JANUS-FACED IMMUNITY TO AN ENTERIC PATHOGEN: HOW CAMPYLOBACTER CAUSES COLITIS AND AUTOIMMUNITY

By

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ABSTRACT

JANUS-FACED IMMUNITY TO AN ENTERIC PATHOGEN: HOW CAMPYLOBACTER CAUSES COLITIS AND AUTOIMMUNITY

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Campylobacter jejuni is an enteric bacterium present ubiquitously in food animals and is one of the top two etiologic agents of gastroenteritis worldwide. While many healthy individuals can be colonized asymptomatically or experience self-limiting gastroenteritis, serious autoimmune disease sequelae can also follow infections with *C. jejuni*. Inflammatory Bowel Disease, Reactive Arthritis and acute neuropathies Guillain Barré Syndrome and Miller Fisher Syndrome, have also been demonstrated to be strongly associated with antecedent *C. jejuni* infection. GBS is now the world's leading cause of acute neuromuscular paralysis, with up to 10,000 cases annually in the USA. Yet very little is known about how *Campylobacter jejuni* interacts with the immune system to establish asymptomatic colonization, induce gastroenteritis and/or lead to autoimmune sequelae. Lack of functional IL-10 is a predisposing factor to IBD, and thus the IL-10 deficient mice reiterate human disease by developing colitis after *C. jejuni* infection, making it a useful model for studying colon inflammation. In these studies, we have demonstrated that *C. jejuni* mediated colitis in specific pathogen free C57BL/6IL-$10^{-/-}$ mice was IFN-γ and IL-17 cytokine dependent. We also showed that both Innate Lymphoid Cells and T cells participate in IFN-γ and IL-17 elicitation, albeit with different kinetics. We also showed that T cells are essential for *C. jejuni* induced colitis. These results yield new cytokine and cellular targets for immunomodulatory therapy against IBD. This work also provides the first demonstration of a time-dependent role of Innate Lymphoid Cells and T cells in mediating Type1 (IFN-γ) and Type17 (IL-17) cytokine responses following infection with a human pathogen. Furthermore, we demonstrated that depleting IFN-γ and/or IL-17 pivots the immune response away from

sustaining inflammation in the colon to inducing a contrasting Type2 (IL-4) cytokine response. Following infection with *C. jejuni* isolates from GBS patients, this T helper cell dependent IL-4 cytokine response lead to elicitation of circulating autoantibodies. These autoantibodies caused histological and phenotypic changes in IL-10 deficient mice that were consistent with this syndrome's manifestation in humans. We further demonstrated that Siglec-1 is a central antigen presenting cell-expressed receptor that mediates uptake of *C. jejuni* and subsequent T_H2 maturation in a sialylation dependent manner. Therefore, IL-4 and Siglec-1 are novel and rational therapeutic targets against this peripheral autoimmunity, against which only invasive, non-specific and inefficient therapies like plasmapheresis exist.

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KEY TO ABBREVIATIONS

ATP: Adenosine triphosphate

AIDP: Acute Inflammatory Demyelinating Polyneuropathy

AMAN: Acute Motor Axonal Neuropathy

AMSAN: Acute Motor Axonal and Sensory Neuropathy

BMDM: Bone marrow derived macrophages

BMDC: Bone marrow derived dendritic cells

CIA: Collagen induced arthritis

cAMP: cyclic adenosine monophosphate

CD: Cluster of differentiation

C-terminus: carboxy terminus

DSS: Dextran sodium sulfate

EAE: Experimental autoimmune encephalomyelitis

GBS: Guillain Barré Syndrome

IFN: Interferon

Ig: Immunoglobulin

IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IL: Interleukin

ILC: Innate Lymphoid Cell

JAK: Janus kinase

JNK: c-Jun N-terminal kinases

LPS: Lipopolysaccharide

LOS: Lipoligosaccharide

MAPK: Mitogen activated protein kinase

MFS: Miller Fisher Syndrome

MMP: matrix metalloproteases

MS: multiple sclerosis

MyD88: Myeloid differentiation primary response gene 88

NFκB: Nuclear factor

NK: Natural Killer

NO: Nitric oxide

NOS: Nitric oxide synthase

PMA: phorbol myristate acetate

PRR: Pattern recognition receptor

ROS: reactive oxygen species

SIRS: Systemic inflammatory response syndrome

STAT: Signal Transducers and Activators of Transcription

TGF: Transforming growth factor

TCR: T cell receptor

TNF: Tumor necrosis factor

TRIF: TIR-domain-containing adapter-inducing interferon-β

Treg: regulatory T

TLR: Toll like receptor

UC: Ulcerative colitis

CHAPTER 1

LITERATURE REVIEW

ABSTRACT

Campylobacter jejuni is a spiral, Gram-negative, microaerophilic bacterium that is the most common bacterial cause of gastroenteritis. Previous infections with *C. jejuni* has been linked with several chronic autoimmune disorders including development and flare-up of Inflammatory Bowel Disease, Irritable Bowel Syndrome, Reiter's arthritis and peripheral neuropathy Guillain Barré Syndrome (GBS). *C. jejuni* affects about 1.4 million individuals in the USA every year, leading to about 10,000 deaths. The occurrence of GBS is temporally associated with development of autoantibodies that target gangliosides on peripheral nerves. The *C. jejuni* strains isolated from GBS patients have modifications of the outer core of the lipooligosaccharide that mimic several peripheral nerve gangliosides. Plasmapheresis and intravenous immunoglobulin (IVIg) treatment are the only known treatments with beneficial effect, but only 60% of GBS patients improve. Thus, new therapeutics are critically needed, but drug development pipelines are limited by lack of appropriate animal models for efficacy testing. In an effort to develop a model to study enteric and systemic manifestations of this bacterium, we showed that a number of isolates of *C. jejuni* from human enteritis patients induce colitis in the IL-10$^{-/-}$ mouse model whereas isolates from human GBS patients colonize these mice without inducing colitis. **Our overarching hypothesis was that fundamentally different immune responses mediate *C. jejuni* induced enteritis, asymptomatic colonization and GBS.** The thesis presented here addresses the *C. jejuni* strain dependent immunological mechanisms behind induction of colitis by gastroenteritis patient-derived isolates versus induction of an asymptomatic colonization by the GBS patient-derived isolates in murine models. Further, we show that infection of mice with *C. jejuni* strains from GBS patients, but not from colitis patients, elicits autoantibodies that react with nerve gangliosides consistent with those seen in human GBS cases. Production of these autoantibodies correlates temporally with a moderate peripheral neuropathic phenotype and causes the histopathological changes that are consistent with this syndrome's manifestations in humans. This review discusses the

considerable recent advances in our understanding of the immunological underpinnings of *C. jejuni* associated enteritis and autoimmunity, along with future challenges.

Basic mechanism of inflammatory reactions. A pathogen typically displays a set of Danger Associated Molecular Patterns (DAMPs) that are recognized by Pattern Recognition Receptors (PRRs, expressed by antigen presenting cells and non-hematopoietic cells like epithelial cells and fibroblasts) leading to production of one or more primary/innate cytokines and chemokines (such as interleukin (IL)-8, IL-12, TNF-α, IL-18, IL-15, IL-1β, IL-6, IL-23 and IL-10)[1]. These primary cytokines perform multiple roles such as mediating the infiltration and activation of immune effector cells, inducing production of anti-bacterial compounds, activating the coagulation cascade to localize the infection and priming a regulated secondary/adaptive immune response. Inflammatory adaptive immune responses can be broadly classified into 4 types – Type1, Type2, Type17 and T regulatory responses. IL-12, IL-15 and IL-18 direct the maturation of naïve T cells and innate (Rag independent) lymphocytes into Type1 cells that are characterized by production of IFN-γ[2]. IL-4 is the most powerful trigger for Type2 cells that are themselves characterized by production of IL-4, IL-5 and IL-13[3]. Therefore, IL-4 both induces and maintains its own expression, although the initial source of IL-4 is not known[3]. Type 17 cells require IL-6, TGF-β and IL-23 for induction and maintenance and are characterized by production of one or more of IL-17A ('IL-17'), IL-17F, IL-21 and IL-22.[4, 5] Type1, 2 and 17 cytokines have both microbiocidal and tissue protective effects. T regulatory (Treg) responses typically involve cytokine IL-10. Innate and adaptive immune cells and also epithelial cells and fibroblasts can produce IL-10. It functions to desensitize APC's, down modulate pro-inflammatory cytokine production and suppress proliferation in mononuclear cells. The nature of the ensuing adaptive immune response depends on the stimulatory innate signals. The first three types enhance infiltration and activation of effector cells and potentiate a humoral response that is in accordance with the nature of the innate response, while Tregs help resolve the inflammation and repair the affected tissue. An immune response may encompass one or more types of innate and adaptive responses in a tissue- and time- dependent manner. A

"successful" immune response rids the body of the pathogenic agent and repairs the affected tissue, while a dysregulated immune response can cause severe morbidity and even mortality.

Inflammatory Bowel Disease. Inflammatory Bowel Disease (IBD) refers to a group of autoinflammatory conditions of the small intestine and colon. Based on pathological manifestations in humans, Inflammatory Bowel Disease (IBD) is often divided into two subsets – Crohn's Disease (CD) and Ulcerative Colitis (UC). While both diseases show epithelial metaplasia, glandular atrophy and architectural distortion, the presence of fissuring ulcers, deep granulomas, transmural inflammation and skip lesions classify it as CD[6]. CD can affect any part of the gastrointestinal tract including the colon. In case of UC, lesions are present throughout the colon and while backwash ileitis may be present, other parts of the gut are unaffected and fissuring ulcers or deep granulomas are not present[6]. Both forms can be relapsing and remitting in some individuals and progressive in others. Both forms of the disease are highly prevalent in developed countries (21 – 246 per 100,00 for UC and 8 – 214 per 100,00 for UC) and represent major burdens for morbidity and the economy[7].

Importance of IL-10 in IBD. Interleukin-10 (IL-10) is a regulatory cytokine that prevents overt self–damaging inflammatory response to pathogens or autoimmune stimulators. IL-10 is produced by many leukocytes (including T regulatory cells, B cells and myeloid cells) after ligation of pathogen recognition receptors and/or co stimulatory molecules and acts on hematopoietic and non–hematopoietic cells to regulate diverse immune responses[8]. IL-10 signals via its cognate receptor, IL-10R, which is composed of two chains, IL-10RA and IL-10RB, which activates Janus kinase family members and STAT transcription factors to mediate its downstream effects[9]. In Antigen Presenting Cells (APCs), it suppresses the release of pro-inflammatory mediators, decreases antigen presentation and decreases expression of MHCII, co-stimulatory and adhesion molecules while enhancing phagocytosis and scavenging

functions[10]. It also acts on $CD4^+$ T cells directly to reduce their proliferation and cytokine synthesis[10]. As the intestine is continuously exposed to dietary antigens and commensal microflora, sophisticated immune regulatory pathways must exist to prevent chronic intestinal inflammation, and IL-10 has been implicated in them[11]. A genome wide association analysis discovered Single Nucleotide Polymorphisms (SNPs) flanking the *IL10* gene as the most significant locus associated with UC, outside the MHC locus[12]. Association of CD with SNPs in *IL10* was also significant. Furthermore, a recently identified monogenic form of CD was shown to be associated with mutations in cognate receptor chains IL-10RA or IL-10 RB[12]. Therefore, impairment of IL-10 associated regulatory pathway is critical for both forms of IBD.

***Campylobacter jejuni* as a causative organism of inflammatory diarrhea.** *Campylobacter jejuni* is a spiral, Gram-negative, bipolar and microaerophilic bacterium and is the most common cause of gastroenteritis worldwide[13, 14]. *C. jejuni* is most commonly associated with poultry and farm animals, and consequently raw/undercooked meat and unpasteurized milk are the most common sources of infection in humans[13]. Enteritis caused by *C. jejuni* is debilitating but usually self-limiting, but can be life threatening in immune-compromised individuals[15]. Histopathological manifestations include colonic crypt distortion, crypt abscesses, mucin depletion of epithelium, edema in the colonic lamina propria (cLP) along with infiltration of granulocytes, plasma cells and lymphocytes[16]. In spite of the medical and economic importance of the pathogen, little was known about the immunological mechanisms initiated and sustained during the course of the disease.

Most of the data regarding *C. jejuni* virulence factors comes from challenge infections in epithelial cell culture or chicken colonization models. In these models it has been shown that CadF (Campylobacter adhesion to Fibronectin) and FlpA (Fibronectin-like protein A) are required for Campylobacter's adherence to the epithelial extracellular matrix, while injection of

flagella mediated Cia (Campylobacter invasion antigens) proteins induced phagocytosis and IL-8 release[17]. Most strains of *C. jejuni* produce CDT (Cytolethal Distending Toxin) that has DNase activity that triggers apoptosis and IL-8 release[18]. PorA (porin), cj0091 (putative lipoprotein), JlpA (a surface-exposed lipoprotein), pldA (phospholipase A), CapA (putative autotransporter), DNAJ (transcriptional regulatory) and FlaA (flagellin subunit A) are some other proteins shown to be involved in epithelial adherence/invasion[19, 20, 21, 22, 23] . The capsule and LOS also play a role in modulating epithelial invasion and cytokine secretion[24, 25]. On the side of the epithelial cells, PI-3 kinase, c-src and Focal Adhesion Kinase (FAK) and Pho GTPases cdc42 and Rac1 are necessary for *C. jejuni* uptake[17, 26]. Recently, we and others have confirmed the positive role of CiaD and HtrA (High temperature requirement A) in mediating enteritis in IL-$10^{-/-}$ mice[27, 28]. However, confirmation of the role of other virulence factors in an *in vivo* model of enteritis has been lacking.

Murine models of *C. jejuni* induced colitis. Natural and mutant isolates of *C. jejuni* have been shown to colonize a large number of in-bred mouse lines, including BALB/c, CBA, C3H and Swiss Webster mice[29, 30, 31, 32]. However colonization in these models was not associated with clinical or histopathological changes typical of campylobacteriosis in humans. Fox et al., showed that mice deficient in NF-κB subunits ($p50^{-/-}$ $p65^{-/-}$) in a C57BL/129 background developed gastritis and proximal duodenitis but large bowel inflammation was mild[33]. Due to the anti-inflammatory and inflammation resolving roles of IL-10, it was hypothesized that IL-10 deficient mice will experience exacerbated Type1, 2 or 17 cytokines in the colon after *C. jejuni* challenge. Consistent with this hypothesis, work in the Mansfield laboratory has showed that wild type (IL-$10^{+/+}$) and IL-$10^{-/-}$ mice of various genetic backgrounds colonize *C. jejuni* while only the IL-$10^{-/-}$ mice develop enteritis[11]. While the IL-$10^{+/+}$ mice of C57BL/6, C3H/HeJ and NOD background stably colonized *C. jejuni* (isolate NCTC11168) for 35 days post oral inoculation without any adverse clinical or histopathological effects, the IL-$10^{-/-}$ mice of all three genetic backgrounds

developed typhlocolitis (inflammation of cecum and colon)[11]. The histopathological features of colitis in IL-10$^{-/-}$ mice also replicated the histopathological features of *C. jejuni* associated colitis in humans[16, 34]. These features include infiltration of mononuclear and polymorphonuclear cells in the colonic lamina propria and occasionally within the muscularis (smooth muscle tissue), necrosis/ulceration of colonic epithelium and edema. A dose range of 10^2 – 10^{10} CFU/mouse caused similar level of pathology in these mice[31, 34]. The immune parameters that sustain inflammation in IL-10$^{-/-}$ mice were also largely unknown before our work.

Several key findings suggest that diet and gastrointestinal microbiome play a role in *C. jejuni* pathogenesis and immunity. In a serendipitous discovery, it was observed that BL/6 IL-10$^{-/-}$ mice maintained on "regular chow diet" (6% fat) during the course of infection after being raised on "breeder diet" (12% fat) had significantly more lethality and pathology than the mice maintained on "breeder diet"[35]. The reason for diet dependence of phenotype remains unknown. Multiple reports in the last few years have demonstrated that mice with reduced/altered microflora are more readily colonized with *C. jejuni* and experience more severe clinical and histological signs of colitis. Chang et al have shown that C3H SCID mice with Limited Flora (LF) experience significant colitis after infection with 81-176, an isolate that does not stably colonize mice with conventional flora[36]. Bereswill et al showed that mice in which conventional microbiota is eradicated by a quintuple antibiotic treatment and then reconstituted with a complete human flora (in contrast with mice reconstituted with mouse flora) develop colitis after *C. jejuni* infection[37]. Furthermore, the colitis response depended upon sensing of *C. jejuni's* LOS and DNA by TLR4 and TLR9[37]. Requirement of TLR4 for full-blown *C. jejuni* induced colitis was also confirmed in a more severe model of colitis - the quintuple antibiotic treated IL-10$^{-/-}$ mice[38]. Direct evidence of microbiota components affecting *C. jejuni* colonization and virulence was provided by Haag et al. by demonstrating that *E. coli* feeding alone could facilitate *C. jejuni* colonization and colitis induction[39]. Stahl et al., have recently developed a new model of *C.*

jejuni colitis that requires vancomycin pretreatment in SIGIRR$^{-/-}$ (Single IgG IL-1 Related Receptor – a negative regulator of MyD88 signaling) mice. This, along with the IL-10$^{-/-}$ mouse model, provides evidence for the requirement of dysregulation of the host immune system and/or perturbation of the host microbiota as pre-requirements for *C. jejuni* to cause colitis in mice, and by extension probably also humans. The vancomycin treated SIGIRR model also confirmed the pro-inflammatory role of TLR4, but demonstrated an anti-inflammatory role of host TLR2 and *C. jejuni's* capsule in colitis induction[40]. Recent studies in germ free or antibiotic pretreated IL-10$^{-/-}$ mice from Sun et al., has demonstrated the pro-inflammatory actions of mTOR (mammalian target of rapamycin)[41] and phosphatidylinositol 3 kinase-γ (PI3-γ)[42] by upregulaing pro-inflammatory cytokine production and neutrophil recruitment[43]. Conversely, an anti-inflammatory role for NOD2 in *C. jejuni* induced colitis has been shown that is mediated by enhancement of Nitrous Oxide (NO) mediated bacterial killing[43]. Thus, these observations tie the microbiome and innate PAMP recognition to *C. jejuni* mediated disease. However, the direct role of inflammatory mediators—particularly lymphocytes and their secreted cytokines—had not been established *in vivo* before our work.

Immune basis of *C. jejuni* induced colitis: Infecting IL-10$^{-/-}$ mice with multiple isolates of *C. jejuni* produces a range of phenotypes, from failure of colonization to colonization without enteritis and colonization with high rate of enteritis[35, 44]. In **chapter 2**, we show that during infection with multiple human enteritis patient isolates, there is a significant increase in the levels of IFN-γ, IL-17, IL-22, IL-6, TNF-α, IL-1β and IL-12, but not IL-4 or IL-13, in the colon and/or mesenteric lymph nodes of IL-10$^{-/-}$ mice[45]. These findings are consistent with elicitation of a mixed Type1 and Type17 cytokine response, with the absence of a Type2 response. Similar findings were made by Edwards et al., that showed the elicitation of Type1 and 17 cytokine responses after challenge of human colon explants and peripheral blood-derived cells with a

hypervirulent isolate of *C. jejuni* 11168[46]. We directly confirmed the pathogenic role of IFN-γ and IL-17 in *C. jejuni* induced colitis by depleting them with neutralizing antibody injections during infection. Depleting IFN-γ and/or IL-17 led to a significant decrease in overt histopathological changes including the extent of neutrophil, macrophage and T cell infiltration in the colon[45].

Strikingly, not all *C. jejuni* isolates are associated with colitis in humans or produce colitis in animal models. But exposure/colonization alone often leads to detectable anti-*C. jejuni* IgG antibodies in circulation[35, 47]. IgG subtype class switching in activated B cells is known to be modulated by Type1, Type2 and Type17 cytokines, and can therefore be used to proxy the specific type of immune response elicited by *C. jejuni in vivo*. Using IFN-γ and IL-4 reporter mice, Reinhardt *et. al.* showed that IFN-γ induces isotype class switching to IgG2a, while IL-4 primarily induces IgG1 class switch[48]. *In vitro*, B cells stimulated with LPS in the presence of IFN-γ preferentially switch to IgG2a and IgG3, while in the presence of IL-4 they preferentially switch to IgG1[49, 50]. IL-17 was shown to be required for induction of autoreactive IgG2b (but not IgG1 or IgG2a) antibodies in an experimental autoimmune myasthenia gravis model by knock out and supplementation strategies[51]. IL-21 is produced by a subset of Type17 cells and has been shown to induce IgG1 and IgG3 switching in CD40L stimulated B cells[52]. Furthermore, TGF-β – a cytokine involved in differentiation of Type17 cells and Tregs, can also induce IgG2b class switching in LPS stimulated B cells[49]. Therefore, Type1, Type17 and Type2 responses seem to have overlapping but distinct class switching ability – Type1 (IgG2a and IgG3) v/s Type17 (IgG2b, IgG3 and possibly IgG1) v/s Type2 (IgG1). Consistent with our finding of upregulation of a Type1 and Type17 but not Type2 response by colon and plasma cytokine analysis, levels of *C. jejuni* specific IgG2b (IL-17 dependent), IgG2c (allelic isoform of IgG2a, IFN-γ dependent) and IgG3 but not IgG1 (IL-4 dependent) were significantly enhanced in infected mice[45]. Depleting IFN-γ (with or without IL-17) led to an increase in the level of colonic

IL-4 and plasma anti-*C. jejuni* IgG1 antibodies. This, along with decrease in IgG2b and IgG2c isotypes, demonstrated a compensatory shift towards a Type2 response.

Elicitation of pro-inflammatory cytokines in response to *C. jejuni* related with progressive infiltration of neutrophils, macrophages and lymphocytes (T cells, B cells and innate lymphocytes) into the three layers of the colon – the epithelium, lamina propria and the muscularis. Innate lymphocytes are functionally heterogeneous and can be broadly divided into NK cells ($CD3^{-}NKp46^{+}$) and Innate-Lymphoid Cells ($Thy1^{hi}CD3^{-}NKp46^{-}$). T cells and innate lymphocytes are known to be major producers of secondary/adaptive cytokines in an inflammatory lesion. T cells can further be divided into two subsets based on the nature of T cell receptor – predominant αβ T cells and less frequent γδ T cells, and into two subsets on the basis of their co-receptor – $CD4^{+}$ T helpers (T_H) and $CD8^{+}$ T cytotoxic cells (T_C). NK cells can be further divided into conventional NK cells (that depend on transcription factor NLIF3 for development and are potent producers of IFN-γ) and NKR cells (that express transcription factor RORγt and are potent producers of IL-22)[53, 54, 55]. ILCs are further categorized as Lymphoid Tissue inducers (LTi's) ($Thy1^{hi}CD4^{+}$) and LTi-like cells ($Thy1^{hi}CD4^{-}Sca\text{-}1^{+}$) and nuocytes ($Thy1^{hi}CD4^{-}CD117^{+}Sca\text{-}1^{+}$)[56, 57]. LTi's are necessary for development of lymphoid follicles and have also been shown to produce IL-17 and IL-22 in dextran sodium sulfate and *Citrobacter rodentium* model of innate colitis[58]. LTi-like cells were discovered as principal IL-17 and IFN-γ producers in *Helicobacter hepaticus* and CD40 ligand induced models of innate colitis respectively[59]. Nuocytes are akin to T_H2 cells as they produce IL-5, IL-9 and IL-13 in response to helminth infection[60] (summarized in Fig 1.1).

Remarkably, we observed an opposite regulation of ILCs and T cells in the colon and draining lymph nodes as the ILCs expand earlier before the expansion of T cells and then contracted with the expansion of T cells. This suggests a “change of hands” of the inflammatory response that seems to be initiated by ILCs and is eventually taken over by adaptive T cells. Intracellular cytokine staining demonstrated upregulated expression of IFN-γ, IL-17 and/or IL-22 from these cell types in a cell type, time and organ-specific manner[45]. We confirmed that non-B cell lymphocytes are necessary for development of colitis as depleting Thy-1^{+} cells during infection completely abrogated inflammation and production of IFN-γ, IL-17 and IL-22 in the gut. Innate immunity alone was found to be insufficient for colitis as T and B cell deficient Rag1$^{-/-}$ nor IL-10R blocked Rag1$^{-/-}$ mice developed colitis after infection. Therefore, T cells are necessary for *C. jejuni* to cause colitis in SPF BL/6 IL-10$^{-/-}$ mice. However, in Rag2/IL-10 double KO germ free mice of 129/SvEV background, it has been shown that 11168 (a human colitis isolate) did induce colitis[42]. This pointed towards a protective role of the interaction between the microbiota and host genetic makeup in determining susceptibility to a pathogen. Furthermore, it has been shown that CD4^{+} T cell depletion in infected gnotobiotic IL-10$^{-/-}$ mice did not decrease the colitis severity[42]. This finding does suggest a role for non CD4^{+} T cell and ILC subsets in driving colitis, but is complicated by the fact that macrophages can also express this marker. Therefore, further targeted studies are needed to resolve the role of each of the cellular subsets in *C. jejuni* induced colitis, and for the development of rational therapeutic interventions that can target particular pathogenic cell subsets.

We also observed that the extent of *C. jejuni* colonization in IL-10$^{-/-}$ mice correlated with the extent of inflammation in the colon. In the kinetic studies, we observed that colonization extent increased with increasing time, and that correlated with increase in the histopathological score in the colon. Furthermore, treatments that decreased the extent of inflammation in the colon, like

depletion of Thy-1$^+$ cells or depletion of IFN-γ and IL-17, decreased the colonization extent to a basal level observed at a time point that was prior to the onset of inflammation. Therefore, in this aspect *C. jejuni* resembles *Salmonella enteric* serovar Typhimurium wherein inflammation facilitates enhancement of the pathogen burden[61, 62]. For salmonella, inflammation has been shown to create a better niche for the pathogen by eliminating competing microbes or by utilizing substrates that are provided by the dying inflammatory cells[52, 53]. Pre-existing enteritis also overcomes colonization resistance of certain *C. jejuni* isolates, but the mechanisms are unknown[39].

Guillain Barré Syndrome: Epidemiology and Clinical Manifestations. With the near eradication of polio, GBS is now the leading cause of acute neuromuscular paralysis with 6,000 – 9,100 cases annually in the US[63]. GBS typically involves an immune attack on peripheral nerves that may lead to irreversible nerve damage and muscle wasting. Unlike most autoimmune diseases, incidence rates do not increase with age, and GBS is 1.5 times more prevalent in men than women[62]. About 1/3rd of the patients require ventilation support and 3-10% die due to diaphragm paralysis[64]. Furthermore, while most patients recover from this syndrome, about 20% are unable to walk after 6 months, and up to 60% suffer pain and fatigue that lasts for years[65]. Incidence rates and mortality is likely higher in developing countries due to inadequate diagnosis, treatment and reporting.

Based on the type of the peripheral nerve fibers affected and the nature of the immune damage, GBS can be divided into multiple subtypes. Acute Inflammatory Demyelinating Polyneuropathy (AIDP) refers to a multifocal immune attack on the myelin sheath that surrounds the axons. Macrophages, neutrophils and T cells invade the myelin and denude the axons[66]. As the myelin sheath acts as an insulator for nerve conductance, its damage leads to decreased nerve signals reaching the neuromuscular junction. As appropriate nerve conduction signals are required for

the integrity of both the axon and muscle, demyelination eventually leads to axon die back and muscle wasting[67]. Acute Motor Axonal Neuropathy (AMAN) and Acute Motor and Sensory Neuropathy (AMSAN) subtypes primarily involve an antibody mediated attack on the axons as macrophages invade the nodes of Ranvier and nerve root ganglia, blocking the nerve conductance but leaving the myelin sheath intact[68]. In severe cases, the whole axon may be permanently damaged[64]. The idea that this pathology is antibody based comes from the observation that only macrophages, and almost no lymphocytes, are present in the infiltrate[69]. Both AIDP and AMAN only affect motor fibers, while AMSAN also has sensory involvement. AIDP is the predominant subtype in North America and Europe accounting for about 90% of the GBS cases[70] while AMAN and AMSAN are more common in South and Central America and Asia accounting for about 50% of the GBS cases[71, 72]. Miller-Fisher Syndrome (MFS) is a variant of GBS that primarily affects oculomotor nerves leading to paralysis of eye muscles[73]. It can also involve ataxia and paralysis of the diaphragm[74]. 5% of the GBS incidences in western countries belongs to this variant, but it is more common in Asia[75].

GBS is frequently preceded by gastrointestinal or lung infections, usually 1-4 weeks before onset of clinical signs[76]. Based on culture and/or serological data, more than 50% of the GBS patients were positive for *C. jejuni* infections[76]. However, many patients that present clinical symptoms of GBS do not present preceding signs of gastroenteritis[75, 76]. Therefore, the correlation, if any, between the extent of gastroenteritis or pneumonia and subsequent GBS is unclear[77, 78]. Other pathogens associated with GBS incidence include *Heamophilus influenzea, Mycoplasma pneumonia,* Epstein-Barr Virus and cytomegalovirus[79]. A slight increase in risk for GBS after swine flu and influenza vaccination have also been suggested[80]. However, for many GBS cases, especially the AIDP variant, no prior infections have been detected. This may be attributable to a different underlying etiology of AIDP and/or transient nature of the preceding infection.

Molecular mimicry and autoimmune basis of GBS. Certain strains of *C. jejuni* (and other GBS associated pathogens) express particular epitopes that are structurally similar to self-molecules present on the nerves[81]. Sialic acids are 9 chain carbon monosaccharides found in terminal position in glycan structures in mammals and some microbes. Gangliosides are sialic-acid containing glycan motifs that are highly presented on the surface of the myelin sheath and axons, including the nodes of Ranvier. Gangliosides are anchored on the outer leaflet of the plasma membrane by ceramide[82]. They can differ based on the position of sialic acid linkage on the galactose residue, and can be mono- or di-sialylated. Gangliosides, namely GM1, GD1a, GM1b and GalNac-GD1a are monosialylated with $\alpha 2 \rightarrow 3$ linkage and commonly found on myelin and axonal membranes of peripheral nerves, while GQ1b is a common disialylated ganglioside with both α,2-3 and α,2-8 linkage and found commonly on cranial oculomotor nerves[79]. These sialylated oligosaccharide motifs are structurally similar to those found on the outer surface of the lipooligosaccharide (LOS) of certain *C. jejuni* strains, thereby providing molecular mimicry for the onset of autoimmunity[81, 83]. *C. jejuni* LOS is a glycolipid entity consisting of a hydrophobic membrane anchor called lipid A, and surface exposed oligosaccharide motif that contains the sialic acid. Thus, it is speculated that after infection, antibodies are elicited against multiple epitopes presented by *C. jejuni*, including the LOS outer surface gangliosides. These antibodies can cross-react with distal limb nerve gangliosides (in case of GBS) or oculomotor nerve gangliosides (in case of MFS), leading to antibody mediated immune attack at these sites[82].

Antibody deposition onto the myelin or axon at the nodes of Ranvier can concievably mount an immune attack by two mechanisms: 1. Classical activation of the complement cascade, wherein C1 complex is activated when it binds to the Fc portion of the antibody, leading to activation of C3 convertase and deposition of C3b and C5b on to the tissue. The released fragments, C3a and C5a, act as chemoattractants for macrophages, while the bound C3b and C5b can lead to

further deposition of complement factors C6, 7, 8 and 9, that together form the membrane attack complex (MAC). MAC causes loss of ionic balance in the cells leading to their apoptosis. IgG and C3b deposition on axolemma in the case of AMAN,[84] and the schwann cell membrane in the case of AIDP patients has been demonstrated[85]. MAC formation on GD1a over-expressing phrenic nerves has been demonstrated ex vivo after deposition of anti-ganglioside antibodies and heterologous complement[86], However its formation in vivo lacks evidence. Antibody deposition at Nodes of Ranvier can cause a block in nerve conductance, that can eventually cause the axon and surrounding schwann cells to under apoptosis. Apoptotic cells can secrete multiple macrophage chemoattractants[87, 88]. Infiltrating macrophages can then be activated further by binding to Fc portion of the deposited antibodies by their Fc receptors. These mechanisms further underscore the idea of using IL-$10^{-/-}$ mice as a model for GBS, because in the absence of IL-10 there is reduced deactivation of inflammatory cells and autoantibody production, that would be expected to intensify the disease.

Interestingly, in humans and our mouse model of GBS, autoantibodies associated with *C. jejuni* associated GBS are predominantly of the IgG1 isotype, while patients with *C. jejuni* associated MFS or *H. influenzea* induced GBS have IgG3 as the predominant autoreactive IgG isotype[89, 90]. Further, in human GBS patients, titers of autoreactive IgG1 correlates with enhanced severity and poor long-term prognosis[89, 91]. After IgM, IgG1 (and to a lesser extent, IgG3) is the isotype most adept at complement fixation and binding to FcR for activation induced degranulation[92]. But because IgM is too big for extravasation from circulation into tissue structures, IgG1 is well suited for the task of carrying out an immune attack in peripheral tissue by complement fixation and inducing activation and degranulation of immune cells. Because isotype class switching to IgG1 or IgG3 isotype requires T helper cells, it is safe to conclude that T cells are necessary for GBS and MFS induction, even if they are not observed as a nerve-infiltrating cell type during AMAN and MFS. Indeed, in **chapter 2** we have directly demonstrated the role of T cells in

antibody elicitation after *C. jejuni* challenge. It is also possible that antibody production is incidental to T cell activation, and that the primary role of T cells is to directly activate the macrophages and other phagocytic cells, and direct their infiltration into the nerve bundle. It is important to note that while IgG3 is predominantly IFN-γ dependent, IgG1 is IL-4 dependent. IL-4 itself is required for T_H2 induction, and is a strong stimulator for T_H2 cells[93]. And because the mucosal organs have a concentration of IL-4 in homeostatic conditions, it is likely that the T_H2 response against *C. jejuni* GBS isolates is initiated there. Indeed, in **chapter 2** we have shown that infection of mice with GBS isolates lead to a small but significant increase in IL-4 transcripts and T_H2 cells in the colon. This suggests that *C. jejuni* induced GBS is IL-4 and T_H2 cell dependent while *C. jejuni* induced MFS is IFN-γ and T_H1 cell dependent. However, it should be noted that IL-21, a cytokine predominantly produced by T follicular helper cells, has also been shown to be independently capable of inducing IgG3 and IgG1 class switching[48, 52]. Therefore, further work is necessary to work out exact T cell, B cell, APC and phagocytic cell interactions that orchestrate GBS and MFS syndromes, and provide therapeutic interventions that can target the central cell types/ cytokines to ameliorate these syndromes.

It is also interesting to note that nerves that innervate the colon, including the myenteric plexus, are often found inflamed (infiltrated with macrophages and T cells) in the *C. jejuni* induced IL-$10^{-/-}$ colitis model[34]. Thus, it is likely that the nervous tissue in colon, the primary site for infection, may provide fodder for further epitope spreading onto the antigens of the limbs and /or craniofacial nerves. However, the exact nature, distribution and density of gangliosides in the enteric and peripheral nervous system, in mice or humans is lacking, and will be important for the development of a robust animal model.

The underlying genetic makeup of LOS biosynthesis locus in a *C. jejuni* isolate determines the sialylation pattern on the LOS[94]. Consequently, the LOS biosynthesis locus determines if the LOS will have molecular mimicry with the limb and/or craniofacial nerve gangliosides and by extension whether the infection may lead to the AMAN/AMSAN or MFS variants of GBS. Atleast 19 different subclasses of LOS biosynthesis loci have been identified and classified in classes A through S[94]. Class A is most commonly associated with GBS, while class B confers structural similarity to craniofacial nerve gangliosides and therefore MFS[95, 96]. Sialytransferase-II (cst-II) is an enzyme central to determining the sialylation pattern of the oligosaccharide in the outer core of the LOS, and is consequently commonly encoded in the genomes of *C. jejuni* strains isolated from GBS patients[97]. The genomes of two GBS isolates that we have utilized experimentally, HB93-13 and 260.94, both contain this gene, while the colitogenic isolates 11168 and CG8421 do not. However, genome of 11168 does contain cst-III, which leads to the presentation of a low level of GM1-, but not GD1a-like sialylation pattern on its LOS[98, 99]. Enhanced fitness can be conferred by surface-structure modifying genes in microbes. Having a sialylated outer core of LOS in this case provides an evolutionary advantage by enhanc bacteriophage resistance when compared to asialylated isolates[100].

Antibody based pathophysiology for *C. jejuni* associated AMAN is supported by the fact that intravenous Ig, alongside plasmapheresis, have been the only successful treatments for GBS. However, these therapies are untargeted and only beneficial in about 60% of the cases[101]. The exact mechanism of action for IVIg is not known, but it can be speculated that it may bind to Fc receptors on phagocytic cells and prevent their activation/infiltration at the site of autoreactive IgG binding, and/or form immune complexes with autoreactive IgG and boost its clearance. A robust mouse model of *C. jejuni* induced GBS will therefore not only be the first demonstration of a bacterial pathogen causing autoimmunity, it will also serve as a model to screen new and targeted therapeutics for this syndrome.

Immune recognition in the context of auto-reactive oligosaccharides.

While innate immune stimulation and recognition is well established for protein based antigens, antigen recognition and stimulation by carbohydrate based antigens, especially for sialic acid containing gangliosides, is less well studied. Sialic acid-binding immunoglobulin-like (SIGLEC) family of receptors are expressed mainly by immune cells that bind to sialic acid containing oligosaccharides[102]. They contain a single N-terminus variable domain that binds to the exposed sialic acid residue, and a number of constant domains that connects the sialic acid binding domain to the plasma membrane anchor, and a variable length cytoplasmic domain[103]. There are 14 different Siglec receptors that have been discovered in humans, and they have different binding specificity towards sialic acid containing oligosaccharides. Based on structural similarity, Siglecs can be divided into two groups: 1. Siglec-1, 2, 4 and 15, that are conserved throughout mammals, and 2. CD33-related Siglecs. Most siglecs contain cytoplasmic domain that contains an immunoreceptor tyrosine-based inhibitor motif (ITIM). ITIM leads to dephosphorylation of activating kinases, thereby providing negative immune stimulatory role to these receptors during proliferation, activation, cytokine production and apoptosis[104]. Humans have nine CD33-related Siglecs while mice have five, and they share 50-90% sequence identity[102].

Siglec-1/Sialoadhesin/Sn (CD169) has a short cytoplasmic chain that is devoid of any known signaling motifs. However, it has 17 extracellular 'C-set' Ig-like domains, placing its sialic acid-binding domain outside the cell's own glycocalyx region. This probably prevents cis-interactions and allows Siglec-1 to make the initial contact with sialylated pathogens or other cells during cell-cell interactions[105]. Siglec-1 is expressed by macrophages located in prime positions for 'first contact' with potential pathogens. For example, it is expressed by marginal zone macrophages in the spleen, the site of afferent lymphatics for incoming blood. In the lymph nodes, Siglec-1 is expressed by the subcapsular sinus macrophages at the site of incoming lymph, and medullary cord macrophages exposed to the efferent lymphatics. In the colon,

Siglec-1 expressing phagocytes are found in the lamina propria surrounding the crypts, a common site for bacterial invasion[106]. Therefore, anatomical locations of Siglec-1 expressing cells suggest its role as a primary sentinel for pathogens or apoptotic and cancer cells.

Consistent with its role as a sentinel, expression of Siglec-1 is usually upregulated under inflammatory conditions, particularly upon exposure to primary interferons. In primary cell cultures, Siglec-1 has recently been shown as a primary receptor for HIV uptake by activated human DCs and macrophages, and its trans-infection into T cells[107, 108]. It has also been shown to act as a specific receptor for phagocytosis of heat-killed *C. jejuni* isolates that carry GBS associated α,2-3 sialylation motifs by human blood-derived macrophages, and also upregulates IL-6 production[99]. Activated murine BMDMs and BMDCs from recently generated Siglec-1$^{-/-}$ mice have also been shown to have decreased uptake of heat-killed and α,2-3 sialylated *C. jejuni*, and decreased expression of Type1 interferons and MyD88 dependent cytokines *in vitro*[109, 110]. Siglec-1 requirement for the rapid IFN-β production was also confirmed by i.v. injection of inactivated sialylated *C. jejuni* in the knockout mice[109, 110]. Recently, a role for Siglec-1 in clearance of intravenously initiated group B streptococcus infection and resulting pathology has also been demonstrated[111]. However, these reports did not examine the role of Siglec-1 in maturation of T cell subsets or IgG isotypes, and its consequence in related diseases.

Multiple recent reports have also suggested a critical role for Siglec-1 in T cell activation during autoimmunity and cancer. Siglec-1 is highly expressed by circulating monocytes during chronic MS and rheumatoid arthritis[112]. It has been shown to play a pro-inflammatory role in the EAE model of MS, by binding to Tregs and preventing their expansion[113]. It has also been shown to play a role in T cell activation and proliferation in coronary artery disease[114]. And the presence of Siglec-1^{+} macrophages in local lymph nodes correlates with enhanced survival from colorectal cancer as well. Siglec-1^{+} macrophages have also been associated with presentation of lipid antigens for activation of iNKT cells[115]. Further, targeting delivery of microbial/tumor

antigens through Siglecs has shown promise for inducing strong T cell activation[116]. A recent publication has also shown that Siglec-1$^+$ macrophages are required to generate and maintain an anti-tumor T_C population. These reports support a view that deregulation of phagocytosis and T cell presentation by Siglec-1$^+$ APCs can lead to activation of auto-reactive T cells or failure to clear tumor cells. In case of pathogenic infections, it can lead to a failure in clearing systemic infection that result in morbidity and mortality. However, the role of Siglec-1 in influencing T_H1/T_H2 balance, and subsequent B cell maturation in the context of a pathogenic infection or an autoimmune disease, has also not been explored before our work.

As described in **chapter 3**, we found that Sigec-1 blocking significantly decreased uptake of GBS isolates by adherent splenocytes and IL-6 production from whole or adherent splenocytes. However, Siglec-1 blocking did not affect elicitation of Type1 cytokines (IFN-γ, TNF-α, IL-22). Siglec-1 blocking also did not affect the uptake or any cytokine elicitation by colitogenic isolates. In vivo, blocking Siglec-1 by neutralizing antibody injections in GBS isolate infected IL-10$^{-/-}$ mice lead to a decrease in T_H2 cells in the colon, and decrease in *C. jejuni* specific and auto-reactive IgG1 isotype antibodies in circulation. However, the effect of Siglec-1 blocking *in vivo* does not appear to be T_H2 specific, as *C. jejuni* reactive IgG2b subtype also trended towards a decrease by Siglec-1 blocking. Furthermore, blocking Siglec-1 mirrored IL-4 neutralization as both lead to a decrease in *C. jejuni* specific as well as auto-reactive antibodies in circulation. Decreased autoantibodies in circulation correlated with decreased macrophage infiltration in the sciatic nerves and its roots. Thus, sialylated oligosaccharide motifs on the LOS of GBS associated *C. jejuni* is unique in the sense that it acts as the ligand for phagocytosis by antigen presenting cells, and also as a cross-reactive epitope that leads to autoimmunity. The role of IL-10 in negatively affecting autoimmunity was also confirmed for GBS as IL-10$^{+/+}$ mice, unlike the IL-10$^{-/-}$ mice, did not develop a significant level of autoantibodies after infection with GBS isolates. IL-

$10^{+/+}$ mice did develop a significant anti- *C. jejuni* IgG2b response, suggesting an IL-10 independent mechanism for its elicitation.

Similar to Siglec-1 binding to α,2-3 mono-sialylated gangliosides that are common in LOS of GBS associated *C. jejuni*, Siglec-7 binds to α,2-3, α,2-8 di-sialylated gangliosides that are common in LOS of MFS associated *C. jejuni*[117]. Siglec-7 is a CD33-related Siglec that contains ITIMs in the cytoplasmic tail and is known to be expressed by human NK cells and myeloid cells[102]. Therefore, ganglioside sialylation pattern 1) forms the basis of autoimmunity, by being conserved between GBS LOS and peripheral nerve gangliosides (mono-sialylation), and MFS LOS and craniofacial nerves (disialylation); 2) it also determines the Siglec receptor interaction and likely subsequent T and B cell maturation (summarized in Fig. 1.2). However, further work is required to pinpoint the role of Siglec-7 in *C. jejuni* uptake, T cell activation and subsequent disease. These Siglecs that are not expressed by the mouse genus, but it is possible that other Siglecs in mice have similar sialic acid binding specificities and perform the same functions. Therefore, other Siglec receptors appear to play a role in GBS and MFS pathogenesis.

APPENDIX

Figure 1.1. Schematic representation of Lymphocyte populations involved in *C. jejuni* infections

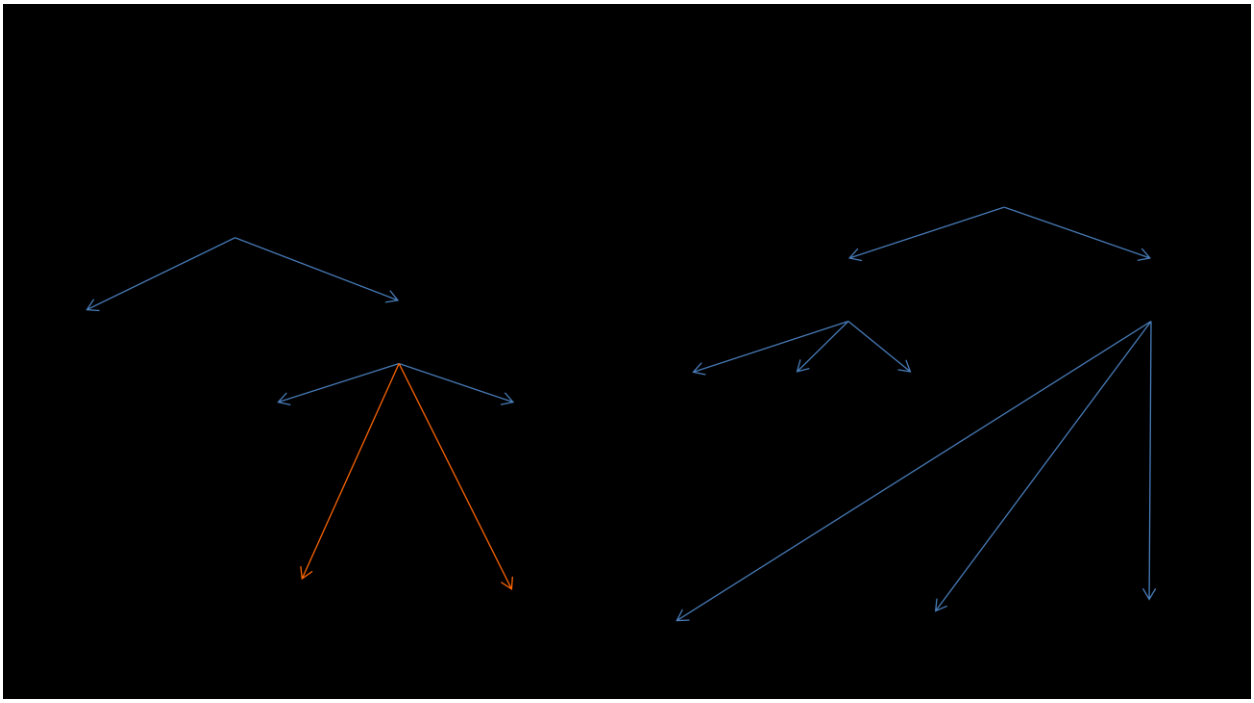

Figure 1.1. On the basis on Rag dependence, lymphocytes can be divided as Adaptive (*Rag* dependent) or Innate (*Rag* Independent). Adaptive lymphocytes can be divided as B cells (CD19+B220+CD3-) and T cells (CD19-CD3+). T cells can divided in to αβ or γδ T cells based on T cell-receptor, or CD4+T_H and CD8+T_C based on co-receptor expression. Innate lymphocytes can be divided as NK cells (NK1.1+) and innate-like lymphocytes (Thy1^{hi}CD3-). NK cells can be divided as conventional NK cells (NK1.1+NKp46+), NKR cells (RORγt expressing NKp46+NK1.1^{lo}) and NKT cells (CD3+NK1.1+). Innate-like lymphocytes can be divided as LTi's (Thy1^{hi}CD4+), LTi-like cells (Thy1^{hi}CD4-Sca-1+RORγt+) and Nuocytes (Thy1^{hi}CD4-Sca-1+IL-13+)

Figure 1.2. Schematic representation of immune basis of *C. jejuni* induced autoimmunity.

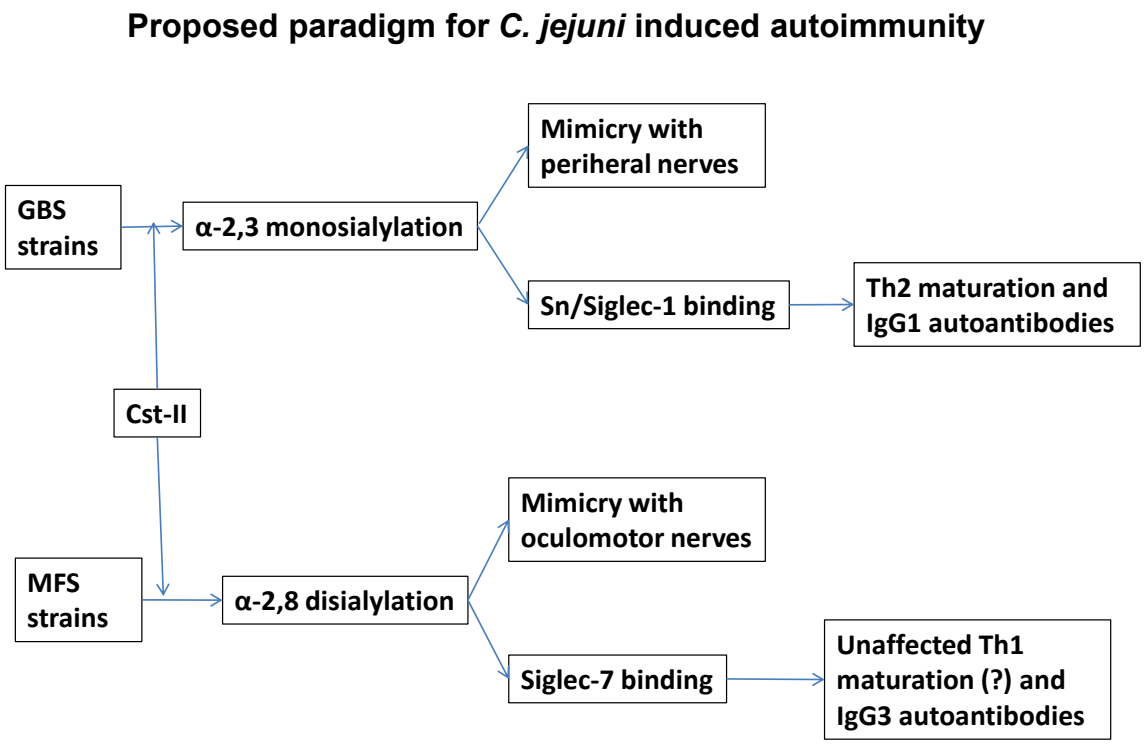

Figure 1.2. The structure of the sialylation of *C. jejuni's* LOS (endotoxin) determines the biological site of it autoreactive potential and specificity towards a Siglec receptor. Almost all sialylation is sialyltransferase (cst)-II dependent. α-2,3 monosialylation mimics gangliosides on nerves of distal limbs and enables binding to Siglec-1, which in turns facilitates *C. jejuni* uptake and T_H2 maturation. α-2,8 disialylation, on the other hand, mimics gangliosides on oculomotor nerves and facilitates binding to Siglec-7. Role of Siglec-7 in bacterial uptake and T cell maturation is not known.

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CHAPTER 2

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ABSTRACT

Campylobacter jejuni is a leading cause of foodborne enteritis that has been linked to the autoimmune neuropathy, Guillain Barré Syndrome(GBS). C57BL/6 IL-$10^{+/+}$ and congenic IL-$10^{-/-}$ mice serve as *C. jejuni* colonization and colitis models, respectively, but a mouse model for GBS is lacking. We demonstrate that IL-$10^{-/-}$ mice infected with a *C. jejuni* colitogenic human isolate had significantly upregulated Type1 and 17 but not Type2 cytokines in the colon coincident with infiltration of phagocytes, T cells and Innate Lymphoid Cells (ILC's). Both ILC and T cells participated in IFN-γ, IL-17 and IL-22 upregulation but in a time- and organ-specific manner. T cells were however necessary for colitis as mice depleted of Thy-1^{+} cells were protected while neither Rag$1^{-/-}$ nor IL-10R blocked Rag$1^{-/-}$ mice developed colitis after infection. Depleting IFN-γ, IL-17 or both significantly ameliorated colitis and drove colonic responses towards Type2 cytokine and antibody induction. In contrast, *C. jejuni* GBS patient strains induced mild colitis associated with blunted Type1/17 but enhanced Type2 responses. Moreover, the Type2 but not Type1/17 antibodies cross-reacted with peripheral nerve gangliosides demonstrating autoimmunity.

INTRODUCTION

Campylobacter jejuni is a spiral, gram-negative microaerophilic bacterium that is the second most common cause of gastroenteritis in the United States with over 2.4 million campylobacteriosis cases reported annually and many sporadic cases unreported[1]. The majority of patients ingesting *C. jejuni* in raw/undercooked meat and unpasteurized milk develop mild to severe gastroenteritis targeting the colon, which is debilitating but self-limiting within 7 to 10 days[2,3]. Histopathological manifestations include colonic crypt distortion, crypt abscesses, mucin depletion, edema of the colonic lamina propria (cLP) and significant infiltration of granulocytes and mononuclear cells[4]. Lesions resolve in most patients, but campylobacteriosis can be life threatening in immune-compromised individuals with systemic spread and multi-organ damage[5,6]. Furthermore, infection with *C. jejuni* has been linked with serious autoimmune sequelae such as development or flare-up of Inflammatory Bowel Diseases[7], Irritable Bowel Syndrome[8], Reiter's Arthritis[9] and Guillain Barré Syndrome (GBS)[10].

Campylobacter jejuni infection is the most common predisposing factor for developing the peripheral neuropathy GBS with 40% of US cases triggered by this bacterium[11,12]. Recently, the GBS disease burden was estimated at 3000 to 6000 cases per year[13]. GBS syndrome consists of at least three different subtypes including acute inflammatory demyelinating polyradiculoneuropthy (AIDP), acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN). AMAN and AMSAN are axonal subtypes associated with development of autoantibodies that target gangliosides on peripheral nerves; these autoantibodies are thought to result from molecular mimicry[10]. Indeed, the lipooligosaccharide (LOS) of *C. jejuni* isolates from GBS patients with antecedent infections have been shown to mimic gangliosides on peripheral nerves including GM1, GD1a and others[10,14,15]. When bound to peripheral nerves, these antibodies are expected to block nerve conduction by activation of complement and/or by cellular mechanisms[16]. At present, plasmapheresis and Intravenous

Immunoglobulin (IVIg) treatment are the only known treatments with beneficial effect, but are effective in only 60% of GBS patients[17]. Little is known about host immunological mechanisms that lead to self-limiting gastrointestinal (GI) disease versus severe enteritis or neurological sequelae.

Our rationale was to utilize inbred mice deficient in IL-10 to study factors mediating the development of *C. jejuni*-induced enteritis and autoimmune manifestations. Innate and adaptive immune cells, epithelial cells and fibroblasts can produce IL-10. It functions to desensitize APC's, down modulate pro-inflammatory cytokine production and suppress proliferation of mononuclear cells [18,19]. Genome wide association analysis studies discovered Single Nucleotide Polymorphisms (SNPs) flanking the *il10* gene as the most significant locus outside the MHC locus to associate with Ulcerative Colitis, a form of IBD affecting 8-24/10,000 individuals in the US and Europe. SNPs in *IL10* also show a significant association with Crohn's Disease, another form of IBD with a similar incidence[20].

We have previously established wild type (IL-$10^{+/+}$) and IL-$10^{-/-}$ mice of various genetic backgrounds as models of *C. jejuni* colonization and colitis respectively[21,22]. While the IL-$10^{+/+}$ mice of C57BL/6, C3H/HeJ and NOD background were stably colonized with *C. jejuni* (strain NCTC11168) for 35 days post oral inoculation without any adverse clinical or histopathological effects, the IL-$10^{-/-}$ mice of these three genetic backgrounds developed typhlocolitis (inflammation of cecum and colon)[22]. Thus, the enteritis model of oral inoculation of IL-$10^{-/-}$ mice with *C. jejuni* essentially involves combining the most strongly associated pathway for susceptibility to IBD (*IL*10) with the most common causative bacterium for colitis (*Campylobacter jejuni*) through the natural route of infection. The histopathological features of colitis in IL-$10^{-/-}$ mice also replicate the histopathological features of *C. jejuni* associated colitis in humans[4,21], including *C. jejuni* invasion of the colonic epithelium followed by ulceration, necrosis and neutrophilic exudates, infiltration of mononuclear and polymorphonuclear cells into the

colonic lamina propria and occasionally the muscularis, and crypt distension with abscesses and edema most prominent in the submucosa. These effects were dose independent as the dose range of 10^2 – 10^{10} CFU/mouse produced similar levels of pathology[21,23]. Furthermore, C57BL/6 IL-$10^{-/-}$ mice inoculated with *C. jejuni* strains obtained from human GBS patients were colonized, but developed little or no colitis[24]. Recent studies have revealed the importance of diet[25], Pattern Recognition Receptors (TLR 2, 4 and 9)[26] and particular signaling molecules (NFκB, mTOR, PI3K-γ)[27,28] in *C. jejuni* colonization and induced pathology in wild type or gnotobiotic IL-$10^{-/-}$ mouse models. However, the role of inflammatory mediators-particularly lymphocytes and their secreted cytokines-has not been established *in vivo*. We hypothesized that differential cytokine responses mediated by lymphocytes in the colon are responsible for *C. jejuni* induced colitis, protection from colitis and initiation of autoimmune sequelae in the IL-$10^{-/-}$ murine host. In humans, autoreactive IgG1 is the commonly associated antibody subtype after *C. jejuni* infection and enhanced IgG1 titers also associate with enhanced severity and a poor long term prognosis for GBS cases[29]. Because IgG1 isotype classically requires T_H2 mediated class switching, we further hypothesized that a *C. jejuni* specific T_H2 response generated by the GBS but not the colitogenic strains will lead to induction of autoreactive IgG1.

RESULTS

C. jejuni induced a mixed Type1 and Type17 cytokine and cellular response in IL-10$^{-/-}$ mice

All mice infected with *C. jejuni* strain 11168 were colonized, with 40-90% of them exhibiting premature mortality[24,25]. Furthermore, colon histopathology scores were significantly enhanced compared to the TSB inoculated controls[24,25]. To assess the type of inflammatory reaction(s) *C. jejuni* induces to cause colitis, C57BL/6 IL-10$^{-/-}$ (IL-10$^{-/-}$) mice were orally infected with 10^9 CFU of *C. jejuni* 11168 or Trypticase Soy Broth (TSB – sham) and observed for clinical signs of enteritis. Mice were euthanized upon showing a severe enteric disease endpoint or at day 35-post inoculation. Tissues were collected during necropsy and analyzed simultaneously. When colon protein extracts were analyzed, primary/innate cytokines (IL-1β and IL-6), Type1 (IFN-γ) and Type17 (IL-17A and IL-22), but not type 2 (IL-4) cytokines were significantly increased in the infected group (**Figure 2.1A**). Similar to the colon, plasma IFN-γ, IL-17A and IL-6 were increased significantly in the infected group (**Figure 2.1C**), while other cytokine were not detectable. The numbers of macrophages (F4/80$^+$) and T cells (CD3$^+$) infiltrating the lamina propria as quantified by IHC were significantly increased in the infected group (**Figure 2.1B**). These data suggest that *C. jejuni* infection induces a mixed Type1 and Type17 response in infected IL-10$^{-/-}$ mice. Furthermore, there were consistently high correlations between histopathology score and colonic IFN-γ, IL-1β, IL-6, IL-17A and IL-22 levels (Spearman rank correlation factor, r_s=0.7-0.9, **Suppl. Table 1**).

IgG subtype class switching in activated B cells is modulated by Type1, Type2 and Type17 cytokines. It has been long established that IFN-γ induces isotype class switching to IgG2a (or IgG2c, an isoform of IgG2a in C57BL/6 background) and IgG3, while IL-4 primarily induces IgG1 class switching[30-32]. IL-17 was shown to be necessary for autoreactive IgG2b (but not IgG1 or IgG2a) antibodies in an experimental autoimmune myasthenia gravis model [33]. Therefore,

antibody responses can inform the nature of *C. jejuni* specific cytokine responses. Consistent with the upregulation of a Type1 and Type17 but not Type2 response by colon and plasma cytokine analysis, plasma levels of *C. jejuni* specific IgG2b (published previously[24]), IgG2c and IgG3 but not IgG1 were significantly enhanced in infected mice (**Figure 2.1D**). Therefore, plasma IgG analysis reinforced our findings of a mixed Type1 and Type17 response, and also demonstrated the *C. jejuni* specificity of the response.

We further analyzed the kinetics of cellular and cytokine changes in a kinetic study wherein mice were sacrificed at days 4, 7 and 11 post *C. jejuni* inoculation. Day 11 was chosen as the end point to avoid the need for premature euthanasia in the infected mice. Histopathology scores evaluated on hematoxylin and eosin stained sections of the ileocecocolic junctions increased with time, reaching significance at day 7 and 11 (**Figure 2.2A)**. On day 7, histological changes were moderate exhibiting slight epithelial hyperplasia and diffuse or patchy infiltration of mononuclear and polymorphonuclear cells in the colonic and cecal lamina propria that sometimes extended into the muscularis. On day 11 histological changes were more drastic with marked increase in mononuclear and polymorphonuclear cells in the lamina propria and the muscularis. The epithelium was ulcerated along with crypt dysplasia and abscess formation. Neutrophilic exudates were also apparent in the lumen. The numbers of infiltrating neutrophils ($CD11b^{hi}Gr\text{-}1^{+}$) and inflammatory myeloid cells ($CD11b^{+}MHCII^{+}Gr\text{-}1^{-}$) were significantly increased at the earliest time point examined (day 4) and continued to increase until the end of experiment (day 11), as assessed by flow cytometry **(Figure 2.2B)**. *C. jejuni* specific IgG2b levels were increased significantly at days 7 and 11 post inoculation, while levels of IgG2c and IgG3 were found to be significantly enhanced on day 11. IgG1 levels (Type2 dependent) were not significantly different at any time point after inoculation (**Figure 2.2C)**. Therefore, *C. jejuni* specific IgG responses corroborate Type1 and Type17 cytokine responses. Also, as ascertained by Q-PCR for *C. jejuni* specific *gyrA* in fecal DNA (**Figure 2.2D**) the degree of colonization did

increase with time. However, it cannot be determined at this stage if increase in colonization is a cause of and/or a consequence of inflammation. Remarkably, levels of IFN-γ, IL-17A and IL-22 were increased in draining lymph nodes as early as day 4, and continued to rise until day 11 (**Figure 2.3A**). However, in the colon, an increase in IFN-γ production was not significant until day 7 and continued to rise at day 11. IL-17, IL-22, TNF-α and MCP-1 were also highest at day 11 in both organs (**Figure 2.3B**). This continuous increase in the levels of pro-inflammatory cytokines from colon and lymph nodes reflects the continuous increase in the number of colon infiltrating neutrophils and inflammatory myeloid cells.

Both Innate Lymphocytes and T cells contribute to Type1 (IFN-γ) and Type17 (IL-17 and IL-22) cytokine production in an organ, time and cell type specific manner

C. jejuni induced colitis is elicited upon infiltration of mononuclear cells and neutrophils in the colon, both in humans and IL-$10^{-/-}$ mice[4,21]. These mononuclear cells can belong to myeloid or lymphoid lineages. Lymphoid cells can further be categorized into adaptive lymphocytes—B cells ($CD3^{-}CD19^{+}$) and T cells ($CD3^{+}CD19^{-}$), and Rag-independent innate lymphocytes. Mucosal innate lymphocytes can further be divided into NK cells ($CD19^{-}NKp46^{+}$) and relatively novel Innate Lymphoid Cells (ILCs) ($Thy1^{hi}Lin^{-}$) where Lin has the following markers ($CD3^{-}CD19^{-}NKp46^{-}Gr\text{-}1^{-}MHC\text{-}II^{-}CD11b^{-}$). T cells, NK cells and ILC's have been shown to be the major producers of IFN-γ, IL-17 and IL-22 in a number of inflammatory diseases[34-36]. Therefore in a kinetic study, we evaluated the relative contribution of T cell subsets, NK cells and ILC's to the increase in production of IL-17, IL-22 and IFN-γ in the colon and mesenteric lymph nodes (MLN). In both organs, there were significant increases in the absolute number of T cells ($CD3^{+}CD19^{-}$) and NK cells at day 7 and/or 11, while ILC's were enhanced at day 4 and 7 but decreased to the level of controls by day 11 (**Figure 2.4 A-C and 2.5 A-C**). The maintenance of elevated numbers of T cells and NK cells and basal levels of ILCs was also confirmed in a

separate experiment at a later time point of day 21 (not shown). Within the T cell compartment, the proportion of $CD4^+$ T_H cells doubled in the colon by day 11, while the proportion of $\gamma\delta^+$ T cells was unchanged during the course of the experiment (**Figure 2.4A**).

The proportion of the IFN-γ^+ cells was increased, in T_H, T_C, $\gamma\delta$ T and NK cell compartments on day 11 post infection in both organs (**Figure 2.4A-B and 2.5A-B**). The proportion of IL-17^+ ILC's (ILC17) increased as early as day 4 in the MLN (**Figure 2.5C**), but decreased to control levels at day 7 while the proportion of IL-17^+ T_H cells increased on days 7 and 11 (**Figure 2.5A**). This pattern of IL-17 production initially from ILCs and subsequently by T cells correlated with the kinetics of infiltration of these cells in the colon and MLN. In contrast, the proportion of ILC17 in the colon did not increase until day 11 **(Figure 2.4C)**, at which time the total number of these cells had decreased to basal level. This demonstrates a selective organ specific activation of this cell type. Furthermore, increases in IL-22^+ T_H cells and NK cells were observed at day 7 and/or 11 in both the colon and MLN (**Figure 2.4A-B and 2.5A-B**). Therefore, a time and cell type specific Type1 and Type17 response was observed in the colon and MLNs of *C. jejuni* challenged IL-$10^{-/-}$ mice.

Thy-1^+ lymphocytes are necessary for *C. jejuni* mediated colitis

Thy-1 (CD90) is a cell surface marker expressed by both innate lymphocytes and T cells and consequently Thy-1 depleting antibody was utilized to deplete these cells in the mouse model. Infected and Thy-1 depleted IL-$10^{-/-}$ mice had significantly lower histopathology scores (**Figure 2.6A**) as well as decreased numbers of neutrophils, inflammatory myeloid cells, T cells and DC infiltration of colonic tissues (**Figure 2.6B**) as compared to mice that were infected but given control antibody injections (11168+CIgG). In fact, the infected and depleted group (11168+α-Thy-1) had numbers similar to the uninfected group (TSB+α-Thy-1). Out of ten mice in the positive control group (11168+CIgG group), two mice had to be euthanized early (day 9 and 21

p.i.) because they reached the threshold of clinical signs. Their tissues were collected during necropsy and appropriately fixed or frozen and analyzed alongside the other samples after the end of the experiment (Day 25 p.i.). Because T cells are necessary for IgG isotype class switching in B cells, Thy-1 depleted and *C. jejuni* 11168 infected mice had lower levels of *C. jejuni* specific IgG2b, IgG2c and IgG3 in the plasma as compared to the 11168+CIgG group (**Figure 2.6C**). Furthermore, colonic levels of IFN-γ, IL-17 and IL-22 were decreased to basal levels in Thy1-depleted infected mice reinforcing that Thy1^{+} cells are the major producers of these cytokines (**Figure 2.6D**). The success of depleting Thy-1 positive cells with antibody treatments was confirmed in the blood and colon by flow cytometry (**Figure S1B**).

Innate immunity is insufficient to mediate severe *C. jejuni* induced colitis

To assess if innate immunity is sufficient for *C. jejuni* mediated colitis, Rag1$^{-/-}$ mice on the C57BL/6 background were challenged with *C. jejuni* alongside C57BL/6 wt mice that serve as a known positive colonization control. While all infected mice were colonized, neither group developed any clinical signs of colitis, and did not have differences in the histopathology scores (**Figure 2.7A**) or the numbers of colonic neutrophils, inflammatory myeloid cells or dendritic cells (not shown) at day 32-35 p.i. as assessed by flow cytometry. To determine if IL-10 was responsible for lack of inflammation in Rag1$^{-/-}$ mice, they were given IL-10R blocking antibody after infection. Even after IL-10R blocking, Rag1$^{-/-}$ mice did not develop any clinical signs of colitis, and had no increase in histopathology scores (**Figure 2.7B**) or numbers of phagocytes in the colon (not shown) as compared to TSB+α-IL-10R or 11168+CIgG treated groups. C57BL/6 wt mice did develop *C. jejuni* specific antibody responses similar in nature to that of IL-10$^{-/-}$ mice with significant increases in the IgG2b, IgG2c and IgG3 isotypes and with no difference in IgG1, IgM or IgA (**Figure 2.7C**), demonstrating that the nature of *C. jejuni* antibody responses are independent of IL-10. Taken together, these data, along with the requirement of Thy-1^{+} cells to

produce colitis, show that T cells are necessary for severe *C. jejuni* induced colitis. *C. jejuni* colonization correlated with colitis induction in as much as the mouse genetic background that supports inflammation (IL-10$^{-/-}$) had higher colonization extent by the end of the experiment than the mouse genotypes/treatments that did not (BL/6wt or Rag1$^{-/-}$ or Rag1$^{-/-}$IL-10R blocked mice) (**Figure 2.7D**).

Both IFN-γ and IL-17 participate in *C. jejuni* induced colitis

To account for the relative contribution of IFN-γ and IL-17 in *C. jejuni* mediated pathology, one or both were depleted during the course of the disease by intra-peritoneal injection of neutralizing antibodies. Results showed that both of these cytokines have a positive role in mediating colitis because depleting either IFN-γ or IL-17 or both led to significant decreases in histopathology scores (**Figure 2.8A**). Furthermore, depleting IFN-γ or IL-17 significantly decreased or trended towards decreasing the extent of colonic infiltration of neutrophils, inflammatory myeloid cells and T cells, while depleting both had a significant effect (**Figure 2.8B**). The number of colon infiltrating ILC's and the proportion of CD4^{+} T_H and γδ^{+} T cells were unchanged between all groups (not shown). The number of NK cells, however decreased in the IFN-γ depleted groups, suggesting a positive feedback mechanism (**Figure 2.8B**). Notably, depleting IFN-γ and IL-17 pushed the response towards Type2 cytokine and antibody induction. IFN-γ depleted groups had decreased levels of *C. jejuni* specific plasma IgG2c but increased plasma IgG1 (**Figure 2.8C**) along with modestly increased levels of IL-4 and IL-13 in the proximal colon (**Figure 2.8D** and not shown). IL-17 depleted groups had decreased IgG2b and increased IgM (**Figure 2.8C**). The double depleted group also had increased levels of *C. jejuni*-specific IgA (**Figure 2.8C**). Thus, depleting IFN-γ and IL-17 after infection with the colitogenic

11168 prevented colitis and shifted the immune response towards Type2 cytokines and antibodies.

Infection with GBS associated *C. jejuni* strains induces Type2 immunity that is protective for colitis but leads to autoimmunity.

Having observed a Type2 response in 11168 infected IL-10$^{-/-}$ mice after IL-17 and IFN-γ neutralization, we hypothesized that the non-colitogenic GBS strains, 260.94 and HB93-13, also induce a blunted Type1/17 but enhanced Type2 response in the IL-10$^{-/-}$ mouse. Indeed, the GBS strains induced significantly blunted Type1/17 mediators (IFN-γ, IL-17, IL-22, IL-6, T-bet) (**Figure 2.9A** and not shown**)** locally in the colon and systemically in the plasma (not shown) when compared to the colitogenic strains 11168, CF93-6 (shown) and CG8421 (not shown). *C. jejuni* specific IgGb (published previously[24]), IgG2c and IgG3 responses by the GBS strains were also trending towards a decrease as compared to the colitogenic strains **(Figure 2.9B**). In contrast, the GBS strains significantly enhanced the *C. jejuni* specific Type2 mediators in the colon (IL-4, IL-13, Gata-3) **(Figure 2.9A)** and antibody (IgG1) responses **(Figure 2.9B)** in the plasma. Remarkably, Type2 but not Type1/17 autoantibodies were detected in plasma of *C. jejuni* GBS strain challenged mice. These IgG1 subclass autoantibodies reacted against the peripheral nerve gangliosides, GM1 and GD1a, in an ELISA format **(Figure 2.9C** and **Figure S2)**. Molecular mimicry along with cytokine milieu was essential for autoimmunity because only the GBS strains induced autoantibodies. Challenge infections with *C. jejuni* 11168 even in Type2 inducing conditions, i.e., after depletion of IFN-γ and IL-17 (from Figure 8C), did lead to Type2 antibody induction against the *C. jejuni* antigen, but these antibodies failed to cross-react with the nerve ganglioside autoantigens (not shown). The GBS strains colonized stably to the same extent as the colitogenic 11168 strain at the early time point of day 4 (from Figure 2D), but had lower fecal colonization at the later time points of day 11 (from Figure 2D) or day 16 (**Figure**

2.9D). Therefore, 11168 has enhanced colonization prowess that may be related to its enhanced colitogenic ability. For the GBS strain HB93-13, all stably colonized mice (8 out of 10), but none of the uncolonized mice (2 out of 10) tested positive for autoantibodies against GD1a, suggesting stable colonization as essential for inducing significant autoantibody responses.

The blunted Type1 responses elicited by the GBS strain HB93-13 were also confirmed *ex vivo* by measuring secreted cytokines from wild type mouse splenocytes challenged with colitogenic 11168 and CF93-6, or GBS associated HB93-13 and 260.94 strains using a gentamicin killing assay. 72 hours post challenge; GBS strains produced significantly less IFN-γ, TNF-α, IL-22 and IL-10 than either of the two colitogenic strains, consistent with the *in vivo* data (**Figure 10A**). However, they induced similar IL-12p40 and more IL-6 which points towards differential innate immune activation and/or T cell maturation downstream of $T_H1/T_H17/T_H2$ pathways. To evaluate IL-12p70 induction by the colitogenic/GBS strains, adherent splenocytes alone were challenged individually (**Figure 2.10B**). Consistent with enhanced IFN-γ production by colitogenic strains, higher IL-12p70 was produced by them when compared to the GBS strains. In contrast the GBS strains produced more IL-6 and IL-10. As invasion is likely an important feature of *Campylobacter*-mediated enteritis, epithelial invasion by gentamicin killing assay was performed using the young adult mouse epithelial cell line (YAMC) (**Figure 2.10C**). While the non colitogenic GBS strain 260.94 invaded to a slightly lower extent as the colitogenic 11168, the other GBS strain HB93-13 invaded significantly more. Therefore, different outcomes to infection by colitogenic versus GBS strains cannot be explained by differences in epithelial cell invasion but rather by the Type1/17 or Type2 induction by individual strains.

DISCUSSION

In this study, we show that *C. jejuni* 11168—a known colitogenic strain—induced a mixed Type1 and 17 cytokine response in the colon and the draining mesenteric lymph nodes of the C57BL/6 IL-10$^{-/-}$ mouse colitis disease model. This is consistent with the observations made by Edwards et al, that demonstrated the induction of Type1 and 17 responses from *C. jejuni* 11168H challenged human colon explants and peripheral blood derived cells *ex vivo*, along with a novel role for IL-17 in reducing intracellular survival in intestinal epithelial cells[37]. In our study, these cytokines orchestrated infiltration of neutrophils, macrophages, T cells, NK cells and ILC's in the colonic mucosa and sub-mucosa that closely resembled lesions seen in patients with *Campylobacter*-induced enteritis. In our model, ILC's were already increased significantly at day 4, peaked at day 7 and decreased to background level by day 11. Increases in T cells and NK cells did not reach significance until day 7 or 11 respectively. By day 4 post infection, the amount of secreted IFN-γ, IL-17 and IL-22 from the MLN's was already increased 2-3 fold, which indicated innate sources for these cytokines since this response is too early for adaptive immunity to arise. We also found that innate lymphocytes as well as T cells participated in the upregulation of IFN-γ, IL-17 and IL-22, but in a time and organ-specific manner. Mirroring the kinetics of their infiltration in the colon, ILC's upregulated IL-17 as early as day 4 in the lymph nodes that switched to an IFN-γ response by day 7 at which time IFN-γ^+ and IL-17$^+$ cells were also increased in the T_H and $\gamma\delta$ cell compartments. Because only adaptive immunity can be antigen specific, it is tempting to speculate that early upregulation of ILC's followed by their downregulation is a mechanism of ensuring an acute response to infection whilst preventing non-specific inflammatory responses that predispose to autoimmunity. However, in the colon, the cytokine production profile from the ILC's over time is similar to that of T cells, and further studies are needed to determine their functional discrepancy in the two organs. Neutrophil and inflammatory myeloid cell numbers in the colon were enhanced significantly at day 4 and

continued to increase until the later time points (e.g. day 11) post infection. This was another reflection of the continuous significant increases in pro-inflammatory factors from the colon and MLN's by a combination of non-specific ILC's and adaptive T cells that maintain and specify the responses.

It should be recognized that although ILCs participated in *C. jejuni* colitis, the innate immune system was unable to induce full blown colitis. Neither Rag1$^{-/-}$ nor IL-10R blocked Rag1$^{-/-}$ mice developed colitis after *C. jejuni* inoculation. However, Thy-1^{+} lymphocytes were necessary for the colitogenic response as significantly decreased pathology was observed in adaptive immune competent IL-10$^{-/-}$ mice that were depleted for Thy-1^{+} cells. These findings demonstrated that T cells were necessary for *C. jejuni* to elicit colitis. In contrast, Jobin et al., have very recently shown that germ-free Rag2 IL-10 double KO mice of the 129/SvEv background develop colitis after *C. jejuni* 11168 inoculation[28]. The difference in outcomes may be attributable to differences in the host genetics (C57BL/6 v/s 129/SvEv) and/or protective effect of host gut microbiota on degree of colonization and immune stimulation. Jobin et al have also recently shown that depletion of CD4^{+} cells does not affect the pathology in *C. jejuni* infected gnotobiotic C57BL/6 IL-10$^{-/-}$ mice[27]. This observation is complicated by the fact that in addition to immature thymocytes and mature T_H cells, CD4 is also expressed by macrophages and therefore CD4 depletion cannot be used as a specific tool for T_H cell depletion. Nevertheless it does suggest a pro-inflammatory role of other lymphocytic populations in *C. jejuni* colitis and, in our model, we indeed observed the increase in numbers of IFN-γ^{+} and IL-17^{+} cells in non-T_H cell compartments, including $\gamma\delta$ and CD4^{-}(CD8^{+}) cells. ILC's can also be further divided into Lymphoid Tissue Inducer cells (Lin^{-}Thy1^{hi}CD4^{+}) and LTi-like cells (Lin^{-}Thy-1^{hi}Sca-1^{+}) that have been shown to perform both pro- and anti-inflammatory functions[38-40]. Early IL-22 production from LTi's in the colon of *Citrobacter rodentium* infected BL/6 IL-10$^{+/+}$ mice was shown to be protective[41] while the production of IFN-γ and IL-17 from LTi-like cells in the colons of

Helicobacter hepaticus infected Rag.129 mice was shown to be pro-inflammatory[42]. Further studies will be necessary to determine the exact role of these subsets in *C. jejuni* mediated colon pathology.

We have shown that enhanced IFN-γ and IL-17 cytokine secretion induces lesions in *C. jejuni* induced colitis. Neutralizing either or both of these cytokines prevented the overt histopathological changes associated with the disease, including the infiltration of neutrophils, inflammatory myeloid cells and T cells into the colon. It was notable that neutralizing these cytokines shifted the immune response towards Type2 cytokines (IL-4 and IL-13) and antibody responses (IgG1), alongside increases in *C. jejuni* specific IgM. The non-colitogenic GBS strains also induced similarly enhanced Type2 and blunted Type1/17 responses, suggesting that *C. jejuni* mediated colitis can be explained by the balance between Type1/17 and Type2 responses. This may also suggest the mechanism underlying the development of autoantibodies and autoimmune diseases after treatment with TNF-α blockers in humans[43-45]. It remains to be seen if blocking the Type2 cytokines in the mouse model could lead to induction of colitis thus diverting the expected autoimmune outcomes after challenge with the non-colitogenic GBS patient strains.

GBS, especially the AMAN form, has most commonly been associated with antecedent *C. jejuni* infection[17]. It has also been published that antecedent gastrointestinal symptoms are only observed in 50% of GBS patients that are serologically positive for *C. jejuni*[46,47]. This suggests that there is no clear cut association between extent of colitis and GBS in humans and at least half of the human GBS cases mirror our mouse model in that infection with GBS strains leads to autoantibodies without antecedent diarrhea. Furthermore, identical to what has been observed in human GBS patients, autoreactive antibodies exclusively of the IgG1 isotype were found in mice infected with the GBS strains but not the colitogenic strains. Molecular mimicry and not just the cytokine milieu is essential for autoantibody development because the colitogenic non-GBS

strain *C. jejuni* 11168 failed to induce autoantibodies even when the immune response was biased towards Type2 after depleting IFN-γ and IL-17. The non-colitogenic GBS strains were colonized to a similar extent as the colitogenic 11168 strain, but only at the early time point (day 4). Colonization with *C. jejuni* 11168 was higher at later time points (day 11/16). Therefore 11168 has enhanced colonization prowess that may be related to its enhanced colitogenic ability and Type1/17 over Type2 maturation. However there is still proof of strain-intrinsic but colonization-independent bias of Type1/17 maturation from ex vivo experiments. Stable colonization was nevertheless necessary to develop autoantibodies because 2 of mice infected with the GBS strain HB93-13 strain that were not colonized failed to develop a significant autoantibody level when compared to their 8 colonized group mates. Consistent with our *in vivo* findings, Bax *et al* have shown *in vitro* that LOS purified from GBS strains induced blunted T_H1 and enhanced T_H2 response when compared to LOS from non-GBS strains[48]. The T_H2 response depended on the nature of sialylation of the LOS and correlated with binding to Sialoadhesin, a DC and macrophage cell surface receptor. The role of Sialoadhesin in *C. jejuni* phagocytosis and primary interferon induction has also been established[49,50]. These results suggest that surface modification of the *C. jejuni* GBS strains play an important role in development of autoimmunity, but more work is needed to determine the relevant ligand-receptor and downstream interactions in this model. Future studies are underway to determine the pathological and histological consequences of developing these autoantibodies, and the innate immune mechanism of differential T cell responses by colitogenic versus GBS strains. It is imperative to understand the host factors and bacterial ligands that are responsible for GBS and IBD in order to develop the needed novel therapeutic interventions for these conditions.

MATERIALS AND METHODS

Mice. C57BL/6J wild type (WT), BL/6.129P2-IL-10^{tm1Cgn}/J (IL-10$^{-/-}$) and B6.129S7-Rag1^{tm1Mom}/J (Rag1$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, MA) and bred in a specific pathogen free breeding colony. Mice at 8 – 12 weeks of age were orally inoculated with Tryptone Soy Broth (TSB – vehicle control) or 10^9 CFU of *C. jejuni* 11168 in 0.2ml TSB, as described previously[21]. All animal protocols were approved by the Michigan State University Institutional Animal Care & Use Committee. Colonization by *C. jejuni* was confirmed and Hematoxylin and eosin stained sections of the ileocecocolic junction were scored as described previously[21].

Antibodies for *in-vivo* neutralization. Anti-IFN-γ (XMG1.2), α-IL-10R (1B1.3A) and α-Thy-1 (30H12) was purchased from Bio-X-Cell, and α-IL-17 (ebioMM17F3) was purchased from eBiosciences. XMG1.2 and ebioMM17F3 injected with 500µg and 200µg i.p. respectively per mouse twice weekly, starting on the day of inoculation. α-IL-10R (1B1.3A) was injected with 1mg/mouse i.p. at the time of inoculation, and weekly thereafter. α-Thy-1.2 (30H12) was injected with 250µg/mouse i.p. twice a week, starting 3 days before inoculation. Rat or mouse IgG (Jackson ImmunoResearch) were used as controls where appropriate.

Immunohistochemical analysis of mononuclear cells. 5 μm serial sections were cut, deparaffinized in two changes of xylene and rehydrated in graded alcohol series. Slides were incubated in 1% H_20_2 in TBS and boiled for 15 minutes in citrate buffer (Vector laboratories) for CD3 or incubated at 37°C in Proteinase K (Genemed biotechnologies) for F4/80. Thereafter, sections were blocked for 1 – 3 hours with 1% BSA + 1.5% Goat serum in TBS and then incubated with respective primary antibody (1:500 for CD3ε, 1:50 for F4/80) at 4°C overnight.

Slides were then stained with the Vectastain ABC kit (Vector laboratories) for CD3 or rat on mouse HRP polymer kit (Biocare medical) for F4/80 according to the manufacturer's instructions. Non-specific IgG (Vector laboratories) for CD3 or irrelevant isotype control (RTK4530, Biolegend) for F4/80 were used as negative controls. From each section, ten non–overlapping 400x magnification fields were chosen randomly, photographed and positively staining cells were counted using the ImageJ cell counter (N.I.H., Bethesda, MD).

Preparation of colonic lamina propria leukocytes. Lamina propria leukocytes were isolated as previously described [51,52]. Briefly, for removal of epithelial cells, the colon was washed; cut into small pieces, and then the pieces were incubated with calcium- and magnesium-free HBSS supplemented with 5% FBS and 5 mM EDTA (Sigma-Aldrich) at 100 rpm at 37°C for 30 min. The tissues were then incubated with RPMI 1640 containing 10% FBS and 0.5 mg/ml collagenase type IV and 0.5mg/ml DNaseI (Sigma-Aldrich) for 1 hour at 37°C with shaking at 150 rpm. The liberated cells were collected by passage through a 70 μm nylon mesh. The isolated cells were separated on a 40/80% discontinuous Percoll gradient (GE Bioscience).

Flow cytometry. The following monoclonal antibodies (eBiosciences or Biolegend) were used: anti-CD3 (clone 145–2C11), anti-CD4 (clone RM 4-5), CD8 (clone 53-67); anti-TCR γδ(clone GL3), CD19 (clone 1D3); anti NKp46 (clone 29A1.4); anti-CD11b (clone M1/70), anti-Gr1 (clone-RB6-8C5), anti-CD90.2 (clone53-2.1) and anti-CD16/CD32 (clone 2.4G2). The cells were preincubated for 20 minutes with anti-CD16/CD32 to block Fc receptors then washed and labeled with appropriate mixture of antibodies or isotype matched controls for 30 minutes, centrifuged at 650g, and resuspended in FACS buffer. To exclude dead/dying leukocytes were gated according to forward and side scatter. For intracellular cytokine staining, cells were restimulated for 4 hours with cell stimulation cocktail (eBioscience) and fixed and permeabilized

using fixation and permeabilization solution (eBioscience). All cells were analyzed on a LSRII flow cytometer (BD Biosciences) using FlowJo software (Tree Star).

Enzyme-linked Immunosorbent Assay. All cytokines were measured according to the manufacturer's protocol (Ready-Set-Go ELISA kits, eBioscience). To prepare protein extracts from colon, flash frozen tissue was homogenized with 0.5 ml HBSS 1% Triton X-100 with the mini protease inhibitor cocktail (Roche). The homogenates were centrifuged at 12,000*g* for 30 min at 4°C, and the supernatants were collected for ELISA. For ex vivo culture, RBC-depleted MLN cells at $5X10^6$ cells/ml or 0.5cm of proximal colon in 0.5ml of RPMI 1640 supplemented with 10% FBS and 100µg/ml penicillin G, 10µg/ml of streptomycin and gentamicin and 5µg/ml amphotericin B were incubated at 37°C for 48 hours. Supernatants were clarified and stored at -70°C. *C. jejuni* specific antibody ELISA has been described previously[21]. The following plasma dilutions were used: 1:10 for IgA, 1:50 for IgG1, IgG2c and IgG3, 1:100 for IgM and 1:400 for IgG2b. Only absorbance values more than 2 SD away from mean of negative control were considered positive. GM1 (Sigma) and GD1a (USBio) were used at 2 and 20µg/ml respectively and handled similarly.

Quantitative and reverse transcriptase PCR. RNA was extracted from flash frozen proximal colon samples using RNaeasy plus extraction kit (Qiagen). cDNA was subsequently synthesized by GoScript Reverse Transcriptase kit (Promega). Real-time PCR was performed for the target and *hprt* genes using Quantifast primers and probe assay (Qiagen) in ABI 7500 PCR machine (Applied Biosystems) or iQ5 iCycler (Bio-Rad). For *C. jejuni* DNA estimation in feces, DNA was extracted as described previously[21]. 50ng of fecal DNA was used in Q-PCR reaction with *C. jejuni* specific *gyr*A primers[53] and iQ SYBR green supermix. *C. jejuni* DNA in fecal DNA was estimated by running a parallel standard curve of pure *C. jejuni* DNA.

Splenocyte challenge by gentamicin killing assay. RBC depleted splenocytes from naïve C57BL/6 wt mouse (10^6 cells/ml) were plated in antibiotic free R10 medium and challenged with indicated *C. jejuni* strains at multiplicity of infection (M.O.I) of 0.1, 1 or 10. One hour after infection, gentamicin (250µg/ml) was added to all the wells to kill extracellular bacteria. Supernatants were collected after 72 hours for cytokine measurement by ELISA. To obtain adherent cells, splenocytes were plated at 10^7 cells/ml for 90 minutes upon which the non-adherent cells were washed off.

Epithelial challenge by gentamicin killing assay. $1.5X10^6$ Young Adult Mouse Colon (YAMC) cells were grown in permissive media (RPMI 1640 with 5% FBS, ITS and 5 IU/ml IFN-γ) in 24 wells plates at 33°C and 5% CO_2. At 80% confluence media is changed to ITS and IFN-γ free RPMI 1640 with 5% FBS and incubated for 18 hours at 37°C. *C. jejuni* is then added at M.O.I of 100 followed by two hour incubation and three washed in PBS. For measuring invasion cells are further incubated for one hour with 250 µg/ml gentamicin, washed in PBS, lysed in 0.1% Triton X-100 and released bacteria is enumerated by serial dilution. For cytokine measurement cells are incubated for further 24 hours in media containing gentamicin and supernatant is clarified. Sensitivity of all strains to this concentration of gentamicin was also confirmed.

Statistical analysis. All statistical tests were performed in Prism 6.0 (GraphPad Software) and described in figure legends. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns not significant.

APPENDIX

FIGURE LEGENDS

Figure 2.1. IL-10$^{-/-}$ mice were inoculated with *C. jejuni* 11168 or TSB and euthanized at humane end point or 35 days post inoculation. **A)** Colon homogenate ELISA. IL-12p70, TNF-α and IL-17F were only detected in colons of 20% of the mice and are not shown. **B)** Colon CD3 and F4/80 IHC, at the time of necropsy, the ileocecocolic junction (junction of ileum, cecum and colon) was fixed in formalin and embedded in paraffin. Its sections were stained for CD3 or F4/80 and the number of positively staining cells in the colonic lamina propria were quantified in 10 random high-powered fields. **C)** Plasma cytokine ELISA. Other cytokines from Figure 1a were not detectable in majority of mice from infected/control group, and are not shown. **D)** Plasma *C. jejuni* specific antibody analysis. Anti *C. jejuni* IgM and IgA was detectable but did not change significantly and is not shown. Data represented is one of three independent experiments with 8 – 10 mice per group. Bar indicates the median, Mann Whitney U test.

Figure 2.2. IL-10$^{-/-}$ mice were inoculated with *C. jejuni* or TSB, and sacrificed at indicated days post inoculation. At the time of necropsy, the ileocecocolic junction (junction of ileum, cecum and colon) was fixed in formalin and embedded in paraffin. **A)** H&E sections were scored in blinded fashion. **B)** Colon leukocytes were prepared and analyzed for indicated populations by flow cytometry**.** Dead/dying cells were excluded on the basis of forward and side scatter. All cells were gated on CD19- gate. **C)** Mice were bled at time of necropsy, and serum was analyzed for indicated antibody subtypes reactive against *C. jejuni* antigen. Anti-*C. jejuni* IgM and IgA was detectable but did not change significantly for any time point and is not shown. **D)** *C. jejuni* colonization was measured in the feces at the time of necropsy by Q-PCR. **E)** Representative photomicrographs from H&E stained proximal colon sections. Data is represented as mean$\pm$s.e.m; two independent experiments; 5-8 mice per group per time point.

Infected group for each time point was compared with control group pooled for each time points by Kruskal-Wallis test and Dunn's post test.

Figure 2.3. IL-10$^{-/-}$ mice were inoculated with *C. jejuni* (open circles) or TSB (filled circles) and euthanized at indicated day post inoculation. **A)** 5 mm of proximal colon or **B)** 2.5X10^6 RBC-depleted cells from MLN were incubated in 0.5ml tissue culture media for 48 hours. Supernatant media was clarified and analyzed for indicated cytokines, as well as IL-1β, IL-4, IL-23 and IL-13, which were not detectable. IL-12p70 was detectable from MLN but not the colon. Data is represented as mean±s.e.m; two independent experiments; 5-8 mice per group per time point. Infected group for each time point was compared with control group pooled for each time points by Kruskal-Wallis test and Dunn's post test.

Figure 2.4. IL-10$^{-/-}$ mice were inoculated with TSB or *C. jejuni* and sacrificed at indicated day post inoculation. Single cell suspension was prepared from colon. T cells, ILCs and NK cells were analyzed for IFN-γ and IL-17 or IFN-γ and IL-22 production by intracellular cytokine staining and flow cytometry following brief restimulation with PMA and ionomycin in presence of brefeldinA. Double positives were relatively rare and data presented here represents total positive. Dead/dying cells were excluded on the basis of forward and side scatter. Data is represented as mean±s.e.m; two independent experiments with 5 - 8 mice per group per time point. Infected group for each time point was compared with control group pooled for each time points by Kruskal-Wallis test and Dunn's post test. Proportion of IL-17+ or IL-22+ cells from CD3+CD4-cells (which are likely CD8+) was not significantly different at any time point, and is not shown.

Figure 2.5. IL-10$^{-/-}$ mice were inoculated with TSB or *C. jejuni* and sacrificed at indicated day post inoculation. Single cell suspension was prepared from MLN. T cells, ILCs and NK cells were analyzed for IFN-γ, IL-17 or IL-22 production by intracellular cytokine staining and flow

cytometry following brief restimulation with PMA and ionomycin in presence of brefeldinA. Data is represented as mean±s.e.m; two independent experiments with 5 - 8 mice per group per time point. Infected group for each time point was compared with control group pooled for each time points by Mann Whitney U test. Proportion of IL-17+ or IL-22+ cells from CD3+CD4-cells (which are likely CD8+) was not significantly different at any time point, and is not shown.

Figure 2.6. IL-10$^{-/-}$ mice were orally inoculated with *C. jejuni* or TSB and injected with α-Thy-1 or CIgG twice weekly starting 3 days before inoculation, and sacrificed at Day 23-24 post inoculation. At the time of necropsy, the ileocecocolic junction (junction of ileum, cecum and colon) was fixed in formalin and embedded in paraffin. **A)** H&E sections were scored in blinded fashion. **B)** Colon leukocytes were prepared and analyzed for indicated populations by flow cytometry. **C)** Mice were bled at time of necropsy, and plasma was analyzed for indicated antibody subtypes reactive against *C. jejuni* antigen. **D)** 5mm of proximal colon was washed and incubated in 0.5ml tissue culture media for 48 hours. Supernatant media was clarified and analyzed for indicated cytokines, as well as other cytokines from Figure.1, which were not significantly different. Bar indicates the median, n=10 mice per group. Kruskal-Wallis test followed by Dunn's post test.

Figure 2.7. A) C57BL/6 wt or Rag1$^{-/-}$ mice were orally inoculated with *C. jejuni* or TSB and sacrificed at Day 32-34 post inoculation. **B)** Rag1$^{-/-}$ mice were injected with α-IL-10R or CIgG after infection. At the time of necropsy, the ileocecocolic junction (junction of ileum, cecum and colon) was fixed in formalin and embedded in paraffin. H&E sections were scored in blinded fashion. **C)** Mice were bled at time of necropsy, and plasma was analyzed for indicated antibody subtypes reactive against *C. jejuni* antigen. **D)** *C. jejuni* colonization was measured in the feces at the time of necropsy by Q-PCR. Data is represented as median with 9-10 mice per group. Kruskal-Wallis test followed by Dunn's post test.

Figure 2.8. IL-10$^{-/-}$ mice were orally inoculated with *C. jejuni* and sacrificed at Day 21-22 post inoculation. IFN-γ, IL-17 or both were neutralized by i.p. injection of neutralizing antibodies twice a week for three weeks, starting on the day of inoculation. **A)** H&E sections were scored in blinded fashion. **B)** Colon leukocytes were prepared and analyzed for indicated populations by flow cytometry. **C)** Mice were bled at time of necropsy, and plasma was analyzed for indicated antibody subtypes reactive against *C. jejuni* antigen. **D)** Level of IL-4 was measured in the proximal colon homogenate by ELISA. Data is represented as medians or mean±s.e.m., with 10 mice per group. Kruskal-Wallis test followed by Dunn's post test. Only comparisons with CIgG group are shown.

Figure 2.9. IL-10$^{-/-}$ mice were orally inoculated with given *C. jejuni* strains and sacrificed at Day 35 post inoculation, or earlier if requiring euthanasia. **A)** Expression of indicated mediators in the colon by real time PCR. **B)** Antibody subtypes reactive against *C. jejuni* antigen. **C)** Peripheral nerve gangliosides autoantigens by ELISA. **D)** *C. jejuni* colonization was measured in the feces at the indicated days post inoculation for the GBS strains and the time of necropsy for all strains by Q-PCR. Data is represented as median or mean±s.e.m., with 10 mice per group. Kruskal-Wallis test followed by Dunn's post test. No other comparisons were significant.

Figure 2.10. Whole splenocytes **A)** or adherent splenocytes **B)** from naïve C57BL/6 wt mouse were challenged with indicated strains and M.O.I's in antibiotic free R10 media. Gentamicin was added after one hour, and cultures were further incubated for 72 hours for whole or 24 hours for adherent splenocytes, upon which supernatants were clarified and analyzed for indicated cytokines by ELISA. IL-17, IL-4 and IL-23 were not detectable with whole splenocytes while IL-12p70 was only detectable with adherent cell culture. **C)** Invasion and IL-6 elicitation by the indicated strains was assessed on YAMC cells at M.O.I of 100. Data represents mean±s.e.m. of three wells and analyzed by two-way ANOVA for splenocytes or one way ANOVA for epithelial

cells followed by Bonferonni's post test. Only comparisons with respect to 11168 for splenocytes or HB93-13 for epithelial cells are shown. One of three independent replicates is shown.

Figure 2.1. Endpoint cytokine, antibody and colonic cellular infiltration analysis

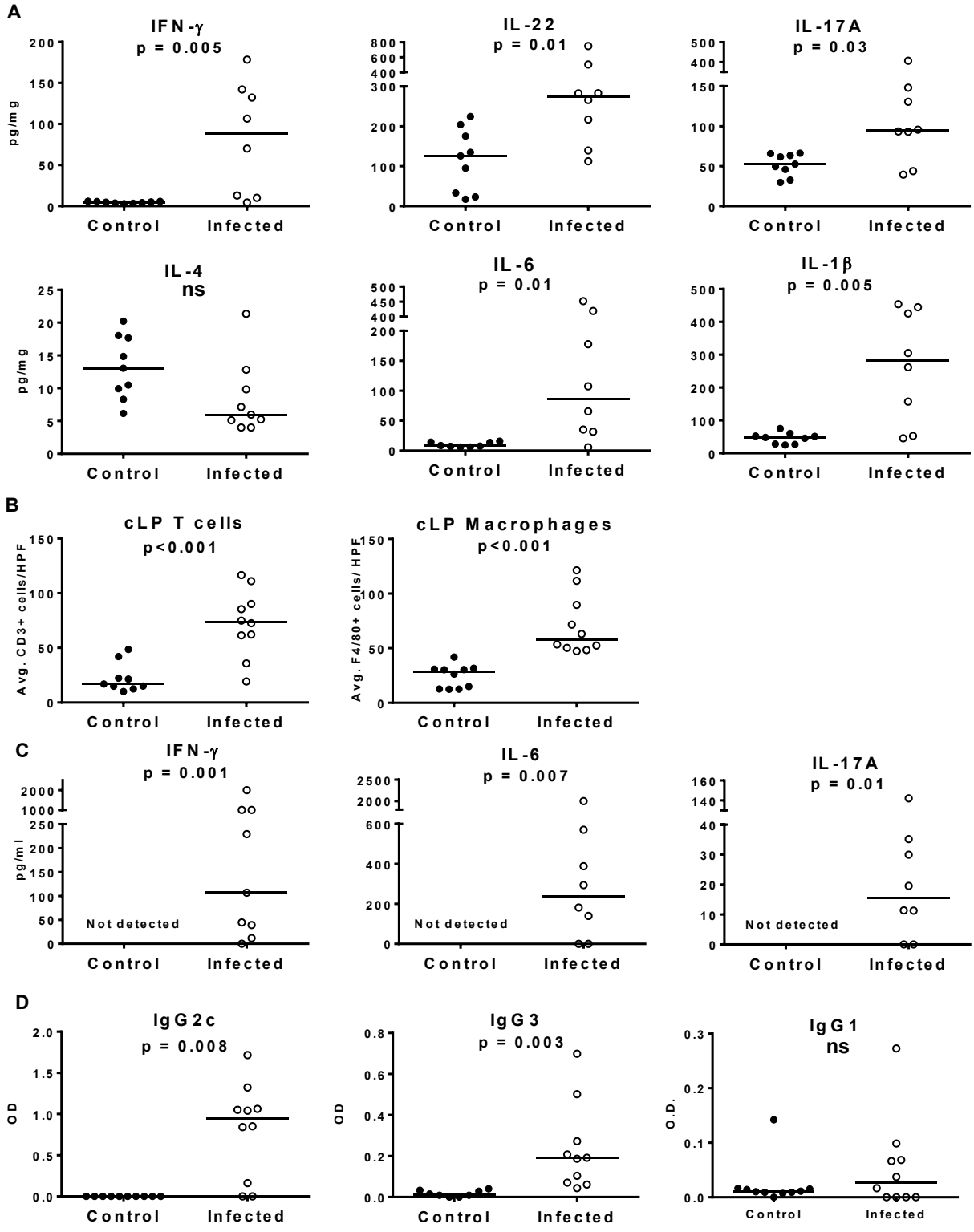

Figure 2.2. Temporal analysis of colon leukocyte and *C. jejuni* specific plasma antibody.

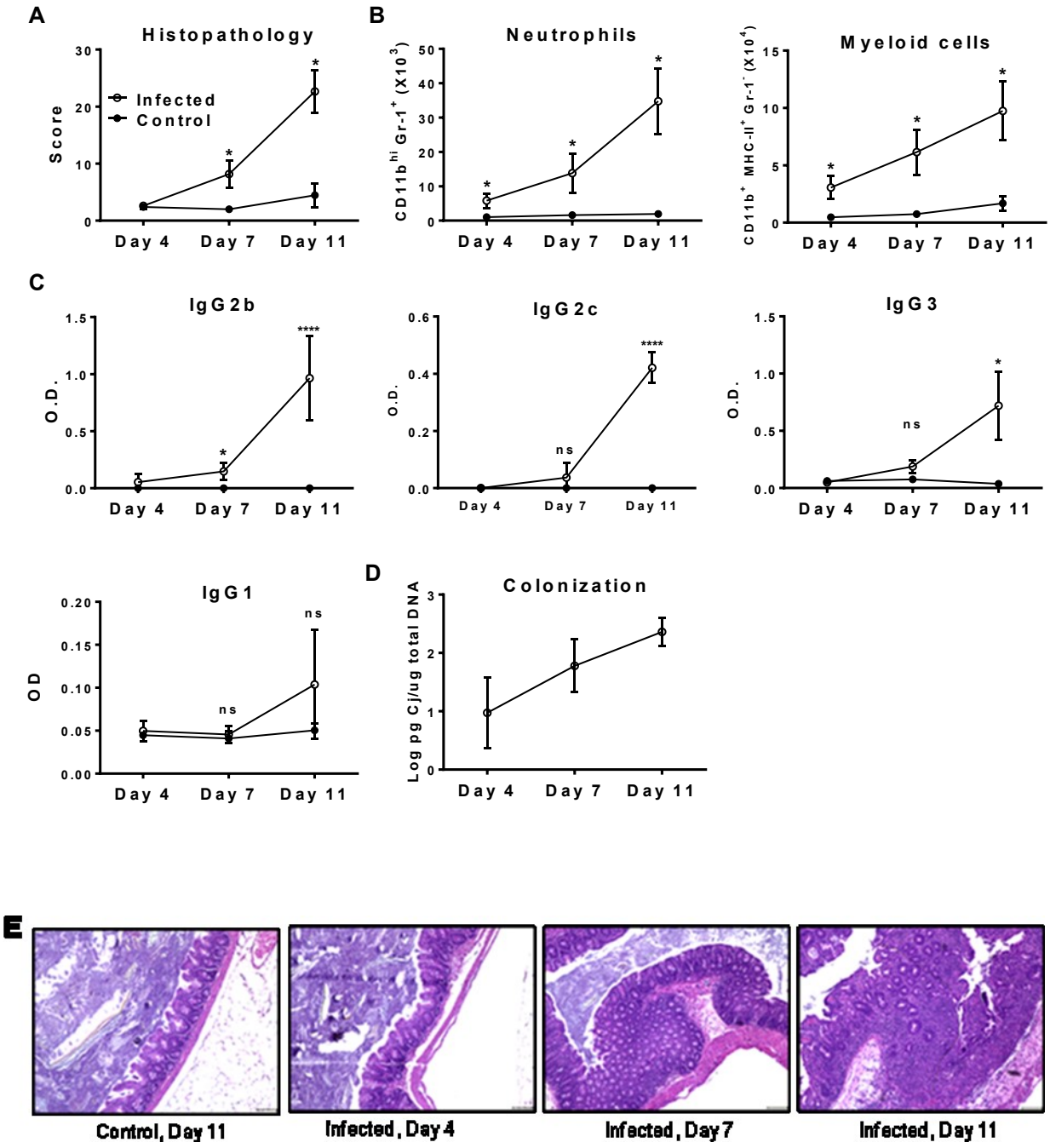

Figure 2.3. Temporal cytokine analysis of colon and MLN.

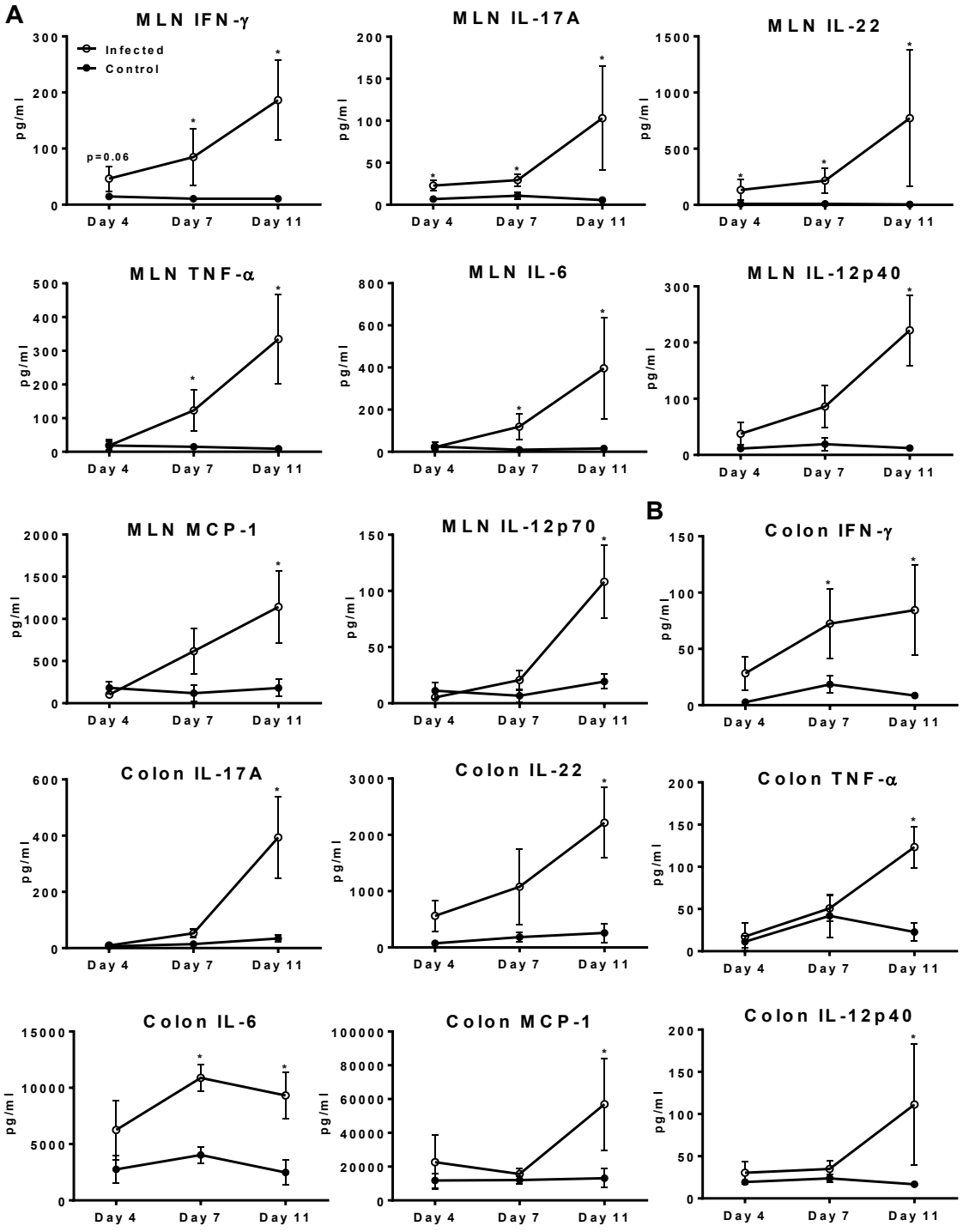

Figure 2.4. Colon cytokine-lymphocyte subset analysis

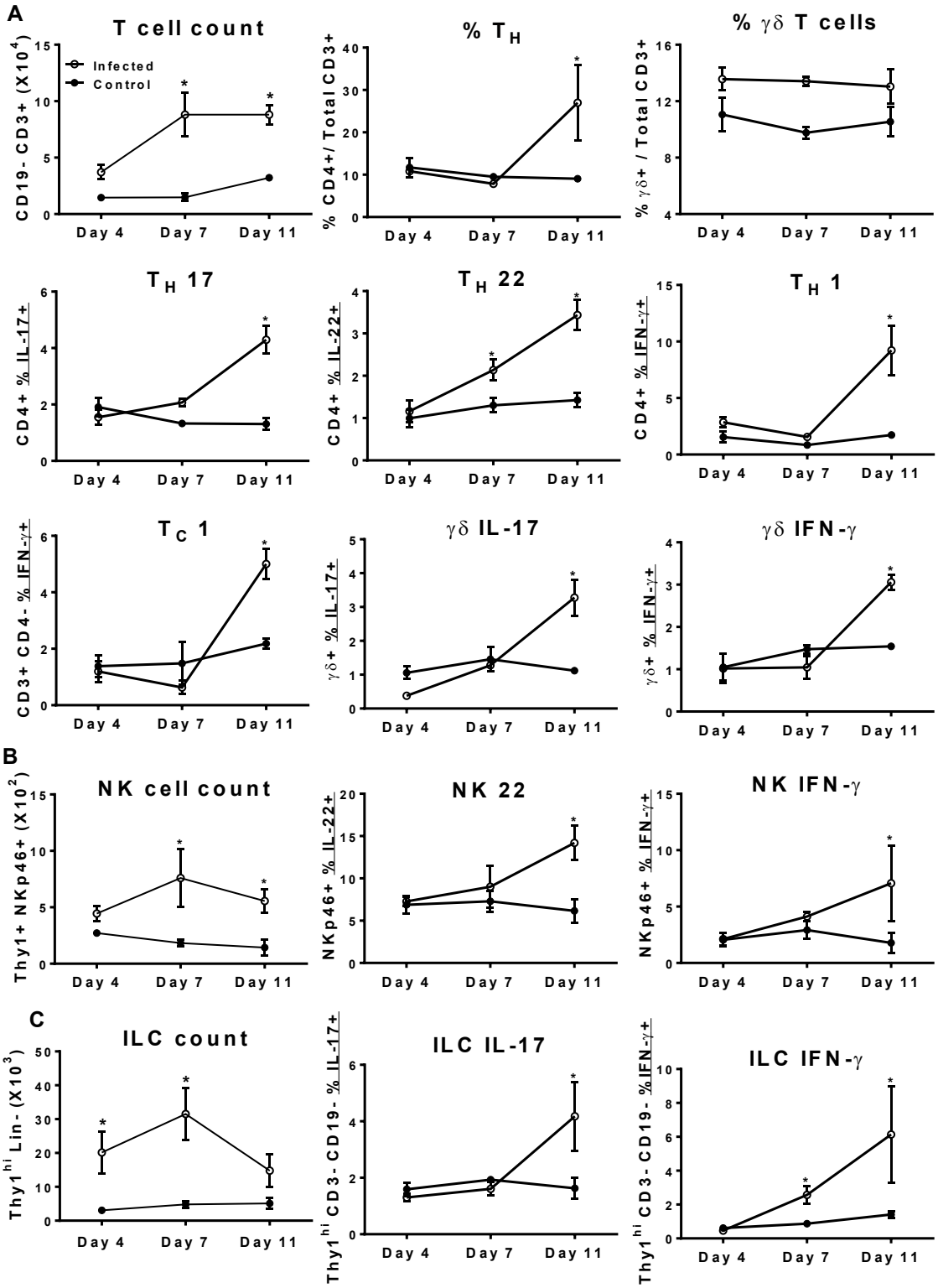

Figure 2.5. Mesenteric Lymph Node cytokine-lymphocyte subset analysis

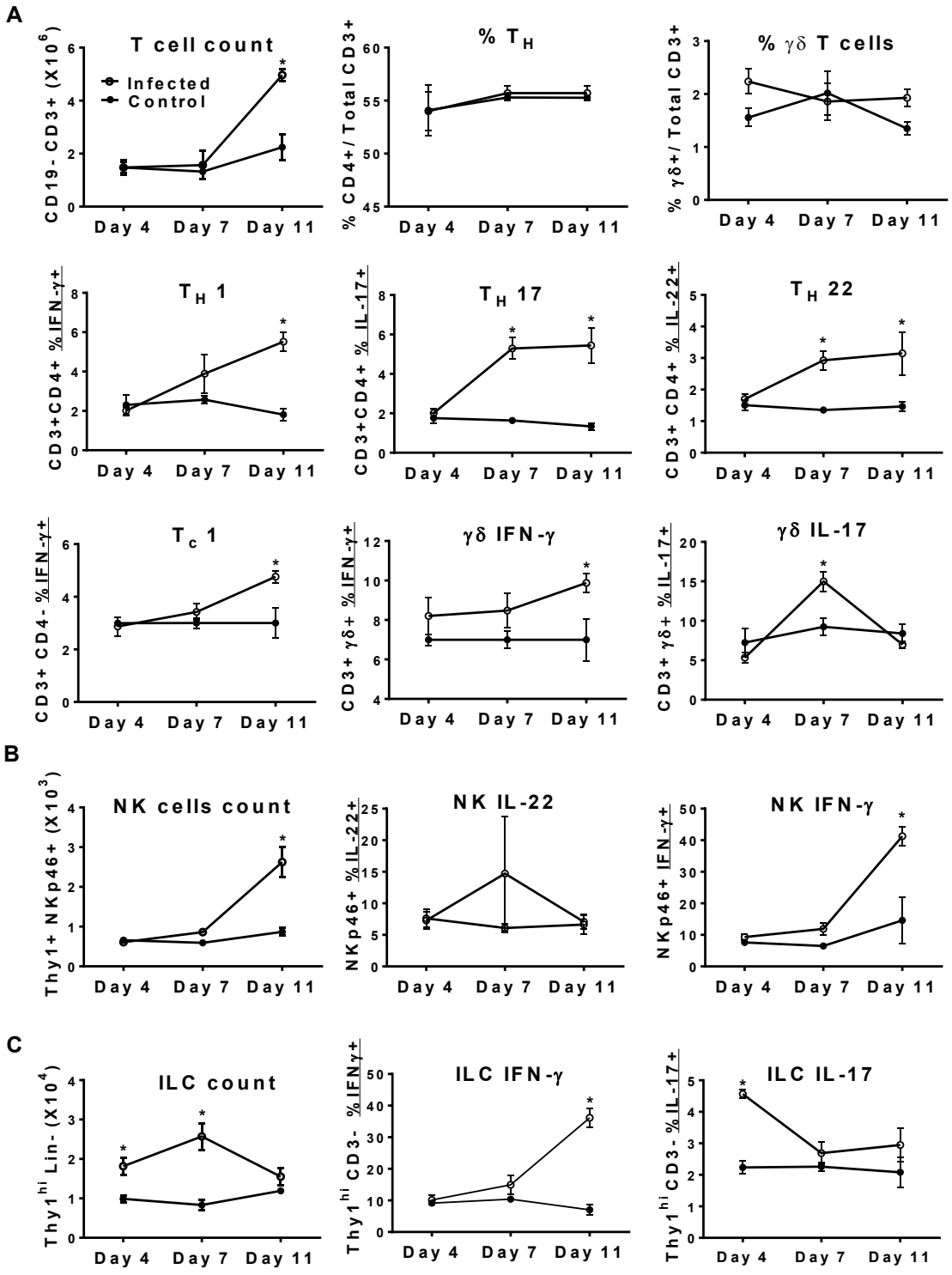

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Figure 2.6. Role of Thy-1^{+} lymphocytes in *C. jejuni* mediated colitis

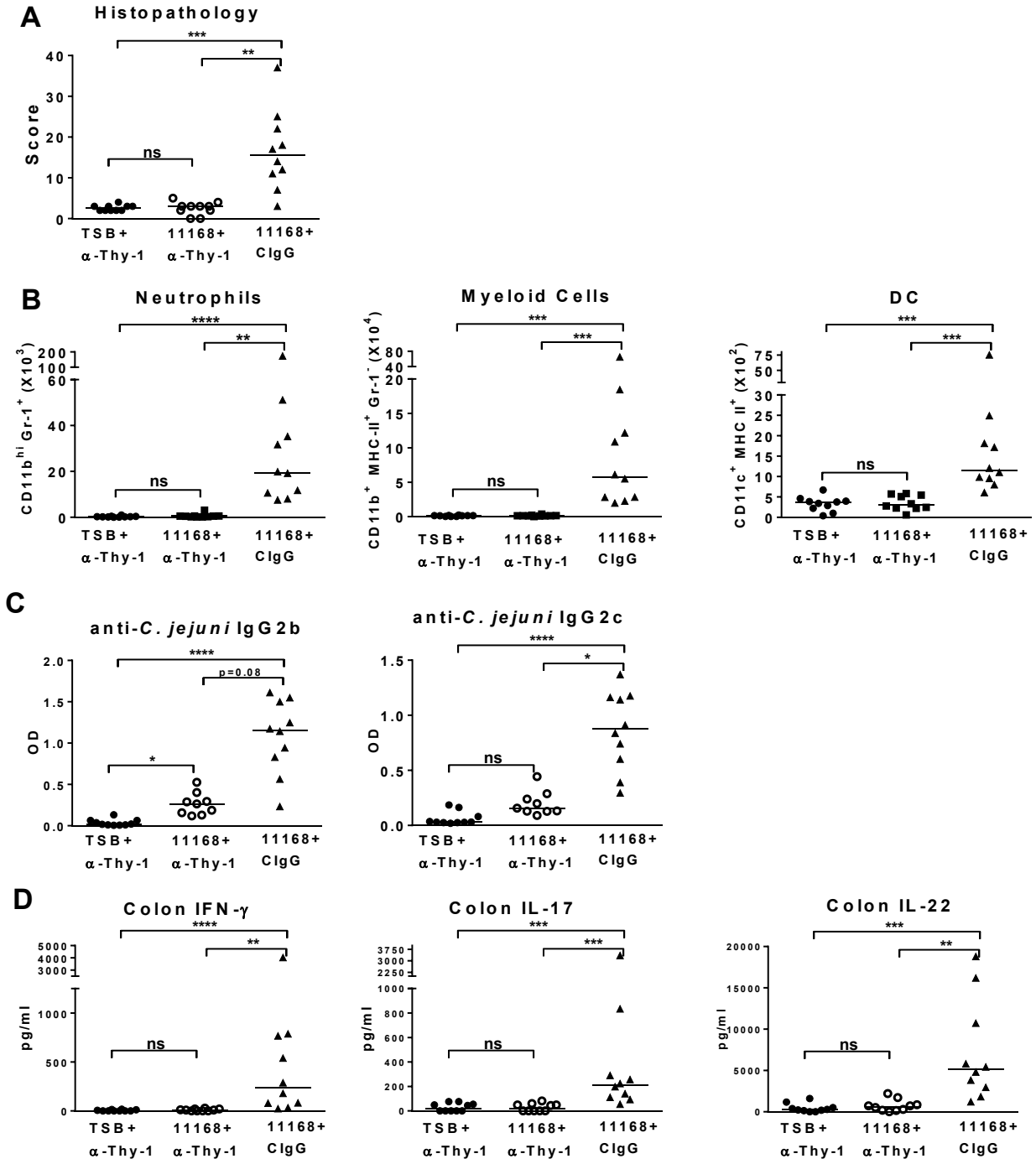


Figure 2.7. Innate immunity is insufficient to induce colitis after *C. jejuni* infection

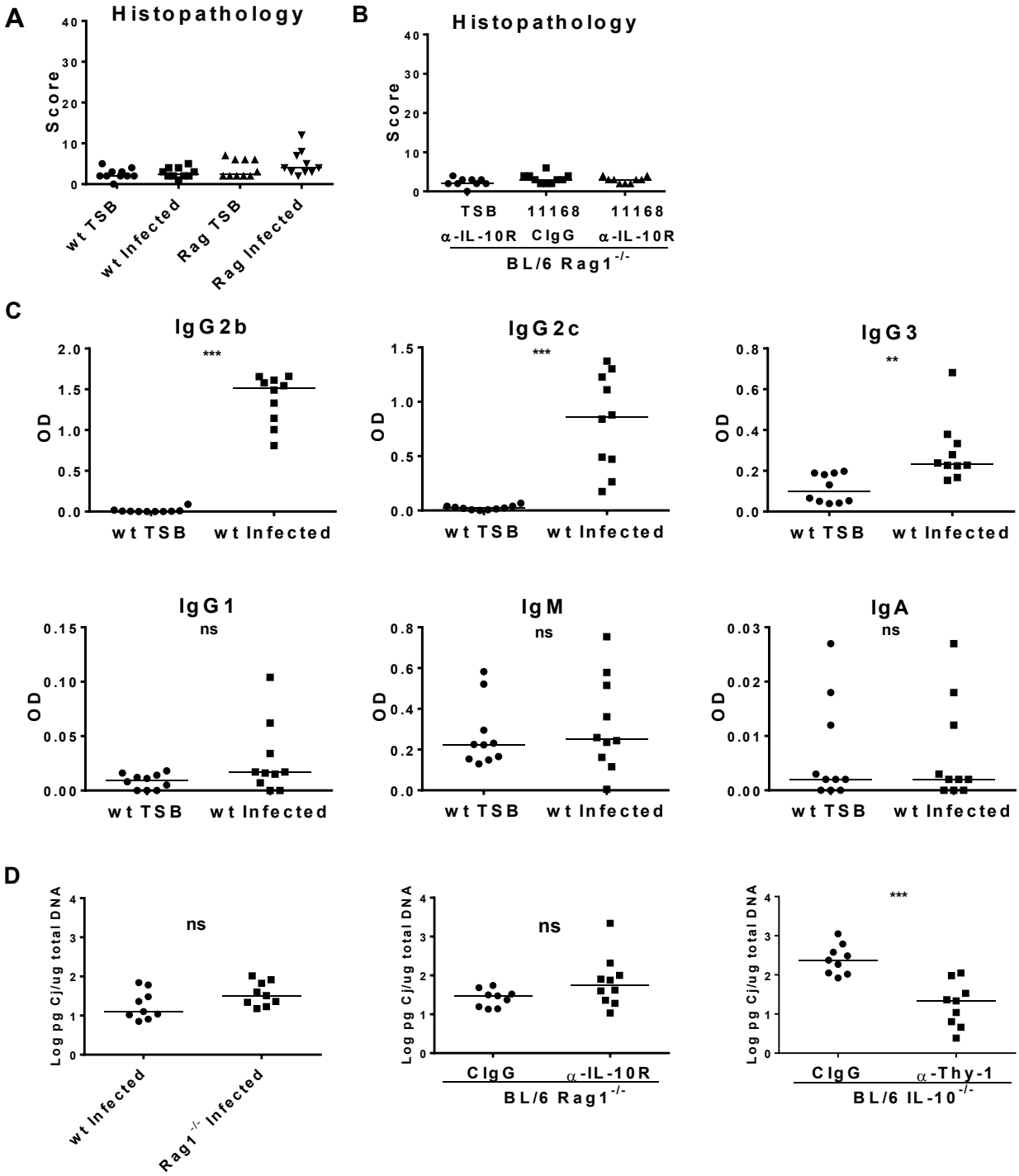


Figure 2.8. Both IFN-γ and IL-17 are involved in *C. jejuni* mediated colitis and humoral responses.

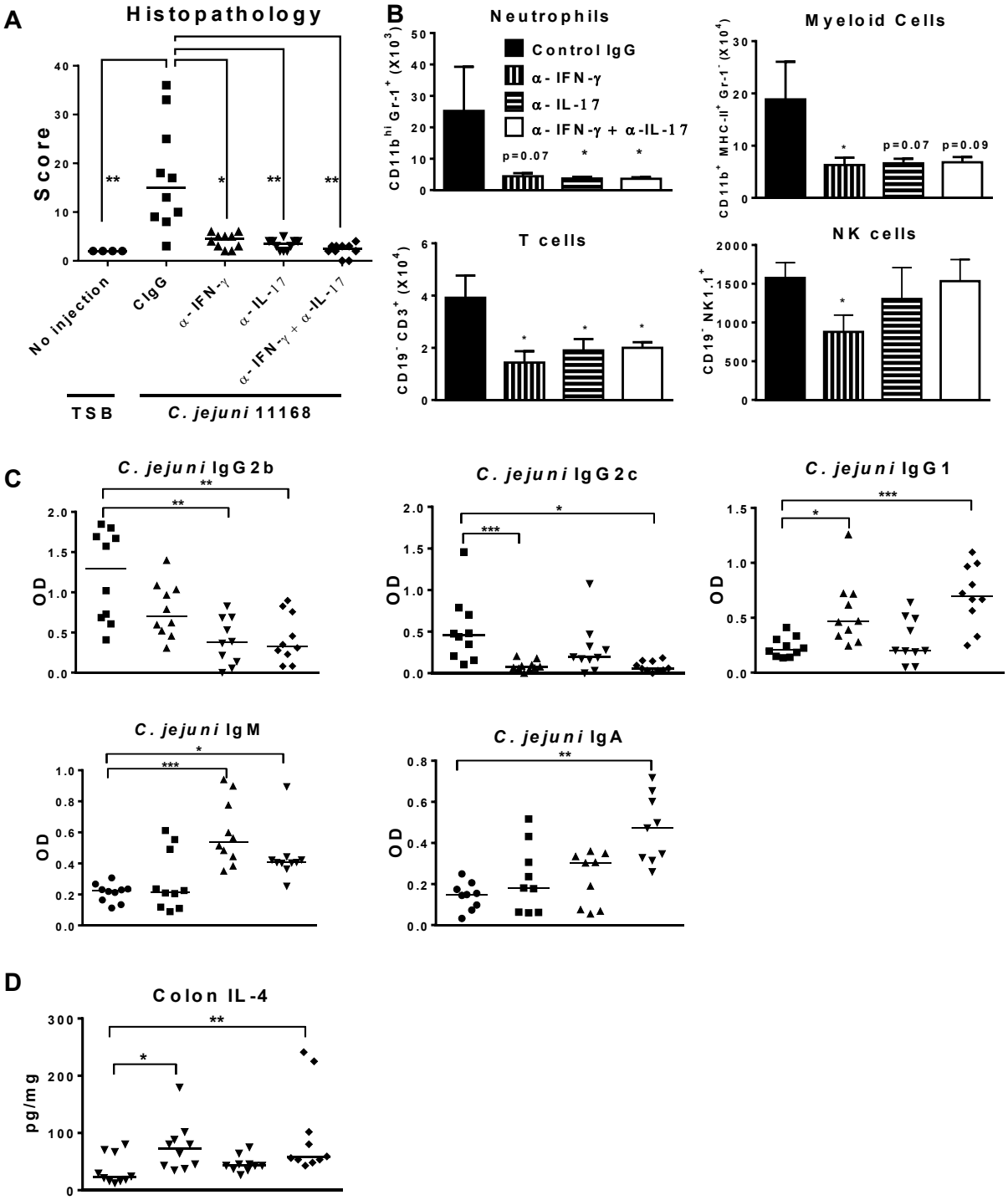


Figure 2.9. Strain dependent colitis and autoimmunity.

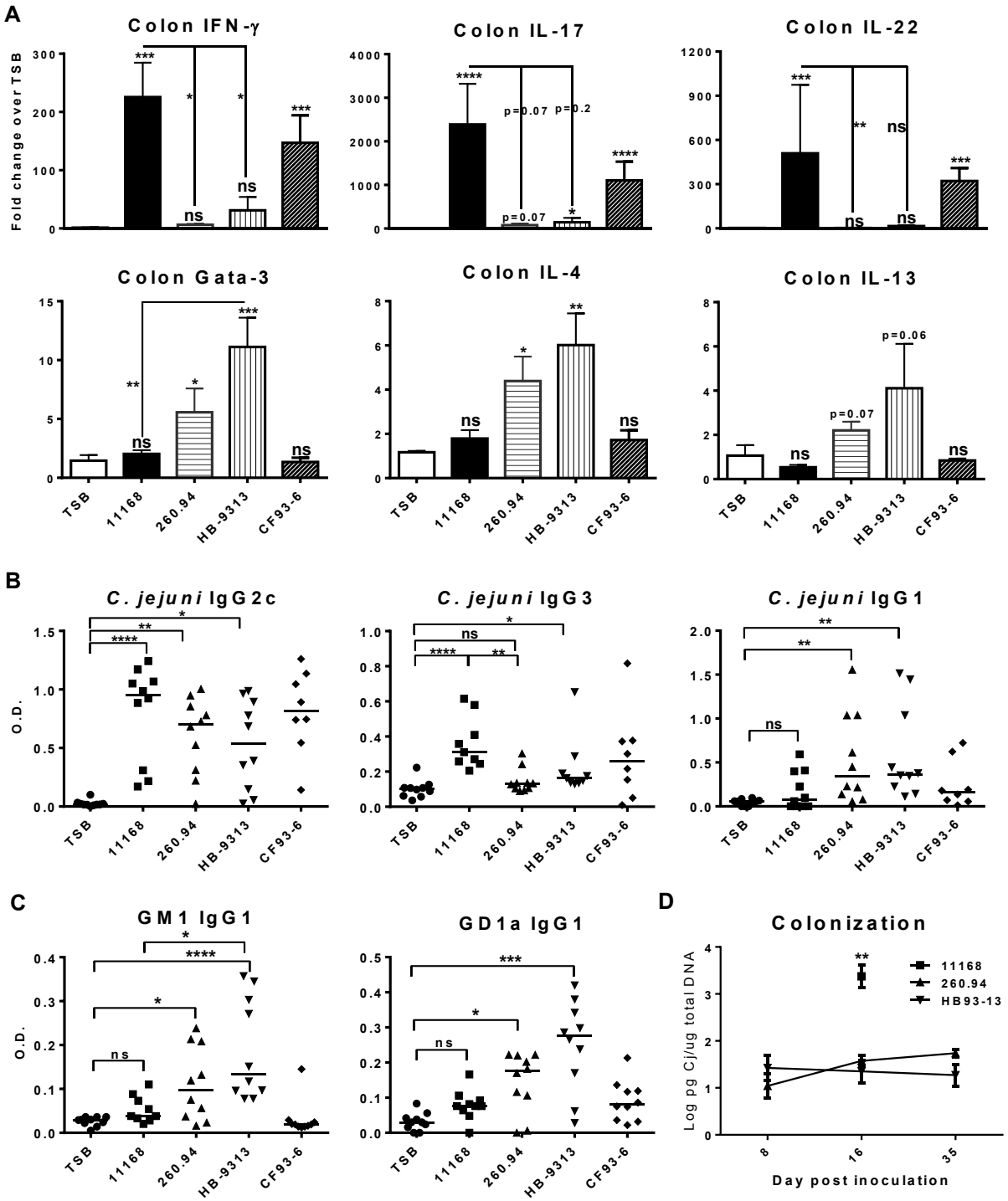
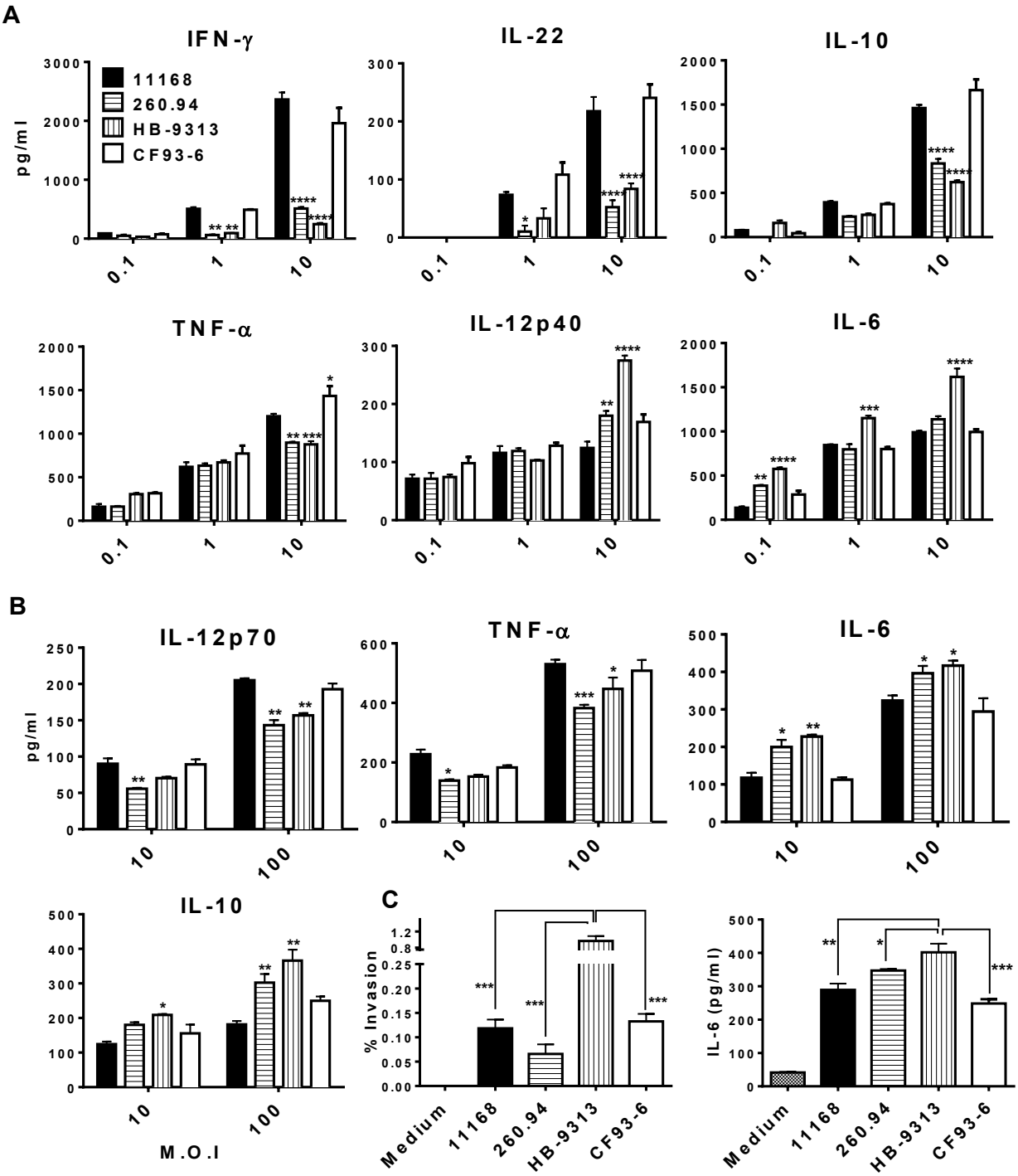


Figure 2.10. Whole splenocyte and epithelial cell challenge by gentamicin killing assay.



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CHAPTER 3

This chapter represents a manuscript titled as **"*Campylobacter jejuni* induces autoimmune peripheral neuropathy via Siglec-1 and IL-4 axes"** that is under preparation for publication.

Contributing authors: Ankit Malik, Barbie J. Gadsden, Alexander Ethridge, Leslie A. Dybas and Linda S. Mansfield.

ABSTRACT

Campylobacter jejuni is a spiral, gram-negative, microaerophilic bacterium that is the most common bacterial cause of gastroenteritis worldwide. *C. jejuni* infection has also been causally linked with development of the peripheral neuropathy called Guillain Barré Syndrome (GBS). We have previously shown that *C. jejuni* isolates from human enteritis patients induce a Type1/17 cytokine dependent colitis response in IL-10$^{-/-}$ mice. In contrast, isolates from human GBS patients colonize the IL-10$^{-/-}$ mice without inducing colitis but instead induce autoantibody elicitation targeted against peripheral nerve antigens. We show here that the autoantibody response is dependent upon blunted Type1/17 but enhanced Type2 cytokine production by T helper cells. Autoantibody elicitation also correlated with enhanced macrophage infiltration in the sciatic nerve and its dorsal root ganglia. Autoantibodies and these histological changes were significantly decreased in mice depleted of IL-4, without leading to colitis induction. Histological damage in the sciatic nerve was associated with abnormal gait and hind limb movements in the IL-10$^{-/-}$ mice, consistent with this syndrome's manifestation in humans. Furthermore, we show here that Siglec1 is a central antigen presenting cell receptor that mediates GBS but not colitogenic isolate uptake, T cell differentiation and autoantibody elicitation. Therefore, this is the first mouse model of an autoimmune disease induced directly by a bacterium and it is dependent upon Siglec1 and IL-4 axes.

INTRODUCTION

Campylobacter jejuni is a gram negative enteric bacterium that is a leading cause of food-borne illness worldwide and affects 1.4 million individuals annually in the United States[1]. It is found ubiquitously in the gastrointestinal tracts of chicken and food animals[2]. Thus, consumption of raw or undercooked poultry, other meats and unpasteurized milk are the most common sources of infection[3]. Campylobacteriosis is an inflammatory diarrhea with polymorphonuclear exudates affecting mainly the colon when the organism invades and induces inflammation. While the majority of healthy adults experience disease for 7-10 days followed by resolution, it has been a cause of mortality in high risk individuals[4]. Histopathological manifestations include colonic crypt distortion, crypt abscesses, mucin depletion of epithelium, edema in the colonic lamina propria (cLP) as well as infiltration of granulocytes, plasma cells and lymphocytes[5]. Infection with *C. jejuni* has also been linked to development and flare-ups of other chronic diseases of the gut like Irritable Bowel Syndrome and Inflammatory Bowel Disease[6, 7].

Besides these effects on the gut, *C. jejuni* infection has also been strongly linked with development of distal autoimmune diseases – Gullian Barré Syndrome (GBS) and Reactive Arthritis[8, 9]. GBS is a term used for a number of autoimmune peripheral neuropathy conditions including Acute Inflammatory Demyelinating Polyradiculoneuropathy (AIDP), Acute Motor Axonal Neuropathy (AMAN) and Acute Motor and Sensory Neuropathy (AMSAN). AIDP primarily involves demyelination of motor axons by inflammatory cell infiltration while AMAN and AMSAN involve axon death without marked inflammatory infiltrates[10]. Early symptoms of GBS appear as tingling or numbness in the extremities that rapidly progresses to ascending paralysis and can cause death due to paralysis of the diaphragm[11, 12]. GBS affects about 9,100 individuals annually in the US and has a mortality rate of 10%[13]. At present, plasmapheresis and intravenous immunoglobulin (IVIg) are the only known treatments with beneficial effect, but only 60% of GBS patients improve[11, 14]. Furthermore, up to 90% of patients face long term disability

after recovery from acute stage of the disease[11]. *C. jejuni* infection is primarily linked with the AMAN form of GBS which has been associated with development of autoantibodies that target gangliosides on peripheral nerves[9, 15]. Gangliosides are sialic acid containing gylcolipids moieties in the outer leaflet of the plasma membrane of the myelin sheath and neurons. Oligosaccharide motifs on the outer surface of *C. jejuni* endotoxin (lipooligosaccharide) isolated from AMAN patients has been shown to mimic the peripheral nerve gangliosides, namely GM1, GD1a and others[16, 17].

The development of effective treatments has been limited by lack of appropriate animal models[18]. We have previously shown that a number of isolates of *C. jejuni* from human enteritis patients induce colitis in IL-10$^{-/-}$ mice whereas isolates from human GBS patients colonize the IL-10$^{-/-}$ mice but do not induce colitis[19]. We have also shown *C. jejuni* induced colitis response depends on an upregulated Type1 and Type17 but not Type2 cytokine response in the colon and draining lymph nodes. Both Innate Lymphoid Cells (ILCs) and T cell subsets participated in this IFN-γ, IL-17 and IL-22 upregulation but in a time and organ specific manner. In contrast, the two *C. jejuni* strains isolated from human GBS patients did not induce colitis and elicited blunted Type1/Type17 cytokine and antibodies, but enhanced Type2 responses. Moreover, these GBS isolates induced Type2, but not Type1/17 antibodies cross-reacted with peripheral nerve gangliosides GM1 and GD1a[20]. However, little is known about the histological or phenotypic consequences of elicitation of these autoantibodies, and what factors control the contrasting T and B cell differentiation following *C. jejuni* colitogenic versus GBS isolate infection *in vivo*.

Siglec-1 or Sialoadhesin is a type1 transmembrane protein, member of the Sialic acid binding Ig-like lectin (Siglec) family. It is expressed by metallophilic macrophages and IFN-α activated dendritic cells and monocytes. It binds to N-acetylneuraminyl alpha 2-3-galactose (α-2-3 Nan-gal) containing glycolipids and glycoproteins, and has recently been shown to be a major

mediator for uptake of HIV by activated dendritic cells and macrophages *in vitro*[21, 22]. Bax et al. have shown that *C. jejuni* LOS has α-2,3 siaylation, which depends on siayltransferase-II and mediates binding to Siglec-1 (CD169). Further, this α-2,3 siaylation is required to induce a significant Type2 response in a human DC-T cell coculture system[23]. This ligand is also present on lipooligosaccharides of GBS associated but not colitogenic isolates of *C. jejuni*[16]. The role of sialoadhesin in *C. jejuni* phagocytosis and primary IFN induction have also been established[24, 25, 26]. Yet, there is no direct evidence of the role for Siglec-1 in regulating adaptive immune Type1/Type2 balance in any inflammatory process. Further, it is well established that IL-10 is a principal anti-inflammatory mediator for many auto-inflammatory diseases including IBD and MS/EAE[27]. It has also been shown that naive IL-$10^{-/-}$ mice have a higher number of T cells with autoreactive TCR in their lymphoid organs[28]. Therefore, we hypothesized that *C. jejuni* induced autoimmune response *in vivo* will depend on IL-4 and Siglec-1 axes, and also that the absence of IL-10 will amplify this autoimmune response.

RESULTS

C. jejuni induced autoimmunity is IL-4 and T helper cell dependent.

We have previously shown that *C. jejuni* isolates from human GBS patients colonize the IL-10$^{-/-}$ mice without inducing colitis but do induce autoantibody elicitation. These responses were associated with decreased Type1/17 cytokine response but an enhanced Type2 cytokine response[20]. We therefore hypothesized that autoimmunity in GBS isolate infected IL-10$^{-/-}$ mice will be blocked by administration of IL-4 neutralizing antibodies. Consistent with our previously published results, GBS isolate HB93-13 infected and control antibody injected mice developed a significant Type2 associated IgG1 response (but not Type1/17 associated IgG2b, IgG2c or IgG3 response) against the *Campylobacter* and peripheral nerve gangliosides GM1 and GD1a, 4 weeks after infection (**Fig.3.1A and B**). The extent of both *Campylobacter* specific and autoantibodies were decreased by administering IL-4 neutralizing antibody injections. We have also previously shown that colitogenic isolate (11168) infection followed by IFN-γ and/or IL-17 depletion leads to reciprocal upregulation of *Campylobacter* specific (but not autoreactive) Type2/IgG1 response[20]. However, IL-4 depletion did not significantly increase the Type1/17 dependent IgG2c, IgG2b or IgG3 isotypes (**Fig.3.1A**).

Because the AMAN form of GBS is associated with damage in the sciatic nerves and their roots[29, 30], we ascertained the histological manifestations of development of these autoantibodies in the sciatic nerve, dorsal root and the phrenic nerve. While no differences were observed upon evaluation of the formalin fixed and H&E stained sections, greater numbers of F4/80^{+} macrophages were found infiltrated into the sciatic nerve and the dorsal root in the infected mice when compared to sham inoculated mice (**Fig. 3.2 A and B**). This macrophage infiltration was ascertained by immuohistochemistry and found to be particularly marked in the

dorsal roots. Furthermore, macrophage infiltration was significantly decreased in mice given IL-4 neutralizing antibodies and correlated with decreased autoantibody titers in circulation.

Previously we have also shown that both Innate and adaptive lymphocytes had contributed towards upregulation of IFN-γ, IL-17 and IL-22 after colitogenic isolate infection. In contrast, after GBS isolate infection, upregulation of IL-4 was exclusively observed in $CD4^+$ T cells and not in other T cell subsets or ILCs, as judged by intra-cellular cytokine staining and flow cytometry from the single cell suspension of the colon (**Fig. 3.3A**). Therefore, *C. jejuni* induced autoimmunity is IL-4 and T helper cell dependent. However, IL-4 depletion did not lead to an increase in T_H1 response nor inflammation in the gastrointestinal tract nor were any clinical signs of colitis exhibited by these or mice injected with control antibody (**Fig. 3.3B**). We have also previously shown that colitis induction by colitogenic isolates correlates with an increase in their colonization extent. However, IL-4 depletion did not lead to any difference in colonization extent n mice infected with GBS isolate (**Fig. 3.3C**). Thus, lack of colitis induction by the GBS isolates is independent of their IL-4 inducing capability.

Siglec-1 is essential for GBS isolate invasion into APCs and cytokine and autoantibody elicitation.

It has been demonstrated that *C. jejuni's* LOS with α-2,3 siaylation, that is structurally similar to mono- or di-sialyated peripheral nerve gangliosides, acts as a ligand for Siglec-1 (CD169)[23]. Also, the ganglioside presentation on the LOS surface is required to induce a significant T_H2 response towards the purified LOS in a human DC-T cell coculture system[23]. However, the role of Siglec-1, if any, in modulating an adaptive Type1/Type2 cytokine response is not known. We used naïve splenocyte (adherent fraction and whole cell preparation) challenge by gentamycin killing assay as an *ex vivo* model to determine the role of Siglec-1 in uptake and immune stimulation by GBS and colitogenic *C. jejuni* isolates. Consistent with our previous observations,

GBS strains induced more IL-6 while the colitogenic strains induced more TNF-α and IFN-γ after challenge to whole splenocytes (**Fig. 3.4A and B**). We found that a 20 min pre-treatment with anti-Siglec-1 antibody, but not isotype control antibody, significantly decreased IL-6 elicitation from GBS isolates but did not affect IL-6 elicitation from colitogenic isolates. This effect was dependent on the antibody dose, a 5 µg/ml dose was found to be sufficient for maximal effect to decrease IL-6 elicitation by GBS isolates (**Fig. 3.4A**). Furthermore, elicitation of Type1 cytokines like TNF-α and IFN-γ were not affected by Siglec-1 blocking at any dose from GBS or colitogenic isolates (**Fig. 3.4A and B**). As Siglec-1 is a cell surface receptor known to be involved in uptake of HIV by macrophages and DCs[21, 22], we also hypothesized that Siglec-1 blocking will lead to similarly decreased invasion of GBS but not colitogenic isolates into the adherent fraction of the splenocytes (that are enriched for phagocytes). Consistent with this hypothesis, Siglec-1 blocking significantly reduced invasion of GBS but not colitogenic isolates into the adherent splenocytes in the gentamycin killing assay (**Fig 3.4C**). To corroborate the specificity of Siglec-1 receptor towards ganglioside presenting *C. jejuni* isolates, we also included an isolate from an enteritis patient that lacks all ganglioside mimics, *C. jejuni* CG8421, in the cytokine elicitation and invasion assay. We have previously shown that CG8421 causes a high degree of colitis in IL-10$^{-/-}$ mice that is consistent with its high Type1 and 17 cytokine elicitation characteristics[20]. This isolate has been shown to lack any ganglioside presentation on its surface[31], and consistent with that Siglec-1 blocking had no affect on its cytokine elicitation (**Fig. 3.4B**) or invasion (**Fig. 3.4C**) properties in whole and adherent splenocytes respectively.

Due to decreased bacterial uptake and IL-6 production after Siglec-1 blocking *ex vivo*, we further hypothesized that Siglec-1 blocking *in vivo* will lead to decreased T cell activation that also leads to decreased *C. jejuni*- and auto-reactive antibody elicitation by GBS isolates. Consistent with the tissue culture data, administering IL-10$^{-/-}$ mice anti-Siglec-1 antibody for 6

weeks significantly decreased T_H2 differentiation in the colon but without affecting T_H1 differentiation, which was similar to sham-inoculated mice (**Fig 3.5A**). Further, both *Campylobacter* specific (**Fig. 3.5B**) and also autoreactive anti-GM1 and anti-GD1a IgG1 antibodies (**Fig. 3.5C**) in circulation were significantly decreased after Siglec-1 blocking *in vivo*. However, the affect of Siglec-1 blocking does not seem to be T_H2 specific as anti-*C. jejuni* IgGb antibody levels also trended towards a decrease after blocking (**Fig. 3.5B**).

Consistent with our previous data, 2/10 mice in the infected + CIgG group that failed to be colonized at the end of the experiment were low/negative for *C. jejuni* specific or autoreactive antibodies. Only one mouse in the infected and Siglec-1 blocked group failed to be colonized at the end of the experiment.

IL-10 is a negative regulator for antibody production.

We have also previously shown that *C. jejuni* induced colitis in mice depends upon absence of IL-10 because IL-$10^{+/+}$ mice become colonized by the colitogenic isolate but do not experience any clinical signs of disease[32]. But colitogenic isolate infected IL-$10^{+/+}$ mice do develop anti-*Campylobacter* antibodies that are of the same classes (IgG2b, IgG2c and IgG3) as that elicited in the IL-$10^{-/-}$ mice[20]. Therefore we asked if infection of IL-$10^{+/+}$ mice with the GBS isolate leads to autoantibody elicitation like that observed in the IL-$10^{-/-}$ mice. To this end, BL/6 IL-$10^{+/+}$ mice were either sham inoculated or inoculated with HB93-13 alongside the IL-$10^{-/-}$ mice from the Siglec-1 blocking experiment, and analyzed for *C. jejuni*- and auto-reactive antibody elicitation 6 weeks post inoculation. We found that the infected IL-$10^{+/+}$ mice did develop a significant anti-*Campylobacter* IgG2b response when compared to sham inoculated mice, and this was not significantly different from the IgG2b response in the IL-$10^{-/-}$ mice (**Fig. 3.5B**). Intriguingly, the T_H2 dependent IgG1 response, both *Campylobacter* specific and reactive to peripheral nerve gangliosides, was not induced to a significant extent in the IL-$10^{+/+}$ mice as opposed to the IL-$10^{-/-}$ mice (**Fig 3.5B and C**). Therefore, IL-10 functions as a negative regulator of *C. jejuni*

induced T_H2 response and consequent autoantibody production, but does not affect *C. jejuni* reactive IgG2b elicitation.

C. jejuni GBS isolate infection leads to abnormal hind limb movements in a subset of IL-10$^{-/-}$ mice.

We have shown that BL/6 IL-10$^{-/-}$ mice infected with GBS isolates of *Campylobacter jejuni* develop T_H2 associated antibodies that bind to peripheral nerve gangliosides GM1 and GD1a, and lead to significant macrophage infiltration in sciatic nerves and its dorsal root ganglia. We wanted to evaluate the phenotypic consequences associated with development of these autoantibodies, and to this end sham and GBS isolate HB93-13 infected IL-10$^{-/-}$ mice were subjected to reaching reflex and open field tests weekly, for up to 17 weeks post inoculation. During the reaching reflex, mice were videotaped while being hung from the tail for 5-10 seconds and scored blinded for abnormal leg splay (mouse will spread legs out to the side and raise them up towards the tail; they will no longer remain in line with the forelimbs) and leg flexing (mouse will pull hind limbs towards its body or will make a 'fist' with its hind limb paw). During open field testing, mice were videotaped for one minute after being put in a rat cage that was divided into 4 quadrants. Videotapes of each mice were scored for wide gait stance, foot drag and knuckling. They were considered affected if they were scored positively for at least three of the above features. The incidence of neurologically affected mice varied from 0 to 6 (out of 18 for infected) and 0 (out of 11 for control), with the highest incidence between weeks 4-7 post inoculation (**Fig. 3.6**). Infected female mice trended towards a higher incidence rate than males but the maximum incidence rate for either sex did not reach above 50%.

The number of rears and quadrants crossed in the open field test were quantified separately during the open field test. While the infected mice trended towards less quadrants crossed and lower number of rears, it did not reach statistical significance (Fig 6B). Digigait©® analysis on

the mice was also attempted but not found useful as these mice gradually stopped cooperating towards running on the treadmill at any speed. After the fourth trial (that includes one trial before infection and 3 after infection) only 36% of the infected group and 45% of the controls ran on the treadmill, consistent with a previous finding of recalcitrance of different strains of mice towards treadmill running[33]. Therefore Digigait testing was discontinued and only the reaching reflex and open field test applied.

We have also previously shown that the extent of colonization in BL/6 IL-$10^{-/-}$mice after infection with colitogenic isolates increases with time, and that this increase correlates with increasing extent of inflammation in the colon. Colonization with this GBS strain was in contrast found to be unstable with time, as the extent of *camplylobacter* DNA in feces decreased significantly at 17 weeks post inoculation when compared to 1, 4 or 8 weeks post inoculation (**Fig. 3.7A**). This decrease in colonization probably explains the absence of elevated numbers of T_H2 (or T_H1) cells in the colon (**Fig.3.7B**), the absence of *campylobacter* specific (**Fig.3.7C**) or autoreactive antibodies (**Fig.3.7D**) in circulation and a decrease in the clinical signs of the disease at the time of necropsy (**Fig.3.6**). We also determined the histological manifestations of long term infection in the sciatic nerve and dorsal roots. While no gross differences were observed upon evaluation of the formalin fixed and H&E stained sections, slightly enhanced number of macrophages were found infiltrated into the sciatic nerve, but not the dorsal root in the infected mice that had demonstrated clinical signs (**Fig.3.7E**). This macrophage infiltration was ascertained by F4/80 immuohistochemistry. Therefore, *C. jejuni* induced peripheral neuropathy in IL-$10^{-/-}$ mice is transient and infrequent.

DISCUSSION

We show here that *C. jejuni* induced autoimmune response in SPF IL-10$^{-/-}$ mice is dependent upon blunted Type1/17 but enhanced Type2 cytokine production. Moreover, these IL-4 and CD4^{+} T cell dependent Type2 antibodies, but not the Type1/17 antibodies, cross-reacted with peripheral nerve antigens. Presence of autoantibodies in circulation correlated with enhanced macrophage infiltration in the sciatic nerve and its dorsal root ganglia, while the phrenic nerve was largely unaffected (not shown). These findings are consistent with human clinical reports that IgG1 isotype is the most commonly associated autoreactive isotype in *C. jejuni* infection, and that its titer directly correlates with severity of clinical signs and worsening prognosis of *C. jejuni* associated GBS[34, 35]. Macrophage infiltration was most marked in DRGs consistent with its weaker nerve blood barrier[36, 37]. This contrasts with our previous findings that *C. jejuni* mediated colitis in specific pathogen free mice is T cell, IFN-γ and IL-17 dependent[20] and demonstrates how differential T cell maturation by different *C. jejuni* strains leads to different disease outcomes in a susceptible host.

Anatomical location of Siglec-1 expressing macrophages suggests its role as a sentinel for primary contact with pathogens, apoptotic and cancer cells. Siglec-1 is expressed by macrophages at the site of afferent lymphatics in the spleen and the lymph nodes, and at the base of the crypts in the colon, which are sites of frequent invasion by pathogens[38, 39]. Consistent with our findings, Heikema et al., have recently also shown that Siglec-1 blocking decreases heat killed-GBS isolate uptake and IL-6 (but not TNF-α) elicitation from human blood monocyte-derived and LPS primed macrophages *in vitro*[26]. Recently it has also been shown that Siglce-1 plays a critical role in phagocytosis and primary IFN and early cytokine production after challenge with sialylated pathogens[24, 25, 40]. We extend these findings to demonstrate that

colonic T_H2 differentiation and autoimmune sequelae after *C. jejuni* GBS isolate infection is also Siglec-1 dependent. Siglec-1 has also been shown to be highly expressed by circulating myeloid and local lymph node cells during MS and other autoinflammatory diseases in humans[41, 42] and plays a critical pro-inflammatory role by binding to Tregs and preventing their expansion, as shown in the EAE model[43]. Siglec-1^+ macrophages were also associated with presentation of lipid antigens for activation of iNKT cells[44]. Further, targeting delivery of microbial/tumor antigens through Siglecs has shown promise for inducing strong T cell activation[45]. Therefore, Siglec-1 is a unique receptor involved in both phagocytosis and cell-cell interaction during antigen presentation to T cells. While we have demonstrated a direct role for Siglec-1 in sialylated *C. jejuni* uptake, it remains to be determined if it has an independent role in blocking APC-T cell or T cell-B cell interactions. Nevertheless, sialylated oligosaccharide motifs on the LOS of GBS associated *C. jejuni* is unique in the sense that it acts as the ligand for phagocytosis by antigen presenting cells, and also as epitopes that lead to autoimmunity.

Histological damage in the sciatic nerve manifested with abnormal gait and hind limb movements in a subset IL-$10^{-/-}$ mice, consistent with this syndrome's manifestation in humans. But the reasons for mild or no disease in some infected mice are not clear. Nevertheless, the extent of macrophage infiltration was significantly decreased in mice given IL-4 neutralizing or Siglec-1 blocking antibodies, without a side effect of colitis. This demonstrates that down modulation of the T_H2 response alone is not sufficient to upregulate a colitogenic Type1/17 response after *C. jejuni* infection. This work is the first demonstration of a role for Siglec-1 in T_H1/T_H2 balance regulation. We also confirmed the negative regulatory role of IL-10 in *C. jejuni* induced autoimmunity. Therefore, SPF BL/6 IL-$10^{-/-}$ mice serve as excellent models of *C. jejuni* induced colitis, autoantibody elicitation and sub-clinical inflammation in the peripheral nervous system, but an insufficient model for studying sever clinical changes associated with *C. jejuni* induced GBS such as paralysis and respiratory insufficiency. As IL-10 is a strong locus of

susceptibility for IBD and other inflammatory diseases[28], this model essentially combines the most frequent genetic perturbation underlying inflammatory disorders with the most common causative organism of colitis and GBS through its natural route of infection.

MATERIALS AND METHODS

Mice, inoculation and antibodies for in vivo neutralization. C57BL/6J IL-$10^{+/+}$ (IL-$10^{+/+}$) and BL/6.129P2-*IL-*10^{tm1Cgn}/J (IL-10-deficient) mice were purchased from Jackson laboratories and maintained in specific pathogen free conditions and transported to University Research Containment Facility at 8 – 12 weeks of age for experimental infection. Mice were housed individually and inoculated with tyrptone soy broth (TSB – vehicle control) or 10^9 CFU of *C. jejuni* in 0.2ml TSB as described previously[32]. α-Siglec-1 (3D6.112) and isotype control (RTK2758) were purchased from Biolegend (San Diego, CA) and injected at 100µg/mouse by retro-orbital injection, weekly for 6 weeks starting at 2 days before inoculation. α-IL-4 (11B11) was purchased from Bio-X-Cell (West Lebanon, NH) and injected intraperitoneally at 400µg/mouse biweekly, starting at the day of inoculation. All animal protocols were approved by Michigan State University Institutional Animal Care & Use Committee and conformed to National Institutes of Health guidelines.

Tissue samples. Colon and spleen was taken from infected and non-infected mice at the time of necropsy and rinsed in PBS. For ELISA or RNA extraction, tissue was frozen immediately using an ethanol-dry ice bath. For immunohistochemistry, sciatic nerves were exposed on the mouse carcass and fixed in 10% buffered formalin for 48 hours, then stored in 60% ethanol followed by paraffin embedment. Ileocecocolic junction was fixed for 20-24 hours. H&E sections were evaluated by a board certified pathologist.

Immunohistochemical analysis of mononuclear cells. The tissue specimens were fixed in 10% formalin buffer pH 7.0 and paraffin-embedded. 3-5 μm thick serial sections were cut and attached to silanized glass slides and stained for F4/80 (BM8, eBiosciences). Sections were deparaffinized in two changes of xylene and rehydrated in graded alcohol series. Endogenous peroxidase was blocked in the sections by incubating in 1% hydrogen peroxide in TBS (50mM tris, 150mM NaCl, pH 7.4). Subsequently, the sections were boiled for 15 minutes in citrate buffer (Vector laboratories). The sections were blocked for 1 hour at room temperature with 1% BSA + 1.5% Goat serum in TBS 0.025% Triton X-100, the incubated with the respective primary antibody at 4°C overnight (Dilution – 1:100, in TBS 0.025% Triton X-100). Sections were then washed twice with TBS 0.025% Triton X-100 and stained with the Vectastain ABC kit (Vector laboratories) as per manufacturer's instructions. The sections were developed with Impress Nova Red (Vector laboratories), counterstained with Gill's hematoxylin, differentiated with 1% acetic acid, dehydrated and mounted with permount (sigma). Negative controls were stained as above, except that primary antibodies were replaced with irrelevant isotype control (RTK4530, Biolegend) for F4/80.

Evaluation of cell densities. Sections were stained and analyzed in a blinded fashion. From each section at 20x magnification contiguous fields of view were photographed so as to include the entire area of the nerve and the root by the micrographs. The area of the nerve/root was calculated with the help of free hand tool of Image J, (N.I.H) and positively staining cells were counted manually with the help of cell counter tool of ImageJ.

Preparation of Lamina Propria lymphocytes. LP lymphocytes were isolated as previously described[46, 47]. Briefly, for removal of epithelial cells, the colon was washed, cut into small pieces, and then the pieces were incubated with calcium- and magnesium-free HBSS

supplemented with 5% FBS and 5 mM EDTA (Sigma-Aldrich) at 140 rpm at 25°C for 30 min. The tissues were then incubated with RPMI 1640 containing 10% FBS and 0.5 mg/ml collagenase type IV for 1 hour at 37°C with shaking at 150 rpm. The liberated cells were collected by passage through a 70 μm nylon mesh. The isolated cells were pooled together and separated on a 40/80% discontinuous Percoll gradient (GE Bioscience). The cell yield was typically 1 – 2 X 10^6 cells per mouse with 90% cell viability, as ascertained by Propidium Iodide staining.

Flow cytometry. The following monoclonal antibodies (eBiosciences) were used in appropriate combinations: anti-CD3 (clone 145 – 2C11), anti-CD4 (clone RM 4-5), anti-TCR γδ (clone GL3), anti CD19(clone 1D3), anti-CD11b (clone M1/70), anti Gr1 (clone-RB6-8C5), eFlour 780 anti-CD90 (clone53-2.1) and anti-CD16/CD32 (clone 2.4G2). The cells were preincubated for 20 minutes with anti-CD16/CD32 to block Fc receptors, thus avoiding nonspecific binding. Cells were then washed and labeled with appropriate mixture of antibodies or isotype matched controls for 30 minutes, centrifuged at 650*g*, and resuspended in FACS buffer. To exclude dead/dying cells and therefore nonspecific antibody-binding cells, leukocytes were gated according to forward and side scatter. The percentages of $CD4^+$, $CD8^+$, $CD4^+$ $CD8^+$ and ϒδT cells subsets were calculated on $CD19^-$ $CD3^+$ gate. For intracellular cytokine staining, cells were restimulated for 4 h with 50ng/ml PMA and 1 μg/ml Ionomycin (Sigma) and Golgi Stop and block (BD biosciences) were added for the last two hours. The cells were fixed and permeabilized using fixation and permeabilization solution (eBioscience). Staining was performed for IL-4 (clone 11B11) and IFN-γ (clone XMG1.2) antibodies, and the cells were analyzed on a LSRII flow cytometer (BD Biosciences) using FlowJo software (Tree Star).

Enzyme-linked Immunosorbent Assay. IFN-γ, IL-6 and TNF-α, were measured in tissue culture supernatants according to the manufacturer's protocol (Ready-Set-Go ELISA kits, eBioscience). *C. jejuni* antibody ELISA was used as described earlier[20]. Briefly, the protein concentration was adjusted to 1.9µg/ml. Nunc-Immuno Maxisorp plates were coated with the antigen overnight at 4°C, blocked overnight at 4°C in blocking buffer (3% BSA in PBS with 0.05% Tween 20). Next day, plates were washed 4 times with wash buffer (PBS with 0.05% Tween 20) plasma samples diluted in blocking buffer (or blocking buffer alone as negative control) were applied to the plate and incubated overnight. Next day, plates were washed 4 times and incubated with biotinylated anti-mouse IgG1, IgG2b, IgG2c, IgG3 or IgM (Jackson Imumunoresearch) at 1:20000 dilution in blocking buffer, incubated for 1 hour at room temperature, washed 4 times with wash buffer and incubated for 1 hour at room temperature with Extravidin Peroxidase reagent (Sigma) diluted 1:2,000 in PBS with 1%BSA and 0.05% Tween 20. Plates were washed four times, developed with TMB (Sigma), stopped with 2N sulphuric acid and absorbance was read at 450 nm with 562 nm as reference wavelength. Serum concentrations were 1:25 for IgG1, IgG2c and IgG3; 1:100 for IgG2b. GM1 (Sigma) and GD1a (US bio) were used at 2 and 20µg/ml respectively and handled similarly.

Splenocyte challenge by gentamicin killing assay. Red blood cell depleted splenocytes from naive C57BL/6 wt mouse ($3X10^6$ cells/ml) were plated in antibiotic-free R10 medium and challenged with the indicated *C. jejuni* strains at multiplicity of infection of 1. One hour after infection, gentamicin (250µg/ml) was added to all the wells to kill extracellular bacteria. Supernatants were collected after 72 h for cytokine measurement by ELISA. To obtain adherent cells, splenocytes were plated at 10^7 cells/ml for 90 min upon which the non-adherent cells were washed off. For measuring invasion, cells are further incubated for 1 h with 250 mg/ml gentamicin, washed in PBS, lysed in 0.1% Triton X-100, and released bacteria was enumerated by limiting serial dilution assay.

APPENDIX

FIGURE LEGENDS

Figure 3.1. IL-10$^{-/-}$ mice were orally gavaged with TSB or *C. jejuni* GBS isolate HB93-13 or colitogenic 11168. Sham and HB93-13 infected mice were injected with CIgG or IL-4 neutralizing antibody biweekly for 4 weeks starting at the time of inoculation. Plasma IgG isotypes reactive to *C. jejuni* antigen (A) or peripheral nerve ganglioside autoantigens (B) were analyzed by ELISA at the time of necropsy. N=8-10 mice per group. Data is represented as mean±s.e.m and was analyzed by Kruskal Wallis test and Dunn's post test.

Figure 3.2. IL-10$^{-/-}$ mice were orally gavaged with TSB or indicated isolates of *C. jejuni* and injected with CIgG or IL-4 neutralizing antibody. Formalin fixed sciatic nerve and root sections were stained for F4/80 (A). Positively staining cells and tissue area were quantified with the help of ImageJ cell counter and area tools respectively. N=8-10 mice per group. Data is represented as mean±s.e.m and was analyzed by Kruskal Wallis test followed by Dunn's post test.

Figure 3.3. IL-10$^{-/-}$ mice were orally gavaged with TSB or indicated isolates of *C. jejuni* and injected with CIgG or IL-4 neutralizing antibody. Single cell suspension of the colon leukocytes was prepared and analyzed for indicated cell populations by ICCS and flow cytometry (A). Formalin fixed illeocecococlic junctions were stained for H&E (B). Colonization load was determined by Q-PCR on fecal DNA with *C. jejuni* specific primers (C). N=8-10 mice per group. Data is represented as mean±s.e.m and was analyzed by Kruskal Wallis test followed by Dunn's post test.

Figure 3.4. Single cell suspension of whole splenocytes from naïve wt mouse was challenged with the indicated *C. jejuni* isolates at M.O.I of 1 by gentamycin killing assay. Cells were pretreated for 20 minutes by anti-Siglec1 or control antibody (1, 5 or 10 µg/ml for A; 5 µg/ml for B). Gentamycin was added 1 hour after challenge and 72 hours later, indicated cytokine levels were determined by ELISA in clarified supernatant media (A and B). For invasion assay cells

were lyzed 1 hour after washing off gentamycin (C). Gentamycin sensitivity to all the strains was also confirmed. Data represents mean±s.e.m of three wells, and was repeated atleast twice independently. Analyzed by two way ANOVA test followed by Bonferroni's post test.

Figure 3.5. Wt or IL-10$^{-/-}$ mice were orally gavaged with TSB or *C. jejuni* HB93-13 and injected with CIgG or anti-Siglec-1 antibody weekly for 6 weeks starting at two days before inoculation. Plasma IgG isotypes reactive to *C. jejuni* antigen (A) or peripheral nerve ganglioside autoantigens (B) were analyzed by ELISA at the time of necropsy. N=8-15 mice per group. Data was analyzed by Kruskal Wallis test followed by Dunn's post test, horizontal bar represents the median.

Figure 3.6. IL-10$^{-/-}$ mice were orally gavaged with TSB or of *C. jejuni* HB93-13 and phenotyped weekly for 17 weeks. N=10-18 mice per group. Error bar represents mean±s.e.m. Data was analyzed by two way ANOVA test followed by Bonferroni's post test.

Figure 3.7. IL-10$^{-/-}$ mice were orally gavaged with TSB or of *C. jejuni* HB93-13 and phenotyped weekly for 17 weeks. Colonization load was determined by Q-PCR on fecal DNA with *C. jejuni* specific primers. Single cell suspension of colon leukocytes was prepared and analyzed for indicated cell populations by ICCS and flow cytometry. Plasma IgG isotypes specific to given antigens were quantified by ELISA at the time of necropsy. Formalin fixed sciatic nerve and root sections were stained for F4/80. Positively staining cells and tissue area was quantified with the help of ImageJ cell counter and area tools respectively. N=10-18 mice per group. Colonization data was analyzed by one way ANOVA test followed by Bonferroni's post test, and the rest by Mann Whitney test.

FIGURE 3.1. IL-4 depletion experiment serum antibodies

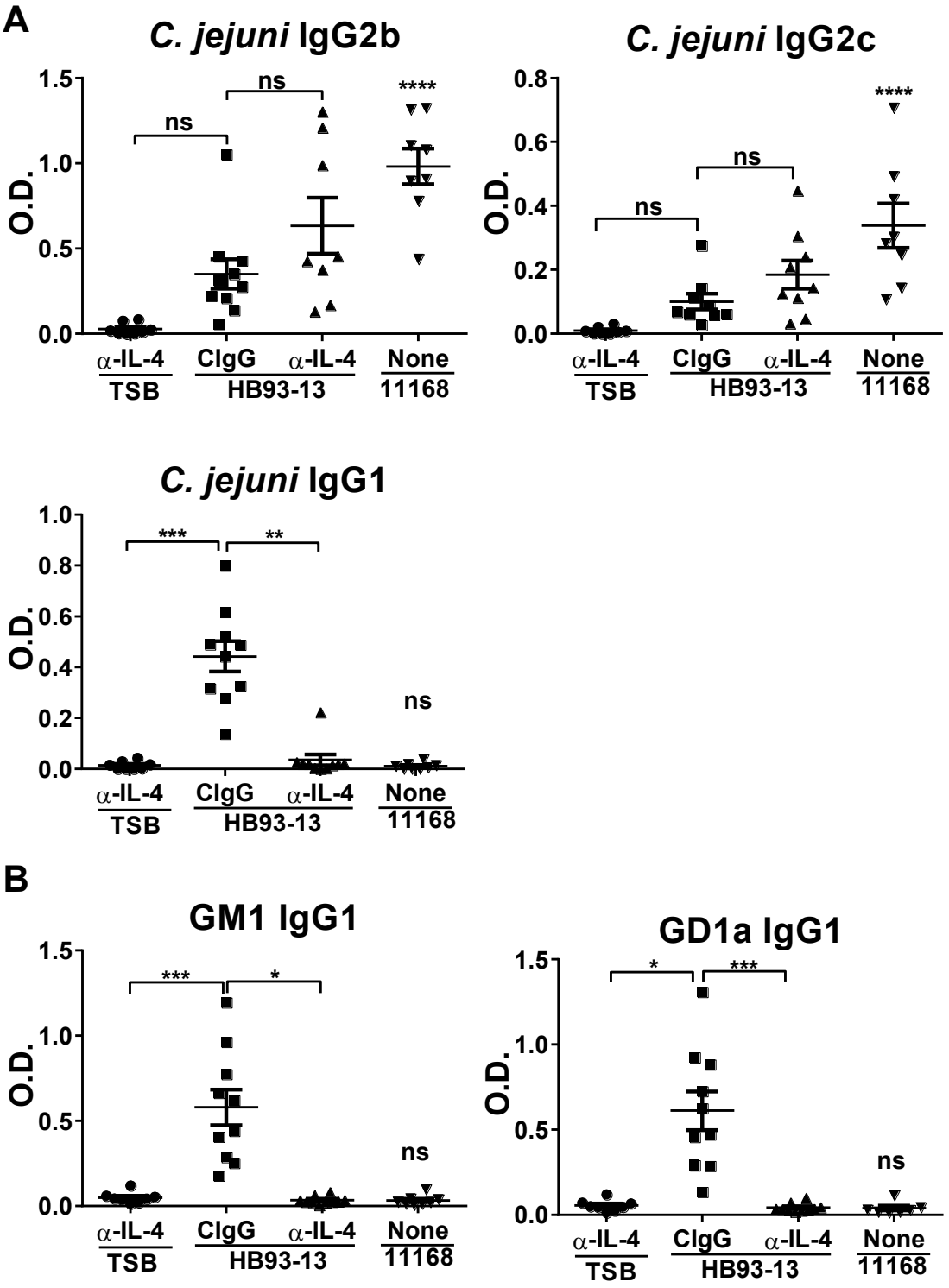
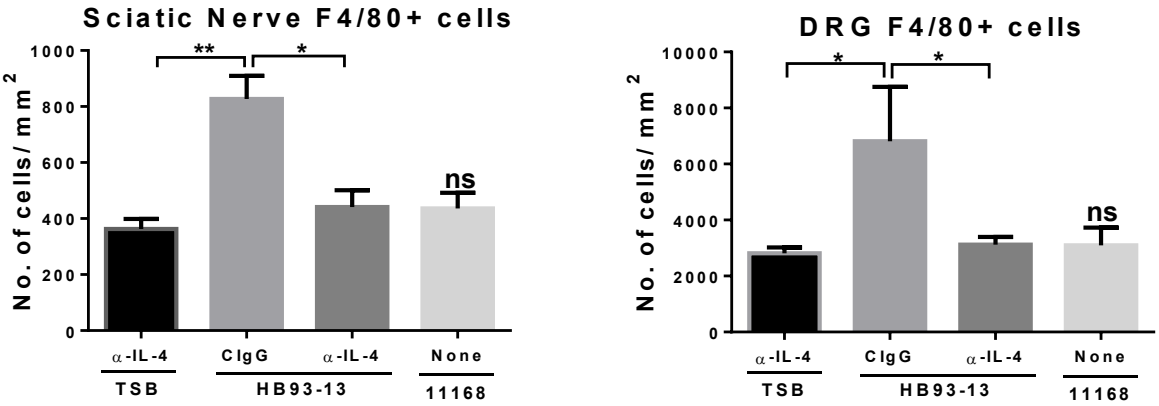


FIGURE 3.2. IL-4 depletion experiment sciatic nerve and DRG macrophage IHC

A



B F4/80

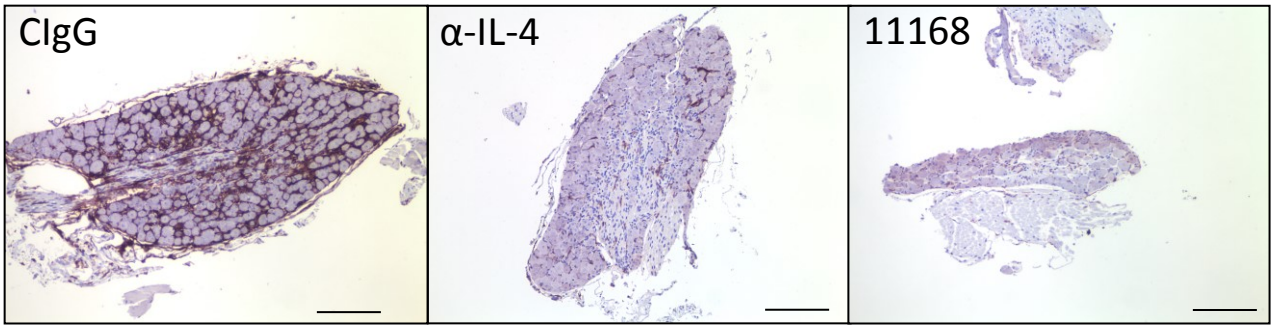


FIGURE 3.3. IL-4 depletion experiment colon flow cytometry, histology and colonization

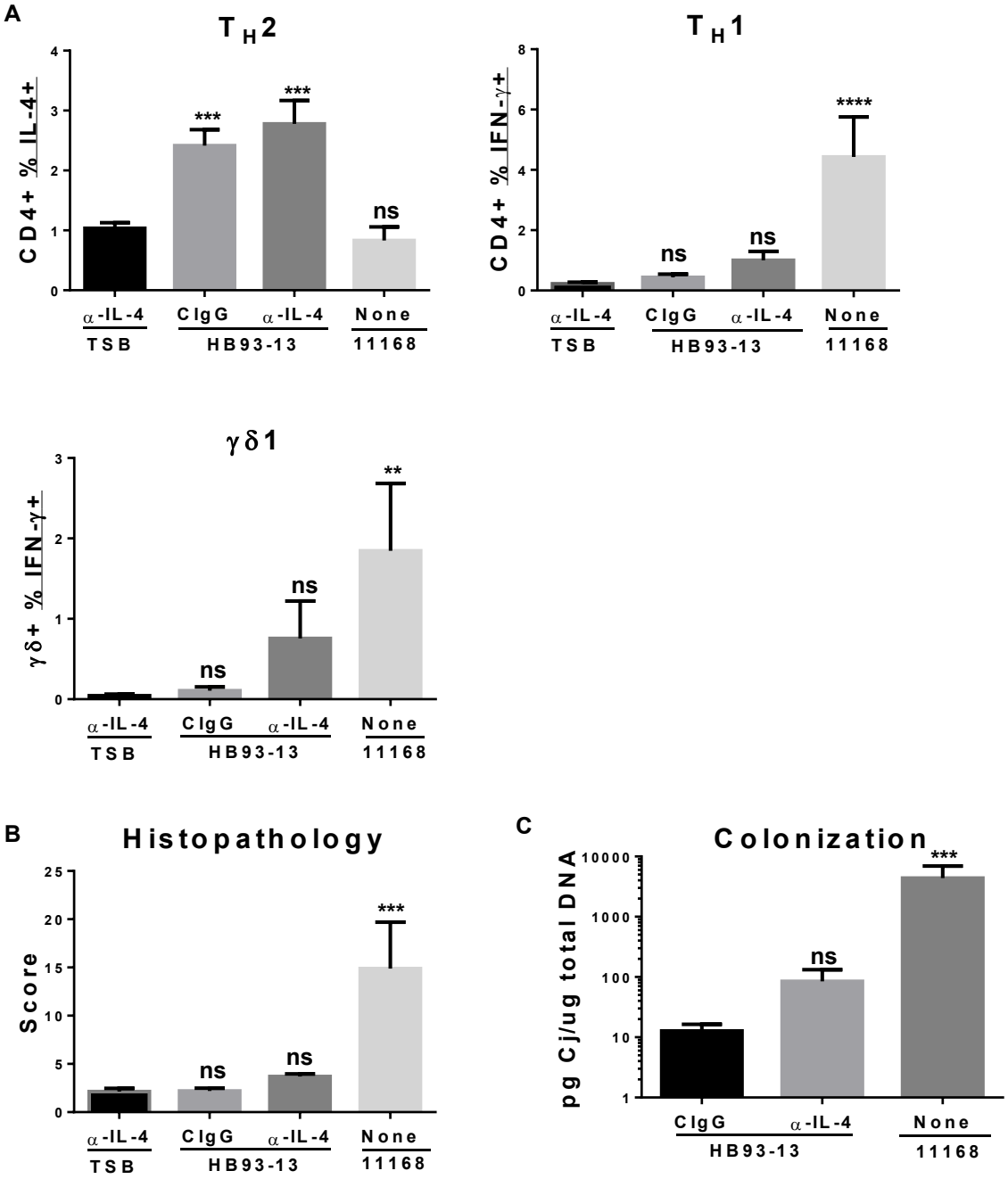


FIGURE 3.4. Siglec-1 blocking in splenocytes ex vivo

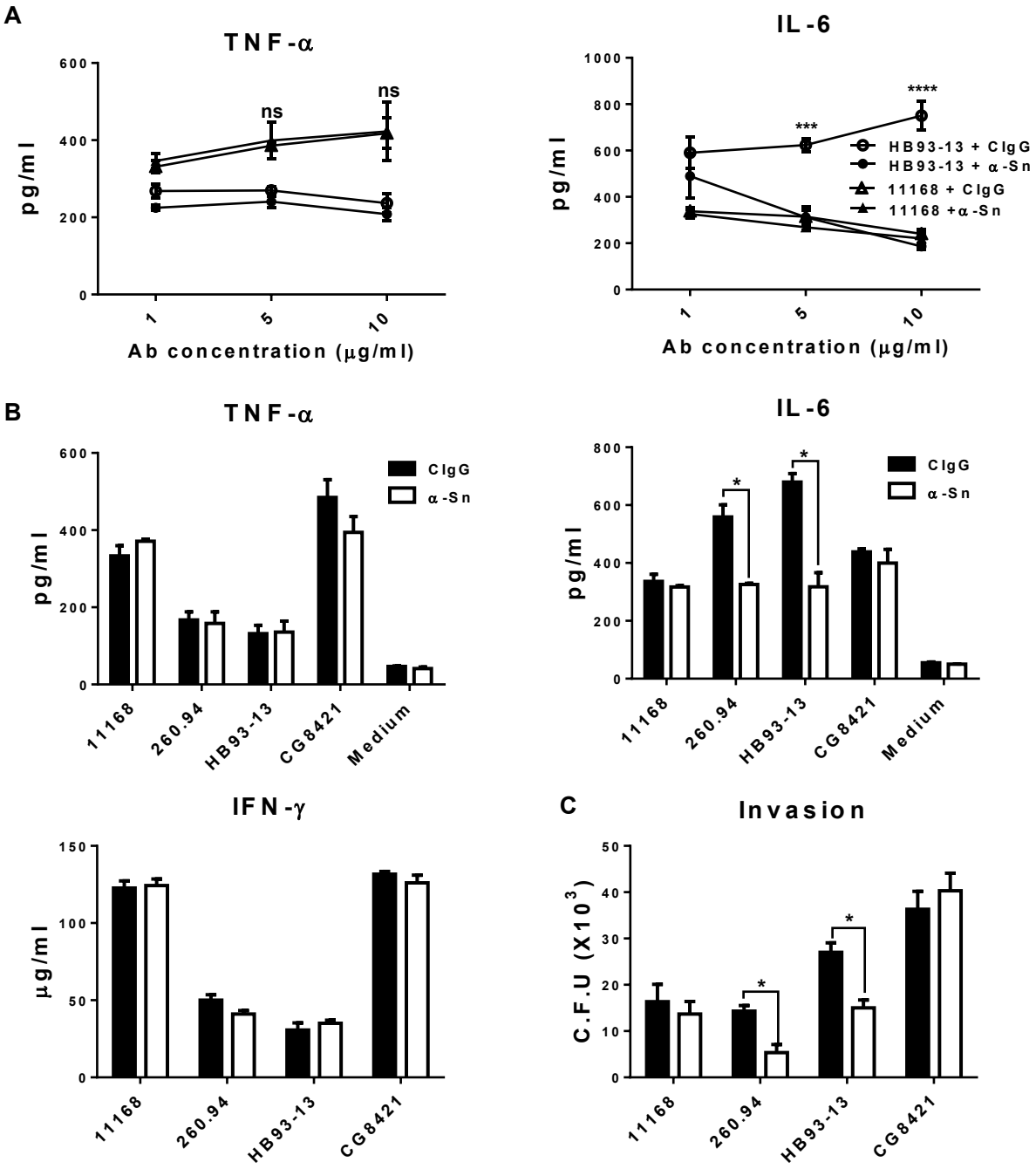


FIGURE 3.5. SIglec-1 blocking in IL-10$^{-/-}$ mice, along with autoantibody analysis in IL-10$^{+/+}$ mice.

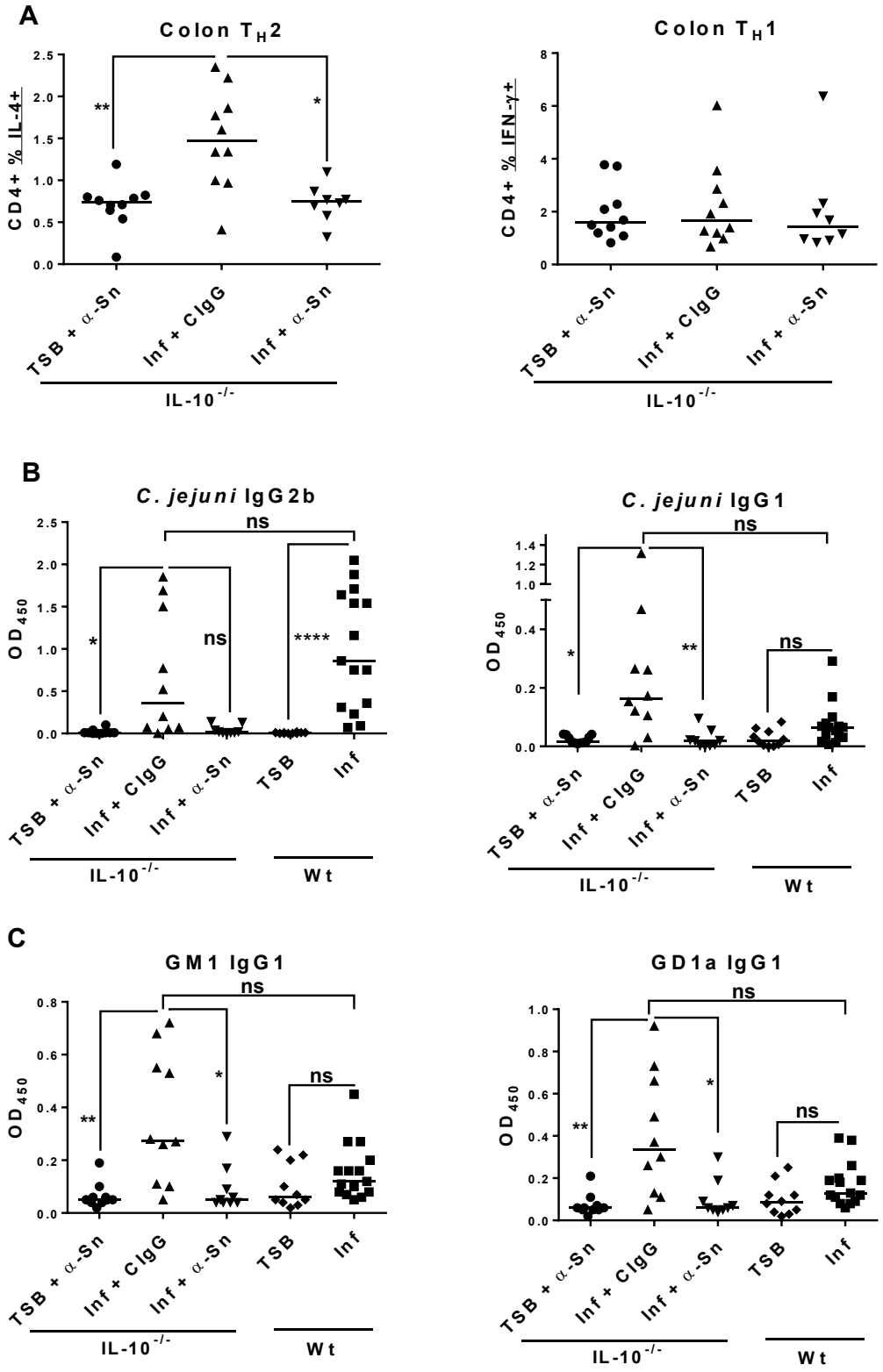


Figure 3.6. Long term phenotyping experiment with GBS isolate infected IL-10$^{-/-}$ mice.

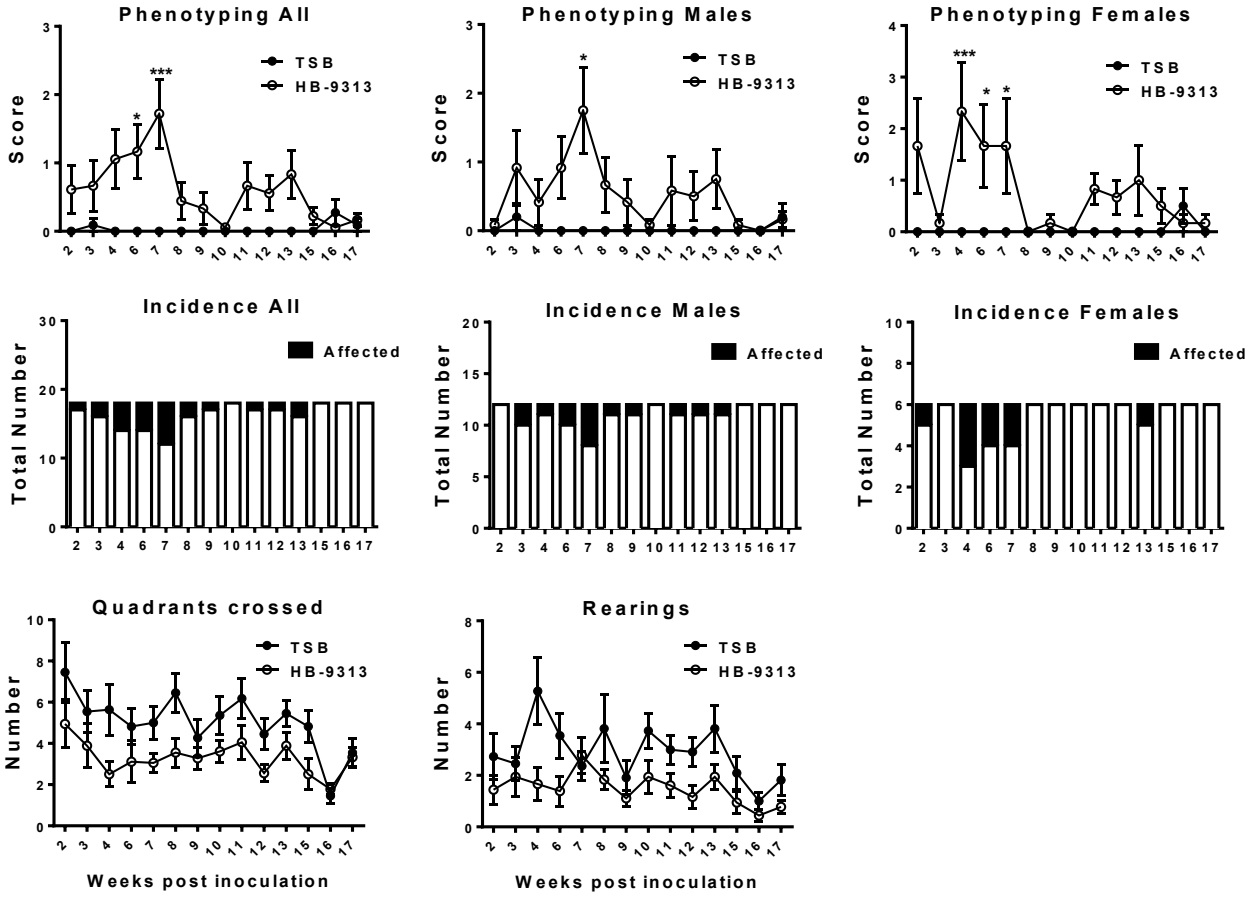
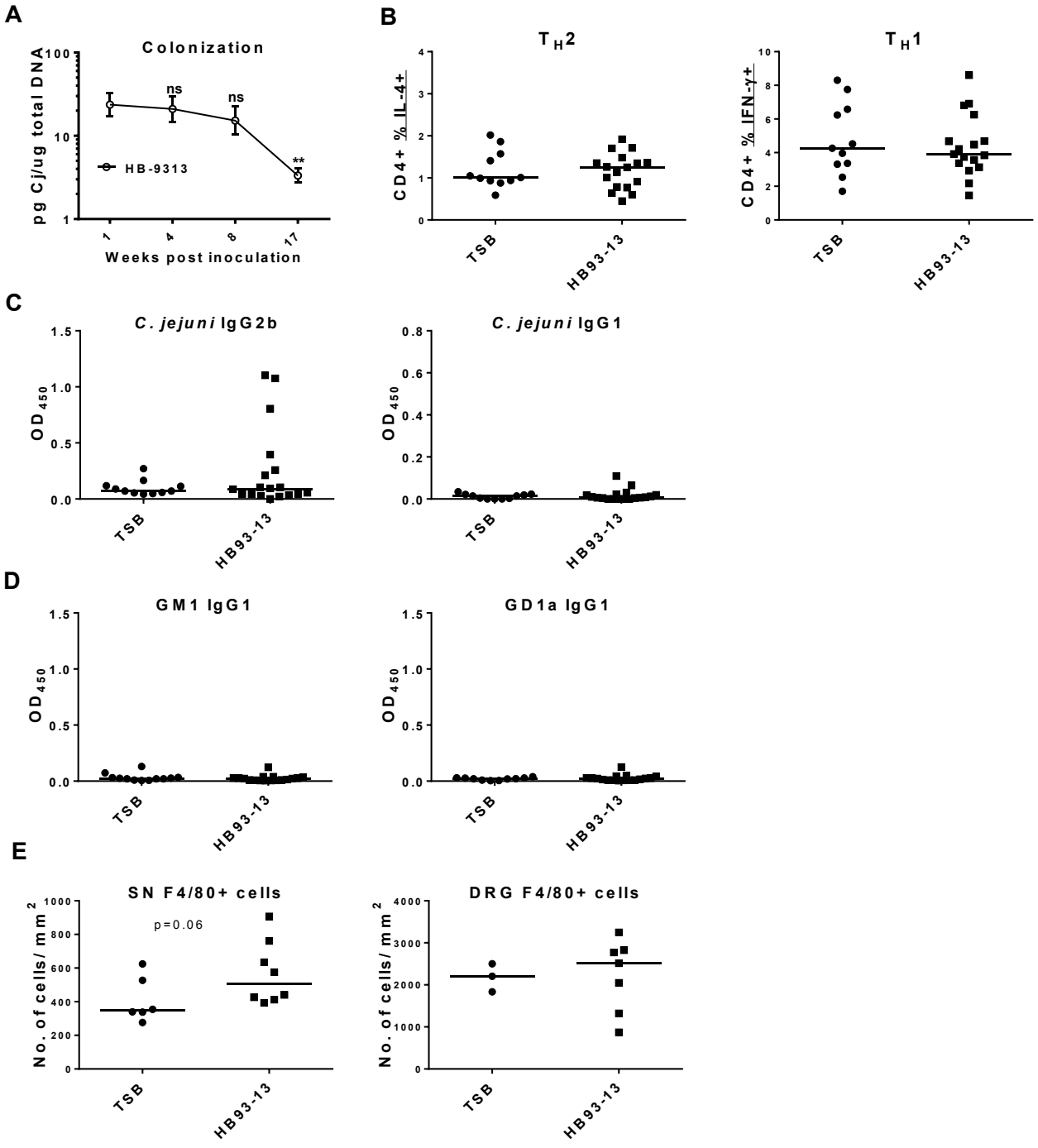


Figure 3.7. Long term phenotyping experiment: colonization, colon flow cytometry and plasma antibody analysis.



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CHAPTER 4

Conclusions, Significance and Future Directions

Conclusions and Significance

Campylobacter jejuni is an ubiquitous enteric bacterium that is the most common bacterial cause of gastroenteritis and has also been linked with development or flare-up of Inflammatory Bowel Disease and Irritable Bowel Syndrome. *C. jejuni* infection has also been causally linked with development of the peripheral neuropathy, Guillain Barré Syndrome (GBS). The occurrence of GBS is associated with development of autoantibodies that target gangliosides on peripheral nerves. The lipooligosaccharide of *C. jejuni* isolated from GBS patients with antecedent infections have been shown to mimic the peripheral nerve gangliosides GM1, GD1a and others. At present, therapies against GBS are untargeted and highly invasive or toxic, like plasmapheresis and intravenous immunoglobulin (IVIg) treatment. They too are not helpful or stop working in almost 40% of the patients[1]. The development of effective treatments is limited by lack of appropriate animal models.

We have previously shown that a number of isolates of *C. jejuni* from human enteritis patients induce colitis in the IL-10$^{-/-}$ mouse[2] whereas the isolates from human GBS patients colonize the IL-10$^{-/-}$ mice without inducing colitis[3]. The thesis presented here tested our overarching hypothesis that differential strain dependent immunological mechanisms are behind colitis induction by a colitogenic strain and asymptomatic colonization by the GBS strains. Further, we show that infection of mice with *C. jejuni* strains from GBS patients elicits autoantibody production that is consistent with that seen in human GBS cases[4]. We found that the colitogenic *C. jejuni* isolate elicits a Type1 and Type17 cellular, cytokine and antibody response as measured in the colon, mesenteric lymph node and plasma. We also determined that Innate Lymphoid Cells as well as adaptive T cells participated in producing a mixed Type1 and Type17 response in colon and draining lymph nodes in a time dependent manner by intracellular cytokine staining and flow cytometry. T cells were found to be necessary for severe colitis as

mice depleted of Thy-1^{+} cells (a common T cell and innate lymphocyte marker) were protected while the T cell deficient mice (Rag1$^{-/-}$ and IL-10R blocked Rag1$^{-/-}$) failed to develop colitis after infection with the colitogenic isolate. The pro-inflammatory role of IFN-γ and IL-17 in *C. jejuni* colitis was demonstrated by neutralizing these proteins *in vivo* which led to colon-protective Type2 cytokine and antibody responses. On the other hand, GBS *C. jejuni* isolates induced blunted Type1 and Type17 but enhanced Type2 colon cytokine and *C. jejuni* specific antibody responses. Furthermore, Type2 but not Type1/17 systemic autoantibodies were produced following infection with *C. jejuni* GBS isolates that also reacted with peripheral nerve gangliosides. These autoantibodies also cause peripheral neuropathic phenotype and histological manifestation that is consistent with this syndrome's manifestations in humans.

Therefore, in support of our overarching hypothesis, we have demonstrated that *C. jejuni* mediated colitis in specific pathogen free C57BL/6IL-10$^{-/-}$ mice is T cell, IFN-γ and IL-17 dependent. This work is also the first demonstration of the time-dependent role of Innate Lymphoid Cells and T cells in mediating Type1 and Type17 responses following infection with a human pathogen. We also show that a contrasting CD4^{+} T cell and IL-4 dependent Type2 immune response pivots the disease away from inflammation in the colon to generation of circulating autoantibodies. This work yields new cytokine and cellular targets for immunomodulatory therapy against IBD. It also suggests a potential mechanism for the situation where patients with rheumatoid arthritis or IBD receive TNF-α blocker treatment and then go on to develop different autoimmune diseases that manifest with autoantibodies. Viral myocarditis is the only known model for studying an intact pathogen's molecular mimicry based autoimmune sequelae. This is the first mouse model of an autoimmune disease induced directly by a bacterium. We further provide IL-4 and Siglec-1 as novel and rational therapeutic targets against this peripheral autoimmunity.

Future direction 1. To determine if early ILC activation facilitates T cell activation in *C. jejuni* mediated colitis.

Thy-1 is a marker common to both T cells and ILCs, and we have shown that depletion of these cells ameliorates *C. jejuni* colitis. Furthermore, since neither Rag1$^{-/-}$ nor IL-10R blocked Rag1$^{-/-}$ mice developed colitis after infection, we can conclude that T cells are necessary for *C jejuni* to induce colitis. This aim is designed to directly determine if T cell activation is dependent on early activation of ILCs. However, as there are no known gene(s) that are exclusively expressed by, or required for the development of innate but not adaptive lymphocytes, *de novo* models that contain adaptive lymphocytes but are devoid of innate lymphocytes do not exist. However, to study the effect of adaptive lymphocytes independent of innate lymphocytes, we can engineer a mouse model that contains adaptive lymphocytes and a 'normal' innate immune system except that it will be devoid of all innate lymphocytes. Rag1$^{-/-}$ $\gamma_c^{-/-}$ (double KO) mice are devoid of all lymphocytes, adaptive and innate, and by injecting positively sorted splenic B and T cells into these mice, one can generate a model of transiently isolated adaptive immunity that is of devoid of all innate lymphocytes. Similar strategy was used by Sonnenberg *et al.,* to show that LTi's are required for protective immunity against *Citrobacter rodentium* infection[5]. These mice can then be inoculated with *Campylobacter jejuni* to evaluate the inflammatory versus protective role of adaptive lymphocytes in *C. jejuni* mediated colitis. T-cell dependent antibody response specific to *C. jejuni* in the adaptive lymphocyte replete and innate lymphocyte deficient host can be used as an internal positive control to check the ability of introduced B and T cells in mounting an immune response similar to that of an unmanipulated host.

Primary Hypothesis. Innate lymphocytes are dispensable for colitis in adaptive lymphocyte-replete host.

Experimental Design. 6 week old Rag1$^{-/-}$ $\gamma_c^{-/-}$ C57BL/6 mice will be injected i.p. with 6X10^7 positively sorted splenic B cells (B220$^+$) and T cells (CD3$^+$) from donor wild type mice. 3 weeks post injection, mice will be orally infected with 10^{10} CFU of *C. jejuni* 11168 and monitored for clinical signs of diarrhea or colitis for up to 5 weeks. Age and sex matched mice inoculated with TSB will serve as controls.

Parameters to be analyzed: Mice will be analyzed for weight loss and clinical signs of diarrhea and colitis, and also for histopathological changes in the colon.

Alternative theory: Adaptive lymphocytes replete but innate lymphocyte deficient host will not develop colitis after *C. jejuni* infection. This could be the case due to two reasons: 1. IL-10 produced in the host was sufficient to prevent colitis and/or 2. ILCs are necessary for T cells to induce colitis. If the alternative scenario turns out to be true, we will distinguish between the two possible reasons by depleting IL-10 in the host during the infection. This will be achieved by injecting 400µg of IL-10 neutralizing antibody every 3 days and starting 6 days before the infection. If IL-10 depleted mice develop colitis, it will suggest that adaptive lymphocytes in the absence of IL-10 and innate lymphocytes are sufficient to induce colitis. If even after IL-10 neutralization mice do not develop colitis, then it will suggest that innate lymphocytes are indeed necessary to cause colitis.

Potential Pitfall: A major cause of concern is that the splenic adaptive lymphocyte population injected into the Rag1$^{-/-}$ $\gamma_c^{-/-}$ host will not be able to self-renew efficiently without continuous reconstitution from the bone marrow. Therefore this is model of *transiently* isolated adaptive immunity only.

Future Direction 2. To determine the role of IL-22 *in C. jejuni* induced colitis and autoimmunity.

In this study, we show that all *C. jejuni* isolates, colitogenic, non-sialylated and GBS associated, induce an IL-22 cytokine response in the colon and mesenteric lymph nodes (*in vivo*) and/or in the splenoctyes (*ex vivo*). IL-22 is a dichotomous cytokine that has been shown to be both pro-inflammatory as well as anti-inflammatory. It has been demonstrated as a major factor in maintaining epithelial barrier integrity in the lung and gut and preventing systemic dissemination of colonized mircobiota[6]. IL-22 is typically upregulated under inflammatory conditions and via STAT3 signaling, it plays a significant role in mucosal wound healing and goblet cell hyperplasia and enhanced mucus production[3]. Especially in conjunction with IL-17, IL-22 leads to elicitation of multiple antimicrobial peptides like β-defensins, S100, lipocalin-2 and Regenerating islet-derived protein (Reg) 3 β and γ from the epithelial cells[7, 8, 9]. Consequently, absence of IL-22 results in increased systemic spread and subsequent morbidity and mortality following gut and lung infections like *C. rodentium* and *K. pneumonia*[5, 10]. IL-22 has also been shown to be protective in ulcerative colitis, hepatitis and lung fibrosis models[11, 12, 13]. We and others have shown that NK cells, ILCs and $CD4^+$ T cells participate in the production of IL-22, with NK cells and ILCs as the dominant source at early time points (within first week post infection), and $CD4^+$ T cells as a dominant source later in the infection[14, 15]. On the other hand, IL-22 has been shown to be pro-inflammatory in experimental psoriasis/atopic dermatitis and collagen-induced arthritis models by upregulating expression of IL-23 and IL-1 family of cytokines by keratinocytes and promoting autoreactive IgG elicitation[16, 17, 18]. It will therefore be interesting to reveal the pro- versus anti-inflammatory role of IL-22 in *C. jejuni* mediated colitis and autoimmunity. Its role can be tested by either depleting IL-22 in IL-$10^{-/-}$ mice by neutralizing antibody injections or generating IL-22 and IL-10 double knock-out mice. It will also be interesting to infect single IL-$22^{-/-}$ mice to determine if it is responsible for preventing systemic dissemination and inflammatory response in IL-10 sufficient hosts.

Primary Hypothesis: IL-22 is primarily protective in *C. jejuni* induced colitis and autoimmunity. Consequently, its absence during colitogenic or GBS isolate infection will lead to systemic dissemination of *C. jejuni* and subsequent systemic inflammation, enhancing morbidity and mortality.

Experimental Design: 8-12 week old IL-10, IL-22 double KO mice will be orally inoculated with *C. jejuni* 11168 or HB93-13. Mice will be monitored for weight loss and clinical signs of colitis and GBS for up to 5 weeks post inoculation. After sacrifice, histopathological changes in the colon, sciatic nerve and its roots, and plasma autoantibody titers will also be evaluated.

Alternative Theory: It is possible that IL-22 is redundant in preventing systemic dissemination of *C. jejuni*. Instead, akin to arthritis and skin inflammation models, its primary role after *C. jejuni* infection is to upregulate pro-inflammatory gene expression from epithelial cells and antigen presenting cells. Consequently, its absence may ameliorate colitis after colitogenic isolate infection and/or decrease autoreactive IgG production after GBS isolate infection.

Future Direction 3. To evaluate the role of complement in *C. jejuni* induced autoimmunity.

It is well established that human GBS patients with antecedent infections usually have antibodies in circulation that cross-react with peripheral nerve gangliosides and the outer core of the pathogen's LOS. We have also demonstrated the proof-of-concept in IL-$10^{-/-}$ mice infected with *C. jejuni* GBS isolates. Autoantibodies against cell surface antigens can lead to cellular damage by complement activation via classical pathway and/or lead to ADCC by NK cells. Using explanted mouse diaphragm nerves, Plomp et al., examined the effect of anti-ganglioside antibodies on neuromuscular transmission. While autoantibody deposition alone did not affect transmission, addition of complement lead to a 300 fold decrease in neuromuscular transmission[19]. Therefore, complement can serve to enhance the effect of antibody deposition in blocking nerve conductance that can eventually lead to axonal death. Furthermore, antibody mediated classical complement activation leads to formation of membrane attack complex and release of C3a and C5a. C3a and C5a are strong macrophage chemoattractants, consequently complement may also be involved in recruitment of macrophages to the peripheral nerves. Deposition of complement factors on the nerves can be evaluated by IHC. Role of complement in *C. jejuni* induced GBS can be tested with $C3^{-/-}$ mice. C3 is a central component of complement, essential for C5 cleavage and membrane attack complex formation.

Primary Hypothesis: Complement activation is necessary for anti-ganglioside antibody mediated nerve damage and macrophage infiltration. Therefore complement deposition will be found in the sciatic nerve of GBS isolate infected IL-$10^{-/-}$ mice, and it will be co-localized with macrophage marker F4/80. Furthermore, infected C3 and IL-10 double KO mice will have reduced infiltration of macrophages in sciatic nerves and its roots when compared to single IL-$10^{-/-}$ mice.

Potential Pitfall. $C3^{-/-}$ mice are hypersusceptible to certain bacterial infections, like intra-venous Group B Streptococcus infection results in increased mortality as compared to

immunocompetent controls (LD_{50} dose: control 6.3 x 10^4 vs. 1.3 x 10^3 in C3-deficient mice)[20]. Therefore, it may be necessary to choose a lower dose of *C. jejuni* for this experiment. We have shown that a dose of 10^2 cfu/mouse is sufficient to obtain stable colonization, and this low dose should be sub-lethal in these mice.

Alternative theory: Another approach to test the importance of complement towards *C. jejuni* induced GBS can be to use a mouse model that lacks complement regulatory checkpoint(s). CD59, also known as MAC-inhibitory protein, is a cell surface expressed protein that prevents formation of MAC complex by inhibiting C9 polymerization with the deposited C5b678 complex[21]. So if MAC formation aids in development of GBS lesions, CD59 IL-10 double KO mice will have a greater extent of phenotypic and histological changes associated with GBS when compared to single IL-$10^{-/-}$ mice. As CD59 deficiency will not affect the elicitation of released products C3a and C5a, this model alongside the $C3^{-/-}$ mice, will also help isolate the role of MAC formation versus chemoattractant generation as the primary function of complement in GBS.

APPENDIX

Table 1. Contrasting *C. jejuni* induced colitis and autoimmune neuropathy

	***C. jejuni* induced colitis**	***C. jejuni* induced autoimmunity**
Pathogenic Cytokine	IFN-γ, IL-17, IL-22?	IL-4, IL-21? IL-6?
T cell dependent?	Yes, including CD4+, CD8+ and γδ+	Yes
Role for ILCs?	Yes, ILC1 and ILC17	No
Siglec dependent?	No	Yes
B cell dependent?	No	Yes
Exacerbated by IL-10 deficiency?	Yes	Yes
Major IgG isotype	IgG2c, IgG3, IgG2b	IgG1, IgG2b

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