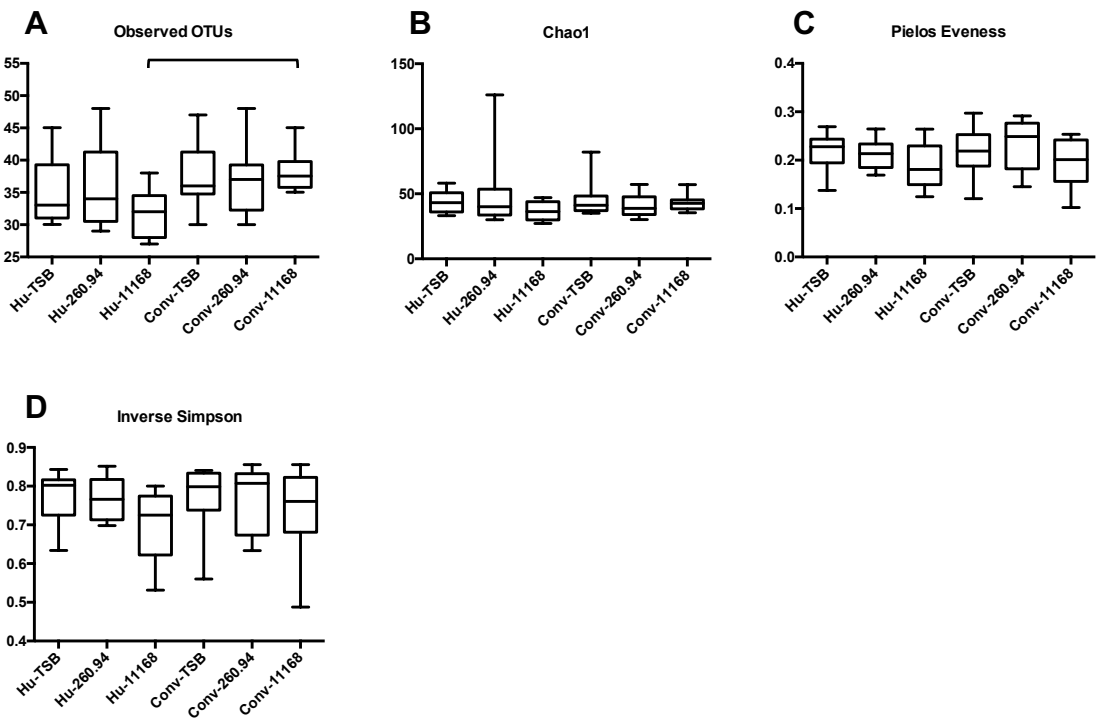


Figure 3.5. Alpha-diversity indices for 16S rRNA gene sequences. Panels represent A) observed OTU's, B) estimated richness (Chao1), C) species evenness (Pielos), and D) species diversity (inverse simpson). Data were analyzed by Kruskal-Wallis test on ranks and Dunn's post-test; $p \leq 0.05$ considered statistically significant. Bars indicate statistical significance.

	Average Abundance								
OTU	Conv-TSB	Hu-TSB	p-value	% Contribution	Phylum	Class	Order	Family	Genus
002	2070.3	32.9	≤0.0001	22.73	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified
003	0.5	1641.2	≤0.0001	23.18	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
004	121.5	76.4	0.0651	0.58	Bacteroidetes	unclassified	unclassified	unclassified	unclassified
006	47.7	515.2	≤0.0001	6.04	Bacteroidetes	Bacteroidia	Bacteroidales	unclassified	unclassified
008	0.6	363.6	≤0.0001	4.28	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
014	14.4	0.4	0.0004	0.38	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Barnesiella
001	522.6	1111.1	0.0619	12.02	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified
005	231.1	69.3	0.0003	2.77	Firmicutes	Clostridia	Clostridiales	unclassified	unclassified
009	73.5	102.5	0.1230	0.75	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified
010	88	5.5	≤0.0001	1.07	Firmicutes	unclassified	unclassified	unclassified	unclassified
011	521.6	167.6	0.0148	5.20	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Turicibacter
012	297.2	0.1	≤0.0001	2.58	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
016	7.6	97.8	0.0010	0.99	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium_XlVa
028	3.7	1.9	0.0453	0.04	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea
032	35.8	0.6	0.0019	0.80	Firmicutes	Clostridia	unclassified	unclassified	unclassified
056	1.6	14.5	0.0004	0.17	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Flavonifractor
060	0.8	0.1	0.0352	0.03	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Johnsonella
025	0.1	12	0.0019	0.23	Proteobacteria	Betaproteobacteria	unclassified	unclassified	unclassified
039	62.5	0.1	0.0223	1.20	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma
007	206.2	25.5	≤0.0001	2.77	unclassified	unclassified	unclassified	unclassified	unclassified

Table 3.3. Contribution of taxa to group differences. The average read abundance OTUs that distinguish the Hu-TSB and Conv-TSB based. P-values determined by paired T-test and Benjamin-Hochberg correction for multiple comparisons. Contribution to variance determined by Similarity Percentages (SIMPER) analysis.

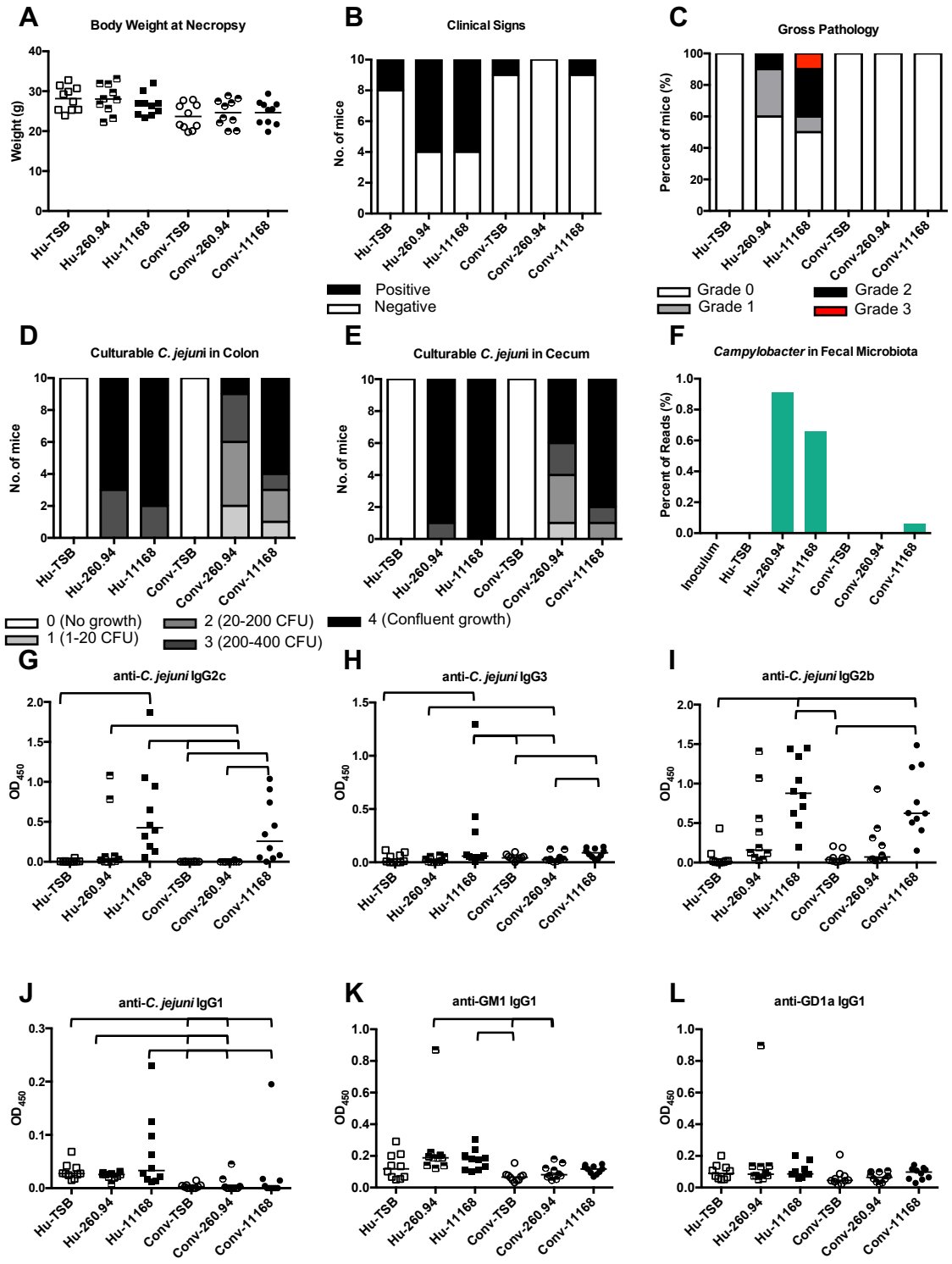


Figure 3.6. Hu mice are more susceptible to *C. jejuni* colonization and GI inflammation. Data represent A) body weight at necropsy, B) clinical signs, C) gross pathology, culturable *C. jejuni* in D) colon and E) cecum, F) percentage of 16S rRNA amplicons assigned to the genus *Campylobacter* G-J) plasma anti-*Campylobacter,* and K and L) anti-ganglioside antibodies

Figure 3.6 (cont'd). detected by indirect ELISA. Data were analyzed by Kruskal-Wallis test on ranks and Dunn's post-test where appropriate; $p \leq 0.05$ considered statistically significant. Bars indicate statistical significance.

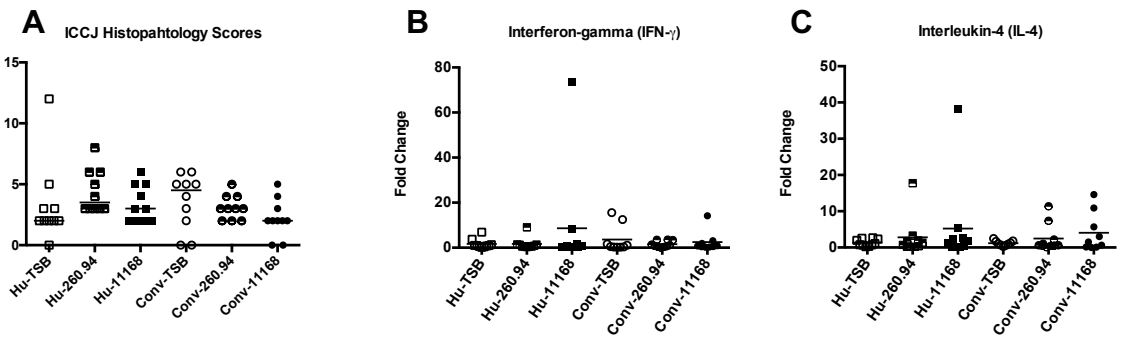


Figure 3.7. Severe gross pathology in ^{Hu}microbiota mice not associated with enteric lesions or IFNγ. Panels represent A) ICCJ Histopathology scores, and colon homogenate B) IFNγ and C) IL-4 mRNA levels.

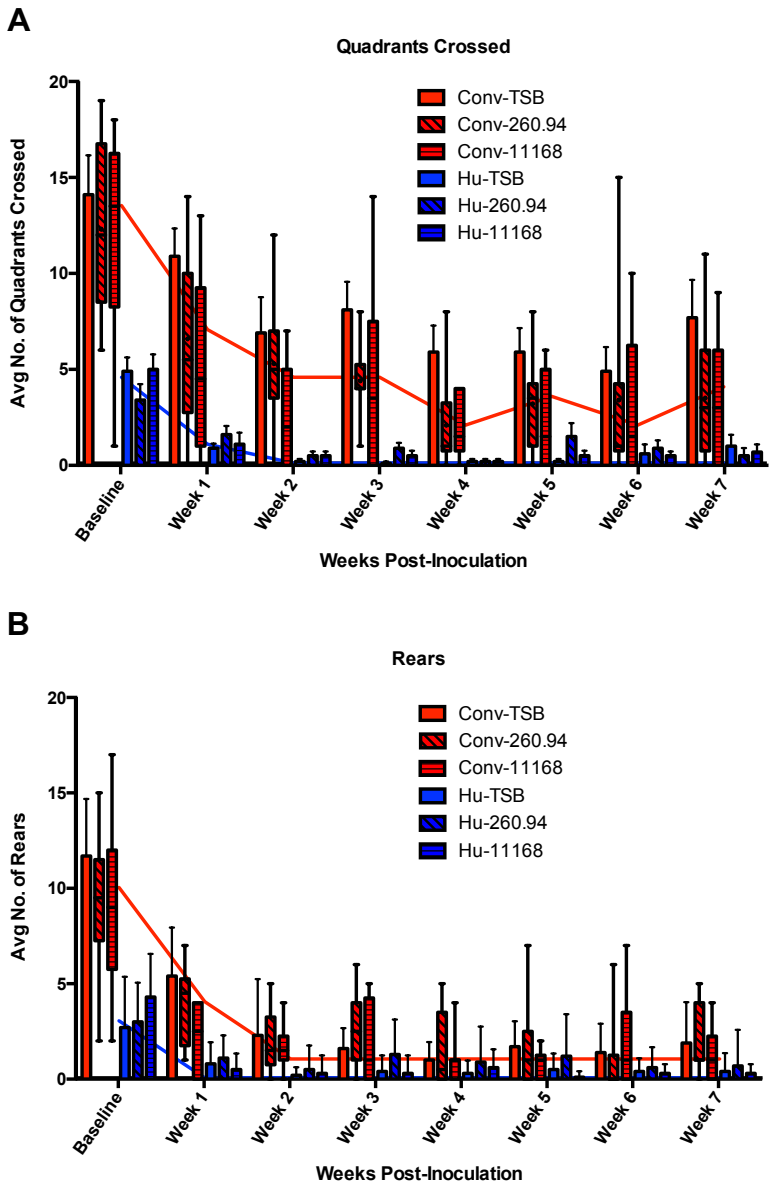


Figure 3.8 Behavioral phenotyping in the open-field. Number of A) Quadrants crossed and B) Rears in the open-field one-week prior to inoculation (i.e. baseline) and 1 to 7 weeks post-inoculation. Boxes represent 95% confidence intervals and whiskers represent range. Lines represent the median of conventional (red) and ^{Hu}microbiota (blue) mice regardless of inoculation status. Data were analyzed by repeated measures two-way analysis of variance (ANOVA) and Tukey's post-test; $p \leq 0.05$ indicates statistical significance (reported in results).

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CHAPTER 4

GENETIC VARIATION IN MULTIDRUG TRANSPORTER GENES *CMEB* AND *CMER* IN *CAMPYLOBACTER JEJUNI* POPULATIONS PASSAGED IN ANTIBIOTIC TREATED MICE

ABSTRACT

Campylobacter jejuni is a gram-negative, motile, rod-shaped bacterial pathogen whose genome is known to undergo substantial variation during passage *in vivo*. Although host microbiota has been shown to influence *C. jejuni* colonization and enteric disease, it is not known whether host microbiota influences the pattern of genetic variation in *C. jejuni*. Passage often results in genetic variation in *C. jejuni* antigenic surface structure biosynthesis loci including capsular, flagellar, and peripheral nerve ganglioside mimicking lipooligosaccharide (LOS) loci. Because LOS ganglioside mimicry evokes anti-ganglioside antibodies associated with the acute peripheral neuropathy Guillain-Barré Syndrome, identifying determinants of genomic adaptation could help predict and potentially prevent poor infection outcomes. We, therefore, aimed to characterize genomic variation in *C. jejuni* populations following passage in mice with conventional murine (^{Mo}microbiota), human-derived (^{Hu}microbiota), and antibiotic treated microbiota compared to ancestral inocula. *C. jejuni* genomic variation was found to be dependent upon both antibiotic treatment and composition of the microbiota. Comparative genomic analysis revealed variant accumulation in all passaged *C. jejuni* populations compared to ancestral populations, indicating host adaptation. Variants were more frequent in populations from ^{Mo}microbiota microbiota mice than in populations from ^{Hu}microbiota or antibiotic treated mice. No distinct patterns of variation were seen following infection in mice harboring a human-derived microbiota. In contrast, following passage in antibiotic treated mice, variants accumulated in *cmeB*, an inner membrane transporter of a multidrug efflux pump, and *cmeR,* a transcriptional repressor of the *cmeABC* multidrug efflux pump operon, but not in populations obtained from untreated mice. This study provides support for the influence of antibiotics and microbiota on *C. jejuni* genomic adaptation.

INTRODUCTION

Campylobacter jejuni is a Gram-negative, spiral rod-shaped, highly motile bacterial pathogen and a leading cause of bacterial gastroenteritis in both the developing and developed world (CDC 2014). Genomic sequencing of *C. jejuni* has a revealed a lack of many classic virulence factors (Parkhill, Wren et al. 2000). Because *C. jejuni* poses a global health burden, identifying determinants of *C. jejuni* mediated disease is of great value. Following passage in mice, comparative genomic analysis of *C. jejuni* populations and their ancestral inocula revealed variation in the length of homopolymeric nucleotide tracts (Jerome, Bell et al. 2011). Similarly, Kivistö et al. (2014) demonstrated that closely related *C. jejuni* strains collected from a farm had little genomic variation, yet the length of several homopolymeric tracts in LOS, capsular, and flagellar loci could not be resolved by next generation sequencing, indicating that several alleles were present. These data provide evidence that host passage promotes genetic variation in antigenic loci. Variation in these loci is widely considered to affect host-pathogen interactions, thus modulating host immune responses and potentially providing a mechanism by which the pathogen evades host immune responses during chronic infections (van der Woude and Baumler 2004, Deitsch, Lukehart et al. 2009). Experimental inoculation of mice with antibiotic depleted microbiota and mice with human derived microbiota (^{Hu}microbiota) resulted in enhanced susceptibility to *C. jejuni*-mediated inflammation and exacerbated antibody responses (Brooks et al. unpublished), raising the question of whether or not these changes are associated with genetic variation in loci encoding antigenic surface molecules.

Experimental *C. jejuni* inoculation models have often been used to study *C. jejuni* host-adaptation, and frequently host passage confers phenotypic variation in *C. jejuni* including increased motility and increased colonization (Ringoir and Korolik 2003, Jones, Marston et al. 2004). Mechanisms of genomic variation during passage include genomic rearrangements (Wassenaar, Geilhausen et al. 1998), recombination (de Boer, Wagenaar et al. 2002), single nucleotide variants (Thomas, Lone et al. 2014), and variation in homopolymeric nucleotide tract

length (Jerome, Bell et al. 2011, Kim, Artymovich et al. 2012, Kivistö, Kovanen et al. 2014). Analysis of passaged populations revealed differential expression of candidate virulence genes (Bell, Jerome et al. 2013), and subsequent passages resulted in enhanced *C. jejuni* strain specific enteric disease (Bell, St Charles et al. 2009). Recently two studies demonstrated that gut microbiota modulates susceptibility to *C. jejuni* colonization and enteric inflammation. Chang et al. 2007 showed that limited flora (LF) mice had increased susceptibility to *C. jejuni* infection and LF severe combined immune deficient (SCID) mice showed persistent *C. jejuni* colonization and severe gastroenteritis. Similarly, Stahl et al. (2014) demonstrated that antibiotic depleted gut microbiota enhanced susceptibility to *C. jejuni* colonization and enteric disease which was exacerbated in Sigirr$^{-/-}$ antibiotic treated mice. Together these data demonstrate that host genetics and microbiota modulate susceptibility to *C. jejuni* colonization and enhanced enteric disease; however, whether increased susceptibility is correlated with genomic variation in *C. jejuni* virulence loci is unknown.

We hypothesized that *C. jejuni* would undergo genomic variation during passage and that the pattern of variation would correlate with antibiotic treatment and microbiota. To test this hypothesis, we experimentally inoculated mice with distinct gut microbiota in two separate experiments, isolated *C. jejuni* populations from fecal or cecal samples upon sacrificing the mice, and sequenced the *C. jejuni* population DNA using next generation sequencing (Fig. 4.1). In our controlled *C. jejuni* inoculation experiments mice with antibiotic depleted microbiota or (^{Mo}microbiota) (experiment 1), and mice with either ^{Hu}microbiota or (^{Mo}microbiota) (experiment 2; ^{Hu}microbiota and ^{Mo}microbiota, respectively) were inoculated with *C. jejuni* 260.94. Using established comparative genomic tools, we assessed genomic variants in passaged populations compared to a frozen aliquot of the initial inoculum for each experiment. To determine whether the expected genomic variation would be altered by antibiotic depletion of gut microbiota, variants in *C. jejuni* populations recovered from antibiotic treated mice were compared to populations from untreated controls. Similarly, genomic variants recovered from ^{Hu}microbiota mice were compared

to those found in populations from ^{Mo}microbiota mice. Here we show that 1) *C. jejuni* genomic variation during passage is influenced by antibiotic treatment, 2) variants in some genes occur in during passage in all mice, and 3) the presence of gross pathological changes does not influence genomic variation during passage in ^{Hu}microbiota mice.

RESULTS

Recovery of *C. jejuni* populations. In experiment 1, C57BL/6 IL-10 deficient mice were continuously given drinking water with or without cefoperazone, inoculated with GBS-associated *C. jejuni* strain 260.94 and infection allowed to proceed for five weeks. This design was chosen because 1) cefoperazone, to which most *C. jejuni* strains are intrinsically resistant, has been shown to significantly deplete the gut microbiota resulting in a starkly different community compared to untreated mice (Antonopoulos, Huse et al. 2009, Theriot, Koenigsknecht et al. 2014), 2) antibiotics may be used to treat severe *C. jejuni* infections in humans (CDC 2014), 3) antibiotic resistance is prevalent in *Campylobacter* including resistance to cefoperazone (Taradon, Byeonghwa et al. 2009), thus we could continue administration for the duration of the experiment, and 4) ^{Hu}microbiota mice serve as a robust experimental "human-like system" (Nguyen, Vieira-Silva et al. 2015). In experiment 2, germ-free C57BL/6 mice that had been colonized with a human fecal slurry and bred for six generations and C57BL/6 mice having conventional mouse microbiota were inoculated with *C. jejuni* 260.94 and infection allowed to proceed for seven weeks. In both experiments, DNA was isolated from *C. jejuni* populations recovered from feces (Experimet 1) cecal contents (Experiment 2) by culture and subjected to deep sequencing. Sequencing data from the *C. jejuni* 260.94 inocula for the two experiments were used to construct reference genomes for analysis of genomic variation generated during passage in mice with different microbiotas.

Assembly statistics. To determine if the entirety of the *C. jejuni* genome was represented in our sequences and the average number of times each base was sequenced we estimated sequencing coverage and mean base coverage. In all cases >99% of the reference genome was represented in our sequences and the average estimated mean base coverage was 74.

The frequency of variants in coding regions is associated with treatment and microbiota. To assess the pattern of genomic variation in our passaged populations, we compared the genomes of our recovered populations to the inoculum using breseq (Daniel and Jeffrey 2014). First, we evaluated the number of single nucleotide variants (SNVs) and structural variants (SV) in coding and non-coding regions. In experiment 1, variants were more prevalent in populations obtained from untreated than treated mice, and the difference between the treatments could be attributed to base substitutions in coding regions (Fig. 4.2 A). In experiment 2, variants were more prevalent in ^{Mo}microbiota mice than ^{Hu}microbiota mice with most of the difference between groups attributable to base substitutions in coding regions (Fig. 4.2 B). Large deletions were prevalent in both experiments and were more likely to occur in non-coding than in coding regions (Fig 4.2).

Antibiotic treatment results in variant accumulation in populations obtained from antibiotic treated mice. Next, we compared genetic variants in *C. jejuni* populations from each treatment group to determine if any patterns were evident. Two candidate virulence loci accumulated variants in all mice in both experiments (Data not shown). Variants in *flaA* (flagellin) and *porA* (major outer membrane protein or MOMP) were present in all mice; two variants in *flaA*, one synonymous mutation (AAT→AGT, N222N) and one non-synonymous mutation (AAT→AGT, N224S), were present in 100% of the population in all mice , and 6 variants in *porA*, 5 synonymous (1) AAA→AAG, K379K, 2) GGT→GGC, G377G, 3) GAT→GAC, D337D, 4) GGT→GGC, G318G and, 5) TAT→TAC, Y364Y) and 1 non-synonymous (AAT→ACT, N373T), were present in at least 65% of the population in all mice in experiment 1. In addition, another 25 candidate virulence loci

were affected by mutations in at least one population, however, no patterns were detected (data not shown). Variants that were unique to treatment or microbiota were detected in experiment 1 and affected antibiotic resistance genes, *cmeR* and *cmeB*, in antibiotic treated mice (Table 1). None of the variants in *cmeB* were identical in all populations; however, three non-synonymous variants, one in each population, were present in at least 59% percent of the reads from that population. In contrast, a single nucleotide deletion in *cmeR* at position 470 was present in 100% of the reads in two of three populations.

Variant accumulation in four genes following passage in ^{Mo}microbiota mice. No unique mutations were detected in ^{Hu}microbiota mice compared to ^{Mo}microbiota mice; however, four genes were more likely to be affected by variants in populations from ^{Mo}microbiota than ^{Hu}microbiota mice (Data not shown). Several variants in two of these four genes were identical. In a hypothetical protein, two variants (AAA→AAT, K53N, and ACA→ATA, T58I) were present in all populations from ^{Mo}microbiota mice. Interestingly, the same variants were also found in two of ten populations from ^{Hu}microbiota mice. These variants were not present in more than 8.60% of these reads in any population; and, when found, the abundances were similar in both ^{Mo}microbiota and ^{Hu}microbiota associated *C. jejuni* populations. A single variant (GAC→GAT, D83D) was present in all ^{Mo}microbiota populations. This variant was also present in two ^{Hu}microbiota populations. Finally, a single variant (AGT→AGC, S53S) in Thiol peroxidase, Tpx-type was present in all ^{Mo}microbiota populations and comprised 5.6–16.8% of the reads from those populations. A single population collected from ^{Hu}microbiota mice had this variant.

Patterns of genetic variation were not influenced by enteric disease in ^{Hu}microbiota mice. To determine if inflammatory immune responses in *C. jejuni* infected mice would affect the pattern of genomic variation in populations obtained from these mice we compared genomic variants in ^{Hu}microbiota mice with and without severe enteric gross pathology. Four of 10 ^{Hu}microbiota mice showed gross pathological changes in their GI tracts; however, there were no

significant differences in the number of variants or the pattern of variants (data not shown). Also, no unique pattern of genetic variation was detected that distinguished populations collected from mice with gross pathological changes versus those collected from mice without such changes.

DISCUSSION

C. jejuni's genome has a high frequency of variation during passage and this variation often affects virulence-associated loci involved in biosynthesis of antigens that interact with the host immune system (Jerome, Bell et al. 2011, Kivistö, Kovanen et al. 2014). Major virulence components of *Campylobacter jejuni* that have been shown to be hypervariable include the LOS, capsule, and flagella. The LOS is unique in that it contains structures that mimic gangliosides found on peripheral nerves that interact with the host immune system to evoke anti-ganglioside antibodies which are hypothesized to result in the acute peripheral neuropathy Guillain-Barré Syndrome. We have experimentally demonstrated autoimmunity following *C. jejuni* infection in murine models with exacerbated anti-ganglioside antibody responses in antibiotic treated and ^{Hu}microbiota mice compared to untreated and ^{Mo}microbiota controls respectively (Brooks et al. unpublished). In this study, we used comparative genomic analysis of deep sequenced, mouse-passaged *C. jejuni* populations to determine if patterns of genomic variation were influenced by passage in ^{Hu}microbiota, ^{Mo}microbiota, or antibiotic depleted microbial communities. We hypothesized that genomic variation in candidate virulence loci would be affected by the ecological structure of the microbiota. Our analysis show that genetic variants in both candidate virulence and non-virulence associated loci occurred frequently during passage. This result is in line with the findings of two previous experimental inoculation studies which found that passage in mice or chickens resulted in genomic variation in *C. jejuni* (Jerome, Bell et al. 2011, Kim, Artymovich et al. 2012). Similarly, our results in this study showed that passage resulted in variation in several candidate virulence loci including those encoding PorA (the major outer

membrane protein), FlaA (the major flagellin), and CmeR (a transcriptional regulator of both the *cmeABC* multidrug efflux pump operon and a number of surface structure biosynthesis enzymes). Notably, variants in *flaA* and *porA*, genes encoding flagellin and major outer membrane were present in all mice highlighting the plasticity of *C. jejuni* genome, indicating laboratory to host adaptation, and providing a mechanism for antigenic variation which may affect host-pathogen interactions. Together these results provide additional evidence that host passage drives variation in antigenic loci.

In our experimental *C. jejuni* infection, antibiotic treated *C. jejuni* infected mice showed exacerbated enteric disease and anti-ganglioside antibody responses compared to untreated *C. jejuni* infected mice (Brooks et al. unpublished). Antibiotic depletion of gut microbiota was confirmed by 16S rRNA gene PCR (Brooks et al. unpublished). We evaluated *C. jejuni* genomic variants in populations obtained from these mice compared to ancestral populations to determine if treatment dictated the pattern of genomic variation. We found that populations obtained from antibiotic treated mice had variants in two antibiotic resistance genes; *cme*R and *cme*B. CmeABC is a multidrug efflux pump that consists of three membrane proteins (CmeA, CmeB, and CmeC) that are present in many *Campylobacter* species and confer resistance to several antibiotics, including cefoperazone (Lin, Michel et al. 2002, Guo, Lin et al. 2010). CmeB is an inner membrane efflux transporter (Guo, Lin et al. 2010), and CmeR is a transcriptional repressor of the *cme*ABC operon (Lin, Akiba et al. 2005). Variants in these genes were not found in untreated mice. In antibiotic treated mice, variants in *cme*B and *cme*R were unique with the exception of a single nucleotide deletion at position 470 in *cme*R which occurred in two of three populations and which was present in 100% of the reads from both populations indicating selection (Table 1). Mutations in repressors of antibiotic resistance genes contribute to overexpression of efflux pumps and multidrug resistance phenotypes (Wang, Dzink-Fox et al. 2001, Webber and Piddock 2001, Adewoye, Sutherland et al. 2002). In contrast, previous studies show that variants in *cme*B are frequent, and an isolate with only 78% percent amino acid sequence identity retained efflux pump

activity (Cagliero, Cloix et al. 2006). In addition, *cme*R is involved in the regulation of at least 28 other genes, including seven genes encoding enzymes involved in capsule biosynthesis and four encoding membrane transporters, and a mixture of other transport related and hypothetical proteins. Furthermore, *cme*R mutants display diminished fitness *in vivo* (Guo, Wang et al. 2008) thus raising the question of whether variants in our populations would influence disease in subsequent infections.

In comparison, *C. jejuni* infected ^{Hu}microbiota mice showed enhanced susceptibility to enteric disease compared to ^{Mo}microbiota mice (Brooks et al. unpublished). This is consistent with findings in two previous studies where ^{Hu}microbiota mice were experimentally inoculated with *C. jejuni* (Bereswill, Fischer et al. 2011) or *Clostridium difficile* (Collins, Auchtung et al. 2015). In contrast to findings in experiment 1, no variants were found in experiment 2 that were unique to the microbiota; however, variants were more likely to occur in ^{Mo}microbiota mice than in ^{Hu}microbiota mice (Data not shown). Variants in foldase protein PrsA precursor, pyruvate-flavadoxin oxidoreductase, thiol peroxidase Tpx-type, and a hypothetical protein were present in all populations obtained from ^{Mo}microbiota mice but no more than 3 of 10 ^{Hu}microbiota microbiota mice. Foldase protein PrsA precursor is an isomerase that belongs to one of three families defined by inhibitors of this enzyme. Pyruvate-flavadoxin oxidoreductase is an enzyme involved in carbohydrate metabolism. Thiol peroxidase, Tpx-type is an enzyme involved in glutathione metabolism (Kanehisa and Goto 2000, Kanehisa, Sato et al. 2016). Thiol peroxidases are associated with stress responses and the reduction of oxidants (Cha, Kim et al. 1996, Horst, Jaeger et al. 2010, Somprasong, Jittawuttipoka et al. 2012); thus selection for variants in the latter two genes may be indicative of a change in the need for protection against reactive oxygen species. Notably, although variants in these genes were found, they were not present in more than 10% of the reads in any population.

Our results serve as the basis for further exploration of rapid genomic adaptation of *C. jejuni* in antibiotic treated mice. A complimentary experiment could include short-term antibiotic

treatment resembling the typical course of treatment in humans to determine if selection pressures applied during short duration antibiotic treatment are sufficient to elicit genomic variation in antibiotic resistance genes. Alternatively, our short read-sequencing approach could be accompanied by (1) long-read sequencing for analysis of genomic rearrangements and closing gaps, and (2) analysis of transcript abundance. Previous reports have shown that *C. jejuni* undergoes large scale genomic rearrangement during passage (Wassenaar, Geilhausen et al. 1998, Nuijten, Berg et al. 2000, Scott, Timms et al. 2007). In the absence of long-read sequencing genomic rearrangements are difficult to predict. In addition, large intergenic deletions were detected and abundant in both experiments. These gaps may result from repeat regions and may be resolved with longer reads. Because our data do show unique mutations in genes involved in antibiotic resistance and surface structure biosynthesis, whether mutations result in differences in infection outcomes could be explored by serial passage (Bell, St Charles et al. 2009). Furthermore, whether genomic variants acquired during passage in our experiment affect transcript or protein expression is unknown. RNA-seq has been used for high-throughput analysis of the *in vivo C. jejuni* transcriptome which led to the discovery of potential regulatory elements (Taveirne, Theriot et al. 2013). Adapting this approach for analysis of *C. jejuni* populations in antibiotic depleted, ^{Hu}microbiota, and ^{Mo}microbiota may link genomic variation in *C. jejuni* to changes in the functional potential of the pathogen or the community.

Infections with *C. jejuni* are often associated with genomic variation in loci associated with biosynthesis of antigenic surface structures interact with the host immune system including LOS, flagellar, and capsular loci (David, Vijay et al. 2010, Jerome, Bell et al. 2011, Kivistö, Kovanen et al. 2014). This often results from variation in the length of homopolymeric tracts of nucleotides; however, SNVs also play a role in genomic variation in *C. jejuni*. Experimentally it has been demonstrated that SNVs can have a significant impact on synthesis of antigenic surface structures. Guerry et al. (2002) showed that site directed mutagenesis of *cgtA* (N-galactosaminyltransferase) resulted in a shift from GM_2 to GM_3 ganglioside mimicry by the LOS.

Similarly, a single amino acid substitution at position 51 in *cst-II*, an acetyltransferase, determines whether the LOS will mimic gangliosides GM1 and GD1a, or GT1a and GD1c ganglioside (Yuki 2007). In the present study, populations passaged in antibiotic treated mice accumulated SNVs in two components of and a repressor of the multidrug efflux pump *cmeABC* operon, *cmeB* and *cmeR* respectively. In contrast, no genetic variants unique to populations obtained from ^{Hu}microbiota mice or their ^{Mo}microbiota controls were detected. Because these populations were not used for subsequent infections, it is not known if fitness advantages were conferred by these mutations. In addition, isolation of *C. jejuni* populations involved two laboratory passages on selective media which may have contributed to further selection. In summary, this study provides evidence that antibiotic treatment affects genomic variation during passage; however, whether microbiota influences patterns genomic of variation cannot be ruled out. In line with previous studies, we demonstrate the plasticity of the *C. jejuni* genome and identify antibiotic treatment as a determinant of genomic variation. Whether *C. jejuni* genomic variation in different microbial communities is a determinant of infection outcomes should be addressed in future experiments.

MATERIALS AND METHODS

Experimental infection model. All *C. jejuni* populations were obtained from experiments described previously (Brooks et al. unpublished). Procedures involving animals were performed in accordance with the recommendations described in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under protocols approved by the Michigan State University Institutional Animal Use and Care Committee (approval numbers 06/12-107-00 and 06/15-101-00). In experiment 1, *C. jejuni* 260.94 populations were passaged in B6.129P2-IL-10^{tm1Cgn}/J (referred to here as C57BL/6 IL-10$^{-/-}$) mice obtained from Jackson Laboratories (Bar Harbor, ME), a breeding colony was established in a *Campylobacter/Helicobacter*-free facility, and offspring were used for all experiments described here. Mice received either sterile drinking

water or 0.5 mg/mL cefoperazone in sterile drinking water ad libitum, for one week prior to experimental inoculation and for 5 weeks post-inoculation. Several mice reached the humane endpoint based on standardized scoring criteria (Mansfield, Bell et al. 2007) prior to 5 weeks post-infection and were sacrificed immediately. All populations used in this study were obtained between 3 and 5 weeks post-inoculation. The populations obtained in experiment 1 were named by treatment and a randomly assigned number ranging from 1 to 3.

In experiment 2, *C. jejuni* 260.94 populations were passaged in age-matched C57BL/6 ^{Hu}microbiota and ^{Mo}microbiota mice. ^{Hu}microbiota mice were generated and maintained as described previously (Collins, Auchtung et al. 2015). All mice in experiment 2 were maintained for 7-weeks post-inoculation and then sacrificed. In all cases, mice were housed in *Helicobacter/Campylobacter* free conditions, fed an irradiated mouse diet (mouse breeder diet 7904; Harlan Teklad, Indianapolis, IN), kept on autoclaved bedding, and given filter sterilized water (autoclaved water in bottles for weanlings) in an MSU limited-access room. A portion of the inoculum from each experiment was set aside and stored at -20°C until DNA was extracted. Inocula from experiment 1 and 2 are referred to as 260.94-CPZ and 260.94-Hu, respectively.

***Campylobacter jejuni* strain recovery.** *Campylobacter jejuni* 260.94 populations were obtained from mice in experiments described previously (Brooks et al. unpublished). Passaged populations were obtained by streaking cecal contents or feces on selective media; tryptone soy agar plus 5% defibrinated sheep's blood and 20 μg/mL cefoperazone, 10 μg/mL Vancomycin, and 2 μg/mL amphotericin B (TSAB-CVA). Plates were incubated in aerobic jars with a *CampyGEN* sachet for 48 to 72 hours at 37°C. In all cases, this yielded a lawn; the entirety of which was collected and suspended in tryptone soy broth (TSB). Because desired purity could not be obtained after a single passage, all populations were plated a second time on TSAB-CVA and grown under identical conditions. Purity was assessed by dark field microscopy and gram-staining. In all cases, purity was estimated to be ≥99%. DNA was extracted using the Qiagen

DNAeasy blood and tissue kit according to the manufacturer's instructions, stored overnight at 4°C, and frozen at -80°C until submitted for sequencing.

Sequencing. *C. jejuni* DNA was sent to the Michigan State University Research and Technology support facility for sequencing. In all, DNA from twenty-two populations, including inocula, were submitted. DNA was prepared for sequencing and libraries constructed with the Rubicon Genomics ThruPLEX DNA Library kit (MI, USA). Libraries were quality checked and quantitated using the Qubit dsDNA assay (Thermo Fisher Scientific, USA), Caliper, LabchipX, and Kapa Library quantitation qPCR kit (Kappa Biosystems, USA). Libraries were pooled, concentrations normalized, and loaded on an Illumina MiSeq flowcell (v2) and sequencing performed in a 2 x 250 base pair paired-end format with a 500 cycle reagent cartridge (v2). Base calling was done by Illumina Real Time Analysis (RTA), output de-multiplexed, and converted to FastQ format with Illumina Bcl2fastq v1.8.4. Sequences will be made available on NCBI prior to publication.

Quality assessment and preparation for analysis. All analyses were performed on the Michigan State University high performance computer unless noted otherwise. We assessed read quality using FastQC v 0.11 (2016) and in all cases, quality scores exceed 26, thus trimming was omitted. To estimate sequencing depth and coverage, the resulting reads were assembled using SPAdes 3.7 and aligned to the *C. jejuni* 260.94 reference contigs (RefSEQ accsssion number = GCA_000168135.1). Estimated mean base coverage was calculated as ((average read length x total number of reads)/estimated genome size) and coverage represents the percent of the reference genome (i.e. genome fraction) recovered as determined by Quast: Quality assessment tool for Genome Assemblies version 3.0 (Gurevich, Saveliev et al. 2013).

Assembly and annotation of reference genomes. DNA obtained from *C. jejuni* 260.94 cultures used to inoculate mice in two separate experiments (i.e. 260.94-CPZ and 260.94-Hu), were used as reference genomes for comparative genomic analysis. As previously mentioned these genomes were assembled with SPAdes version 3.7, read quality assessed with FastQC

version 0.11, mean read quality calculated, genome coverage determined with Quast version 3.0. Assembled contigs were annotated using the genomic annotation tool Rapid Annotation Using Subsystem Technology (RAST) version 2.0 (Accessed May 2016) (Aziz, Bartels et al. 2008).

Comparative genomics. DNAs from populations were then analyzed using breseq version 0.26 in polymorphism mode with assembled and annotated 260.94-Hu and 260.94-CPZ as reference genomes when appropriate. To be considered for analysis, variants must 1) be present in 5% or more of the reads in at least one population, and 2) have a minimum base coverage of 40X. A putative *C. jejuni* 260.94 virulome was constructed based on the presence of genes in a modified *C. jejuni* 11168 virulome originally proposed by Bell et al. (2012). In all, the modified *C. jejuni* virulome contains 381 loci of which at least 351 are present in our *C. jejuni* 260.94 reference genomes (Bell, Jerome et al. 2013).

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APPENDIX

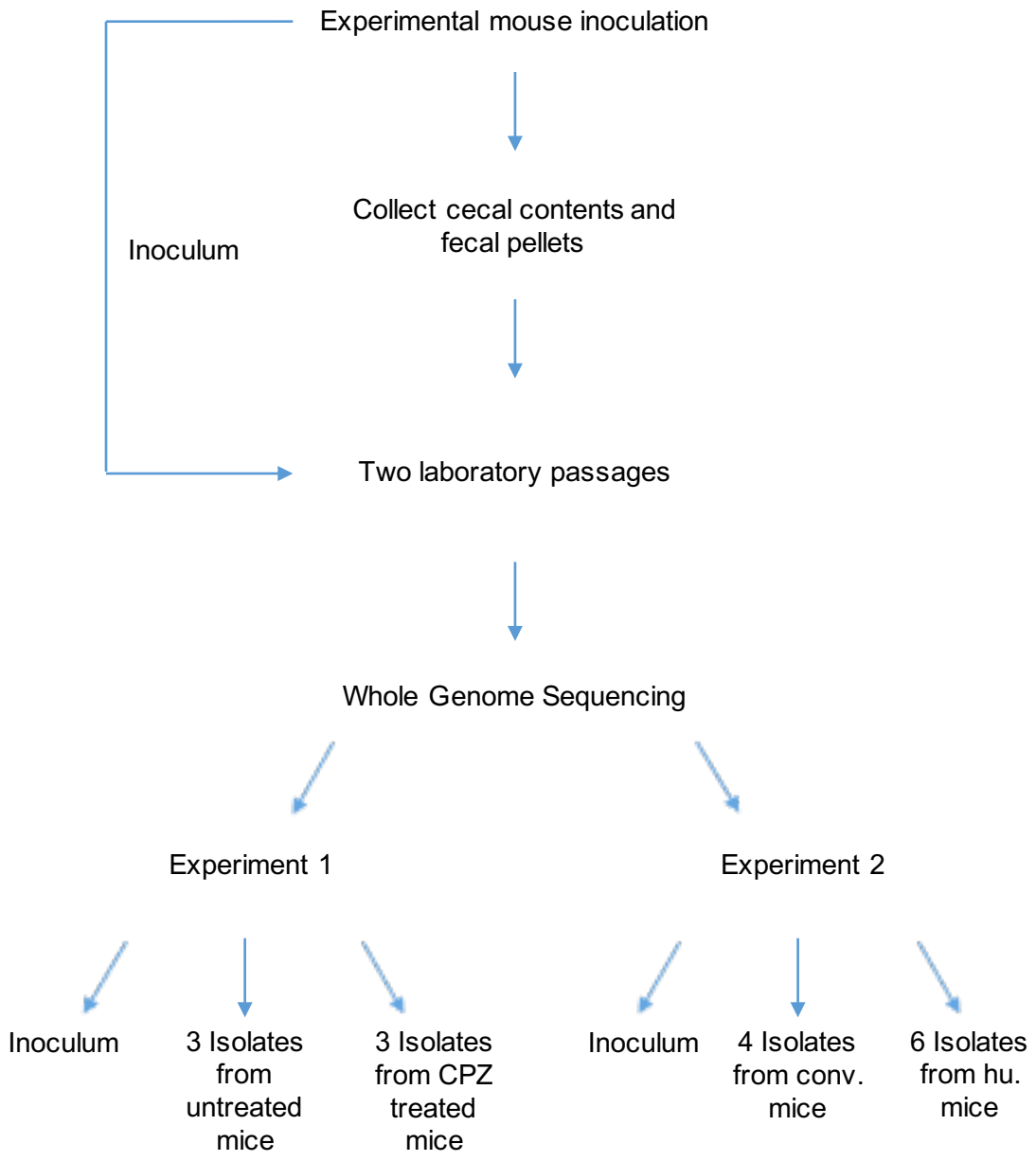

Figure 4.1. **Experimental design.** *C. jejuni* 260.94 populations were obtained from mouse fecal pellets (Experiment 1) and cecal contents (Experiment 2) and following experimental inoculation and 5 or 7 week passage. Populations were streaked for growth on selective media; the entirety of the resulting growth was collected and assessed for purity by microscopy and gram straining. DNA was extracted from resulting populations and sequenced. Comparative genomic analyses were performed comparing passaged populations to their ancestral population (i.e. inoculum).

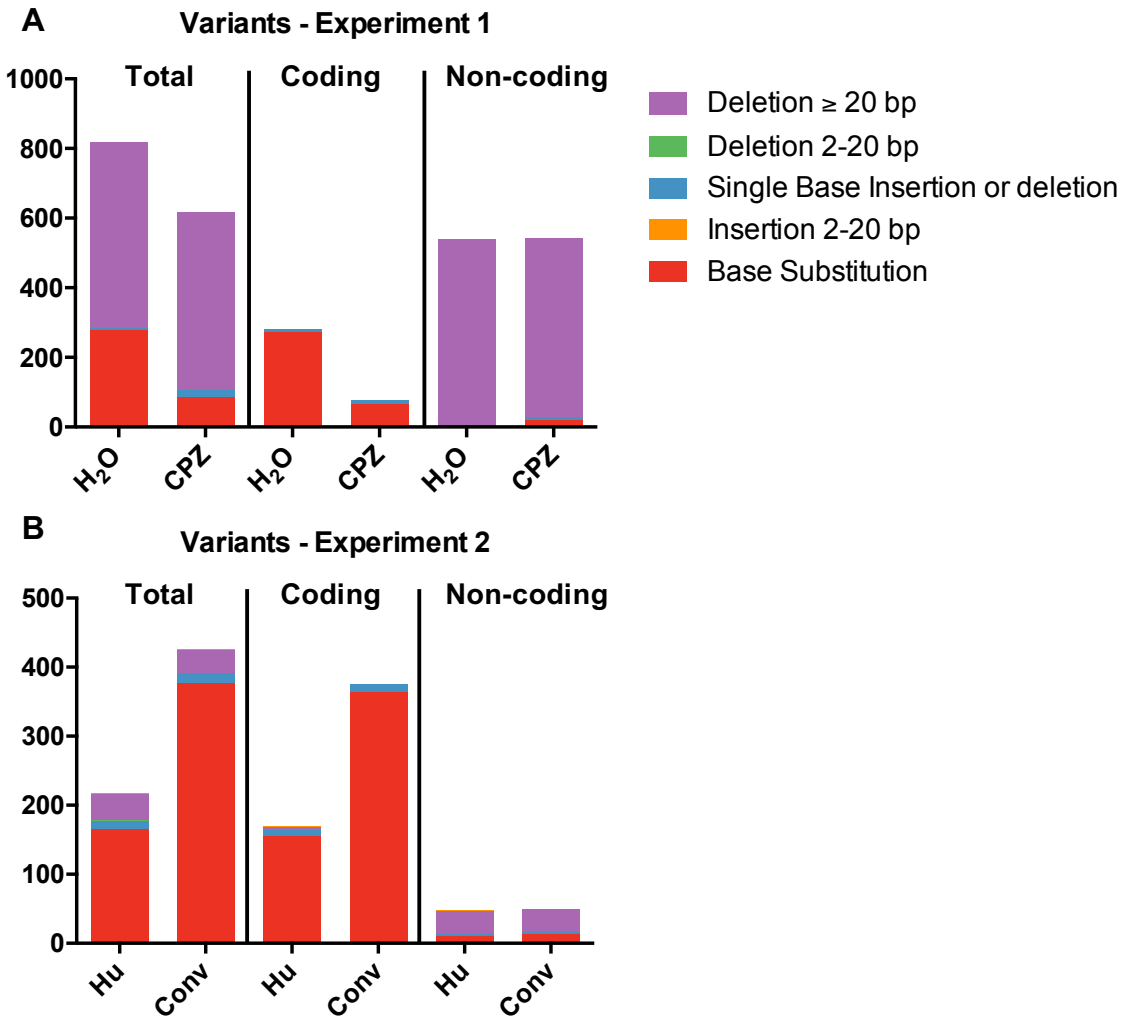

Figure 4.2. Variants in untreated and antibiotic treated mice compared to inoculum. Data represent total genomic variants, variants in coding regions, and variants in non-coding regions compared to ancestral populations in both experiment 1 and 2.

	Experiment 1					
	H_2O			CPZ		
Description	H_2O^1	H_2O^2	H_2O^3	CPZ^1	CPZ^2	CPZ^3
RND efflux system, inner membrane transporter CmeB				T105I (ACC→ATC) 59.90%	G989E (GGA→GAA) 14.00%	G568S (GGT→AGT) 100.00%
RND efflux system, inner membrane transporter CmeB					R131G (AGA→GGA) 73.10%	L385F (CTT→TTT) 6.80%
Transcriptional repressor of CmeABC operon, CmeR				P172S (CCT→TCT) 100.00%	coding (470/633 nt) 100.00%	G45E (GGA→GAA) 20.80%
Transcriptional repressor of CmeABC operon, CmeR						coding (470/633 nt) 100.00%

Table 4.1. Variant accumulation in multidrug efflux pump component and repressor following passage in antibiotic treated mice. All populations recovered from antibiotic treated (CPZ) mice accumulated variants in multidrug efflux pump transporter gene *cmeB* and its repressor *cmeR***.** No variants were detected in either gene in populations recovered from untreated (H_2O) mice. Data represent nucleotide variants in passaged populations compared to inocula and the percent of the population with each variant. Superscript indicates population number from untreated (H_2O) or antibiotic treated (CPZ) mice.

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CHAPTER 5

DISCUSSION AND LITERATURE REVIEW

DISCUSSION

A pathogen-induced disease state in general is the product of interacting factors: pathogen characteristics, host immune responses, microbiota, and environmental influences such as treatment. In this study we sought to determine how pathogen induced immune responses would be affected by the presence of human microbiota or antibiotic treatment. Towards that end we assessed several interacting factors: a) pathogen colonization, b) degree of disease expression, c) immune responses, and d) within-host evolution of the pathogen, which in turn influences colonization, disease, and immune responses. All of these questions were addressed in two murine models; *Campylobacter jejuni* infected antibiotic treated mice and mice harboring a human-derived intestinal microbiota (^{Hu}microbiota).

At the center of this study is *Campylobacter jejuni;* a highly-motile, gram-negative, microaerophilic bacterial pathogen that poses a significant health burden in the developed and developing world (Scallan, Griffin et al. 2011, CDC 2014). Often times infection with *C. jejuni* results in self-limiting nausea, vomiting, and diarrhea that resolves in 7−10 days; however, long-term sequelae including Guillain-Barré Syndrome (GBS) can arise (Young, Davis et al. 2007, CDC 2014, Willison, Jacobs et al. 2016). GBS is an acute peripheral neuropathy characterized by rapid symmetrical progression, muscle numbness or weakness, and in severe cases partial or complete paralysis (van den Berg, Walgaard et al. 2014). The most common form of GBS associated with prior *C. jejuni* infection is acute motor axonal neuropathy (AMAN). AMAN is a pure motor subtype of GBS characterized by rapid progression, severe weakness, prolonged recovery, and sometimes pain (van den Berg, Walgaard et al. 2014). Evidence from experimental and investigative histological studies in AMAN cases suggest that molecular mimicry of peripheral nerve gangliosides by the outer core of the *C. jejuni* LOS elicits an anti-ganglioside antibody response upon host infection that results in complement mediated nerve damage, macrophage scavenging of damaged axons, and diminished nerve conductivity (Yuki, Taki et al. 1993, Griffin,

Li et al. 1996, Hafer-Macko, Sheikh et al. 1996, Hughes, Hadden et al. 1999, Keiichiro, Matthew et al. 2007, Yuki 2012, van den Berg, Walgaard et al. 2014).

Experimental inoculation of mice with *C. jejuni* patient strains revealed cross-reactive anti-ganglioside antibodies consistent with human GBS (Malik, Sharma et al. 2014); however, whether these antibodies resulted in histological changes in peripheral nerves was not investigated. Furthermore, host determinants of *C. jejuni*-induced autoimmunity are understudied compared to factors modulating enteric disease. As microbiota have been shown to mediate *C. jejuni* colonization and enteric disease (Chang and Miller 2006, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015), we hypothesized that altered gut microbiota might exacerbate anti-ganglioside antibody levels in *C. jejuni* infected mice and potentially increase severity of nerve lesions. Thus, experimental inoculation of antibiotic treated and ^{Hu}microbiota mice with *C. jejuni* GBS patient strains may increase host-susceptibility to *C. jejuni*-mediated autoimmunity including GBS. The overarching goal of this study is to determine if the composition of gut microbiota affects *C. jejuni*-triggered autoimmunity in murine models. In this study, we present the first evidence that the host-microbiota modulates *C. jejuni*-mediated autoimmunity, and exacerbated autoimmune responses coincide with severe enteric disease. This experimental model was chosen because infection outcomes in *C. jejuni* infected C57BL/6 wild-type and C57BL/6 IL-10$^{-/-}$ mice are well characterized (Mansfield, Bell et al. 2007, Mansfield, Patterson et al. 2008, Malik, Sharma et al. 2014, Stahl, Ries et al. 2014), antibiotic treatment is an established method of microbiota depletion (Antonopoulos, Huse et al. 2009), and ^{Hu}microbiota mice may serve as human-like models for study of enteric disease.

First, we analyzed C57BL/6 IL-10$^{-/-}$ mice experimentally inoculated with *C. jejuni* GBS patient strains receiving either sterile drinking water or 0.5 mg/mL cefoperazone (CPZ) in sterile drinking water and found that gut microbiota mediate *C. jejuni* inflammation and autoimmunity in mice. In line with previous studies (Chang and Miller 2006, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015), our results show that depleted gut microbiota enhance susceptibility to

C. jejuni-mediated inflammation; however, this is the first study to show this effect with GBS patient strains. Histological investigation of ileocecocolic junctions in infected mice harboring conventional mouse microbiota (^{Mo}microbiota) revealed that enteric disease was mild following infection with GBS patient strains 260.94 and HB93-13. Only a trend towards significant enteric disease without antibiotic treatment was evident in D8942 infected mice, but this result did not achieve statistical significance. In striking contrast, antibiotic depletion of gut microbiota drove severe colitis correlating with a mixed T_H1/T_H2 response. Surprisingly, T cells were not elevated in the colon of some mice with enteric disease, indicating a role for other cell types in *C. jejuni*-mediated inflammation. Previous reports show that innate lymphoid cells play a role in colitis in C57BL/6 IL-$10^{-/-}$ mice (Malik, Sharma et al. 2014); innate lymphoid cells were not investigated in this study. In addition, in previous work with ^{Mo}microbiota C57BL/6 IL-$10^{-/-}$ mice, we found that that GBS patient strains could not elicit severe inflammation (Bell, Jerome et al. 2013, Malik, Sharma et al. 2014); yet, our results show that under the right conditions these strains can elicit severe inflammatory responses. Together with previous reports, these data show that *C. jejuni* infection outcomes are dictated by multiple factors including host-microbiota and *C. jejuni* genetics.

In our experimental model, C57BL/6 IL-$10^{-/-}$ mice showed anti-ganglioside antibody responses when infected with GBS patient strains confirming *C. jejuni*-mediated autoimmunity. This is consistent with a previous report by Malik et al. (2013) showing anti-ganglioside antibody responses following infection with *C. jejuni* 260.94 and HB93-13; however, we report here that in addition to T_H2 associated anti-ganglioside antibodies, both T_H1 and T_H17 associated antibodies reacted with peripheral nerve gangliosides. These results were strain specific, and only C*. jejuni* HB93-13 elicited significant T_H2 associated anti-ganglioside antibody responses. Strikingly, we show that antibiotic treatment exacerbated anti-ganglioside antibody responses; however, the results varied depending upon the infecting *C. jejuni* strain. Variation in antibody responses elicited by *C. jejuni* strains was expected because biochemical analysis has shown that

ganglioside mimics vary among strains as a result of genetic variation in LOS loci (Gilbert, Karwaski et al. 2002). To date biochemical analysis of D8942 has not been conducted. It is known that anti-ganglioside antibodies can bind to peripheral nerves in ganglioside immunized rabbit models (Susuki, Nishimoto et al. 2003) consistent with IgG deposition in AMAN cases (Griffin, Li et al. 1996); therefore, we hypothesized that anti-ganglioside antibodies found in our study may bind to peripheral nerves and lead to compliment mediated destruction of nerves and subsequent macrophage scavenging. Recently, results in our lab have revealed increased macrophage numbers in dorsal root ganglia of non-obese diabetic (NOD) mice infected with GBS patient strains (St. Charles et al. unpublished). Because macrophage numbers were enhanced in a comparable model and antibody binding is not sufficient to confirm neuropathy, we hypothesized that histological assessment of peripheral nerves would provide a better evaluation of neurological disease, which is described below.

Notably, our results also show that depletion of gut microbiota is sufficient to elicit anti-ganglioside antibodies in the absence of infection. Antibiotic treatment increased GM1 and GD1a anti-ganglioside antibodies in uninfected, antibiotic treated mice (uninfected + CPZ) compared to uninfected, untreated mice (uninfected + H_2O) in both experiment 1 and 2. It is well known that gangliosides are present throughout the nervous system including the myenteric plexus, thus inflammatory processes may have liberated antigen that contributed to anti-ganglioside antibody responses; however, inflammation is likely not the cause of the anti-ganglioside antibodies in uninfected, antibiotic treated mice because investigative histopathology studies of ileocecolic junctions from these mice revealed that inflammation was rare, and very mild when present. Alternatively, some members of the gut microbiota havestructural mimics on their surface (Cress, Englaender et al. 2014, Ruff and Kriegel 2015). Though not determined here, some commensals may possess structural mimics with GM1- and GD1a-like groups that contribute to anti-ganglioside antibody responses not found in sham-inoculated, untreated mice due to antibiotic dependent alterations in the environment of the gut. In line with this idea, several autoimmune

diseases including type 1 diabetes and rheumatoid arthritis are associated with shifts in the ecological structure of the gut microbiota (Campbell 2014, Sargent 2014). In the absence of data collected from C57BL/6 IL-10$^{-/-}$ mice treated with antibiotics other than CPZ we are unable to determine if these results are specific to CPZ treatment; however, similar data showing anti-ganglioside antibodies have been collected in sham inoculated chloramphenicol treated Non-Obese Diabetic (NOD) mice (St. Charles et al. unpublished). In this study, our findings provide an immunologic basis for autoimmunity in uninfected, antibiotic treated mice; thus assessment of peripheral nerve lesions compared to our infected and control mice was warranted and is described below.

To determine if a human microbiota upregulated interferon gamma (IFNγ)-dependent immune responses to infection with *C. jejuni* in a mouse model we experimentally inoculated ^{Hu}microbiota and ^{Mo}microbiota C57BL/6 genetically wild-type mice with a GBS patient strain (260.94) and an enteric disease strain (11168). In our experimental inoculation of ^{Hu}microbiota C57BL/6 mice, *C. jejuni* colonization was enhanced in ^{Hu}microbiota mice compared to ^{Mo}microbiota mice as determined by culture and 16S rDNA gene amplicon sequencing; this enhanced colonization was correlated with increased inflammation at the ileocecocolic junction as determined by gross pathological changes observed at necropsy. In contrast to our antibiotic treated mice, severe enteric lesions were not detected in ^{Hu}microbiota mice; however, it is important to note that these mice were genetically wild-type mice and microbiota were not depleted. Therefore, whether or not depletion of gut microbiota in C57BL/6 wild-type mice results in severe enteric disease is unknown.

Taken together with previous reports showing that non-murine microbiotas enhance susceptibility to *C. jejuni*-mediated inflammation but mutations in immune regulatory genes including IL-10, Sigirr$^{-/-}$, and Prkdc^{scid}are required for severe enteric disease in murine models (Chang and Miller 2006, Mansfield, Bell et al. 2007, Stahl, Ries et al. 2014) these data reinforce the idea that both host microbiota and host-genetics affect host susceptibility to *C. jejuni*-mediated

inflammation in murine models. Because this correlation is so strong in mice, an underlying genetic basis for severe enteric disease in *C. jejuni* infected humans should be investigated. Along those lines, polymorphisms in IL-10 have been identified as potential determinants of inflammatory bowel disease (IBD) in humans (Glocker, Kotlarz et al. 2009, Moran, Walters et al. 2013, Engelhardt and Grimbacher 2014).

Consistent with results in antibiotic treated mice compared to untreated mice, anti-ganglioside antibodies were exacerbated in *C. jejuni* infected ^{Hu}microbiota mice compared to ^{Mo}microbiota infected mice. *C. jejuni* 260.94 but not 11168 infected mice showed elevated anti-ganglioside antibodies compared ^{Mo}microbiota controls infected with the same strain. Notably, ^{Hu}microbiota mice presented a biased T_H2 associated *C. jejuni* antibody response. Consistent with a previous report (Bereswill, Fischer et al. 2011) ^{Hu}microbiota mice were more susceptible to enteric disease after *C. jejuni* infection*;* however, direct comparisons to that study cannot be made due to substantial differences in study design including methods for generating ^{Hu}microbiota and ^{Mo}microbiota mice involving long-term antibiotic treatment. Despite these differences, both studies indicate that ^{Hu}microbiota mice have exacerbated inflammatory responses to *C. jejuni* infection. In our study, gross inflammatory changes were detected in the cecum and colon of ^{Hu}microbiota infected mice, but no significant histological changes nor increases in the inflammatory cytokine IFNγ in colon homogenates. Gross pathologic changes in ^{Hu}microbiota mice primarily consisted of enlarged ICC lymph nodes and occasionally enlarged spleens.

Notably, our study is the first to investigate *C. jejuni*-mediated autoimmunity in a ^{Hu}microbiota mouse model and our data show that ^{Hu}microbiota mice display a biased T_H2 antibody response. Since plasma samples had already been exhausted in the course of the study, T_H1 and T_H17 anti-ganglioside antibodies were not investigated. Furthermore, because no single human microbiota exists, our results are confined to comparisons in microbiota structure between our ^{Hu}microbiota and ^{Mo}microbiota mice. Our findings show that ^{Hu}microbiota mice were more susceptible to enteric disease, showed a biased T_H2 antibody response, and had differences in

the abundance of *Bacteroidetes* and *Firmicutes;* thus these taxa may play a critical role in modulating *C. jejuni*-mediated inflammation. Moreover, the microbiota of ^{Mo}microbiota mice was enriched in *Lactobacillus,* showing at least a 6000-fold increase in reads assigned to this genus compared to ^{Hu}microbiota mice. *Lactobacillus acidophilus* has been shown to inhibit the growth of *C. jejuni* in *in vivo* and *in vitro* models; thus this population may influence *C. jejuni* loads in our ^{Mo}microbiota mice. Gnotobiotic mice could be used to study the effects of each of these taxa in mediating *C. jejuni* outcomes.

Next, we analyzed peripheral nerves and dorsal root ganglia from experimentally inoculated C57BL/6 IL-10$^{-/-}$ mice to determine if macrophage counts were increased in infected mice. This is in line with the hypothesis that anti-ganglioside antibodies evoke complement mediated immune responses and increased macrophage recruitment in AMAN cases. Our results show that anti-ganglioside antibody elicitation was not sufficient to promote increased macrophage recruitment to peripheral nerves in a manner corresponding with experimental groups. Although we are unable to confirm our original hypothesis because macrophage counts did not achieve statistical significance in infected groups, macrophage counts in nervous tissue were elevated in many individual infected mice compared to controls. Furthermore, many untreated infected and antibiotic treated infected mice showed increased macrophage counts compared to uninfected, untreated controls. In general, macrophage counts were exacerbated in the dorsal root ganglia or sciatic nerve and were generally less prevalent in C57BL/6 mice in experiment 2. Taken together these results indicate both infection and antibiotic depletion of gut microbiota are independently sufficient to exacerbate macrophage counts in peripheral nerves of C57BL/6 IL-10$^{-/-}$ mice compared to untreated controls but the factors underlying these responses are unclear. Currently, many investigators are probing the role of the microbiota in autoimmunity, including molecular mimicry (Rodríguez-Reyna, Martínez-Reyes et al. 2009, Cress, Englaender et al. 2014, Ruff and Kriegel 2015); thus future investigations may shed light on our findings.

Microbiota are not the only factors mediating *C. jejuni* pathogenesis as both enteric disease and autoimmunity varied depending upon the infecting strain regardless of the microbiota. Bell et al. 2009 showed that enteric disease in *C. jejuni* infected mice and enhanced pathogenicity following passage depended upon the infecting *C. jejuni* strain. Consistent with these results we found differences in the ability of each *C. jejuni* strain to cause enteric disease as determined by the severity of enteric lesions. In the absence of antibiotic treatment only *C. jejuni* strain D8942 resulted in significant inflammation which was exacerbated by antibiotic treatment. Furthermore, mild inflammation in mice infected with *C. jejuni* 260.94 and HB93-13 is consistent with previous reports (Malik, Sharma et al. 2014). Notably, Bell et al. (2013) also determined that infection outcomes were associated with differences in the expression of some known virulence genes, loci with unknown function, and loci present in all experimental strains that were shown to cause colitis in mice. Collectively these loci comprise the *C. jejuni* virulome (Bell, Jerome et al. 2013). Unfortunately, *C. jejuni* virulence factors are understudied, with the exception of cytolethal distending toxin; thus the proposed virulome provides the best platform for the analysis of virulence determinants.

Next, we analyzed the population genomes of *C. jejuni* isolated from experimentally *C. jejuni* inoculated mice with antibiotic treated, ^{Hu}microbiota, and ^{Mo}microbiota to determine if treatment or microbiota dictated patterns of genomic variation compared to ancestral isolates. We hypothesized that genomic variants would be present in virulence associated loci and that these variants would correlate with antibiotic treatment or microbiota. Our results from comparative genomic analysis show that *C. jejuni* isolates obtained from antibiotic treated mice accumulated variants in antibiotic resistance genes *cme*R (transcriptional repressor of CmeABC operon) and *cme*B (inner membrane transporter of CmeABC multi-drug efflux pump when compared to ancestral isolates. No variants were present in antibiotic resistance genes of *C. jejuni* isolates obtained from untreated controls. Because CmeR also regulates the expression at least 28 other genes encoding capsule biosynthesis enzymes, membrane transporters, and a mixture of other

transport related and hypothetical proteins, *cme*R variants that affect function may have sweeping effects. Interestingly, our results show that mutations were frequent in virulence associated genes, but no microbiota dependent pattern of mutations could be discerned. Whether microbiota or antibiotic treatment influences genetic adaptation in *C. jejuni* has not been investigated prior to this study; however, our results indicate that single nucleotide variants and structural variants play a role in *C. jejuni* adaptation which is consistent with previous reports (Revez, Zhang et al. 2014, Thomas, Lone et al. 2014). Because mutations in antibiotic resistance genes were only present in strains obtained from antibiotic treated mice and these adaptations were retained following two laboratory passages, these data suggest that this is a model for rapid and heritable adaption of *C. jejuni* during *in vivo* passage. Whether these variants contributed to enhanced enteric disease in mice or whether they confer a fitness advantage to *C. jejuni* requires further investigation. To test this our *C. jejuni* isolates could be used to infect mice in subsequent experiments similar to previous *C. jejuni* serial passage experiments (Bell, St Charles et al. 2009).

Several reasons may account for the differences in infection outcomes seen in ^{Hu}microbiota and antibiotic treated mice. First, it is known that C57BL/6 wild-type mice are less susceptible to enteric disease than IL-10$^{-/-}$ mice (Mansfield, Bell et al. 2007). IL-10$^{-/-}$ is an anti-inflammatory cytokine produced by both innate and adaptive cells that downregulates the expression of pro-inflammatory T_H1 cytokines and regulates the activity of natural killer cells and macrophages (Couper, Blount et al. 2008). Our ^{Hu}microbiota mice were IL-10+/+; thus milder inflammatory responses were expected. Second, antibiotic treatment drastically depleted the gut microbiota, thus eliminating an important immune regulatory component in an already susceptible host. Our data show that ^{Hu}microbiota mice have diverse microbiota with differences that lead to increase susceptibility to inflammation yet are not sufficient to allow severe disease. A cefoperazone treatment experiment could be performed to determine if depleted microbiota results in enhanced disease severity in C57BL/6 ^{Hu}microbiota mice.

In summary, Guillain-Barré Syndrome is a post-infectious disorder which is most often preceded by infection with *C. jejuni*. This likely results as a consequence of molecular mimicry of host gangliosides by the LOS of *C. jejuni* which elicit cross-reactive anti-ganglioside antibodies that bind to peripheral nerves activating complement mediated responses which targets nerves in classic autoimmunity. *C. jejuni*-mediated-inflammation in the gut is modulated by the gut microbiota, *C. jejuni* genetics, and host-genetics in mice. In the present study, mice infected with *C. jejuni* and treated with antibiotics had greatly exacerbated enteric lesions and antiganglioside antibody titers compared to infected, untreated and sham inoculated mice. Additionally, anti-ganglioside antibodies were not predictive of increased macrophage presence in peripheral nerves and dorsal root ganglia. Similarly, ^{Hu}microbiota mice displayed enhanced susceptibility to enteric disease when infected with *C. jejuni*; yet anti-ganglioside antibody responses were mild and peripheral nerve macrophages were sparse and not elevated in infected mice. Surprisingly, anti-ganglioside antibodies were evoked in the sham inoculated antibiotic C57BL/6 IL-10-/- treated mice compared to sham inoculated untreated mice thus requiring further investigation. In addition, *C. jejuni* infection initiated significant anti-ganglioside antibody responses which were exacerbated by antibiotic treatment. In all cases, *C. jejuni* could be isolated from antibiotic treated mice with statistically significant anti-ganglioside antibody responses. Hence, this study indicates C57BL/6 IL-10 deficient mice provide a model for the study of *C. jejuni*-mediated inflammation and autoimmunity after infection with a single strain. Together with data collected from ^{Hu}microbiota mice we provide evidence that gut microbiota modulates *C. jejuni*-mediated inflammation and autoimmunity. Assuming that similar immune responses occur in humans, therapeutic approaches that alter the microbiota of *C. jejuni* infected patients may increase susceptibility to inflammation and GBS. Finally, these results suggest that the growing prevalence of antibiotic resistant *C. jejuni* will be accompanied by an increase in severe gastroenteritis and higher rates of GBS in human populations.

FUTURE DIRECTIONS

Guillain-Barré Syndrome is an autoimmune disorder in which it is hypothesized that cross-reactive antibodies promote immune mediated destruction of peripheral nerves. Because infectious organisms can often be isolated from patients with GBS the syndrome has been classified as a post-infectious disorder, and current evidence suggests that *Campylobacter jejuni* is the leading antecedent infection. In line with this finding, experimental inoculation of mice with *C. jejuni* GBS patient strains elicited an anti-ganglioside antibody response consistent with GBS in humans (Malik, Sharma et al. 2014). Strikingly, data presented in this dissertation show that antibiotic depletion of gut microbiota exacerbates both enteric lesions and anti-ganglioside antibodies in *C. jejuni* infected mice. In contrast to data collected in human GBS patients our data do not indicate an increase in macrophages in peripheral nerves; thus the question remains whether these antibodies in fact lead to peripheral nerve damage. Furthermore, antibiotic depletion of gut microbiota did not facilitate identification of specific microorganisms that regulate *C. jejuni*-mediated inflammation and/or autoimmunity. Finally, our data show that ^{Hu}microbiota mice display diminished *C. jejuni* colonization resistance and increased susceptibility to enteric disease correlating with ecological shifts in gut microbiota compared to ^{Mo}microbiota mice; however, functional differences in these communities that influence colonization resistance, enteric disease, or behavior are still undetermined. To address these knowledge gaps I propose the following future directions:

Future direction 1: Determine if experimental inoculation of antibiotic treated ^{Hu}microbiota C57BL/6 wild-type mice with GBS associated isolates evokes excerabated anti-ganglioside antibody responses. This aim will determine if enteric disease in ^{Hu}microbiota mice can be exacerbated by antibiotic treatment and if this correlates with anti-ganglioside antibody responses similar to results in IL-10$^{-/-}$ mice. The experimental approach will include antibiotic depletion of

^{Hu}microbiota mice and ^{Mo}microbiota microbiota mice and subsequent infection with GBS patient strains. I expect that both ^{Hu}microbiota and ^{Mo}microbiota antibiotic treated mice will exhibit exacerbated inflammatory responses and anti-ganglioside antibodies.

Future direction 2: Determine if there are differences in the functional potential of the microbiotas of ^{Hu}microbiota mice compared to ^{Mo}microbiota mice. This aim will test the hypothesis that humanized mouse microbiota modulate *C. jejuni* colonization through the secretion of anti-microbial substances. The experimental approach will include comparative transcriptomic and metabolomics analysis of gut microbiota from ^{Hu}microbiota and ^{Mo}microbiota mice; I expect that these profiles will differ corresponding with differences in the ecological structure (numbers, identities, and functions of organisms present) of these communities. The broader impacts of these data could include a better understanding the mechanisms that govern *C. jejuni*-mediated inflammation and autoimmunity.

Future direction 3: Determine if anti-ganglioside antibodies elicited by *C. jejuni* infection in IL-10$^{-/-}$ mice bind to peripheral nerves. This aim will test the hypothesis that *C. jejuni* infection elicits anti-ganglioside antibodies that bind to peripheral nerves. The experimental approach will include experimental inoculation of C57BL/6 IL-10$^{-/-}$ mice with *C. jejuni* 260.94 and HB93-13 with a time course to sacrifice (7, 21, 35, and 70 days) coinciding the onset of adaptive responses, previously studied time points, and long-term time points. Nerves will be collected and immunohistochemically labeled with anti-IgG. The significance of these findings is to determine whether *C. jejuni*-mediated autoimmunity in mice results in *in vivo* cross-reactivity

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