Innate Immune Recognition of Glycosylated Surface Determinants of *Campylobacter jejuni*

By

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Declaration:

I, Holly Nicole Stephenson confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Campylobacter jejuni, a commensal in poultry members, causes acute gastroenteritis in humans. Understanding of *Campylobacter jejuni* pathogenesis lags far behind that of other gastrointestinal pathogens despite *Campylobacter sp.* being a leading cause of bacterial gastroenteritis in the developed world.

In this study *C. jejuni* was shown to induce high levels of IL-10 from dendritic cells, a potent anti-inflammatory cytokine. IL-10 secretion was induced by the flagella of *C. jejuni,* a protein based filament structure that is modified by sialic-acid like structures. These sugar structures were shown to be critical in the induction of IL-10. *C. jejuni* lacking flagella induced lower levels of p38 activation, and inhibition of p38 reduced IL-10 secretion. Interestingly Myd88-dependant TLR signalling was shown to be critical for the induction of IL-10 despite the inability of *C. jejuni* flagellin to activate TLR5. We showed that *C. jejuni* can bind to Siglec-10, an interaction that was dependent on the glycosylation of the flagella. We speculate that the addition of glycan structures to *C. jejuni* flagellin proteins may be a host-subversion strategy via the induction of IL-10.

C. jejuni strains isolated from infected humans can be sub-divided into two distinct phylogenetic clades. We sought to investigate whether the lipooligosaccharide (LOS) structure is distinct between the two clades. The structures of the LOS from 15 different strains were analysed. Variation in the oligosaccharide (OS) structure, amide linkages connecting the acyl chains to the lipid A backbone, and phosphorylation of the lipid A between the strains were observed. Phosphorylation of the lipid A and sialylation of the OS correlated with the induction of TNF-α from monocytes. Interestingly, the sialylation of the OS correlated with the phylogenetic clade.

Collectively the data presented highlights the importance of glycosylated surface determinants on pathogenic bacteria to manipulate the host innate immune response.

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Abbreviations

AMP	Antimicrobial peptide
BA	Blood agar
CDT	Cytolethal distending toxin
CFU	Colony forming unit
CHO	Chinese hamster ovary cell line
C. jejuni	*Campylobacter jejuni*
CPS	Capsular polysaccharide
DC	Dendritic cell
DHB	2,5-dihydroxybenzoic acid
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
FACS	Fluorescence-activated cell sorting
FAM	6-carboxyfluorescein
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GBS	Guillain-Barré Syndrome
GI	Gastrointestinal
GlcN	D-glucosamine
GlcN3N	2,3-diamino-2,3-dideoxy-D-glucose
HBD	Human beta defensin

HEK	Human embryonic kidney cell line
Hex	Hexose
HexNAc	N-acetyl hexosamine
HI	Heat inactivated
IBD	Inflammatory Bowel Disease
IEC	Intestinal epithelial cell
IFNγ	Interferon-gamma
KDO	3-Deoxy-D-manno-oct-2-ulosonic acid
L	Litre
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
IVOC	*In-vitro* organ culture
Leg	Legionaminic acid
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
µL	Micro-Litre
µM	Micro-Molar
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MAPK	Mitogen-activated protein kinase
min	Minute
mL	Milli-Litre
MLST	Multi-locus sequence type
mRNA	messenger ribonucleic acid

MOI	Multiplicity of infection
MS	Mass spectrometry
NeuAc	Sialic acid
NF-κB	Nuclear factor kappa light-chain-enhancer of activated B cells
NOD	Nuclear-binding oligomerisation domain
OD	Optical density
P	Phosphate
PAGE	Poly-acylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEA	Phosphoethanolamine
PGN	Peptidoglycan
Pse	Pseudaminic acid
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
PRR	Pattern recognition receptor
RT	Room temperature
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS	Sodium-dodecyl-sulphate
SEAP	Secreted embryonic alkaline phosphatase
sec	Second

SEM	Standard error of the mean
ST	Sequence type
TAMRA	Tetramethylrhodamine
TBS	Tris-buffered saline
THAP	2,4,6-trihydroxyacetophenone
THP-1	Human acute monocytic leukaemia cell line
TLR	Toll-like receptor
TNF-α	Tumour-necrosis factor alpha
UC	Ulcerative colitis
VAIN	Variable atmosphere incubator
VSV-G	Vesicular stomatitis virus glycoprotein G
WT	Wild-type

Chapter 1.

Introduction

1.1 Campylobacter jejuni

Campylobacteriosis is the most frequent cause of food-borne gastroenteritis in the European Union with over 190,000 cases reported annually, although the actual number of cases are estimated at 9 million (European Food Safety Authority; EFSA). *Campylobacter sp.* are a major cause of bacterial-associated gastroenteritis in the UK with rates as high as 9.3 cases per 1000 person-years for community acquired infections (Tam et al. 2012). The economic cost of infection in the EU is estimated to be 2.4 billion Euros therefore not surprisingly considerable efforts are being made to limit infection. *Campylobacter jejuni* is the cause of ~90% cases of human *Campylobacter* enteritis. In the majority of cases, disease is self-limiting with acute gastroenteritis lasting less than 1 week; however in a small minority of cases post-infection immune pathologies develop. Despite the heavy disease and economic burden presented by *C. jejuni*, far less is known about its pathogenesis when compared to other enteropathogens such as *Salmonella typhimurium, Shigella sp.,* and *Escherichia coli.*

1.1.1 *Taxonomy*

C. jejuni belongs to the genus *Campylobacter,* order Campylobacterales, which are members of the epsilon class of proteobacteria. The *Campylobacter* genus was first identified as separate from *Vibrio* in 1963 after Sebald and Véron noted the distinct biochemical properties of *Vibrio fetus* and *Vibrio bubulus* from other Vibrio species (Sebald and Veron. 1963). In 1973, further analyses of microaerobic/anaerobic bacteria in the *Vibrio* taxa resulted in the re-classification of these bacteria into the *Campylobacter* taxon (Veron and Chatelain R 1973). Pre-1973 *Campylobacter sp.* were primarily seen as veterinary pathogens, and a 1957 report on related *Vibrio* in human diarrhoeal disease was believed to be an anomaly (King 1957). Throughout the 1970's the importance of *Campylobacter sp.* in human gastroenteritis became apparent as new techniques allowed the culturing of these micro-aerophillic bacteria from patients presenting with diarrhoea (Skirrow 1977). There are currently 25 known species of *Campylobacter* which have been isolated from a diverse array of hosts, both colonised without apparent symptoms and from those presenting with enteritis.

Campylobacter jejuni was first described in 1931 by Jones and colleagues in cattle suffering from spontaneous diarrhoea (termed *Vibrio jejuni*) (Jones et al. 1931). There are two subspecies of *Campylobacter jejuni: C. jejuni* subsp*. jejuni* (referred to as *C. jejuni*) which is the major cause of human *Campylobacter* enteritis (campylobacteriosis), and *C. jejuni*

subsp. *doylei.* The two sub species differ considerably in their distribution and ecology. As yet no known animal host has been identified for subsp. *doylei* (Parker et al. 2007). *C. jejuni* subsp. *doylei* has been isolated from patients suffering from both gastritis and enteritis, and is more frequently associated with bacteraemia than subsp. *jejuni*. Interestingly, the genes encoding the cytolethal distending toxin (CDT) are absent in subsp. *doylei* and it is hypothesised that a suboptimal host pro-inflammatory response increases chances of systemic infection. Overall, subsp. *doylei* is the cause of very few cases of gastroenteritis in comparison to subsp. *jejuni.*

The second most common cause of campylobacteriosis is *C. coli,* which is genetically the closest relative of *C. jejuni*. *C. coli* accounts for <20% of human disease and often colonises pigs (Gurtler et al. 2005). Over the past decade the importance of other *Campylobacter sp.* in human disease has increasingly been appreciated (Man 2011). *C. concisus* was first isolated from a patient suffering from periodontal disease in the 1980's, its role in disease however remains unclear as it has subsequently been isolated from healthy patients (Macuch and Tanner 2000). *C. concisus* and *C. upsaliensis* have been isolated from patients suffering from campylobacteriosis in Belgium, South Africa, Denmark, and Sweden. It has been hypothesised that the fastidious growth conditions of these *Campylobacter sp.* has resulted in an under representation of these organisms in clinical diagnosis (Man 2011).

Helicobacter, *Wolinella*, *Arcobacter*, *Sulfurospirillium* and *Campylobacter* belong to the order Campylobacterales. *Helicobacter pylori* was originally termed *Campylobacter pylori*, however in 1989 *Helicobacter* was assigned to a new genus as increasing structural and biochemical differences between the two genus's were identified; such as the sheath that encases the flagellum of *Helicobacter sp.* but is absent in the *Campylobacter sp.* (Goodwin et al. 1989). *H. pylori* colonises the human stomach and is the leading cause of gastritis and related complications. *Wolinella succinogenes* is a non-pathogenic commensal of cattle rumen; interestingly it retains many of the virulence factors associated with *H. pylori* and *C. jejuni* (Baar et al. 2003). Intricate crosstalk between host and bacterial factors most likely determines the potential outcome of host-microbe interactions which may include commensalism, long-term existence with potential pathology or acute infection with or without additional chronic clinical manifestations.

1.1.2 *Clinical Manifestations*

1.1.2.1 Gastroenteritis

Acute gastroenteritis is the major clinical manifestation in humans infected with *C. jejuni*; clinical symptoms include watery or bloody diarrhoea, often accompanied by abdominal cramps and fever (Blaser 1997). Symptoms often resolve within 1 week, although they may persist longer than this. Incubation times of between 24-72h generally occur but incubation periods of >1 week have been reported, the latter may be associated with low inoculum of infection. In the majority of cases in the developed world campylobacteriosis induces inflammatory diarrhoea, with polymorphonuclear (PMN) cells and erythrocytes present in the stools (Blaser et al. 1982). Symptoms are generally self-limiting, although immunocompromised patients, such as HIV sufferers, are more likely to have extended gastroenteritis and develop bacteraemia (Manfredi et al. 2002).

Clinical manifestations in developing *versus* developed countries

Clinical manifestations of *C. jejuni* infection differ in people from developing countries than those from developed countries, a fact which may aid in improving our understanding of its pathogenesis. Principally, people in developing countries are exposed to multiple *C. jejuni* strains far more frequently throughout their lifetimes which correlates with a decrease in the symptoms associated with infection (Blaser 1997). Gastroenteritis is more commonly seen in young children, and it is rare for adults to suffer from symptomatic disease (Blaser 1997). Additionally, people from developing countries may asymptomatically shed *C. jejuni,* a phenomena often associated with a prior enteritis episode (Blaser et al. 1980; Pazzaglia et al. 1991). Symptomatic disease is often watery rather than bloody diarrhoea. This suggests repeated exposure to different *C. jejuni* strains in early childhood allows the development of protective immunity which limits symptomatic disease in older individuals (Havelaar et al. 2009). Interestingly, travellers from developed countries with no pre-existing *C. jejuni* immunity develop the more serious symptoms of bloody diarrhoea/abdominal cramps when infected with *C. jejuni* in developing countries (Walz et al. 2001). Additionally, the same isolates associated with watery diarrhoea in children in developing countries have been isolated from travellers presenting with bloody diarrhoea (Ketley and Konkel 2005). This suggests strain variation is not the major cause for the differences observed between developed and developing countries. In addition, no differences have been found between strains isolated from symptomatic and asymptomatic infection (Champion et al. 2005; Sjogren et al. 1989). In a human volunteer study however *C. jejuni* strains showed differential ability to induce pathology (Black et al. 1988). Taken together, strain variation

may account for some but not all of the differences in clinical manifestations observed around the world.

The protective immunity acquired by repeated exposure to different *C. jejuni* strains protects only from symptomatic disease but not colonisation in developing countries (Havelaar et al 2009; Sjogren, Ruiz-Palacios, & Kaijser 1989; Taylor et al. 1988). *C. jejuni* has also been isolated from asymptomatic long-term abattoir workers (Cawthraw et al. 2000). Calculations based on multiple longitudinal cohort studies predicts that even in developed countries only 1:120 people who shed *Campylobacter* species actually present with clinical manifestations (Havelaar et al. 2009). Host immunity is therefore predicted to be critical in the outcome of exposure to *C. jejuni* in humans.

Invasive disease

Invasive disease and bacteraemia is very uncommon in *C. jejuni* infected individuals. HIV-patients and the elderly are more likely to suffer from bacteraemia which suggests host immunity plays a major role in limiting systemic spread (Fernandez-Cruz et al. 2010).

The spectrum of clinical manifestations associated with human *C. jejuni* infection allows a basic model of infection to be predicted which is largely dependent on the host immune status. In an immune naïve individual a sufficient *C. jejuni* inoculum with a virulent strain causes acute inflammation and diarrhoea; this acute inflammation accounts for the bloody diarrhoea however it also prevents systemic spread. An individual who has acquired immunity to multiple *C. jejuni* strains can mount a robust adaptive immune response and limiting pathology but not colonisation.

1.1.2.2 Intestinal and Extra intestinal sequelae of C. jejuni infection

Inflammatory Bowel Disease

Crohn's Disease (CD) and Ulcerative Colitis (UC) are collectively termed Inflammatory Bowel Disease (IBD); they represent major chronic inflammatory disorders of the gastrointestinal (GI) tract. Increased risk of developing IBD has been associated with both *Campylobacter* and *Salmonella* infection (Gradel et al. 2009). However, this association remains controversial as a more recent study has shown increased sampling bias around the onset of IBD is responsible for the increased detection rates of *Campylobacter* and *Salmonella* in newly diagnosed IBD patients (Jess et al. 2011).

Guillain-Barré Syndrome

Guillain-Barré Syndrome (GBS) is an autoimmune neuropathy resulting from the production of auto-antibodies that cause de-myelination of nerve gangliosides in the peripheral nervous system. Symptoms often begin in the lower extremities, and can ascend through the body resulting in full paralysis requiring ventilator-assisted breathing. The majority of GBS cases are preceded by gastroenteritis, and the discovery of molecular mimicry between *C. jejuni* lipooligosaccharide (LOS) and nerve gangliosides identified *C. jejuni* infection as a major aetiological agent of GBS (Aspinall et al. 1994; Mishu and Blaser 1993). Host factors are also important as GBS-associated *C. jejuni* strains have been isolated from patients suffering from uncomplicated enteritis (Nachamkin et al. 1999).

Miller Fisher Syndrome

Miller Fisher Syndrome (MFS) is a rare variant of GBS which primarily affects the nerves involved in eye movement. *C. jejuni* infection has also been linked to the development of MFS (Yuki 1997).

Reactive Arthritis

Reactive Arthritis (RA) is a post-infectious inflammatory arthritic disease associated with the HLA-B27 allele. Along with other enteropathogens such as *Salmonella, C. jejuni* is associated with the onset of this disease (Doorduyn et al. 2008).

1.1.3 *Disease and colonisation in other species*

C. jejuni can cause enteritis in many animal species. *C. jejuni* has been isolated from dogs, cats, pigs, and ferrets suffering from diarrhoea (Babakhani et al. 1993; Davies et al. 1984; Fox et al. 1986). *C. jejuni* can reside in pigs as a commensal or cause diarrhoeal disease (Ketley & Konkel 2005). Outbred pigs with no prior exposure to *C. jejuni* develop disease after inoculation (Mansfield et al. 2003). Immunocompetent pigs with no alterations to enteric bacteria do not suffer enteritis on exposure to *C. jejuni,* in contrast colostrum-deprived new born piglets can suffer similar symptoms to human pathology (Babakhani 1993). These findings suggest that adaptive immunity is important in the clinical manifestations of *C. jejuni* infection in pigs, as noted for humans. *C. jejuni* is not considered to be a major cause of enteritis in cattle and sheep even though it is implicated with increased incidence of abortion (Manser and Dalziel 1985).

Zoonotic transmission is the major route of *C. jejuni* infection in humans, in particular transmission from contaminated poultry is estimated to cause between 20-70% of all human infection (Allos 2001). For this reason understanding *C. jejuni*/chicken GI

interactions has received increased attention in recent years. Approximately 75% of all commercially reared chickens are infected with *C. jejuni* (EFSA). *C. jejuni* is generally considered a commensal in poultry although *C. jejuni*-mediated diarrhoeal disease has been reported in inoculated 3 day-old chicks (Ruiz-Palacios et al. 1981). *C. jejuni* spreads rapidly in broiler chickens, which can be colonised at very high numbers of between 10^6-10^8 colony forming units (CFU)/gram in the cecum (Meade et al. 2009). Surprisingly for a commensal bacterium, *C. jejuni* has been isolated from multiple organs in the broilers, including the spleen and liver, which indicates systemic spread is possible from the cecum (Meade et al. 2009). This also raises questions about its commensal status in chickens. Evidence suggests that *C. jejuni* translocates through the epithelial barrier to enable systemic spread by a process that does not elicit an inflammatory response (Van et al. 2008). The lack of an inflammatory response in chickens is considered to be a result of an "immune-dampening" strategy by *C. jejuni* (Hermans et al. 2011). *C. jejuni* reduces anti-microbial peptide (AMP) expression in the chicken gut highlighting one of many potential immune evasion mechanisms it employs (Meade et al. 2009).

In addition to contaminated poultry, other, non-livestock sources, such as contaminated milk and water are significant reservoirs of *C. jejuni.* A study comparing the phylogenetic relationship of "livestock" and "non-livestock or environmental" sources found that over 50% of human clinical strains tested clustered with the "environmental" strains (Champion et al. 2005). It is probable that this source of infection may have been under-represented in other studies.

1.1.4 *C. jejuni Surface Structures*

The surface structures of *C. jejuni,* like many other mucosal pathogens are heavily glycosylated; ~8% of its genome encodes proteins implicated in surface carbohydrate structures (Figure 1-1) (Parkhill et al. 2000). *C. jejuni* encodes both N-linked and O-linked glycosylation pathways for the modification of periplasmic proteins and flagellin proteins respectively. The first genome sequence highlighted the ability of *C. jejuni* to mediate phase-variation of many genes involved in the glycosylation pathways (Parkhill et al. 2000). Long stretches of repeated C or G bases called "homopolymeric tracts" increase slip-strand mispairing which can result in genes being "switched on" or "switched off". The importance of these glycosylation systems in the pathogenesis of bacterial-driven human disease is just beginning to be appreciated.

Figure 1-1: *C. jejuni* surface structure features

(a) The flagellum is anchored into both the inner and outer membrane. Flagellin proteins, FlaA and FlaB, are O-linked glycosylated with sialic-acid like structures. (b) The capsule is a highly branched polysaccharide structure. (c) Lipooligosaccharide (LOS) is an essential feature of the outer membrane. (d) Periplasmic and outer-membrane embedded proteins are modified by the N-linked glycosylation system. Modified from (Young et al. 2007).

1.1.4.1 Lipooligosaccharide

Lipopolysaccharide/lipooligosaccharide (LPS/LOS) forms an integral part of the outer membrane of Gram-negative bacteria. *C. jejuni* expresses LOS which consist of a hydrophobic lipid A that anchors into the outermembrane and an extracellular carbohydrate oligosaccharide (OS) that lacks the repeating carbohydrate O-antigen of LPS molecules (Figure 1-2). The lipid A anchor is essential for membrane integrity and complete mutation of *C. jejuni* LOS is lethal (Phongsisay et al. 2007). The OS structure can vary in length and composition; this property is a key strategy employed by Gram-negatives' that allows them to evade and modulate the immune system of the intended host. Interestingly, severe truncation of the OS to just one sugar moiety proximal to the 3-deoxy-α-D-manno-oct-2-ulopyranosic acid (KDO) residue does not affect bacterial growth, although it does increase the sensitivity of *C. jejuni* to antibiotics (Figure 1-2b) (Kanipes et al. 2006).

The sugar components of the disaccharide lipid A backbone can be 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N) or a D-glucosamine (GlcN) moiety (Moran et al. 1991). The

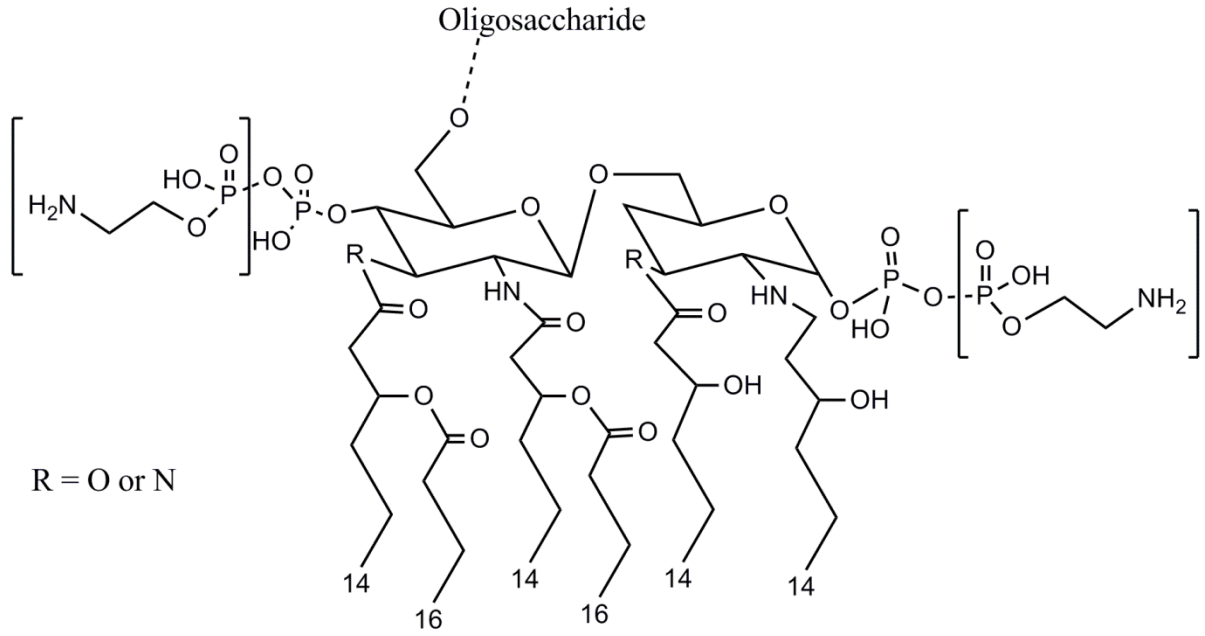
lipid A backbone is hexacylated with either palmitic (14 carbon) or myristic (16 carbon) acid (Moran et al. 1991). GlcN3N contains two amide linked palmitic/myristic acid chains, in comparison GlcN contains one amide and one ester linked fatty acid chain (Figure 1-2a). The two additional palmitic acid chains are attached to the palmitic and myristic acid of the sugar proximal to the OS attachment, making the disaccharide unsymmetrical. The most common backbone composition is GlcN3N-GlcN, although both GlcN3N-GlcN3N and GlcN-GlcN structures have been detected (Szymanski et al. 2003). Mutation of two genes, *gnnA* and *gnnB,* eliminates the production of the GlcN3N precursor, UDP-GlcNAc3N, resulting in the production of *C. jejuni* LOS bearing only GlcN-GlcN backbone (Van Mourik et al. 2010). GlcN3N sugars reduce both the ability of *C. jejuni* LOS to activate Toll-like receptor 4 (TLR4) and the sensitivity of live *C. jejuni* to cationic AMPs. The disaccharide backbone can be modified with the addition of both phosphate (P) and phosphoethanolamine (PEA) on either or both sugars (Moran et al. 1991; Szymanski et al. 2003). The gene responsible for the addition of PEA groups onto the lipid A, *Cj0256*, is also responsible for the modification of the flagellar rod protein, FlgG, with PEA (Cullen and Trent 2010). The phosphorylation pattern and disaccharide composition of *C. jejuni* varies between strains and can also differ between different growths of the same strain; how these properties alter virulence remains to be determined (Szymanski et al. 2003).

The *C. jejuni* OS consists of a mainly conserved inner core OS proximal to the lipid A backbone, and an outer core OS which varies significantly between strains suggesting the two OS cores are under differential selective pressure. The OS structure of *C. jejuni* strain NCTC 11168 is shown in Figure 1-2b. The inner core OS contains two KDO residues, two heptose (Hep) residues, with two branched glucose (Glu) residues on each heptose, and one PEA/P residue on the heptose proximal to the KDO (St Michael et al. 2002). The outer core OS of 11168 and other *C. jejuni* strains are composed of galactose (Gal), glucose, N-acetyl-D-galactosamine (GalNAc), and N-acetylneuraminic acid (Neu5Ac; sialic acid) residues. The number of each sugar varies between strains and some strains lack sialic acid and GalNAc residues completely. The LOS biosynthesis locus in the *C. jejuni* genome is hypervariable and is composed of between 10 – 20 genes (Parker et al. 2008). To date 19 different LOS classes have been identified with varying levels of homology (Parker et al. 2005; Parker et al. 2008). Figure 1-3 shows the original 6 LOS classes described in 2005 (Parker et al. 2005). A further 11 loci described in 2008 evolved by the deletion/ insertion of single or whole cassettes of genes from either other LOS loci or capsular polysaccharide (CPS) loci (Parker et al. 2008). *waaC* (open reading frame (orf) 1) and *waaF* (orf13) encode

heptosyltransferases for the addition of the inner core Hep residues (Kanipes et al. 2006; Oldfield et al. 2002). *lgtF* (orf3) is predicted to be a potential glycosyltransferase; the two domain protein (e.g. class A) transfers a Glu residue to both Hep residues in the inner core, the one-domain protein (e.g. class D) transfers only a single Glu to the Hep proximal to the KDO residue (Kanipes et al. 2008). Orfs 4, 5, and 6 are predicted to function in the addition of Gal residues to the outer core (Karlyshev et al. 2001). Classes A, B, and C contain genes responsible for the sialylation of LOS. *neuA1* (orf 10) encodes a CMP-Neu5Ac synthase; *neuB1* (orf 8) encodes a Neu5Ac synthase; *neuC1* (orf9) encodes a UDP-GlcNAc 2-epimerase; and *cstII* (orf 7) encodes a sialyltransferase (Gilbert et al. 2000; Gilbert et al. 2002; Linton et al. 2000). Sialic acid may also be modified by an O-linked acetyl group catalysed by sialic-acid O-acetyl transferase encoded by *soat* (orf 11; class A and B) (Houliston et al. 2006). *cgtA* (orf 5) encodes a GalNAc-transferase critical for the addition of GalNAc to the outer core (Guerry et al. 2002). Sequencing of multiple LOS loci has revealed minor genetic alteration within strains of the same LOS class as an additional mechanism which *C. jejuni* employs to vary the outer core OS. Phase-variation, gene inactivation by single base insertion/deletion and missense mutations are all operational at the *C. jejuni* LOS loci (Parker et al. 2008).

The high degree of variability in the outer core OS structure suggests that it may be under differential selective pressure compared to the inner core. Indeed many structural features of the outer core OS are critical in host-interactions (Jeon et al. 2009; Kanipes et al. 2006; Naito et al. 2010). An important consequence is the molecular mimicry observed between the OS outer core structure and host gangliosides and other glycans (Aspinall et al. 1994; Houliston et al. 2011). In addition to gangliosides, *C. jejuni* LOS can share structural features with P-blood group, paragloboside, lacto-*N*-biose and sialyl-lewis-c units (Houliston et al. 2011). Presumably, mimicry of host glycans is a potential immune evasion strategy, as adaptive immune cells with specificities for these glycans will be deleted leading to increased peripheral tolerance.

(a)



(b)

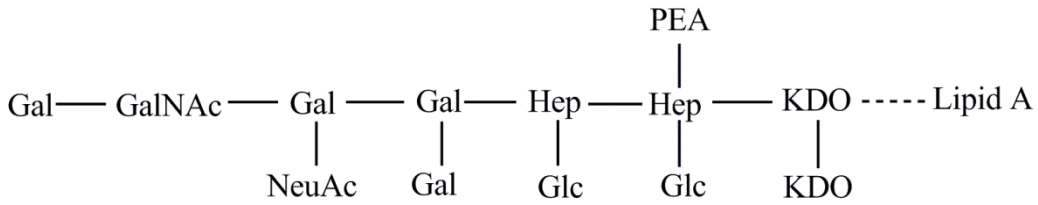


Figure 1-2: Structure of *C. jejuni* Lipooligosaccharide (LOS)

(a) Hexacylated *C. jejuni* lipid A. (b) Oligosaccharide structure from *C. jejuni* strain 1116H (LOS class C). Hep = heptose; KDO = 2-keto-3-deoxyoctulosonic acid; Glc = glucose; Gal = galactose; GalNAc = N-acetylgalactosamine; PEA = phosphoethanolamine; NeuAc = 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosonic acid.

Figure 1-3: *C. jejuni* LOS classes

Characterisation of 6 *C. jejuni* LOS classes. Common genes to all known LOS classes are coloured mid-grey; genes involved in sialic acid modification are coloured dark grey; other glycosyltransferases are coloured light grey (Parker et al. 2005)

1.1.4.2 Flagella

C. jejuni possess a single flagellum at either pole; this structure is critical for motility and the colonisation of the avian host (Hendrixson and DiRita 2004). The flagellar filament is composed of two structural proteins, FlaA and FlaB. Mutation of the *flaA* gene results in a non-motile phenotype and severe truncation of the flagellum indicating that FlaA is a major structural component (Guerry et al. 1991). In contrast, a *ΔflaB* mutant bears full length flagella and shows only partial reduction in motility. The flagellar filament is embedded into both the inner and outer membrane via a basal body and hook complex composed of multiple structural proteins which allow free rotation of the filament to drive motility (Figure 1-1). The flagellar filament of *C. jejuni* is a coiled-coiled structure composed of 7 protofilaments with a central lumen, unlike *Salmonella typhimurium* which is composed of 11 protofilaments (Galkin et al. 2008).

The flagellin proteins are O-linked glycosylated with sialic-acid like structures which accounts for ~10% of its total mass. O-linked glycosylation involves the addition of a sugar moiety to the hydroxyl group of a serine or threonine residue of a target protein. Unlike N-linked glycosylation there is no known conserved amino acid sequence that dictates which residues are to be modified. O-linked glycosylation of flagellin proteins is considered to be critical for the assembly of functional flagella and for colonisation in chickens (Howard et al. 2009). Initial binding studies utilising sialic-acid specific lectins led to the conclusion that *C.*

jejuni flagellin contains sialic acid moieties, however more recent data indicates that these modifications are derivatives of sialic-acid like structures Pseudaminic acid (Pse) and Legionaminic acid (Leg) encoded by the Pse and Ptm biosynthesis pathways respectively (Figure 1-4) (Doig et al. 1996; Howard et al. 2009; Thibault et al. 2001). All *C. jejuni* strains studied to date express either Pse and its derivatives (e.g. strain 81-176) or derivatives of both Pse and Leg (e.g. strain 11168). In strain 11168 the flagellin glycosylation genetic cluster is composed of ~50 genes which encode Pse and Leg pathways (Parkhill et al. 2000). In contrast, strain 81-176 contains only the Pse synthesis pathway. Mutational analysis of strain 81-176 found only 3 of the 19 O-linked glycosylated residues are critical for the assembly of functional motile flagella, 5 residues are important for auto-agglutination, and 11 residues at present have no ascribed function (Ewing et al. 2009). The lack of phenotypic alteration upon the removal of ~50% of the glycosylation sites and the exposure of these residues on the external surfaces of the flagellin monomers suggest that these modifications may play a role in interaction with the host. In fact, mutation of the Leg structures in strain 11168 dramatically decreases the ability of *C. jejuni* to colonise the chicken GI tract (Howard et al. 2009). Interestingly, "livestock" strains show a greater propensity to encode the Leg synthesis genes compared to "non-livestock/environmental" strains (Champion et al. 2005). At present, the role and contribution of the glycosylation moiety of *C. jejuni* flagella in modulating human host-pathogen interactions is unknown.

Unlike many other well-known Gram-negative enteropathogens (e.g. *Salmonella*, *Yersinia sp.*), *C. jejuni* lack a functional type III apparatus, instead *C. jejuni* secrete effector proteins, invasion antigens (Cia proteins) and FlaC through the flagella apparatus (Konkel et al. 2004). This process requires the basal body and hook structure, and one of the FlaA or FlaB proto-filaments but not both.

C. jejuni flagellin proteins are major immunomodulatory antigens during infection and significantly contribute to the heat-labile (HL) serotyping of the bacterium (Lior et al. 1982). *C. jejuni* is able to switch between flagellated and aflagellated phenotype; this property may allow the flagellin to perform its dual contrasting role(s), firstly in promoting colonisation and yet allowing immune evasion (Caldwell et al. 1985). Only motile flagellated bacteria were recovered from the stools of human volunteers when the inoculum administered contained a mixture of both flagellated and aflagellated bacteria, this data highlights the necessity for flagella expression for colonisation of the human GI tract (Black et al. 1988). As an immune evasion strategy, *C. jejuni* has evolved mutations in the FlaA

protein such that it no longer interacts with TLR5; how the loss of this interaction renders a selective advantage for *C. jejuni* is not clear (Andersen-Nissen et al. 2005).

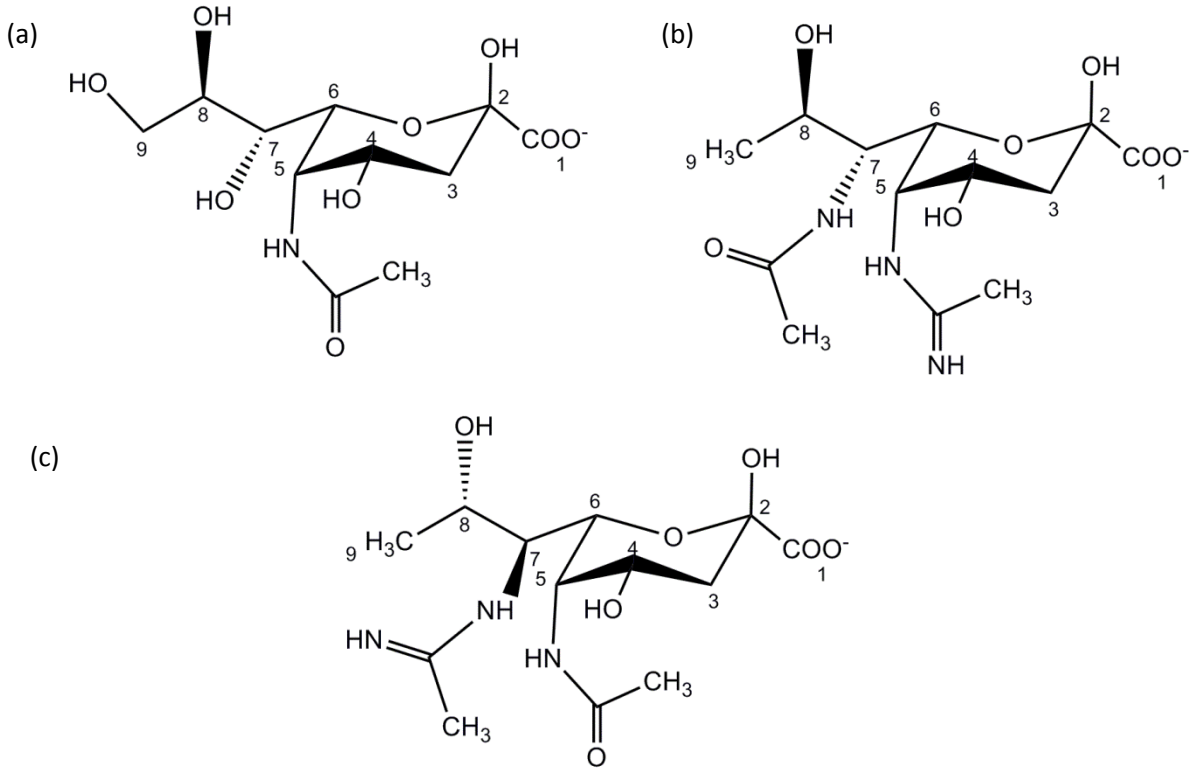


Figure 1-4: Structure of Neuraminic acid, Legionaminic acid, and Pseudaminic acid derivatives

(a) N-acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosonic acid; Neu5Ac) (b) Legionaminic derivative (5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-d-*glycero*-d-*galacto*-nonulosonic acid; Leg5Am7Ac) (c) Pseudaminic acid derivative (5-acetamido-7-acetamidino-3,5,7,9-tetradeoxy-l-*glycero*-l-*manno*-nonulosonic acid; Pse5Ac7Am).

1.1.4.3 Capsule

The high molecular weight polysaccharide component of the capsule was originally thought to be LPS, it was only identified as capsular polysaccharide (CPS) upon the elucidation of the genome sequence (Karlyshev et al. 2000; Karlyshev, McCrossan, & Wren 2001). The CPS and not the LOS was also found to be the determinant in the heat-stable Penner serotyping scheme. The genetic locus for strain 11168 CPS contains 38 genes but this varies widely between strains. The genes involved in CPS polymerisation and translocation are conserved between strains, however the internal region encoding the CPS repeating units are highly

variable (Karlyshev et al. 2005). CPS encoding region can range between 15.2kb and 34.1kb in size. Similar to the LOS, phase-variable *cps* genes have also been identified. The high degree of variation implies interaction(s) may occur between *C. jejuni* CPS and the host. Indeed, CPS is important for dampening pro-inflammatory cytokine responses in dendritic cells (DCs) as well as invasion of intestinal epithelial cells (IECs) (Bacon et al. 2001; Rose et al. 2011)

1.1.4.4 N-linked Glycosylated Proteins

C. jejuni was the first bacteria in which an N-linked glycosylation system was identified (Wacker et al. 2002). N-linked modified structures bear a sugar moiety attached to the amide nitrogen of an asparagine residue. Unlike the glycosylation of other *C. jejuni* surface structures, N-linked glycosylated proteins are highly conserved between strains (Young et al. 2002). A heptasaccharide composed of a single bacillosamine moiety attached to a linear chain of 5 GalNAc residues with one tertiary glucose residue forms the structure of all *C. jejuni* N-linked carbohydrates. The 12-gene *pgl* (protein glycosylation) locus encodes enzymes required for the synthesis of carbohydrate structures in the cytoplasm and subsequent linkage of the heptasaccharides to ~30 target proteins in the periplasm.

The biological role of the N-linked glycosylation system is not fully understood although studies indicate a role in colonisation of the chicken gut, adherence and invasion of human IECs and modulation of DC inflammatory cytokine responses (Karlyshev et al. 2004;Rose et al. 2011; van Sorge et al. 2009).

1.1.5 *Additional Virulence Factors*

1.1.5.1 Cytolethal Distending Toxin

Cytolethal Distending Toxin (CDT) is a tripartite complex that causes cell cycle arrest at the G_1/S or G_2/M transition stage (Lara-Tejero and Galan 2000). The active subunit CdtB translocates to the nucleus where it acts as a DNase causing DNA damage and cell cycle arrest. The role of CDT in pathogenesis is unclear as *C. jejuni* that lack CDT are still able to induce disease in humans (Mortensen et al. 2011). CDT may have a potential role in asymptomatic infection as CDT from other species show immunosuppressive capacity via induction of apoptosis of T-cells (Shenker et al. 2000).

1.1.5.2 Invasion Proteins

C. jejuni secretes Cia proteins via its flagella apparatus. Cia proteins are important in the invasion of IECs via the recruitment of Rho GTPase, Rac-1 (Eucker and Konkel 2011). FlaC is

structurally similar to FlaA and FlaB. FlaC is secreted via the flagella apparatus and plays a role in the invasion of IECs via as yet undefined mechanism(s) (Song et al. 2004).

1.1.6 *Infection Models*

Understanding of *C. jejuni* pathogenesis lags behind that of other enteric pathogens in part due to a lack of good small animal models of disease. Chick models are used for *C. jejuni* colonisation studies, however the pathology associated with human disease is not observed. Wild-type (WT) mice of multiple genetic backgrounds show limited colonisation of *C. jejuni*, and pathology is even more infrequent (Chang and Miller 2006; Rathinam et al. 2008). Until recently, mice with the genetically altered immune systems such as SCID mice or IL-10 knock-outs were the most frequently used animal model systems (Chang & Miller 2006; Lippert et al. 2009; Rathinam, Hoag, & Mansfield 2008). Recently a model replacing the enteric flora of WT mice with human flora was described (Bereswill et al. 2011). These mice were colonised with *C. jejuni* and developed inflammatory responses. Larger animal models, such as new-borne piglets, are also used for pathogenesis studies however, high cost and limited genetic tools make them less desirable (Babakhani, Bradley, & Joens 1993).

1.2 Gastrointestinal tract

1.2.1 *Ingestion to infection*

The structure of the GI tract, from the mouth to the anus, can be divided into four major layers: the mucosa, submucosa, muscularis externa, and serosa. The mucosa broadly functions in absorption and secretion: this layer is composed of a single cell epithelial lining, beneath which is the “non-organised” lamina propria (LP) containing the majority of the immune cells, and a layer a smooth muscle (muscularis mucosae).The structure of the mucosa varies widely along the length of the GI tract depending on the function of the organ.

Low pH in the stomach acts as a major chemical defence against ingested pathogens. *C. jejuni* is relatively susceptible to low pH conditions *in vitro*, surviving less than 60mins at pH3.0 (Rotimi et al. 1990). This is in discordance with the low bacterial inoculums of 1000-10,000 CFU required to cause campylobacteriosis in humans (Black et al. 1988). It has been proposed that *C. jejuni* can withstand the acidic environment via association with food and even by inhabiting amoebae as a route into the lower GI tract (Axelsson-Olsson et al. 2010; Waterman and Small 1998).

The entire GI tract is lined with a single IEC layer that plays a critical in absorption of nutrients. The ileum and jejunum lining is organised into crypt and villus macrostructures that increase the surface area for absorption of nutrients. The colon lacks villi and functions to absorb water and fat-soluble vitamins. Intestinal epithelial cells (IECs or enterocytes) are columnar cells with apical invaginations (villi and microvilli) that markedly enhance the surface area for optimal absorption. Enterocytes are interspersed with goblet cells that secrete mucin, the major component of the mucus layer, which provides a physical and chemical barrier keeping commensal and pathogenic microbes at bay and simultaneously smoothing the peristaltic movement of passing bolus. Microfold (M) cells are largely found in the ileum and lie above the Follicle-Associated Epithelium (FAE) of the Peyer's Patch (PP). M cells allow direct uptake of luminal bacteria and antigens for sampling by the underlying immune cells. Stem cells are located in the crypt surrounded by AMP secreting Paneth cells (PCs) which provide sterility in a microbe-rich environment. Stem cells differentiate into four epithelial lineages which include the enterocytes, PCs, goblet cells, and the neuro-endocrine cells. Gut-associated lymphoid tissue (GALT) can be subdivided into the "organised" tissue of the PPs, isolated lymphoid follicles (LFs), and mesenteric lymph nodes (MLNs).

C. jejuni initially colonises the jejunum and ileum where pathology can occur, however the colon is considered to be the major site of pathology in the majority of campylobacteriosis cases (Black et al. 1988; Russell et al. 1993). Motility is important in the colonisation of both the human and avian gut (Black et al. 1988; Nachamkin et al. 1993). Mucin(s) act as a chemoattractant for *C. jejuni* in part via the L-fucose glycans which can be an energy source for specific virulent *C. jejuni* strains (Hugdahl et al. 1988; Stahl et al. 2011). L-fucose triggers the downregulation of CPS which may aid attachment and invasion of IECs. The ability of *C. jejuni* to translocate the IEC layer is considered important in inducing colitis compared to eliciting watery diarrhoea (Everest et al. 1992). *C. jejuni* may translocate the epithelial barrier by a number of mechanisms. In a rabbit ileal loop model of infection *C. jejuni* preferentially binds and translocates M cells over enterocytes (Walker et al. 1988). This mechanism of translocation is also important for other enteropathogens such as *Salmonella,* and *Yersinia*. Both transcellular and paracellular routes of bacterial translocation have been demonstrated for *C. jejuni in vitro.* The ability of *C. jejuni* to invade IECs varies depending on the cell type, however most studies agree that bacterial invasion precedes disruption of IEC tight junctions (Bras and Ketley 1999; Wine et al. 2008). The advantages for *C. jejuni* to translocate the epithelium lie in the access to a new ecological

niche with limited competition from other bacteria; however the low rates of septicaemia associated with infection and the inability to survive within mono-nuclear phagocytes suggest *C. jejuni* is not a particularly successful pathogen within the mucosa (Allos 2001; Hu et al. 2006). Recent reports showing the ability of *C. jejuni* to survive within IECs highlights this cell type as a potential harbour for the bacterium in the human intestine (Watson and Galan 2008).

1.3 Epithelial Responses

1.3.1 *Epithelial Adhesion, Invasion & Intracellular Survival*

The ability of *C. jejuni* to adhere to and invade IECs has been demonstrated in several cell-lines as well as in human colonic biopsies (Bras & Ketley 1999; Edwards et al. 2010; van Spreeuwel et al. 1985; Wine, Chan, & Sherman 2008; Zilbauer et al. 2007). *C. jejuni* manipulates both the microtubular and actin cytoskeleton to gain entry into IECs (Eucker & Konkel 2011; Watson & Galan 2008). *C. jejuni* binds to fibronectin and integrin complexes on IECs via fibronectin-binding proteins triggering epidermal growth factor receptor (EGFR)-mediated-signalling (including phosphinositide 3-kinase (PI3K) activation) and subsequent endocytosis (Eucker & Konkel 2011), the latter depending upon lipid rafts and caveolin for formation of a *C. jejuni*-containing vacuole (CCV) (Watson & Galan 2008). The CCV follow a non-canonical endocytic pathway and avoid lysosomal fusion allowing *C. jejuni* to survive within IECs for >24h.

1.3.2 *Pathogen Recognition*

Adhesion and invasion of IECs by *C. jejuni* is associated with the secretion of the pro-inflammatory cytokines, IL-8 & IL-6, and the induction of AMPs human beta-defensins 2 & 3 (hBD-2 & 3) (Friis et al. 2009; Watson and Galan 2005; Zilbauer et al. 2005). *C. jejuni*-mediates activation of multiple IEC signalling pathways; these include nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinases (MAPKs), and PI3K activation. The MAPK, extracellular signal-related kinase (ERK), is critical for IL-8 responses (Watson & Galan 2005). Interestingly, the *ΔflaA* mutant exhibits reduced ERK-stimulating capability, suggesting receptors independent of TLR5 are important for the recognition of *C. jejuni* flagellin in IECs. The only IEC pattern recognition receptors (PRRs) identified to date that recognises *C. jejuni* are NOD-1, which reduces intracellular bacterial survival and drives IL-8 and hBD-2 expression, and TLR2 which induces IL-6 secretion (Friis et al. 2009; Zilbauer et al. 2007).

1.4 Innate Immunity to *C. jejuni*

1.4.1.1 Host Factors

Host genetics and acquired immunity play important roles in the clinical manifestations of *C. jejuni* infection. Acquired immunity to *C. jejuni* limits symptomatic disease but not eliminate colonisation. In addition, genetic factors have been implicated as important in the development of campylobacteriosis and post-infection sequelae (Janssen et al. 2008). A single-nucleotide polymorphism (SNP) in the first intron of the interferon gamma (IFNγ) gene is associated with *C. jejuni*-associated enteritis (Nielsen et al. 2012). This association implicates T helper 1 (Th1) responses in host immunity to *C. jejuni* infection. Two possible scenarios could explain this association. Firstly, an inappropriate overt IFNγ-mediated increase in pro-inflammatory responses may directly promote immune-mediated enteritis and pathology or an initial suboptimal IFNγ response may lead to unsuccessful bacterial clearance, a trigger for exacerbation of inflammation. As the impact of this SNP on cytokine induction is unknown theoretically both scenarios are plausible explanations for this association. Interestingly, the same SNP is associated with the development of RA, however again either of these two scenarios could explain this association. Interestingly, IL-10 promoter and TLR4 promoter polymorphisms have been associated with GBS which also implicates the regulation of the immune response to the development GBS (Myhr et al. 2003; Nyati et al. 2010).

1.4.2 *Innate Immune Cells of the GI mucosa*

After translocation through the epithelial barrier, *C. jejuni* is sensed by innate immune cells of the LP. In addition, infected IECs signal to underlying immune cells. Chemokines, such as IL-8, drive an influx of neutrophils which promote bacterial clearance and halt systemic spread and yet overt presence of neutrophils is characteristic of the inflammatory pathology (Figure 1-6) (van Spreeuwel et al. 1985). Activated professional antigen presenting cells (APCs) trigger an adaptive immune response which further stimulates the innate system to clear *C. jejuni* and limit bacteraemia (Fernandez-Cruz et al. 2010).

APCs that reside in the LP under homeostatic conditions generally express a more "tolerogenic" phenotype and sense commensal bacteria in the lumen either directly or indirectly via the epithelium without activating an inflammatory response. However, during infection migrating APC are able to activate pro-inflammatory adaptive immunity either in the draining lymph nodes (LN) or within the LP.

1.4.2.1 Dendritic Cells

Dendritic cells (DCs) bridge the innate and adaptive immune response by acting as APCs that have the capacity to promote naïve $CD4^+$ T helper cell differentiation and functions. Upon encountering bacteria DCs phagocytose, process and present antigen to T-cells in the context of MHC class II or cross-presentation on MHC class I. In addition, DCs undergo a maturation process that involves the upregulation of co-stimulatory molecules and secretion of a plethora of cytokines that tailors the adaptive immune response to the nature of the infectious insult. Although the IEC layer acts as a barrier between the lumen and the GALT, DCs can make direct contact with luminal antigens by sampling via DC protrusions (Rescigno et al. 2001) or indirectly as antigens pass through M-cells and IECs.

Functionally, DCs have the challenging task of remaining tolerogenic to gut microflora whilst retaining the capability to mount appropriate immunity to potential pathogens. It is therefore logical that multiple subsets of DCs exist with more specialised functions. In the murine mucosa $CD103^+$ (the receptor for E-Cadherin, an IEC adhesion molecule) DCs migrate to the MLN via expression of CCR7 and induce the differentiation of regulatory T-cell (Tregs) (Coombes et al. 2007). However, under inflammatory conditions $CD103^+$ DC induce Th1 and Th17 responses (Laffont et al. 2010). $CX3CR1^+$ positive-DCs are able to extend dendrites and directly sample luminal antigens (Niess et al. 2005). Interestingly, $CX3CR1^+$ DCs are programmed with more pro-inflammatory capabilities than $CD103^+$ DCs. $CX3CR1^+$ DC lack migratory properties, they are therefore hypothesised to play a greater role in modulating local mucosal immunity (Schulz et al. 2009). Absence of enteric microflora reduces $CX3CR1^+$ but not $CD103^+$ DC numbers (Niess and Adler 2010). This suggests that under inflammatory conditions an increase in pro-inflammatory $CX3CR1^+$ DCs may drive T-cell polarisation directly in the LP towards a Th1/Th17 phenotype.

For purpose of investigation, human peripheral blood monocyte-derived DCs or murine bone marrow-derived DCs (BMDCs) are frequently used in *in vitro* co-culture assays. Both DC types model a more inflammatory monocyte-derived DC subset, which is more likely to be present during intestinal infection and inflammation. *C. jejuni* is readily phagocytosed and killed by human monocyte-derived DCs and murine BMDCs (Hu et al. 2006; Rathinam, Hoag, & Mansfield 2008). In contrast other enteropathogens such as *Salmonella enterica* survive within APCs which may explain greater incidence of systemic disease (Abrahams and Hensel 2006) when compared to campylobacteriosis. *H. pylori* can also replicate within BMDCs for a limited time. A >1 log fold-increase between 2 and 6h post-innoculation has been observed, declining thereafter (Wang, Gorvel et al. 2010).

1.4.2.2 Macrophages

Resident gut–associated macrophages largely reside within the LP and lack inflammatory responsiveness to many microbe-associated molecular patterns (MAMPs), this is due to low expression of TLR-associated adaptor molecules (Smythies et al. 2010). Intestinal macrophages are able to phagocytose whole bacteria and cellular debris but secrete the anti-inflammatory cytokine IL-10 and not pro-inflammatory cytokines (Denning et al. 2007). This is in contrast to the chronic inflammatory setting of IBD where a $CD14^+$ subset of macrophages secretes a number of pro-inflammatory cytokines that contribute to disease pathogenesis (Kamada et al. 2008). This suggests that macrophages that migrate to the gut retain pro-inflammatory capabilities that may activate adaptive immunity whereas resident macrophages may function to clear IECs debris or commensal bacteria without eliciting a strong inflammatory response.

Studies performed to date analysing macrophage responses to *C. jejuni* have utilised phorbol 12-myristate 13-acetate (PMA)-stimulated THP-1 cells which display a phenotype between the monocyte/macrophage stages of differentiation. Unlike resident intestinal macrophages, PMA-stimulated THP-1 cells are capable of eliciting pro-inflammatory responses to TLR ligands. *C. jejuni* induces IL-1β secretion from PMA-stimulated THP-1 cells (Siegesmund et al. 2004). In addition *C. jejuni* induces apoptosis via a caspase-1 and -9 independent pathways which is partially reliant on the ability of *C. jejuni* to produce Cia invasion antigens. This suggests that bacterial driven macrophage apoptosis may be a potential immune evasion strategy, however at present the effects of *C. jejuni* on resident intestinal macrophages is unknown. Phagocytosed *C. jejuni* is readily killed by murine BM macrophages; earlier studies however suggested *C. jejuni* can survive within monocytes/macrophages for up to 7 days (Kiehlbauch et al. 1985; Watson & Galan 2008). The use of different cell types may account for this discrepancy, it is however interesting to speculate that a potential difference in the bactericidal activity of resident intestinal macrophages may allow them to harbour viable *C. jejuni.*

1.4.2.3 Polymorphonuclear Cells

Polymorphonuclear (PMN) cells or granulocytes include neutrophils, eosinophils, basophils, and mast cells. Eosinophils, basophils, and mast cells play an important role in Th2 mediated pathology of allergic disease and shall not be discussed further. Neutrophils engulf and rapidly kill bacteria via production of reactive oxygen species (ROS) within the phagolysosome. Neutrophils play a critical role in resolution of many infections as they have the most potent phagocytic and bactericidal capability of all the phagocytes. The level

of PMN infiltration in the GI mucosa of children during *C. jejuni* infection correlates with the degree of inflammation (Everest et al. 1992), this evidence implicates PMNs in the acute inflammatory pathology seen in some *C. jejuni* infections. A similar association is also seen in a rabbit ileal loop model of *C. jejuni* infection (Everest et al. 1993). Interleukin 8 (IL-8) and leukotriene B4 (LTB4), are neutrophil chemoattractants secreted by IECs and neutrophils respectively and are both induced in response to *C. jejuni* infection (Everest et al. 1993; Watson & Galan 2005). Although the main function of neutrophils is to limit systemic spread of *C. jejuni*, they may also contribute to pathology by the release of toxic ROS (Figure 1-6). In addition, neutrophils can migrate between IECs and into the lumen thus disturbing barrier integrity (Sun et al. 2011).

Interestingly, colonisation of the avian GI tract by *C. jejuni* induces IL-8, and yet this increase is not associated with an increase in heterophil numbers (the avian equivalent to neutrophil) (Meade et al. 2009), although an increase in circulating monocytes/macrophages is observed. Potentially the lack of heterophils may account for the persistent colonisation and yet disease free status of the avian gut in response to *C. jejuni.*

1.4.2.4 Natural Killer cells, Lymphoid Tissue-Inducer Cells & Gamma-Delta T cells

Local, mucosal sources of IFNγ, IL-22, and IL-17 provide an early host defence shield in response to infection, long before the adaptive immune system comes into play. Natural killer (NK) cells, lymphoid tissue-inducer (LTi) cells, and gamma-delta (γδ) T cells are innate immune cells that reside within the LP and GALT and are able to produce an array of host-defence peptides. In an *ex vivo* human intestinal biopsy model of infection *C. jejuni* induced IFNγ, IL-22, and IL-17A within 8h of infection suggesting a robust source of these cytokines is present in the LP and GALT (Edwards et al. 2010). *C. jejuni* expands γδ T cells in the presence of $CD4^+$ T cells, suggesting this cell population may also be activated during infection (Van Rhijn et al. 2003). At present there is no data available on the cytokine responses of this cell population. *C. jejuni* LOS bearing a α2, 8-linked sialic acid moiety interacts with the inhibitory sialic-acid binding immunoglobulin-like lectin 7 (Siglec-7) on NK cells but the functional consequence of this interaction remains unclear (Avril et al. 2006).

1.4.3 *Soluble Mediators of Innate Defense*

1.4.3.1 Complement

Complement activation is a key part of the innate immune defence towards limiting systemic spread of pathogens. *C. jejuni* is susceptible to human serum suggesting sensitivity

towards complement components (Blaser et al. 1985). However, *C. fetus* is less sensitive to human serum. It has been hypothesised that the discrepancy between the sensitivity of the two species may account for why *C. fetus* is more frequently found in patients suffering from septicaemia.

1.4.3.2 Anti-microbial peptides

Anti-microbial peptides (AMPs) are a diverse group of small cationic peptides that possess bactericidal activity by targeting the cell membrane of microbes. Two IEC AMPs, HBD-2 and -3 are induced in response to *C. jejuni* (Zilbauer et al. 2005). *C. jejuni* is sensitive to the effects of HBD-2 and -3 which cause a "peeling" of the cell wall and loss of cytoplasmic contents. Surprisingly elimination of the capsule does not increase sensitivity to AMPs. Indeed, other studies have shown the importance of the LOS structure but not the capsule for resistance to the antibiotics erythromycin and polymixin B (Jeon et al. 2009; Naito et al. 2010). The PEA modification of the lipid A and oligosaccharide of *C. jejuni* LOS are important for AMP resistance, which is likely to involve charge interactions as both PEA and sialic acid residues are negatively-charged (Cullen & Trent 2010; Naito et al. 2010). Interestingly, *C. jejuni* colonisation of chicken reduces expression of 7 different AMP genes in comparison to *Salmonella typhimurium* which induces expression (Meade et al. 2009). Reduced AMP expression is hypothesised to contribute to the observed high *C. jejuni* bacterial load and may also contribute to systemic spread. In humans, a robust AMP response may limit *C. jejuni* numbers in the intestine and therefore halt systemic spread.

1.4.4 *Cytokine Responses to C. jejuni infection*

C. jejuni-mediated infection induces a pro-inflammatory response from IECs, innate, and adaptive immune cells. Chemokines such as IL-8, monocyte chemoattractants chemokine (C-C motif) ligand 2 (CCL2; MCP-1) and CCL4 (MIP1β) are secreted from *C. jejuni*-stimulated IECs (Hu and Hickey 2005; Watson & Galan 2005). Co-culture experiments with human *ex vivo* intestinal biopsies also show elevated IL-8 levels (MacCallum et al. 2006).

C. jejuni also stimulates pro-inflammatory cytokine induction in DC and macrophages (Edwards et al. 2010; Hu et al. 2006;Rathinam, Hoag, & Mansfield 2008). Increase in acute-phase response inducers tumour necrosis factor-alpha (TNF-α) and IL-6, as well as Th1 and Th17 polarising cytokines IL-12 and IL-23 respectively has also been noted (Edwards et al. 2010). Similar to IEC responses, no dramatic difference between human and chicken *C. jejuni*-stimulated macrophages/monocytes has been observed (de Zoete et al. 2010). Minimal levels of IL-1β have also been detected in *ex vivo* stimulated biopsies (Edwards et

al. 2010). A recent report suggested that induction of the potent anti-inflammatory cytokine IL-10 may be flagellin-dependent (Rose et al. 2011). Whether the induction of IL-10 by *C. jejuni* is an immune evasion strategy has yet to be investigated.

1.4.5 *Microbial detection by the innate immune system*

To detect microbes, immune and non-immune (e.g. IEC) cells express a plethora of PRRs that bind to evolutionary conserved microbe-associated molecular patterns (MAMPs) leading to appropriate cellular activation. This model was first proposed by Charles Janeway in 1989 (Janeway, Jr. 1989) but it is now widely accepted that the same system can also detect endogenous danger signals as suggested by Polly Matzinger (Matzinger 2001). It is now recognised that components from both commensal bacteria MAMPs and pathogens (PAMPs) activate PRRs. It is therefore predicted that availability of MAMPs/PAMPs for their cognate receptors largely detects activation of inflammatory responses in the gut. The thick mucus layer likely goes some way to minimise contact between commensal microflora and cells of the mucosa.

1.4.5.1 Toll-like receptors

Toll-like receptors (TLRs) were the first PRR family to be implicated in mediating anti-fungal immunity in drosophila (Lemaitre et al. 1996). Subsequently a human homolog of TOLL, TLR4, was identified (Medzhitov et al. 1997). TLRs are type-1 transmembrane proteins, comprised of a C-terminal Leucine-Rich Repeat (LRR) domain that is ligand (MAMP)-binding, a single membrane spanning domain, and an N-terminal cytoplasmic Toll/IL-1R (TIR) homologous interacting domain that is capable of binding adaptor molecules to orchestrate signalling cascades. There are 11 known mammalian TLRs that function as homo- or hetero-dimers. TLRs are expressed either on the cell surface or within endosomes. TLRs are known to bind a diverse array of microbial products of viral, bacterial and fungal origin. The best characterised of these is the receptor for LPS/LOS, TLR4. Jules Hoffmann and Bruce Beutler were awarded a share of the 2011 Nobel Prize for Medicine for the discovery of the TLR-mediated antifungal cascade and elucidation of the interaction between LPS/LOS and TLR4 respectively; these discoveries are seen as pivotal to our current understanding of fundamental signals employed by the innate immune system to detect and respond to infection (Poltorak et al. 1998).

Signalling downstream of TLRs is mediated by a number of scaffolding proteins that contain TIR-containing adaptor molecules; myeloid differentiation primary response protein (MyD88), MyD88 adaptor-like (Mal), TIR domain-containing adaptor inducing IFN-β (TRIF),

and TRIF-related adaptor molecule (TRAM). TLR signalling cascades result in activation of MAPK, NF-κB, and interferon regulatory factor (IRF) pathways which are central master regulators of inflammatory response(s) (Figure 1-5) (Kawai and Akira 2006). TLR signalling has also been implicated in the phagocytosis and the maturation of the phagolysosome (Blander and Medzhitov 2004).

C. jejuni activates human TLR1/2/6, and TLR4/MD2 (de Zoete et al. 2010). *C. jejuni* di- and tri-acylated lipoproteins are the predicted ligands for TLR 1/2 and TLR 2/6, and LOS is the known ligand for TLR4/MD-2 (Kuijf et al. 2010). TLR signalling is a critical regulator in the gut. $TLR5^{-/-}$ mice have increased bacterial load and develop spontaneous colitis which is associated with elevated pro-inflammatory cytokine levels (Vijay-Kumar et al. 2007). Interestingly, $TLR5^{-/-}$ $TLR4^{-/-}$ double knock-out mice have increased bacterial load but do not develop colitis suggesting TLR signalling within the gut is important for regulating the enteric flora but under certain circumstances can also drive inflammatory pathology. *C. jejuni* can successfully colonise $MyD88^{-/-}$ but not WT mice highlighting the role for TLR signalling in colonisation resistance in this model (Watson et al. 2007). Expression of TLRs in the gut may vary between species which may contribute to the different outcomes observed. *C. jejuni* is able to stimulate chicken TLR2 and TLR4 homologs (de Zoete et al. 2010). Interestingly, the chicken TLR9 ortholog, chTLR21, is able to sense *C. jejuni* DNA unlike the human counterpart. In addition, activated chicken TLR4 is unable to induce a type 1 IFN response unlike human TLR4. Whether these discrepancies play a role in the different outcomes of *C. jejuni* infection between humans and chickens requires clarification.

1.4.5.2 Nucleotide Oligomerisation Domain-like receptors

The nucleotide oligomerisation domain (NOD)-like receptors (NLRs) are a group of cytoplasmic microbial sensors. NLRs are composed of an effector domain, a nucleotide-binding domain (NBD), and an LRR ligand binding domain. The NBD is implicated in complex oligomerisation which is necessary for NLR function. NLRs can be subdivided based on the presence of a caspase-recruitment effector domain (CARD) or pyrin effector domain (PYD). NOD1 and NOD2 are two NLRs that recognise muropeptide iE-DAP and muramyl dipeptide (MDP) respectively, two subcomponents of peptidoglycan (PGN) which is found in the cell wall of both Gram-positive and Gram-negative bacteria. Activation of NOD1 and NOD2 triggers the formation of a signalling complex containing receptor-interacting serine/threonine kinase-2 (RIP2) and cellular inhibitor of apoptosis (cIAP) 1 and 2 which leads to the activation of NF-κB and MAPK cascades (Bertrand et al. 2009). NOD1 and

NOD2 can synergise with TLR signalling to either dampen or stimulate pro-inflammatory responses depending on the cellular context (Fritz et al. 2005; Watanabe et al. 2004).

In IECs, *C. jejuni* activates NOD1 but not NOD2 to induce the secretion of HBD2 and IL-8 (Zilbauer et al. 2007). NOD1 activation also minimises intracellular *C. jejuni* numbers. *C. jejuni* can also activate NOD2 in a reporter cell-line but the functional consequences of this is unknown (Al-Sayeqh et al. 2010; Zilbauer et al.2007).

The inflammasome

A subfamily of the NLRs activates caspase-1 rather than the NF-κB signalling pathways. Upon activation these NLRs form a complex called the "inflammasome" in the cytoplasm that cleaves pro-caspase-1 to active caspase-1 (Martinon et al. 2002). NLR proteins utilise their PYD and CARD domains to form the inflammasome, the complex formation culminates in the recruitment of apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1. Autocatalytic cleavage of the latter releases active caspase-1, the enzyme responsible for cleaving pro-IL-1β, pro-IL-18, and pro-IL-33 resulting in their release from the cell. Bioactive IL-1β and IL-18 are potent pro-inflammatory activators with multifactorial properties, not surprisingly overt expression and release is tightly regulated at multiple levels. Transcription levels are controlled by TLRs and NOD1/NOD2 pathways and post-translation modification by the inflammasome. This is called the "two-hit" hypothesis. Activation of the inflammasome can also trigger cell death via pyroptosis. The activation of the inflammasome is triggered by a diverse variety of PAMPs and also by endogenous danger-associated molecular patterns (DAMPs). The presence of circulating binding proteins (*e.g.* IL-1 receptor antagonist and IL-18 binding protein) provides another level of regulation for this family of cytokines.

The Inflammasome has been implicated in the sensing of multiple enteropathogens including *Salmonella typhimurium* and *Escherichia coli* (Miao et al. 2010b). Inflammasome activation is critical for clearing pathogens when cleavage of IL-1β/IL-18/IL-33 and/or the induction of pyroptosis are required. In a murine model of *Salmonella typhimurium,* infection leads *to* systemic disease and death. However, infection with *S. typhimurium* which constitutively expresses the flagellin protein (flagellin is recognised by the NLR member, IPAF, causing pyroptosis of infected macrophages and clearance by PMNs) does not lead to death, mainly due to inflammasome function (Brodsky et al. 2010; Miao et al. 2010a). It is not surprising that some pathogens have evolved mechanisms to suppress inflammasome function. *Mycobacterium tuberculosis* produces a zinc metalloprotease

which inhibits inflammasome activation in macrophages (Master et al. 2008). *Yersinia sp.* secretes an effector protein YopK, which interferes with recognition of its type III secretion system by macrophages (Brodsky et al. 2010). To date the potential role of the inflammasome during infection with the order Campylobacterales has not been reported

1.4.6 *Glycan Receptors*

Glycosylation of surface structures lends microbes with potential fitness advantages. These include survival in harsh and varying environments; assembly of surface structures; adherence and invasion of target cells; and disguise antigenic surface proteins during engagement with the host immune system. Glycans are less immunogenic than protein counterparts as they are not generally presented on MHC molecules to T cells, and are therefore T-cell independent antigens with some noted exceptions. For example, recent findings have demonstrated the capacity of polysaccharides from commensal bacteria to influence the mucosal immune system. Polysaccharide A (PSA) is a component of the CPS of the human commensal bacterium *Bacteroides fragilis.* PSA can bind directly to TLR2 on mucosal $CD4^+$ T cells and generate Treg differentiation by inducing IL-10, TGF- β, and Foxp3 which subsequently inhibit Th17 responses (Round et al. 2011). The induction of Tregs allows closer association of *B. fragilis* with the epithelium in a mouse model, which suggests this could also be a mechanism exploited by pathogens although this has not been investigated. Bacterial glycan structures can bind to a wide variety of host receptors expressed on immune and non-immune cells. These interactions have been shown to both enhance and dampen pro-inflammatory immune responses to *C. jejuni* (Kuijf et al. 2010; van Sorge et al.2009).

1.4.6.1 Sialic-acid binding Ig-like receptors

Sialic-acid binding immunoglobulin-like receptors (Siglecs) are a group of receptors largely expressed on the surface of haemopoeitic cells. Siglecs are composed of a single trans-membrane domain, multiple extracellular immunoglobulin (Ig) domains that bind ligand, and a cytoplasmic intracellular inhibitory motif (ITIM) domain which exerts regulatory function. Some Siglecs contain an intracellular growth factor receptor binding protein-2 (Grb2) binding motif and ITIM-like motifs that do not conform to canonical ITIM sequences, the functional consequences of these domains upon receptor ligation remains largely uncharacterised (Crocker et al. 2007). There are two sub-categories of Siglec receptors based on sequence similarity. The first includes Sialoadhesin, CD22, Siglec-4 and -15; the second subset share sequence homology with CD33 and are termed the CD33-related siglecs (Crocker, Paulson, & Varki 2007; Crocker and Redelinghuys 2008). The CD33-related

Siglecs are rapidly evolving, with variation in the numbers of receptors between species, even in higher order mammals. There are currently 10 known human CD33-related Siglecs which are expressed on both innate and adaptive immune cells (Lock et al. 2004). Although humans display the most rapid rate of Siglec evolution, lower Siglec expression in human compared to chimpanzee T cells has been implicated in the heightened reactivity of human T cells to many stimuli (Soto et al. 2010). Additionally, the lower expression is hypothesised to contribute to the "over-reactivity" of the human immune system which leads to diseases such as asthma and RA.

Siglecs bind sialylated structures (most often sialylated glycans) via the terminal V-set Ig domain, and have varying specificities for the linkage of the sialic acid and the underlying glycan structure which can be present on both host cells and microbes. Interestingly, recent reports have also highlighted the ability of Siglecs to bind to both host and pathogen non-sialylated ligands. Siglec-5 can bind the β-protein of group B *Streptococcus* (Carlin et al. 2009a), and Siglec-10 can bind vascular adhesion protein-1 (Kivi et al. 2009). However it is unlikely that these interactions occur via the sialic-acid binding pocket.

Siglec engagement has been implicated in many cellular processes including endocytosis, apoptosis, cellular activation, and proliferation (Crocker, Paulson, & Varki 2007). The immunomodulatory capability of Siglecs is often via the modulation of TLR signalling (Boyd et al. 2009). Siglecs can bind to host cell surface sialic acid both via *cis* (on the same cell) and *trans* (different cell) interactions. Binding of Siglec-10 (murine Siglec-G) by the receptor CD24 (ligated to the DAMPs, high mobility group box 1 (HMGB1) or heat shock proteins) leads to down-regulation of NF-κB signalling, protecting mice in a inducible liver necrosis model (Chen et al. 2009). This suggests that Siglecs play a role in limiting immune responses to DAMPs. This immune modulatory capability of Siglecs may have been exploited by pathogens during evolution as binding to these receptors potentially reduces immune activation and therefore increase chances of establishing infection. Many sialylated pathogens engage Siglecs, including *C. jejuni* (Avril et al. 2006; Heikema et al. 2010). Engagement of Siglec-9 by sialylated CPS and Siglec-5 by β-protein by Group B *Streptococcus* reduces the oxidative burst of neutrophils and increases intracellular survival (Carlin et al. 2009a; Carlin et al. 2009b). Engagement of murine Siglec-E by *Trypanosome cruzi* reduces DC IL-12 production which alters subsequent T-cell activation (Erdmann et al. 2009). *C. jejuni* strains that contain terminal α2,8-linked and α2,3-linked sialic-acid on the LOS bind to Siglec-7 and Sialoadhesin respectively (Avril et al. 2006; Heikema et al. 2010).

The differential expression of these linkages drives alternative T cell responses, a factor which is hypothesised to impact on the development of GBS post *C. jejuni*-infection (Bax et al. 2011).

Multiple hypotheses have been proposed for the cause of rapid evolution of CD33-related Siglecs. One hypothesis is a need to keep pace with the changing host sialome (Crocker, Paulson, & Varki 2007). As high percentage of sialic-acid containing pathogens infect humans; this raises a second hypothesis that rapid evolution may allow Siglecs to act as potential PRRs aiding in microbe detection (Crocker, Paulson, & Varki 2007). However, the evolution of Siglec "paired-receptors" which share structural identity of the ligand binding domain but lack the intracellular signalling domain suggest this could be a decoy strategy by the host to counteract the immune inhibition pathogens achieve on Siglec engagement, therefore supporting the first hypothesis (Crocker & Redelinghuys 2008). Until further studies detailing the functional consequences of Siglec engagement during infection are performed their exact role in either microbe recognition or/and immune evasion strategies remains open.

1.4.6.2 C-type Lectin Receptors

Similar to Siglecs, C-type lectin receptors (CLRs) are group of receptors that are prominently found on the surface of immune cells. CLRs contain a single trans-membrane domain, a C-type lectin-like domain (CTLD) involved in ligand binding, and often an intracellular signalling domain (Osorio and Reis e Sousa 2011). Upon engagement CLRs potentially initiate or dampen/modulate immune responses. Similar to Siglecs, engagement of CLRs often leads to modulation of TLR signalling, and has therefore been described as an immune evasion strategy in some instances. *Mycobacterium tuberculosis* and *Candida albicans* are able to induce IL-10 secretion by engaging the CLR DC-SIGN, via prolonged NF-κB activation (Gringhuis et al. 2007). As a consequence of modulating innate immune effector responses, engagement of CLRs can have downstream effects on T-cell polarisation. *H. pylori* interaction with DC-SIGN reduces DC cytokine induction which stunts Th1 responses (Bergman et al. 2004). *Neisserria gonorrhoea* activates DC-SIGN or the CLR macrophage-binding galectin (MGL) on DCs depending on the terminal sugar moiety on its LOS (van Vliet et al. 2009). Engagement of MGL skews T helper responses towards an unproductive Th2 response.

GalNAc residues on N-linked glycosylated proteins on the surface of *C. jejuni* bind the CLR MGL. In addition the LOS from certain strains bearing a terminal GalNAc residue can also

bind MGL. This interaction with MGL has shown to reduce DC IL-6, indicating that certain *C. jejuni* surface structures may actively promote immune suppression (van Sorge et al. 2009).

1.4.7 *Signalling*

Linking microbial recognition to innate immune cell effector responses and T helper cell polarisation involves a complex network of intracellular signalling cascades. Many cellular events are triggered in innate immune cells by microbes; three of the major pathways that are activated downstream of MyD88-dependent TLR signalling are the NF-κB, MAPKs, IRFs (Figure 1-5).

1.4.7.1 Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

The NF-κB family is comprised of five related transcription factors, p50, p52, p65, RelA, and RelB. In unstimulated cells, inhibitor of NF-κB (IκB) sequesters inactive NF-κB in the cytoplasm. Upon cellular activation, IκB is degraded allowing NF-κB to undergo nuclear translocation and initiate transcription of NF-κB responsive genes by binding to κB sites in gene promoter or enhancer regions. NF-κB molecules form functional heterodimers before translocation to the nucleus (Ghosh and Hayden 2008).

In vitro inhibitor studies have revealed a critical role for NF-κB signalling in the induction of inflammatory cytokines both in *C. jejuni*-stimulated IECs and DCs (Lippert et al. 2009). In a gnotobiotic IL-$10^{-/-}$ NF-κB-reporter murine model, pathology is associated with NF-κB activation in lamina propria mononuclear cells (LPMC) and induction of IL-12p40 and TNF-α (Lippert et al. 2009). However, the use of NF-κB inhibitors did not ameliorate *C. jejuni*-mediated pathology suggesting this signalling pathway alone is not responsible for the inflammatory nature of campylobacteriosis in this model.

1.4.7.2 Mitogen-Activated Protein Kinases

MAPKs are a family of serine/threonine kinases that have multiple roles in cellular biology including: cellular proliferation/survival, transcription induction, cellular movement, as well as induction of inflammatory responses after microbe recognition. MAPKs are activated by upstream MAPK kinases, which in turn must be activated by MAPK kinase kinases (MAP3K). Once activated MAPKs regulate a number of different transcription factors that are involved in immune function. Regulation of activation is tightly controlled, for example MAPKs can regulate their own activation by negative feedback mechanisms involving activation of one of three classes of protein phosphatases (Jeffrey et al. 2006).

There are three major MAPK family members: Extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK), and p38. They exert cell-specific effects on cellular processes. p38 and JNK are linked to pro-inflammatory responses. p38 was first identified as the target of pyridinyl imidazoles, a group of compounds capable of inhibiting monocyte TNF-α and IL-1 secretion (Lee et al. 1994). ERK has been linked to pro-inflammatory responses in DCs (Boele et al. 2009), but it can also influence IL-10 and TGF-β expression, and the development of Tregs (Escors et al. 2008). MAPKs activate transcription factors that differentially regulate inflammatory gene expression. For example TLR-mediated ERK-dependent activation of AP-1 is essential for IL-23 p19 gene expression, although this pathway does not elevate IL- 12/IL-23 p40 expression (Liu et al. 2009). The profile of transcription factor activation by these pathways tailors a specific immune response. TAK1 is an important MAP3K, downstream of many PRRs including TLRs, and is capable of activating all three MAPK members. In addition, TAK1 can activate NF-κB directly through the ubiquitination and degradation of inhibitory IKK molecules (Sato et al. 2005). In addition to gene regulation, MAPK can also participate in microbe uptake. Syk is a tyrosine kinase downstream of many CLRs and is involved in multiple immune functions including cytoskeletal rearrangement for phagocytosis. ERK-mediated Syk activation has been linked to the phagocytosis of *Francisella tularensis* by macrophages (Parsa et al. 2008). *C. jejuni* induces ERK and p38 activation in IECs leading to IL-8 production (Watson & Galan 2005). No studies to date have investigated the role of MAPK signalling in *C. jejuni*-mediated innate immune cell activation.

1.4.7.3 Interferon-regulatory transcription factors

Interferon-regulatory transcription factors (IRFs) are a family of transcription factors involved in the regulation of type 1 interferon genes. IRF3 is downstream of TRIF-mediated signalling (Figure 1-6). IRF3 phosphorylation in response to *C. jejuni* occurs in a TLR-dependent manner, this molecular signature is associated with an upregulation of IFN-β secretion (Rathinam et al. 2009). Much less is known about the role of type 1 interferons during intracellular bacterial infection than viral infections, although evidence suggests that IFN-β may enhance anti-bacterial effects through a positive feedback mechanism (Gautier et al. 2005).

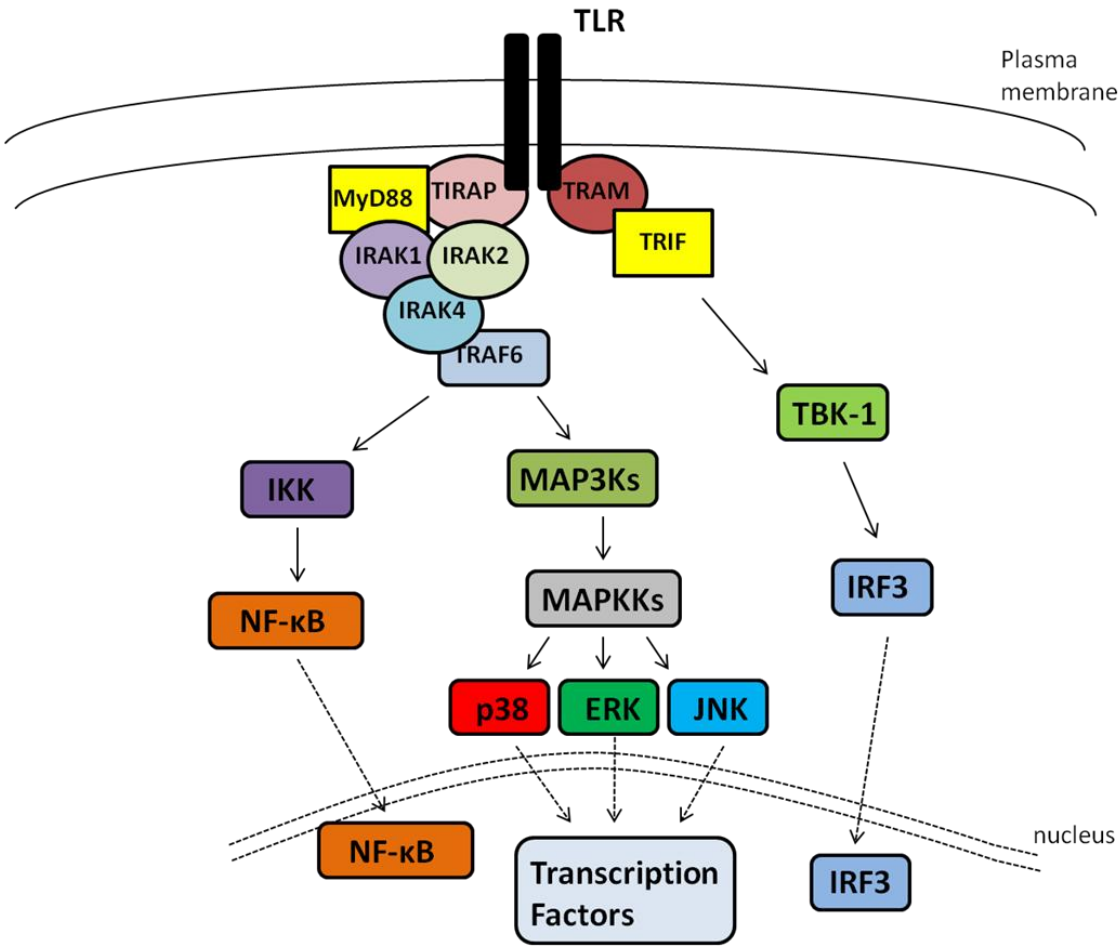


Figure 1-5: TLR signalling pathways

TLR engagement leads to activation of two major downstream signalling pathways. The **MyD88-dependent pathway** is downstream of all TLRs except TLR3 and leads to the activation of NF-κB and MAPK. The **TRIF-dependent pathway** (or MyD88-independent pathway) is downstream of TLR3 and TLR4 and involves the activation of IRF3 and induction of type 1 IFN inducible genes.

1.4.7.4 Phosphoinositide-3-Kinase

PI3K signalling pathways have been implicated in a diverse array of cellular function including growth, proliferation, survival, and motility. Recent findings have implicated PI3K in the dampening of pro-inflammatory NF-κB signalling in response to LPS (Weichhart et al. 2008). Mammalian target of rapamycin complex-1 (mTORC1) is downstream of PI3K activation, and is inhibited by rapamycin. Interestingly, treatment with rapamycin, an inhibitor of mTOR signalling, inhibits colitis in the gnotobiotic IL-$10^{-/-}$ murine model of infection (Sun, Threadgill, & Jobin 2011). The inhibition of mTORC1, contrary to the LPS reports, decreases NF-κB activation. However, it is unlikely that it is this effect which

reduces pathology as NF-κB inhibition alone does not ameliorate disease (Lippert et al. 2009). Rapamycin inhibits production of IL-17 and IL-1β in this model which is associated with a decrease in neutrophil infiltration. IL-17 is a potent chemoattractant for neutrophils therefore IL-17 levels may be responsible for the observed reduction in neutrophil infiltration. Interestingly, the source of IL-17 in this model is not $CD4^+$ T-cells as depletion of these cells had no effect on pathology. Other potential sources of IL-17 in the LP include γδ T cells, NK cells and LTi cells.

1.5 Adaptive Immune Response to Gastrointestinal Pathogens

A critical role for adaptive immunity in limiting *C. jejuni* infection in humans is demonstrated by the prolonged infection and increase in bacteraemia seen in immunocompromised individuals (Fernandez-Cruz et al. 2010). Both humoural and cellular immunity has been implicated in bacterial clearance and subsequent protection from re-infection (Janssen et al. 2008).

Dimeric secretory IgA (sIgA) cross the IEC monolayer and directly interact with bacteria in the GI lumen. sIgA is abundant in breast milk and is passed to infants during feeding, conferring protection against microbes including *C. jejuni* (Ruiz-Palacios et al. 1990). Hypoagammaglobulinemic patients, who lack antibodies, suffer from prolonged and often systemic campylobacteriosis suggesting a critical role for antibodies in limiting infection (van den Bruele et al. 2010). Interestingly, IgM but not IgG antibody transfer reportedly clears infection in these patients, implying a role for IgM in clearance of *C. jejuni* (Borleffs et al. 1993).

In addition to a correlation with fecal IgA, a human volunteer study highlighted a significant association between protection from *C. jejuni*-mediated pathology during re-infection and IFNγ levels (Tribble et al. 2010). The susceptibility of HIV patients to systemic *C. jejuni* infection also implicates cellular-mediated immunity in bacterial clearance during infection (Fernandez-Cruz et al. 2010).

APCs present antigen on MHC class II which together bind to the T-cell receptor (TCR) complex on $CD4^+$ T cells. In the presence of co-stimulatory molecules naïve $CD4^+$ T cells differentiate into T helper effector cells upon TCR ligation. The T cell effector phenotype generated is dependent on the cytokine milieu in the local environment, primarily derived from activated APCs. T helper (Th) 1 cells broadly activate professional phagocytes by producing IFNγ, increasing their phagocytic capability and bactericidal activity; in contrast,

Th2 cells secrete IL-4 which is necessary for B-cell activation and Ig-class switching. In addition to the conventional Th1/Th2 paradigm, the recently discovered Th17 cells are associated with immune responses to pathogenic bacteria and also autoimmunity (Harrington et al. 2005). The production of IL-17 by Th17 cells recruits neutrophils to the site of inflammation. In addition to these T helper subsets there are multiple subsets of suppressor T cells that can either be generated in the thymus (regulatory T cells – Tregs) or induced in the periphery (inducible (i)Tregs). Regulatory T cells play an important role in limiting inflammation-associated damage during infection.

In vitro assays with *C. jejuni*-stimulated human DCs show the potential of these cells to expand both Th1 and Th17 memory cells as well a unique IFNγ^{+} IL-17^{+} double-positive T cell subset (Edwards et al. 2010). Murine DCs also show the potential for *C. jejuni*-stimulated cells to differentiate naïve CD4^{+} T cell into Th1 effector cells (Rathinam, Hoag, & Mansfield 2008). In a gnotobiotic IL-10$^{-/-}$ murine model IL-17 production in the mucosa was associated with colitis, although this was also not produced by CD4^{+} T cells (Sun, Threadgill, & Jobin 2011). During infection, IFNγ likely aids the clearance of *C. jejuni* however as seen in IBD overt activation of cellular immunity may contribute to the severity of inflammation. The contribution of elevated IFNγ levels driven by prior exposure to *C. jejuni* to clearing bacteria more rapidly upon re-infection is a likely explanation for the observed association between IFNγ levels and protection (Tribble et al. 2010).

Multiple bacterial pathogens have been shown to manipulate T cell polarisation in order to evade the host immune response. *H. pylori* promote Treg skewing via induction of DC IL-10 and TGF-β (Kao et al. 2010). One hypothesis is that *C. jejuni* actively inhibits cellular mediated immunity in chickens resulting in impaired heterophil chemotaxis thus promoting colonisation (Meade et al. 2009). Current understanding of chicken mucosal T-cell responses remains limited although systemic spread of *C. jejuni* triggers a T-cell response in the liver (Jennings et al. 2011; Meade et al. 2009). In contrast to the human mucosal response to *C. jejuni*, chickens produce limited IL-6 and IL-1β upon colonisation (Meade et al. 2009; Shaughnessy et al. 2009); this may limit the activation of T-cells in the chicken mucosa which subsequently may limit heterophil influx.

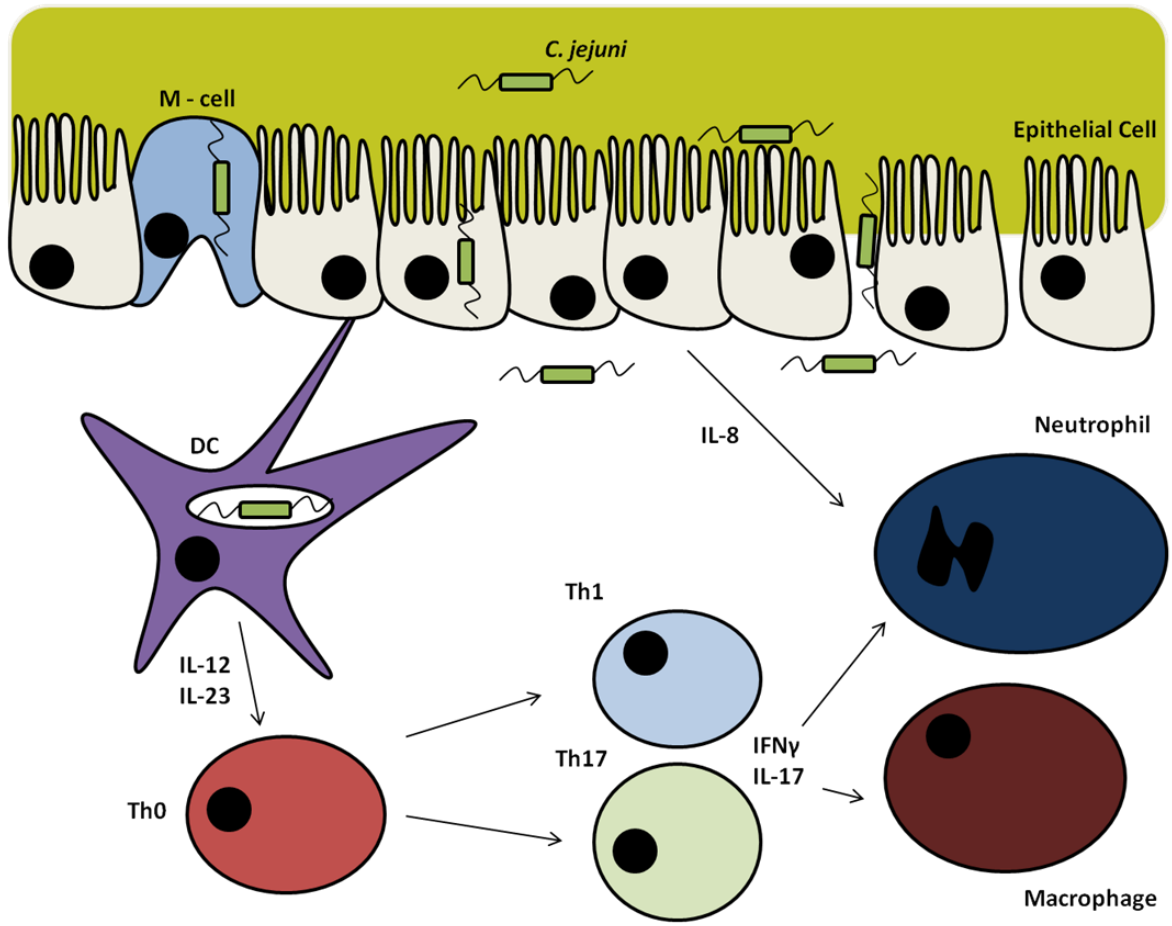


Figure 1-6: Model for *C. jejuni* pathogenesis in the human intestine

C. jejuni motility is critical to traverse the thick mucus lining of the epithelium. *C. jejuni* invade IECs leading to s IL-8 production and barrier disruption. *C. jejuni* may also invade the LP via M-cells. DCs phagocytose *C. jejuni* in the LP and trigger T cell differentiation. Cytokines and chemokines attract and activate innate professional phagocytes such as neutrophils and mononuclear cells to the site of infection. Professional phagocytes clear infection but this may come at a cost of pathologic inflammation in susceptible individuals.

1.5.1 *Vaccine Development*

The high socioeconomic cost of campylobacteriosis means significant efforts are being made to develop vaccines against infection primarily in chickens, but also in humans. The finding that chicks up to the age of 3 weeks are protected from *C. jejuni* colonisation by maternal IgG antibodies suggest a protective humoural response can be induced in chickens (Sahin et al. 2003). Epidemiological studies suggest the ability of humans to mount a protective immune response to *C. jejuni*-mediated pathology but not colonisation, suggesting a human vaccine could limit disease. A human volunteer study showed short-term veterans (<50 days since initial *C. jejuni* challenge) were protected from pathology and

partially to colonisation when re-infected with the same *C. jejuni* strain, observed by lower excretion levels (Tribble et al. 2010). Approximately half of long-term veterans (1 year since initial *C. jejuni* challenge) were protected from pathology when re-infected. This suggests minimal length of protection of *C. jejuni* adaptive immune responses, and therefore repeated exposure in developing countries most likely boosts immune responses in adults.

Studies to date have looked at multiple methods of vaccination in multiple species (Jagusztyn-Krynicka et al. 2009). A commercially available inactivated *Campylobacter* whole-cell vaccine against *Campylobacter fetus* and *Campylobacter jejuni* (CampyVax) is available for protection against *Campylobacter*-mediated abortion and diarrhoea in sheep. A similar vaccine was used for a Phase III human vaccine trial started after a successful Phase II trial performed in 2002 however results have not been released from the company, Antex. Whole-cell vaccines of *C. jejuni* may be considered risky due to the association of *C. jejuni* with post-infectious neuropathies, therefore other vaccination strategies are being researched but are in early stages of design. Subunit vaccines based on flagellin, flagellin-exported proteins, and CPS-conjugate vaccines are currently being researched.

1.6 Project Aims & Hypotheses

In the present study we wished to test the hypothesis that *C. jejuni* modulates host innate immune responses via glycosylated surface determinants which may favour either pro- or anti-inflammatory responses

The specific aims of the project were:

1. To investigate the role of the *C. jejuni* capsule, N-linked glycosylation system, and O-link glycosylated flagellin proteins in the activation of DC responses.
2. To identify potential host innate immune receptors that may be involved in recognition of *C. jejuni* and modulation of subsequent immune outcome.
3. To determine whether differences in *C. jejuni* LOS structures aid in defining the livestock and environmental phylogenetic clades, and whether LOS structural differences alter innate immune responses.

Chapter 2.

Materials & Methods

2.1 Bacterial Culture

2.1.1 *Campylobacter jejuni Strains*

The hyper-motile strain 11168H is a variant of NCTC 11168 wild-type (WT) *C. jejuni*, a strain that was originally isolated from a diarrheic patient by Martin Skirrow in 1977 (Karlyshev et al. 2002). 81-176 is a strain isolated from a milk-borne outbreak in the USA (Korlath et al. 1985). Isogenic mutant bacteria were the kind gift of Professor Brendan Wren (LSTMH). Isogenic mutants were constructed by inserting a kanamycin resistance cassette into the gene of interest and introducing the construct into the chromosome by double homologous recombination, leading to a disruption of the gene. The isogenic mutant strains utilised in this study are listed in Table 2-1.

Gene	Phenotype	Reference
ΔflaA	Lacks FlaA flagellin protein, one of two structural proteins constituting the flagella. Aflagellate bacteria; secretion positive.	(Wassenaar et al. 1994)
ΔrpoN	Disruption of the alternative sigma factor σ54 (RpoN). Aflagellate bacteria; secretion negative.	(Jagannathan et al. 2001)
ΔpglB	Lacks the conserved N-linked glycosylation system thus denuding periplasmic and outer membrane proteins	(Young et al. 2002)
ΔkpsM	Capsular polysaccharide (CPS) negative	(Karlyshev et al. 2001)
ΔCj1316	Flagellin monomers modified with only pse5Ac7Ac but not pse5Ac7Am (or further derivatives)	(Guerry et al. 2006)
ΔwaaF	Lacks a LOS heptosyltransferase truncating the OS to just one heptose residue proximal to the KDO residue	(Oldfield et al. 2002)

Table 2-1 *C. jejuni* Isogenic Mutants

2.1.2 *C. jejuni Culture*

C. jejuni was routinely cultured on 7% blood agar (Oxoid, Basingstoke, Hampshire, UK) under micro-aerobic [Variable Atmosphere Incubator (VAIN; Don Whitley Scientfific, Shipley, UK); 85% N_2, 10% CO_2, and 5% O_2] conditions at 37^{o}C. Alternatively, *C. jejuni* was grown in 2.5L gas jars (Oxoid) using micro-aerobic generating sachets, CampyGen (Oxoid) in a 37^{o}C incubator.

2.1.2.1 Growth on Blood Agar Plates

Agar was prepared by dissolving 18.5g Columbia blood agar (BA) base (Oxoid) in Milli-Q 0.22μm-filtered water (Millipore, London, UK) made up to 500mL and subsequently sterilising by autoclaving at 121^{o}C for 15mins. 35mL of pre-warmed (to 37^{o}C) defibrinated

horse blood (Oxoid) and one vial of *Campylobacter* selective supplement (a cocktail of vancomycin, trimethoprim, and polymyxin B; Oxoid) was added to 500mL Columbia blood agar base prior to plate (Fisher Scientific, Leicestershire, UK) preparation. For growth of isogenic mutant strains, 50µg/mL kanamycin (Sigma, Gillingham, UK) was also included. Frozen bacterial stocks were used as inoculums; cultures were passaged bi-weekly for up to 4 weeks (stock plates). To obtain bacterial cultures in log phase growth for experiments, fresh BA plates were inoculated with *C. jejuni* from the stock plates and grown for 24h prior to experimentation. *C. jejuni* were subsequently resuspended in phosphate buffered saline (PBS; Invitrogen, Paisley, UK) and adjusted to the correct colony forming units (CFU)/mL using optical density (OD) measurements at 600nm.

2.1.2.2 Growth in Brucella Broth

Large scale preparation of *C. jejuni* (for lipooligosaccharide (LOS) isolation) was achieved by growing *C. jejuni* in Brucella broth. 28g BBL™ Brucella broth (Becton Dickinson (BD), Oxford, UK) was dissolved in 1L Milli-Q water and autoclave sterilised. Starter cultures were grown overnight by inoculating 3mL Brucella broth with a streak of *C. jejuni* from a fresh culture plate (<24hrs old) under micro-aerobic conditions with shaking at 200rpm at 37^{o}C. Starter cultures were used to inoculate 500mL cultures which were subsequently grown to stationary phase under micro-aerobic conditions, at 37^{o}C for 24-48h.

2.1.3 *Bacterial Quantification*

Bacterial cultures were resuspended in PBS and quantified in cuvettes (10mm path length; Fisher Scientific) using a spectrophotometer (Jenway 6300 Spectrophotometer; Jenway, Essex, UK) at 600nm. The OD_{600} was determined for individual bacterial suspensions.

2.1.3.1 CFU count

CFU counts were performed to determine the absolute number of bacteria. 10-fold serial dilutions of bacterial suspensions were performed in PBS. 10µL aliquots of each dilution were spotted in duplicate onto BA agar plates with up to 12 spots per plate. Biological replicates were also performed for each serial dilution. Colonies were counted for the lowest two serial dilutions with colonies present from both plating replicates and biological replicates, which were then used to calculate CFU counts. For both WT and isogenic mutant strains an optical density (OD) reading of 1 at 600nm equalled 3 x 10^9 colony forming units (CFU).

2.1.4 *Preparation of Frozen Bacterial Stocks*

Using a sterile 10µL plastic loop, a streak of *C. jejuni* (grown on BA plates for 24h) was used to inoculate a microbank vial (Pro-lab, Neston, Cheshire, UK) and subsequently stored at -80^{o}C.

2.1.5 *FITC labelling of C. jejuni*

A fresh saturated solution of Fluorescein isothiocyanate (FITC; Sigma) dissolved in sterile PBS was filter sterilised (0.22µm). Resuspended *C. jejuni* (OD 1) was mixed 1:1 with the saturated FITC solution. The mix was incubated in the dark at 37^{o}C for 1h with gentle agitation. After incubation bacteria were pelleted by centrifugation at 10,000g for 2mins. The supernatant was discarded and the bacterial pellet resuspended in fresh PBS. The wash step was repeated three times before resuspending the pellet in PBS to a desired OD for co-culture studies.

2.2 *C. jejuni* gene analysis

2.2.1 *Isolation of Bacterial Genomic DNA*

Bacteria grown overnight were harvested into 1mL PBS then pelleted at 10,000g for 2mins. Supernatant was decanted and the pellet was resuspended in 467µL TE buffer (10mM TRIS pH 7.6, 1mM EDTA) by repeated pipetting. 30µL of 10% sodium dodecyl sulphate (SDS) and 3µL of 20mg/mL proteinase K (Sigma) was added prior to incubation for 1h at 37^{o}C to allow bacterial lysis and protein digestion. An equal volume of Phenol:Chloroform (Sigma) was added and the suspension mixed gently to avoid shearing the genomic DNA. The mixture was spun at 10,000g for 10mins and the upper aqueous phase transferred to a fresh 1.5mL eppendorf. An equal volume of Phenol:Chloroform was added and the suspension gently mixed prior to centrifugation at 10,000g for 10mins. 1/10 volume of 3M sodium acetate (pH 5.2) was added to the collected aqueous layer followed by addition of 6/10 volume of isopropanol to precipitate the DNA. DNA was pelleted at 10,000g for 5mins and resuspended in the appropriate volume of water. DNA was quantified by NanoDrop (Thermo Scientific, Wilmington, USA). DNA was stored at -20^{o}C until required.

2.2.2 *PCR analysis of gene content*

To confirm the presence of specific genes in different *C. jejuni* strains, PCR analysis was performed utilising gene specific primers. Reaction mixtures constituted: 10µL of Biomix Red PCR master mix (containing 1.5mM $MgCL_2$, 200µM dNTPs, and *Taq* polymerase; Bioline, London, UK), 2pmol of both forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany), and 100ng bacterial genomic DNA template. Reactions were made to

20µL in volume with MilliQ water. Primers utilised in this study are listed in Table 2. For multiple reactions, master mixes were prepared to reduce intra-experimental variation. Reaction contents were vortexed briefly then centrifuged. PCR amplification was performed in a thermal cycler (Techne TC-512, Staffordshire, UK) utilising the following cycling conditions:

Initial denaturation: 95°C for 5min - 1 cycle

Denaturation: 95°C for 1 min
Annealing: 55°C for 30sec
Extension: 72°C for 90sec
} 30 cycles

Final extension: 72°C for 10min – 1 cycle

PCR products were separated by agarose gel electrophoresis. 1g agarose (Sigma) was dissolved in 1 x Tris Borate EDTA (TBE) buffer (Sigma) by heating in the microwave. Once cooled to ~50°C 1/10,000 Gel Red (Sigma) was added to the solution. The agarose was poured into a gel caster and allowed to set. Gels were placed in a gel electrophoresis tank containing 1 x TBE buffer (Sigma). Samples were separated at 100V for ~30mins. Bands were visualised using a UV imager.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)
lgtF (Cj1135) set 1	ACTTAACAATGAAAGTAGCGATAATACCCTAA	GCCACCAACCACATGCCTTTATCC	290
lgtF (Cj1135) set 2	ACTTAACAATGAAAGTAGCGATAATACCCTAA	GCCAAATGTGTTTTAAAGGGCAAGGA	854
lgtF (Cj1135) set 3	CCATCAAGATTGGCTTGGTTTTGG	ATTGTGCATAAAGCTTTGCTATGATAAAACCTC	518
NeuB1 (Cj1140)	GCAGGnGCTAAGATnATAAAnCAnCAAAC	TAATnCTnACTACnCTnGCAAAnGCAAAATCAAT	748
orf 8ab*	ATTATAGCCATTTGCTCACTTTG	AAAGCACCCTTAGTCGTACCTG	755
orf 7ab*	ACTACACTTTAAAACATTTAATCCAAAATCA	CCATAAGCCTCACTAGAAGGTATGAGTATA	579
orf 8c* (Cj1140)	CCTTTGATAATCCCTGAAATAGGT	TCCTTTGCACTTATACCACCTT	910
orf 7c* (Cj1139)	TTGAAGATAGATATTTTGTGGGTAAA	CTTTAAGTAGTGTTTTATGTCACTTGG	745
SOAT	CCTCATTATGCAAAAGTTGGAAGGTATTG	ATCAGCAAAATGATATTTCCACCATTGAAT	424
htrB* (Cj1133)	GTTACTTTTATGCCTGAGTGTATCTTGCA	CGCTTTCGTTTTCTACACAGTCTTGATC	546

Table 2-2 PCR primers used to study genetic composition of *C. jejuni* strains

n = G, C, A, or T * denotes primer pairs where annealing temperature was 52°C

2.3 Mammalian cell Culture

2.3.1 *Heat-inactivation of Foetal Calf Serum*

Foetal calf serum (FCS; Invitrogen, Paisley, UK) was defrosted overnight at 4^{o}C. Heat inactivation (HI) was achieved by incubating FCS at 55^{o}C for 30mins in a water bath in order to inactivate heat-labile complement proteins present in the serum. FCS was aliquotted and stored at -20^{o}C.

2.3.2 *Cell-lines*

2.3.2.1 THP-1 cell line

The non-adherent human acute monocytic leukaemia cell line THP-1 was cultured in RPMI 1640 medium (Invitrogen) containing 2mM L-glutamine supplemented with 10% HI FCS, 100U/mL penicillin and 100mg/mL streptomycin (Invitrogen). This media is referred to as complete RPMI media hereafter. Cells were passaged bi-weekly and maintained at 1 x 10^5 – 1 x 10^6 cells/mL in T75 culture flasks. Cells were stimulated with 10ng/mL phorbol myristate acetate (PMA; Sigma, Poole) for 18h to differentiate the cells from a pro-monocytic to a monocyte/macrophage phenotype (Daigneault et al. 2010). PMA-stimulation resulted in the majority of cells becoming adherent.

2.3.2.2 HEK/TLR cell lines

The semi-adherent Human Embryonic Kidney (HEK) cell-line stably transfected with human TLR4/CD14/MD2 and a inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene (HEK-blue^{TM} hTLR4; InvivoGen, Nottingham, UK) were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) containing 2mM L-glutamine supplemented with 10% HI FCS, penicillin/streptomycin and 100µg/mL Normicin (InvivoGen). This media is referred to as complete DMEM media hereafter. Cells were grown to 80% confluency, and passaged on average bi-weekly. Cells were used up to passage 15.

2.3.2.3 Siglec-CHO cell-lines

The adherent Chinese Hamster Ovary (CHO) cell-line either non-transfected or transfected with human Siglec-10 or Siglec-7 were the kind gift of Prof. Paul Crocker (University of Dundee, UK). Cells were cultured in F12 Ham media containing 2mM L-glutamine supplemented with 10% HI FCS, penicillin/streptomycin. Cells were grown to 80% confluency and passaged bi-weekly. Cells were washed in sterile PBS before addition of 2mM EDTA in PBS. Cells were incubated at 37^{o}C for 5mins to detach the cells. On addition

of complete media cells were pelleted at 1600rpm for 7mins. Cells were resuspended in complete media and cultured in T75 flasks.

2.3.3 *Counting viable cells*

10µL of cell suspensions was mixed 1:1 with 0.4% Trypan blue (Sigma, Poole) for 1min. 10µL of this mixture was placed onto a haemocytometer counting chamber (Hawksley, Sussex, UK). Live cells (clear) were counted (40X magnification) with a light microscope (Phase Contrast 2, Nikon). The viable cell count was calculated as follows:

Cell number within a 25 field box x dilution factor = total cell number x 10^4/mL

A minimum of four 25 box fields were counted.

2.3.4 *Freezing cells*

Cells were pelleted (1600rpm, 7mins) and resuspended at ~1 x10^6/mL in 90% FCS containing 10% dimethyl sulphoxide (DMSO; Sigma). 1mL aliquots were made in Nunc cryovials (Fisher Scientific) and slowly frozen in a Nalgene Mr. Frosty (Sigma) containing isopropanol at -80^{o}C. For long term storage cells were stored in liquid nitrogen.

2.4 Primary Cell culture

2.4.1 *Mice*

A colony of WT C57BL/6 mice were kept at ICH Western labs animal facility. MyD88$^{-/-}$, TRIF$^{-/-}$, single and double knock-out mice were the kind gift of Dr. Caetano Reis e Sousa (Cancer Research UK). IPAF$^{-/-}$, ASC$^{-/-}$, NOD2$^{-/-}$, and NALP3$^{-/-}$ knock-out mice were the kind gift of Dr. Claire Bryant (University of Cambridge).

2.4.2 *Generation of Murine Bone-Marrow Derived Dendritic Cells (BMDCs)*

The muscle from femurs and tibias of 6-12wk old C57BL/6 mice were removed by scalpel, and bones sterilised in 70% ethanol. The bone marrow was flushed out with sterile PBS containing 2% FCS and 10µg/mL gentamicin (Invitrogen). Bone marrow was resuspended by gentle pipetting, and pelleted by centrifugation at 1600rpm for 7mins. Red blood cells were lysed in Red Blood Cell Lysis Buffer (Sigma) for 5mins, pelleted and washed twice in PBS. Cells were resuspended and plated at 0.5 x 10^6 cells/mL in Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen) containing 10% HI FCS, penicillin/streptomycin, 2mM L-glutamine, 50µM β-Mercaptoethanol (Invitrogen), 10µg/mL gentamicin, (termed complete media) with 20ng/mL murine GM-CSF (Peprotech, London, UK, or from Invitrogen). Cells were seeded at ~1 x 10^5 cells/mL in 6-well plates. On day 3 or 4, non-adherent cells were

collected, spun down and resuspended in fresh IMDM complete media containing GM-CSF. Fresh media was added when necessary on day 6. Cells were used on days 7 or 8. BMDCs were ~80% positive for CD11c as assessed by flow cytometry from two independent experiments.

2.4.3 *Murine Naïve T cell Isolation*

Spleens were removed from 6-12 wk old C57BL/6 mice. Single cells suspensions were achieved by pushing spleens through nylon 70µm cell strainer (BD Biosciences, Oxford, UK) with the plunger of a 5mL syringe with PBS. Splenocytes were pelleted by centrifugation at 1600rpm, 7mins. Red blood cells were lysed by resuspending the cell pellet in 1mL of Red Blood Cell Lysis Buffer and incubating at room temperature (RT) for 5mins. Splenocytes were subsequently washed twice in PBS. $CD3^+$ $CD4^+$ $CD62L^+$ $CD44^{low}$ Naïve $CD4^+$ T cells were isolated using a negative selection MagCellect Mouse Naïve $CD4^+$ T cell Isolation kit (R&D systems, Abingdon, UK) according to manufacturer's protocols. Typically 1 x 10^8 splenocytes were extracted from one spleen. Splenocytes were resuspended in chilled 1 x MagCellect Buffer at a density of 2 x 10^8/mL and transferred to a 5mL sterile polystyrene tube. 200µL of MagCellect Mouse Naïve CD4+ T Cell Biotinylated Antibody Cocktail was added per mL and gently mixed by pipetting and incubated at 4^{o}C for 15mins. 250µL of MagCellect Streptavidin Ferrofluid was added to the suspension per mL, mixed gently by pipetting, and incubated at 4^{o}C for 15mins. The suspension was subsequently adjusted to a total of 3mL volume with 1 x MagCellect buffer and mixed gently by pipetting. The suspension was placed in an EasySep magnet (Stemcell Technologies, Grenoble, France) for 6mins at RT to allow the magnetically labelled cells to migrate to the magnet leaving the naïve T cells in suspension. Naïve T cells were collected by inverting the tube whilst still in the magnet and collecting the suspension into a fresh tube. Naïve T cells were pelleted and washed twice in PBS. Naïve T cells were used immediately for subsequent experimentation. Cells were >95% $CD3^+$ $CD4^+$ as assessed by flow cytometry from two independent experiments.

2.4.4 *Human Peripheral Blood Monocyte Isolation*

50 – 100mL blood was obtained from consented healthy adults at the Institute of Child Health and collected in tubes containing heparin. Blood was mixed 1:1 with RPMI then carefully layered onto 2/3 volume lymphoprep without disrupting the interface (Axis Shield, Uxbridge, UK). Peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation at 2,200rpm for 25mins, with acceleration set at grade 4 and the brake off. PBMCs were carefully collected from the interface of the lymphoprep through the upper

media layer using a sterile Pasteur pipette. PBMCs were pelleted at 1,600rpm for 7mins, washed in RPMI and re-pelleted. Monocytes were extracted by $CD14^+$ magnetic bead isolation (Miltenyi Biotec, Surrey, UK). PBMCs were carefully resuspended in 80µL MACS buffer (pre-chilled PBS containing 2mM EDTA, 1% FCS) per 10^7 cells. 20µL of magnetically labelled anti-CD14 beads were added per 10^7 cells and mixed gently prior to incubation at 4^oC for 20mins. 10mL MACS buffer was added to the PBMCs and mixed before pelleting. PBMCs were resuspended in 500µL MACS buffer. An LS MACS column (Miltenyi Biotec) was placed in a MACS magnet (Miltenyi Biotec) and primed by passing 3mL of MACS buffer without collection. PBMCs were passed through the column which was subsequently washed three times with 3mL MACS buffer. The column was removed from the magnetic field and the $CD14^+$ eluted from the column by passing 5mL MACS buffer through it using the sterile plunger provided. $CD14^+$ cells were washed in RPMI and pelleted, and were ready for further experimentation. Cells purified were >95% positive for CD14 as assessed by flow cytometry from two independent experiments.

2.4.5 *Generation of Human Monocyte-derived Dendritic Cells*

Isolated $CD14^+$ monocytes were cultured in complete RPMI containing 50ng/mL recombinant human IL-4 and 100ng/mL GM-CSF (R&D systems) for 5-7 days at a density of 2.5×10^5 cells/mL in 6-well plates. DCs were harvested and used immediately for further experimentation. Cells were >95% positive for CD11c as assessed by flow cytometry from two independent experiments.

2.5 Co-culture experiments

2.5.1 *BMDC Co-cultures*

BMDCs were seeded at 1×10^6/mL in RPMI containing 10% HI FCS in poly-propylene tubes to limit cell adhesion upon activation unless otherwise stated. Cells were treated with *C. jejuni* multiplicity of infection (MOI) 100, PBS alone, or 1µg/mL *E. coli* 0111:B4 LPS (Sigma), all prepared in 100µL PBS. Cells were incubated for 24h at 37^oC, and pelleted by centrifugation at 1600rpm for 7mins. Supernatants were collected and stored at -80^oC for cytokine analyses, cells were used immediately for surface marker analyses.

2.5.2 *Cytokine Secretion*

Cytokine levels were assessed using Ready-SET-Go! Enzyme-linked immunosorbent assay (ELISA) (eBioscience, Hatfield, UK) following manufacturer's protocols. Plates were read at

450nm on a Multiskan EX Multiplate Photometer (Thermo Scientific). Data was analysed using Ascent software (London, UK).

2.5.3 *Surface Marker Analyses*

Cells were assessed for expression of maturation markers that increase upon DC maturation. Cells were washed in FACS wash buffer (PBS, 2% FCS, 0.02% sodium azide) then blocked with 1/100 anti-murine Fc Receptor antibody (BD Bioscience, Oxford, UK) for 20mins on ice to eliminate binding of antibodies utilised to Fc receptors. Cells were then either stained for maturation markers (ebioscience; Table 0-3) or with an isotype control, or left unstained for 30mins on ice in staining buffer (FACS wash buffer containing 10% FCS). Cells were pelleted and washed with FACS wash buffer twice, and fixed in 4% paraformaldehyde (PFA) for 20mins. Cells were pelleted and resuspended in PBS. Surface marker expression was investigated by flow cytometry on a FACScan machine (BD Bioscience). Analysis was performed using FlowJo software (Tree Star, Oregon, USA).

Antibody	Fluorochrome	Concentration
Anti-CD40	PE	1/40
Anti-CD80	PE-Cy5	1/200
Anti-CD86	FITC	1/200
Anti-MHC Class II	FITC	1/200

Table 2-3 Antibodies for BMDC maturation markers expression

2.5.4 *C. jejuni-mediated Activation of DC Signalling Pathways*

BMDCs were seeded at 1 x 10^6/well in 1mL media RPMI containing 2mM L-glutamine and 0.05% FCS a day prior to infection; this was to reduce growth related mitogen-activated protein kinase (MAPK) signalling. BMDCs were stimulated with *C. jejuni* at an MOI 100 in 100µL PBS. 100µL PBS was added to unstimulated controls. After inoculation, co-cultures were spun at 1500rpm for 5mins to promote association of *C. jejuni*/BMDCs. Co-cultures were incubated at 37^{o}C for the duration of the infection. Cells were lysed in 50µL lysis buffer [1% Triton-X 100 (Sigma), 150mM sodium chloride, 50mM Tris pH 8.0, complete-mini protease inhibitors (Roche, Sussex, UK), phosphatase inhibitor cocktail (Roche)] at specified time points. After 5mins, lysates were collected and incubated on ice for a further 30mins to ensure complete lysis. Lysates were spun at 13,000rpm for 2mins to pellet insoluble membranes and DNA, then aliquoted and stored at -80^{o}C.

2.5.5 *DC/T cell Co-cultures*

1 x 10^5/mL BMDCs were co-cultured with *C. jejuni*, media alone, or *C. jejuni* LOS (100ng/mL) for 24h in RPMI containing 2mM L-glutamine and 10% FCS. Cells were pelleted (1600rpm 6mins) and supernatants were collected and passed through a 0.22µm filter. BMDCs were resuspended in complete RPMI containing 150µg/mL gentamicin and incubated at 37^{o}C for 2h to kill extracellular bacteria. BMDCs were subsequently pelleted and the supernatant discarded. BMDCs were washed once in PBS to remove residual dead bacteria then resuspended in the original sterile filtered supernatants. BMDCs were plated into 96-well plates at 1 x 10^4 BMDCs/well in 100µL. Naïve T cells were suspended at 1 x 10^6/mL in RPMI (10% FCS) containing 20µg/mL gentamicin, 10U/mL IL-2 (Peprotech), and anti-CD3/CD28 Dynabeads (bead to cell ratio 1:1; Invitrogen). 1 x 10^5 Naïve T-cells (1:10 DC:T cell ratio) were added to the wells in 100µL making a final volume of 200µL per well, and 10µg/mL gentamicin. Media alone was added to BMDC only and naïve T cell only control wells. DC/T cell co-cultures were incubated for 4 days. Supernatants were collected and stored at -80^{o}C prior to cytokine analyses.

2.5.6 *Human DC co-cultures*

1 x 10^5 monocyte-derived DCs were seeded into 96-well plates in 200µL RPMI containing 2mM L-glutamine and 10% FCS. DCs were stimulated with WT *C. jejuni* and various isogenic mutants at an MOI 100. 24h post-infection supernatants were collected and stored at -80^{o}C for further cytokine analysis.

2.5.7 *Siglec-CHO co-cultures*

Adherent Siglec-CHO cells were washed once in PBS then detached in PBS containing 2mM EDTA (Sigma) for 5mins at 37^{o}C. Cells were pelleted at 1600rpm for 7mins and resuspended in F12 media containing 0.5% FCS at 1 x 10^6/mL cell concentration. 300µL of the resuspended cells was placed in a 1.5mL eppendorf and chilled to 4^{o}C. Cells were co-cultured with various FITC-labelled *C. jejuni* strains at an MOI 100 for 2h with gentle rotation on a MACSmix Tube Rotator (Miltenyi Biotec) at 4^{o}C. Cells were collected and pelleted (1500rpm, 5mins, 4^{o}C) in 5mL polystyrene FACS tubes. Cells were carefully washed once in 0.5mL chilled PBS re-pelleted and fixed in 4% PFA for 15mins. Cells were pelleted and resuspended in 100µL PBS alone. Adherence of *C. jejuni* strains was assessed by flow cytometry.

2.5.8 *THP-1 co-cultures*

Adherent PMA-stimulated THP-1 cells were washed once in PBS then detached in 1mL trypsin (Invitrogen) for 5mins at 37^{o}C. 200µL (1 x 10^5 cell/ml) of cell suspension in RPMI containing 2mM L-glutamine and 10% FCS was seeded into individual wells of a 96-well plate. Cells were allowed to adhere for 2h prior to stimulation with either various live strains of *C. jejuni* or isolated LOS. 20h after stimulation supernatants were collected and stored at -80^{o}C for further analysis.

2.5.9 *Monocyte co-cultures*

1 x 10^5 $CD14^+$ monocytes were seeded into 96-well plates in 200µL RPMI containing 10% FCS. Monocytes were stimulated with various concentrations of LOS purified from different *C. jejuni* strains. 20h post-infection supernatants were collected and stored at -80^{o}C for further analysis.

2.5.10 *HEK-TLR4 co-cultures*

HEK-TLR4 SEAP cells (Invivogen) were detached by agitation and subsequently pelleted at 1,600rpm for 7mins. Cells were counted and seeded into 96-well plates at 2.5 x 10^4 cells/well in 200µL DMEM containing 2mM L-glutamine and 10% FCS. Cells were stimulated with purified *C. jejuni* LOSs' for 18h. At the end of the co-culture 20µL of supernatant was added to 180µL of HEK-Blue Detection Reagent (180µL) in a fresh 96-well ELISA plate (Nunc). Plates were incubated at 37^{o}C for 15min-2h until colour change from pink to purple was observed. Colour changes were read on an ELISA plate reader at 650nm.

2.6 Gene expression assays

2.6.1 *Total RNA Isolation*

Cells were washed in PBS and either had RNA isolated immediately or stored at -80^{o}C until required. Cell pellets were resuspended in 1mL of monophasic phenol and guanidine isothiocyanate based-TRIzol® in a 1.5mL eppendorf. 200µL chloroform (Sigma) was added to the tubes and vigorously vortexed for 10 seconds to ensure complete mixing. This mixture was allowed to stand for 3mins at RT before centrifugation (12000rpm, 15mins, 4^{o}C) to allow separation of the organic (lower) and aqueous (upper) phases. The aqueous phase (containing nucleic acids) was carefully extracted into a clean 1.5mL eppendorf without disturbing the denatured proteins found at the interface between the two layers. 600µL of isopropanol was added to the nucleic acid solution, mixed well, and left overnight at -20^{o}C to allow maximal precipitation of the total RNA. Tubes were then centrifuged (12000rpm, 10mins, 4^{o}C) to pellet the precipitated RNA. Waste was removed and RNA

pellets were washed twice in 1mL 70% ethanol to remove salts. RNA pellets were re-spun and the 70% ethanol removed. Pellets were air-dried completely to remove remaining ethanol, then resuspended in 30µL 1 x ambion DNAse turbo buffer (Ambion, Paisley, UK) made in RNAse free water. 1µL of DNAse enzyme (Ambion) was added and the reaction was allowed to proceed at 37^{o}C for 30mins to ensure complete removal of contaminating DNA. 3µL of DNAse Stop Solution (Ambion) was added and incubated at RT for 5mins with gentle flicking. Tubes were centrifuged (10,000g, 1.5mins) to separate the Stop Solution from the RNA. The aqueous layer containing the RNA was carefully removed into a fresh eppendorf. RNA was quantified using a NanoDrop machine. RNA was either used directly for conversion to cDNA or stored at -80^{o}C for later use.

2.6.2 *Generating cDNA from total RNA*

In an RNAse free PCR tube, 1-5µg of total RNA was added with 1µL oligo dT (Bioline, London, UK), 1µL random hexamers (Bioline). The mixtures were heated at 65^{o}C for 5mins in a thermal cycler and directly quenched on ice. For each sample a master mix containing 4µL 5 x Bioscript buffer (Bioline), 4µL dNTPs (Bioline), 1.5µL RNAse free water, 0.5µL Bioscript (Bioline) was made, and added directly to the PCR tubes containing the quenched total RNA solution. Contents were gently mixed, cDNA was synthesised using the following parameters in a PCR machine: 25^{o}C for 10mins (primer extension), 42^{o}C for 1 h (reverse transcription), 75^{o}C for 10mins (enzyme inactivation). cDNA was stored at 4^{o}C or -20^{o}C for short- and long-term storage respectively.

2.6.3 *Real-time PCR*

To quantify the relative levels of transcripts for specific genes, real-time PCR was performed on cDNA from BMDCs from co-culture studies. Reaction master mixes contained 10µL SYBR Green (Invitrogen), 5pmol forward primer, 5pmol reverse primer (Table 0-4), brought to 20µL total volume with MilliQ water. Master mix was pipetted into the bottom of the PCR tube (Qiagen). 2µL of cDNA was added to the master mix in the PCR tube. PCR was performed in duplicate using a Rotor-Gene 6000 Real-time PCR machine (Qiagen) utilising the following conditions:

Initial denaturation: 95^{o}C for 10min - 1 cycle

Denaturation: 95^{o}C for 15sec
Annealing: 58^{o}C for 30sec
Extension: 72^{o}C for 30sec
} 40 cycles

Melt Step: Increasing temperature by 1^{o} each cycle from 70^{o}-95^{o}. 90sec wait after first step and a 5sec wait after subsequent steps.

Lasers used: Green 470nm (excitation) 510nm (emission) Gain 10
Yellow 530nm (excitation) 555nm (emission) Gain 5

Data were analysed using Rotor-Gene 6000 Series Software 1.7. Relative levels of transcripts were determined using delta delta cycling threshold method (ΔΔct). ct levels were determined from duplicates of individual samples, and were expressed as a relative number to GAPDH transcript levels using the following equation:

$\Delta ct = 2^{(\text{GAPDH ct value} - \text{transcript of interest ct value})}$

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
p35	CCTCAGTTTGGCCAGGGTC	CAGGTTTCGGGACTGGCTAAG
p40	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAAT
IL-6	GTTCTCTGGGAAATCGTGGA	TGTACTCCAGGTAGCTATGG
IL-1β	CCAAAAGATGAAGGGCTGCT	AGAAGGTGCTCATGTCCTCA
GAPDH	CCTGGAGAAACCTGCCAAGTATG	AGAGTGGGAGTTGCTGTTGAA

Table 2-4 Real-time PCR primers

2.7 Intracellular Bacteria Counting

2.7.1 *Gentamicin Protection Assay*

BMDCs were seeded at 2 x 10^5/well in 24-well plates in a total volume of 1mL RPMI containing 2mM L-glutamine and 10% FCS. Cells were infected with *C. jejuni* at an MOI 100. Plates were spun at 1500rpm for 5mins to initiate bacterial/DC interactions. Co-cultures were incubated at 37^{o}C for various time periods to allow internalisation of *C. jejuni*. Infection media was removed and cells washed twice with sterile PBS. Media was replaced with 1mL RPMI containing 150μg/mL gentamicin, and incubated at 37^{o}C for 2h to kill extracellular bacteria. Media was removed and cells washed twice with sterile PBS. Cells were subsequently lysed in 0.5mL 0.1% Triton-X 100 (Sigma) in PBS for 5mins prior to undergoing 10-fold serial dilutions. CFU analysis was performed on BA plates. Plates were

incubated at 37^{o}C in gas jars containing a CampyGen sachet (Oxoid) to generate a micro-aerophillic atmosphere. Colonies were counted after 48h.

2.8 Activation of host signalling pathways

2.8.1 *SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot Analysis*

10% SDS-polyacrylamide gels were prepared on the day of use as follows:

Resolving Gel:
2.5mL R-Buffer (1.5M TRIS, 0.4% SDS, pH8.8)
3.5 mL 30% Acrylamide
4mL Milli-Q Water
100µL 10% Ammonium Persulphate
10µL Tetramethylethylenediamine (TEMED)

Stacking Gel:
1.2mL S-Buffer (0.5M TRIS, 0.4% SDS, pH 6.8)
1.2mL 30% Acrylamide
2.2mL Milli-Q Water
120µL 10% Ammonium Persulphate
10µL TEMED

Resolving gel was poured first into a gel cast filling ¾ of the space. 200µL of Milli-Q water was placed onto this layer to reduce contact with the air during polymerisation. After polymerisation the water was removed and the Stacking gel was poured onto the resolving gel with the addition of 10-well combs. Gels were allowed to fully polymerise before the combs were removed.

Lysates were mixed 1:1 ratio with 2x laemmli buffer [4% SDS (Sigma), 10% β-mercaptoethanol (Sigma), 20% glycerol (Sigma), 0.004% bromophenol blue (Fisher Scientific), 0.125M Tris HCl, pH 6.8]. Samples were boiled at 95^{o}C for 5mins. Samples were loaded and gels subjected to electrophoresis at 125V for 1.5 h in electrophoresis buffer (25mM Tris, 190mM glycine, 0.1% SDS). Gels were routinely run in a mini-protean 3 cell unit (Bio-Rad, Hertfordshire, UK). Proteins were transferred onto nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) by the wet transfer method. Transfers were conducted at 200mA for 60mins in transfer buffer (48mM Tris, 39mM glycine, 0.04% SDS, 20% methanol). Membranes were blocked in 5% milk (Marvel, Premier Foods, Hertfordshire, UK) in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS/T) for 2h at RT with gentle agitation. Phospho-specific primary antibodies (all from Cell Signalling, MA, USA) were used at 1/1000 concentration or anti-IL-1β (1/250; Santa Cruz, Heidelberg,

Germany) in 5% milk in TBS/T (except P-ERK and P-p38 2% BSA in TBS/T). All primary antibodies were incubated at 4°C for 8h/overnight. Membranes were washed three times for 5mins in TBS/T. Secondary goat anti-rabbit conjugated to horseradish peroxidase (DAKO, Stockport, UK) was used at 1/1000 in TBS/T (except P-ERK and P-p38 3% BSA in TBS/T). Secondary antibodies were incubated for 2h at RT for P-ERK and P-p38, and overnight at 4°C for P-JNK and IκBα detection. Membranes were washed 3 times for 5mins in TBS/T. 1mL Enhanced chemiluminescence (ECL; GE Healthcare, Buckinghamshire) was prepared according to manufacturer's protocol and added to the membranes for 2mins. Membranes were exposed to X-ray film (ECL hyperfilm; GE Healthcare) for various lengths of time and developed. To ensure equal loading of samples, membranes were stripped in stripping buffer (2% SDS, 0.8% β-mercaptoethanol, 0.0625M Tris pH 6.8) at 50°C in the fume-hood for 20mins, stripping buffer was discarded and this step repeated to ensure complete removal of bound antibodies. Membranes were re-blocked in 5% milk in TBS/T for 2h then re-probed with total ERK primary antibody (Cell Signalling).

2.8.2 *Analysis of NF-κB activation in BMDCs using an NF-κB reporter plasmid*

2.8.2.1 Lentivector Production

Lentiviral vectors were produced in a HEK 293T packaging cell line. A combination of 3µg lentivector plasmid, 1.5µg Vesicular stomatitis virus glycoprotein G (VSV-G) plasmid (pMD-G; (Naldini et al. 1996)) 1.5µg packaging plasmid (pCMVR8.91; (Naldini et al. 1996)) was made to 100µL in sterile water. 27µL Fugene 6 (Promega, Southampton, UK) was added to 100µL OptiMEM media (Invitrogen). Fugene/OptiMEM was added to the DNA and mixed thoroughly by vortexing and incubated at RT for 15mins to allow complex formation. DNA/Fugene complexes were added to a 70% confluent T75 flask of HEK 293T cells in complete media. Media was collected and replaced daily for up to 5 days. Collected media containing lentivectors was placed on top of a 3mL sterile 20% sucrose cushion in a 38.5mL Ultra-Clear™ centrifuge tube and centrifuged at 25,000rpm for 2h (Beckman Coulter, High Wycombe, UK). Pelleted lentivectors were carefully resuspended in 300µL PBS by repeated pipetting, aliquoted, and stored at -80°C.

2.8.2.2 Lentivector Titration by qPCR

5 x 10^5 HEK 293T cells were transduced with 2µL lentiviral vectors in 24-well plates. 24h post-transduction cells were collected and genomic DNA extracted using DNeasy Blood & Tissue Kit following manufacturer's protocol (Qiagen). A lentivector plasmid of known concentration was used to make the standard curve. A stock concentration of 1mg/mL

plasmid was diluted 2:175 in water to give a copy number of 10^9. From this stock a 10-fold serial dilution was made from 10^5-10^1 copies/µL. Master mixes were made as follows: 7.5 pmol fwd primer GT 249: 5' GAGTCCTGCGTCGAGAGAGC 3', 7.5pmol rev primer GT 248: TGTGTGCCCGTCTGTTGTGT, 3.75pmol probe FAM-CAGTGGCGCCCGAACAGGGA-TAMRA, 12.5µL QuantiTect PCR mix (Qiagen) made to 20µL with MilliQ water. 5µL of sample or standard was added to 20µL master mix in real-time PCR tubes. qPCR was performed as described in 0. Titres were calculated from the standard curve.

2.8.2.3 Lentivector Transduction of NF-κB reporter plasmid

On day 4 of differentiation, BMDCs were seeded at 1 x 10^5/well in 24-well plates in a total volume of 300µL of complete IMDM media containing 20ng/mL murine GM-CSF. Cells were transduced with lenti-viruses containing the NF-κB reporter plasmid that drives luciferase production depicted in Figure 2-1 the kind gift from Dr. David Escors (UCL) at an MOI 10. 5µg/mL protamine sulphate (Sigma) was used in transduction mixtures to neutralise the negative charge of the plasma membrane and enhance transduction efficiencies. 6h post-transduction, complete IMDM media containing GM-CSF was added to a total volume of 1mL. Cells were grown for an additional 4 days. Media was replaced when necessary by removing the top 0.5mL and replacing it with fresh complete media containing GM-CSF.

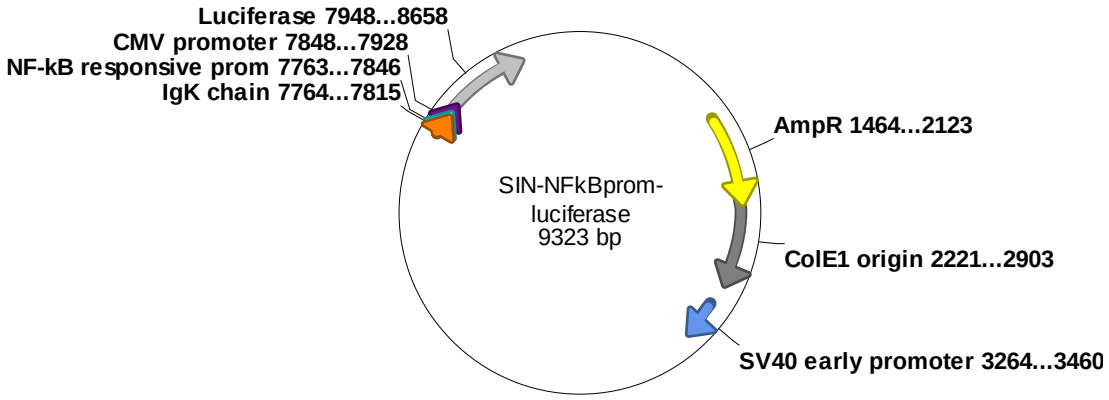


Figure 2-1: NF-κB inducible lenti-viral plasmid

An NF-κB responsive element drives expression of luciferase protein from a minimal cytomegalovirus (CMV) promoter.

2.8.2.4 NF-κB reporter Co-cultures

1 x 10^5 transduced BMDCs in 100µL RPMI containing 10% FCS were stimulated with *C. jejuni* (MOI 100 or 10) or purified *C. jejuni* LOS (100ng/mL) for 6h in sterile white 96-well plates.

Cells were equilibrated to RT before the addition of 100µL Bright-Glo™ (Promega) reagent. Cells were incubated at RT for 2mins to allow for complete lysis before luciferase activity was measured in a luminometer (FLUOStar Optima, BMG Labtech, Aylesbury, UK).

2.9 Lipooligosaccharide Structural Analysis

2.9.1 *Lipooligosaccharide (LOS) Isolation*

Bacterial pellets were resuspended in 5mL water in 50mL falcon tubes. Cultures were frozen at -80°C before being freeze dried. After complete freeze drying bacteria were resuspended in 30mL water and mixed 1:1 with 90% phenol (made fresh with water; Sigma). Bacterial/phenol mixtures were stirred at 68-70°C for 2h to ensure removal of the LOS from the outer membrane. Bacterial/phenol mixtures were dialysed with in 1KD cut-off Spectra/POR dialysis tubing (Spectrum Laboratories, California, US) for 3-4 days, water was replaced twice daily, at the end of the dialysis, the lack of phenol smell was indicative of its removal. After dialysis supernatants were spun at 5000rpm for 20mins to remove cell precipitate. The clear supernatant was frozen at -80°C in 50mL falcons and freeze-dried. The freeze dried bacterial constituents' were resuspended in 30mL 1mM TRIS-EDTA containing 60µg/mL DNAse (Sigma) and 30µg/mL RNAse (Sigma) and incubated at 37°C with shaking (100rpm) for 4h. 30µg/mL proteinase K (Sigma) was subsequently added and the mixture incubated overnight at 37°C with gentle shaking prior to O/N dialysis in 1KD cut-off tubing. The dialysed solution was spun at 5000rpm for 20mins to remove any residual precipitate. Supernatants were frozen at -80°C in 50mL falcons and freeze-dried. Freeze-dried material was resuspended in 10mL milliQ water and spun at 35,000rpm for 4h at 4°C to pellet the LOS. Pelleted LOS was resuspended in 5mL water and freeze-dried overnight. Purified LOS was quantified using a micro-balance and stored either in the lyophilised form at -20°C or re-suspended in milliQ water at 1mg/mL at -80°C.

2.9.2 *Silver Stain*

1µg/mL of purified LOS was run on pre-made 7-15% TRIS-glycine gradient gels (BioRad). Gels were fixed O/N at 4°C in methanol: acetic Acid: water mixture at a 2:1:11 ratio. Gels were washed twice in water for 5mins with shaking and then incubated for 10mins with 1.4% periodic acid. Gels were washed four times in water for 10mins. Silver stain solution was prepared fresh: 5mL 20% silver nitrate (freshly made in water) was added dropwise to 2.8mL 1M NaOH, 2.5mL concentrated NH_4OH with swirling; final volume was made to 150mL with water. Gels were stained for 10mins with silver stain solution and then washed three times in water for 20mins. Gels were developed in development solution: 10mg citric

acid, 100µL concentrated formaldehyde made to 200mL in water. Gels were developed to the desired strength of staining; development was stopped by incubating gels in 5% acetic acid for 30mins. Gels were washed twice in water for 20mins and photographed.

2.9.3 *O-deacylation of LOS*

Samples were O-deacylated prior to Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry (MS) analysis to aid acquisition of highly resolved peaks. ~300µg of LOS from each strain was placed in a 1.5mL eppendorf and 200µL of anhydrous hydrazine (Sigma) was added. The samples were incubated at 37^{o}C in a water bath for 2h with intermittent vortexing. The solution was cooled to RT and the reaction stopped by the addition of 1mL of pre-cooled acetone (-20^{o}C). The precipitated O-deacylated samples were pelleted by centrifugation at 12,000g for 20mins. Supernatants were removed and the pellet washed in 1mL chilled acetone before re-pelleting at 12,000g for 20mins. Supernatants were removed and the pellet resuspended in 500µL of MilliQ water. Samples were frozen at -80^{o}C before being lyophilised.

2.9.4 *Mass Spectrometry*

MS was performed in the linear mode on a Voyager-DE STR model TOF instrument equipped with a 337-nm nitrogen laser and delayed extraction by Dr. Constance John, UCSF, USA. Spectra were obtained in the negative-ion mode with an average of 500 pulses per spectrum. The acceleration voltage was −20 kV. External calibration of the mass spectrometer was performed using the average masses of the molecular ions of the peptides porcine renin substrate, bovine insulin, and oxidized insulin chain B (Sigma). During each acquisition period internal calibration was performed using LOS isolated from *Neisseria menigitidis* of known mass. Spectra were analysed using Data Explorer Software (Applied Biosystems). All spectra were smoothed at 39 points Gaussian Smooth before analysis.

2.9.4.1 Intact LOS Sample Preparation

Intact samples were resuspended in methanol: water (1:1) containing 5mM EDTA at 1-2µg/mL concentration. Samples were de-salted to remove sodium ions using Dowex 50WX8-200 cation-exchange beads (Sigma) on a piece of parafilm. The sample was mixed 1:1 with 20mM dibasic ammonium citrate (Sigma) on a piece of parafilm and 0.5µL was placed onto the MS sample plate on top of a thin layer of matrix using a glass capillary pipette. The make the MS matrix, nitrocellulose transblot membrane (Bio-Rad) was dissolved in acetone:isopropanol (1:1) to a concentration of 15mg/mL; to this 2,4,6-

trihydroxyacetophenone (THAP; Sigma) dissolved in methanol to 200mg/mL was added in a 4:1 ratio. Small drops of this matrix (0.3-0.9µL) was deposited onto the MS sample plate and allowed to air dry before the intact sample was placed on top.

2.9.4.2 O-deacylated Sample Preparation

O-deacylated samples after desalting as described above then were mixed 1:1 in 2,5-dihydroxybenzoic acid (DHB) matrix. The matrix was prepared by dissolving 10 mg DHB in acetone. Samples were spotted onto the MS plate using a glass capillary pipette and allow to air dry.

2.10 Statistics

Data for experiments of >5 repeats which showed normal distribution (parametric data) when plotted on a box and whisker plot were subjected to analysis by paired t-test. Experiments of <5 repeats where normal distribution of the data points was unknown Wilcoxon matched pairs test was used to analyse the data. For comparison of more than two groups in the same experiments Repeated Measures ANOVA was used for parametric data using the Tukey post test to compare all columns of data. For non-parametric data when comparing more than two groups in the same experiment Friedman test analysis was performed with Dunns post test to compare all columns of data. All statistical analysis was performed using GraphPad 5 software.

Chapter 3.

The Role of *Campylobacter jejuni* Surface Determinants in Mediating Dendritic Cell Activation

3.1 Background

Upon recognition of microbial products APCs undergo a maturation process that allows them to trigger T cell immunity via the presentation of three activation signals: antigen presentation on MHC class II; the upregulation of co-stimulatory receptors; and the secretion of cytokines. The collective impact of all these three signals plus local environmental factors together shape the type and extent of T-cell response(s) induced. Upon encounter, multiple microbe-host (MAMP/PRR) interactions come into play leading to APC activation. Evidence suggests that TLR engagement promote specific cellular functions; *e.g.* the engagement of TLR2 from various pathogens (*Yersinia pestis* and *Candida albicans)* preferentially induces IL-10 (Netea et al. 2004; Sing et al. 2002). However, other PRRs are also capable of inducing IL-10, often working synergistically to amplify responses (Gringhuis et al. 2007). It is a challenge to decipher whether the tailoring of specific innate responses are beneficial to the host and/or pathogen. For instance, the induction of IL-10 may be beneficial to the pathogen as it may aid colonisation by immune suppression as demonstrated with *H. pylori* infection (Kao et al. 2010; Panthel et al. 2003).

PRRs bind to specific microbial components that are generally surface exposed. The availability of *C. jejuni* isogenic mutants has greatly aided in improving our understanding of host/pathogen interactions involved in both the *in vitro* and *in vivo* settings. Several surface structure mutations of *C. jejuni* have been characterised, interestingly many of these mutations have little impact on bacterial growth rate and viability (Dr. Nick Dorrell & Professor Brendan Wren, LSHTM, UK; personal communication). The capsule, N-linked glycosylation system, and the flagella have been implicated in the adherence and invasion of IECs (Bacon et al. 2001;Golden and Acheson 2002;Karlyshev et al. 2004). Additionally the N-linked glycosylation of *C. jejuni* proteins can modulate DC cytokine responses via interaction(s) with the PRR MGL (van Sorge et al. 2009). In this chapter we sought to identify the role of specific surface determinants, in particular the role of CPS, N-linked modifications and the O-linked flagella on DC activation. Murine bone-marrow derived dendritic cells (BMDCs) were used as an *in vitro* model for DC activation in response to *C. jejuni.*

C. jejuni isogenic mutants employed in this study are listed in Table 2-1. A *ΔkpsM* isogenic mutant strain of *C. jejuni* 11168 lacks the capsule as determined by electron microscopy of alcian blue stained whole bacteria (Karlyshev, McCrossan, & Wren 2001). A *ΔpglB* isogenic mutant of *C. jejuni* 11168 lacks the conserved N-linked glycosylation, thus denuding

multiple periplasmic proteins and outer-membrane proteins (Young et al. 2002). A *ΔflaA* mutant of *C. jejuni* strain 81116 is non-motile and contains only truncated flagella bearing no FlaA structural filaments, this mutant has not been characterised in strain 11168 (Wassenaar et al. 1994). Mutation of the flagellar transcription activator *rpoN* encoding the alternative sigma factor 54 results in aflagellated bacteria (Jagannathan, Constantinidou, & Penn 2001). Due to the elimination of transcription of multiple proteins involved in the flagellum hook structure the *ΔrpoN* is secretion negative in contrast to the *ΔflaA* mutant which is secretion positive (Konkel et al. 2004).

C. jejuni strains potentially contain two separate biosynthesis pathways for the production of derivatives of pseudaminic acid (Pse) and legionaminic (Leg) acid residues which are subsequently attached to flagellin proteins either on serine or threonine residues (O-linked glycosylation). The Ptm and Pse pathways encode proteins necessary for the generation of Leg and Pse derivatives respectively. Although the pathways are distinct in their apparent specificities i.e. strains that encode the Pse pathway only contain Pse modifications, mutations in one pathway however alters the production of the alternative flagellin modification. For example, mutation of the *Cj1324* gene (involved in the synthesis of Leg modifications) leads to a lack of both Pse5Ac7Am as well as Leg5Am7Ac structures (and other legionaminic acid derivatives) (Howard et al. 2009). Mutations in genes involved in the Pse biosynthesis pathway lead to truncation of the flagella structure. This is due to the requirement of some but not all of the Pse-Ser/Thr modifications in filament polymerisation (Ewing, Andreishcheva, & Guerry 2009). Although complete ablation of the Pse pathway results in a non-motile phenotype; mutation of *pseA* (*Cj1316*) results in flagellin monomers (strain 81-176) with only pse5Ac7Ac present and not pse5Ac7Am (or further derivatives) (Guerry et al. 2006).

3.2 The role of *C. jejuni* capsular polysaccharide, N-linked glycosylation system, and flagella in mediating BMDC responses

Bacteria are readily phagocytosed by professional APCs such as DCs. Successful pathogens have evolved multiple mechanism(s) to evade host immunity including manipulating the process of phagocytosis. In a series of experiments the ability of BMDCs to phagocytose and subsequently kill WT *C. jejuni* was assessed by the standard gentamicin protection assay (Figure 3-1a and b respectively). *C. jejuni* were phagocytosed by BMDCs within 2h of co-culture (Figure 3-1a). The number of intracellular bacteria increased in a progressive

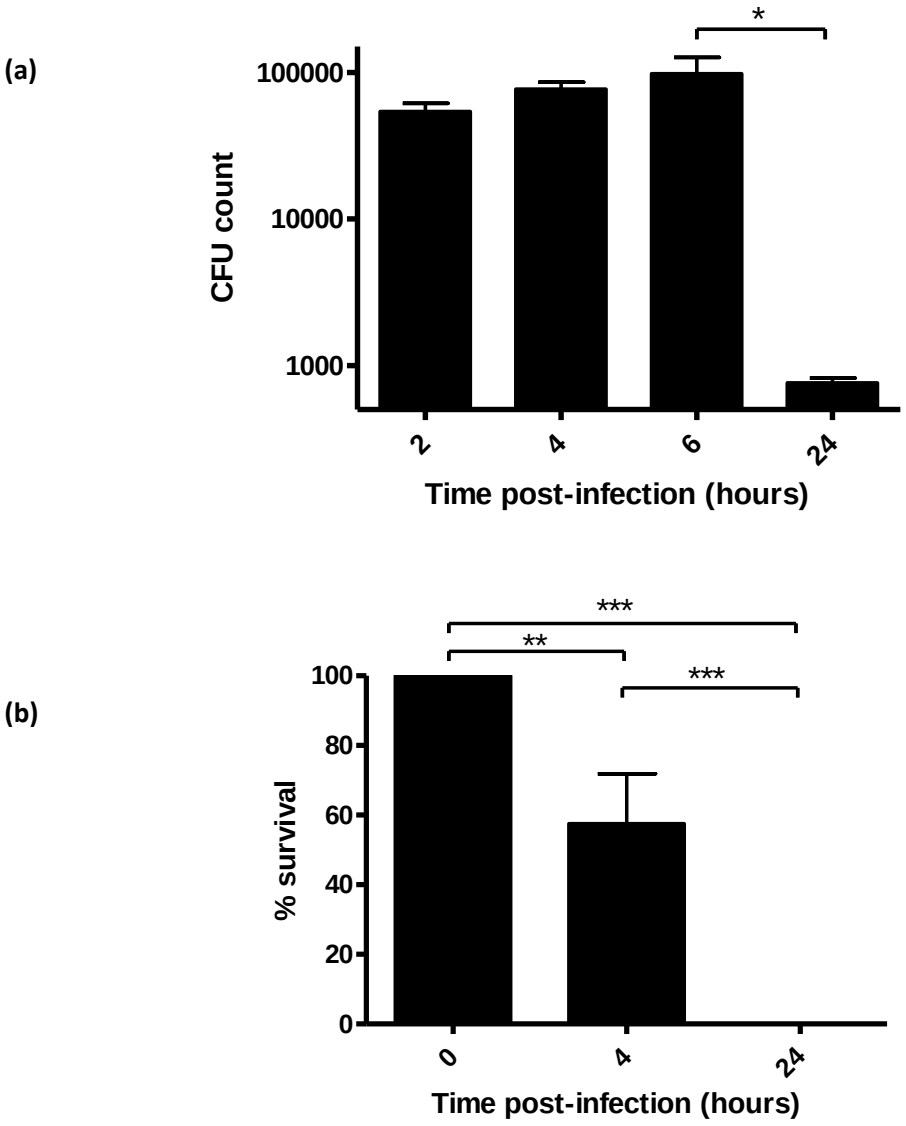


Figure 3-1: *C. jejuni* are phagocytosed and killed by murine bone-marrow derived dendritic cells

(a) 2 x 10^5 BMDCs were infected with WT *C. jejuni* strain 11168H at an MOI 100 for the indicated times, treated for 2h with 150µg/mL gentamicin to kill extracellular bacteria then subsequently lysed in 0.1% triton-X. CFU counts were performed to enumerate intracellular bacteria numbers. (b) 2 x 10^5 BMDCs were infected for 2h with *C. jejuni* MOI 100, treated for 2h with 150µg/mL gentamicin then subsequently lysed (0h time-point) or incubated in media containing a lower concentration of gentamicin for the indicated times before lysis. Rate of survival was calculated and are given as a percentage of the bacterial numbers enumerated at the 0h time point. Values are expressed as mean ± standard error of the mean (SEM) from three independent experiments performed in duplicate. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

manner up to 6h post-infection, where a ~ ratio of 1 intracellular bacterium/DC was observed. The number of viable intracellular bacteria decreased from ~ 100,000 CFU at 6h to 1,000 CFU at 24h (Figure 3-1a). In contrast to the reduction in internal bacteria at 24h post-infection the CFU count in the supernatant increased by ~10-fold (data not shown). This data showed that BMDCs phagocytosed *C. jejuni* in a time-dependent manner, however this phagocytic capability decreased over extended time periods ($p<0.05$).

The ability of BMDCs to kill intracellular *C. jejuni* was assessed (Figure 3-1b). The number of intracellular bacteria enumerated after 2h co-culture and subsequent killing of extracellular bacteria (Figure 3-1a 1st bar) was considered 100% (Figure 3-1b 1st bar). After a subsequent 4h incubation a significant reduction (45%; $p = 0.03$) in viable intracellular bacterial numbers was observed. After 24h incubation no viable bacteria were recovered. Taken together, BMDCs phagocytosed and exerted bactericidal activity against *C. jejuni*. This signified *C. jejuni* was unable to replicate within this cell-type.

The 'glycosylation' moieties of microbe surface structures can engage and modulate the phagocytic process. We hypothesised that glycosylated surface determinants of *C. jejuni* may alter BMDC function by interfering with phagocytosis. To analyse whether three known glycosylated components of *C. jejuni* play a role in BMDC mediated phagocytosis, co-cultures with isogenic mutant strains lacking specific constituents were set-up for 4h and viable intracellular bacteria enumerated (Figure 3-2). No significant difference in phagocytosis of the capsule mutant, *ΔkpsM* (Figure 3-2a), N-linked glycosylation mutant, *ΔpglB* (Figure 3-2b), or the flagellin mutant, *ΔflaA* (Figure 3-2c) was observed. These experiments clearly showed that the glycosylation system alone or its modifications on the capsule and flagella did not affect bacterial uptake.

Upon activation by MAMPs DCs upregulate the expression of co-stimulatory molecules (CD86, CD40, CD80) and MHC class II in order to engage T-cells leading to their differentiation and activation. The contribution of *C. jejuni* glycosylation to modulate these maturation markers was assessed by flow cytometry 24h post-infection (Figure 3-3). Unstimulated BMDCs did not express CD40 (Figure 3-3a). Upon stimulation with WT or *ΔkpsM C. jejuni* a ~1.8 fold increase in CD40 MFI was observed (Figure 3-3b). In contrast, both the *ΔpglB* and *ΔflaA* mutants increased CD40 expression ~1.5 fold which was comparable to LPS-stimulation (positive control). The difference between the WT and *ΔflaA* strain was significant ($p<0.05$). Induction of CD80 was low, showing a ~1.3 fold increase for the WT strain and all the mutants tested. The WT strain induced MHC class II expression by

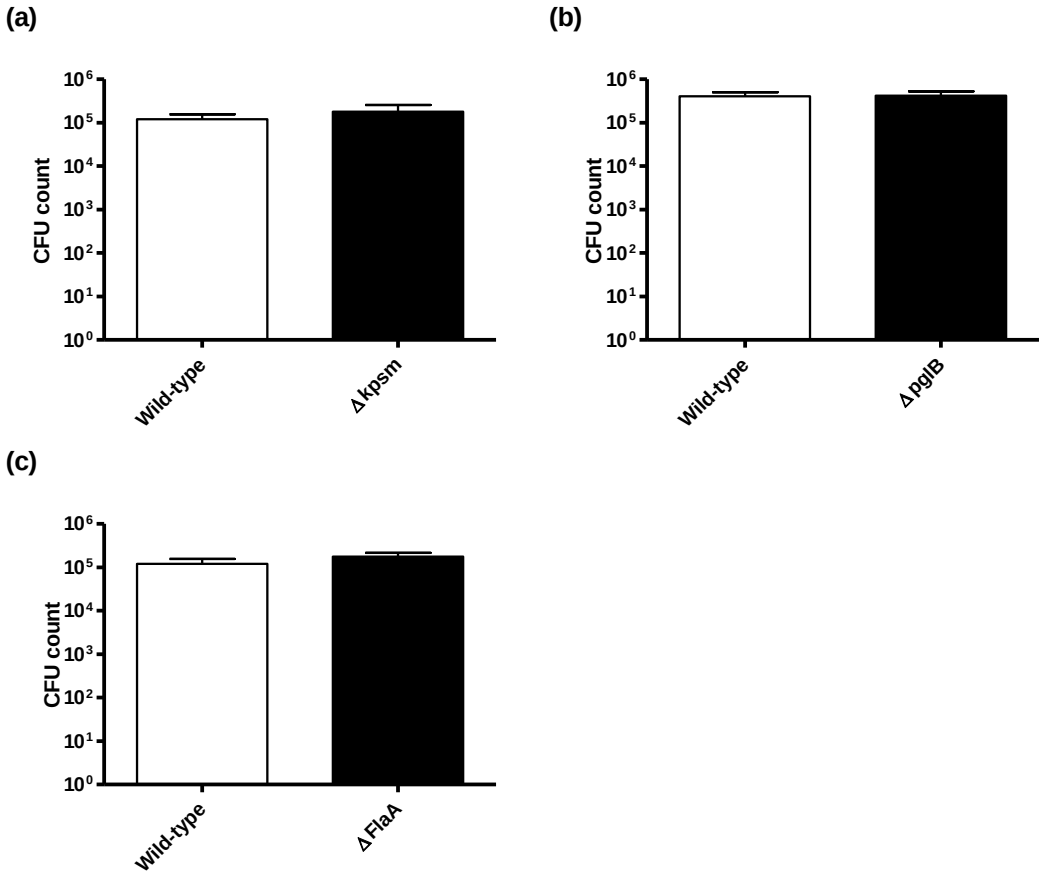


Figure 3-2: *C. jejuni* capsule, N-linked glycosylation system, and flagella do not alter phagocytosis rates

2 x10^5 BMDCs were co-cultured for 4h with WT *C. jejuni* strain 11168H and various isogenic mutants at an MOI 100 and intracellular bacteria were enumerated by gentamicin protection assay 4h post-infection (a) WT and *ΔkpsM* strains (b) WT and *ΔpglB* strains (c) WT and *ΔflaA* strains. Values are expressed as means ±SEM from three independent experiments performed in duplicate. Paired t-test statistical analysis performed. No stars = not significant.

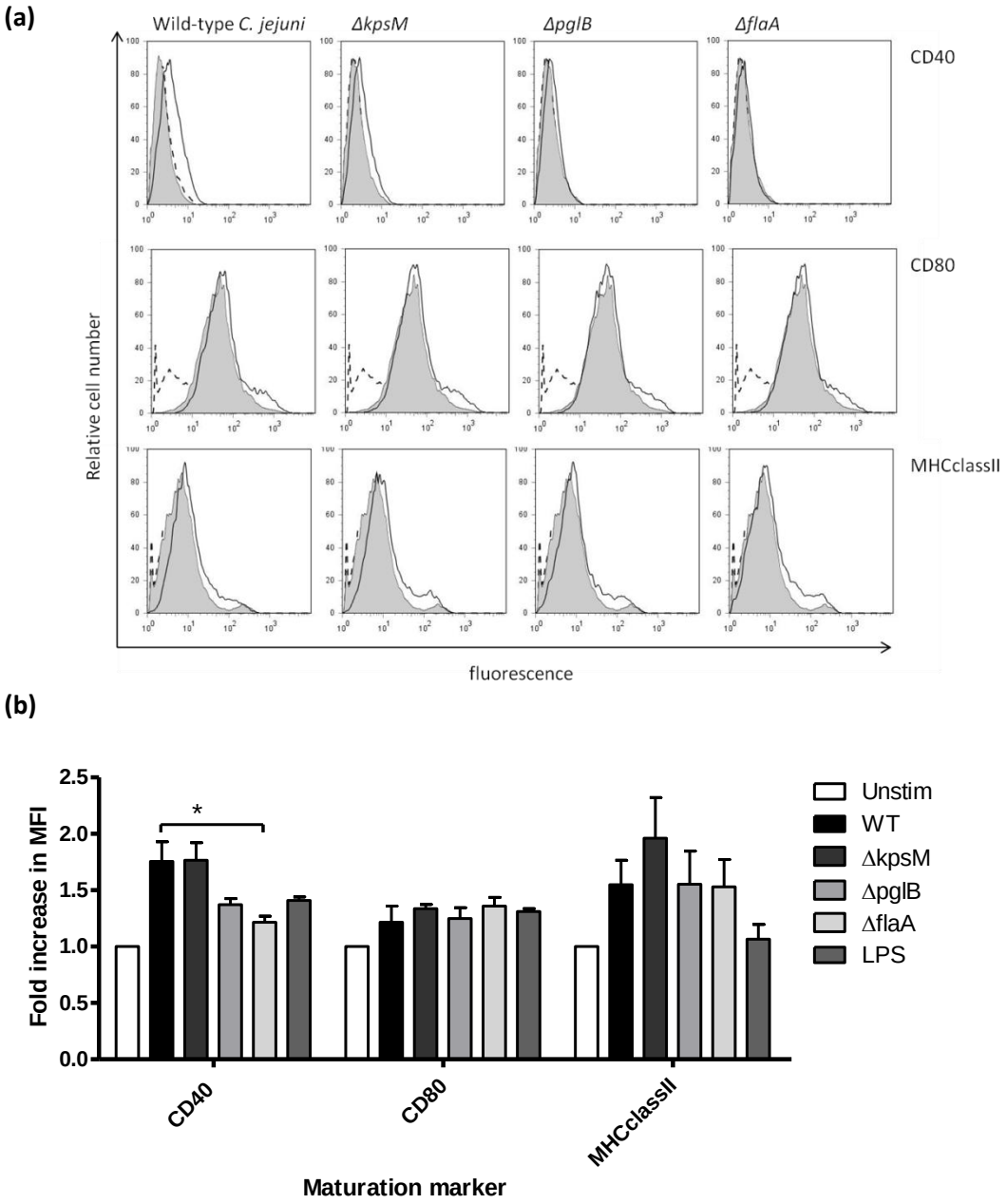


Figure 3-3: Stimulation of BMDCs with *ΔflaA C. jejuni* strain alters CD40 expression

1 x 10^6 BMDCs were co-cultured for 24h with WT *C. jejuni* strain 11168H and various isogenic mutants at an MOI 100. The expression of maturation markers CD40, CD80, and MHC class II was analysed by flow cytometry. (a) Representative histograms for the maturation markers are shown. Unstimulated isotype control (dashed line); unstimulated stained control (solid grey); *C. jejuni* stimulated (black line) (b) Average data for fold induction of geometric mean fluorescence intensity (MFI) over basal expression on unstimulated cells. Data from at least 3 independent experiments. Data are means ± SEM. One-way ANOVA statistical analysis performed with Tukey post-test. * <0.05; no stars = not significant.

~1.5 fold over the unstimulated cells. The *ΔkpsM* mutant induced higher levels of MHC class II, at ~2 fold; however this difference was not statistically significant. The *ΔpglB* and *ΔflaA* mutants showed comparable levels to that noted with the WT strain. This data showed that BMDCs upregulate multiple co-stimulatory markers and MHC class II in response to *C. jejuni*. The flagella directly impacted on the upregulation of CD40 whilst having little impact on other maturation markers.

In addition to the upregulation of maturation markers, DCs secrete an array of both pro- and anti-inflammatory cytokines in response to stimuli. BMDCs were co-cultured with WT and isogenic mutants of *C. jejuni* and the level of cytokines secreted was assessed. WT *C. jejuni* induced both pro- and anti-inflammatory cytokines as measured 24h post-infection (Figure 3-4; Figure 3-5; Figure 3-6). IL-6 was markedly induced by *C. jejuni* at ~35000pg/mL (Figure 3-4b). High levels of TNF-α were also detected at ~5000pg/mL (Figure 3-4a). Interestingly, IL-1β levels (<500pg/mL) in comparison were low (Figure 3-4c). IL-12 family members, IL-12, IL-23, and IL-27 were modest at ~ 100pg/mL, 20pg/mL, and 100pg/mL respectively in response to infection (Figure 3-4d). Importantly, high levels of IL-10 at ~2500pg/mL were detected (Figure 3-4e). *E. coli* LPS, a potent inducer of TLR4, was used as both a positive control for individual experiments and also for comparison in the levels of cytokines induced by *C. jejuni*. Interestingly, levels of particular cytokines induced by *C. jejuni* differed from that of LPS alone. LPS induced higher levels of IL-12 family cytokines, and significantly higher levels of IL-6 ($p < 0.05$) (Figure 3-6). In contrast, WT *C. jejuni* induced significantly higher IL-1β ($p < 0.05$) and IL-10 ($p < 0.05$) than LPS alone. These observations revealed the cytokine milieu generated by BMDCs in response to *C. jejuni.* Additionally, the cytokine profile differed considerably when compared to LPS-mediated response(s).

To assess the contribution of particular surface components, isogenic mutants of *C. jejuni* were co-cultured with BMDCs and cytokines quantified. The capsule mutant, *ΔkpsM,* induced similar levels of TNF-α, IL-1β, and IL-10 to WT *C. jejuni* (Figure 3-4). There was however a trend towards higher levels of both IL-6 and all IL-12 family in response to the *Δkpsm* strain. *ΔpglB* showed little difference compared to the WT strain (Figure 3-5). IL-1β levels were reduced with the *ΔpglB* mutant, however this was not statistically significant. The *ΔflaA* strain induced similar levels of TNF-α and IL-6 compared to the WT strain (Figure 3-6). Similarly, comparable levels of IL-12 family were induced by the flagella mutant. Interestingly, there was a significant reduction in the induction of both IL-10 ($p < 0.01$) and

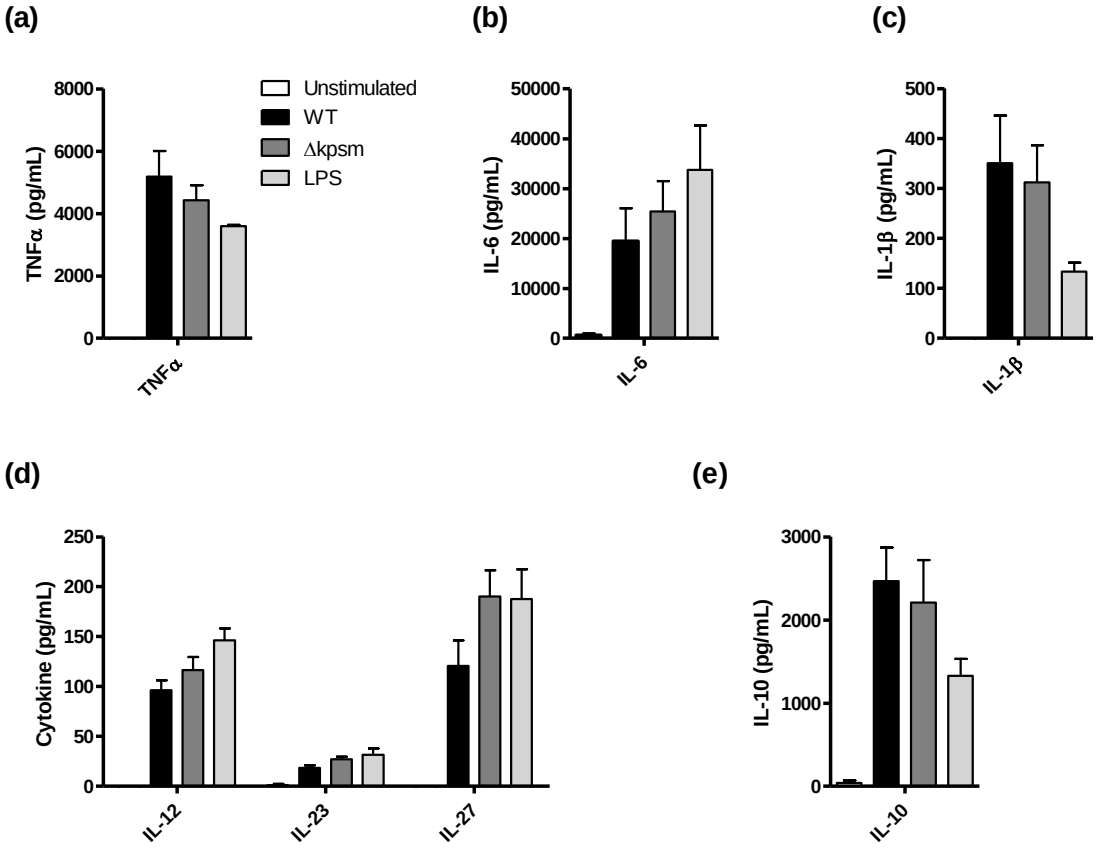


Figure 3-4: Cytokine release by BMDCs exposed to a *C. jejuni* capsule mutant

1 x10^6 BMDCs were co-cultured with WT *C. jejuni* and capsule mutant *ΔkpsM* at an MOI 100 for 24h. Cytokine analysis was subsequently performed by ELISA. (a) TNF-α and IL-6 (b) IL-1β (c) IL-12 family cytokines: IL-12, IL-23, and IL-27 (d) IL-10. Values are means ± SEM from a minimum of three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant

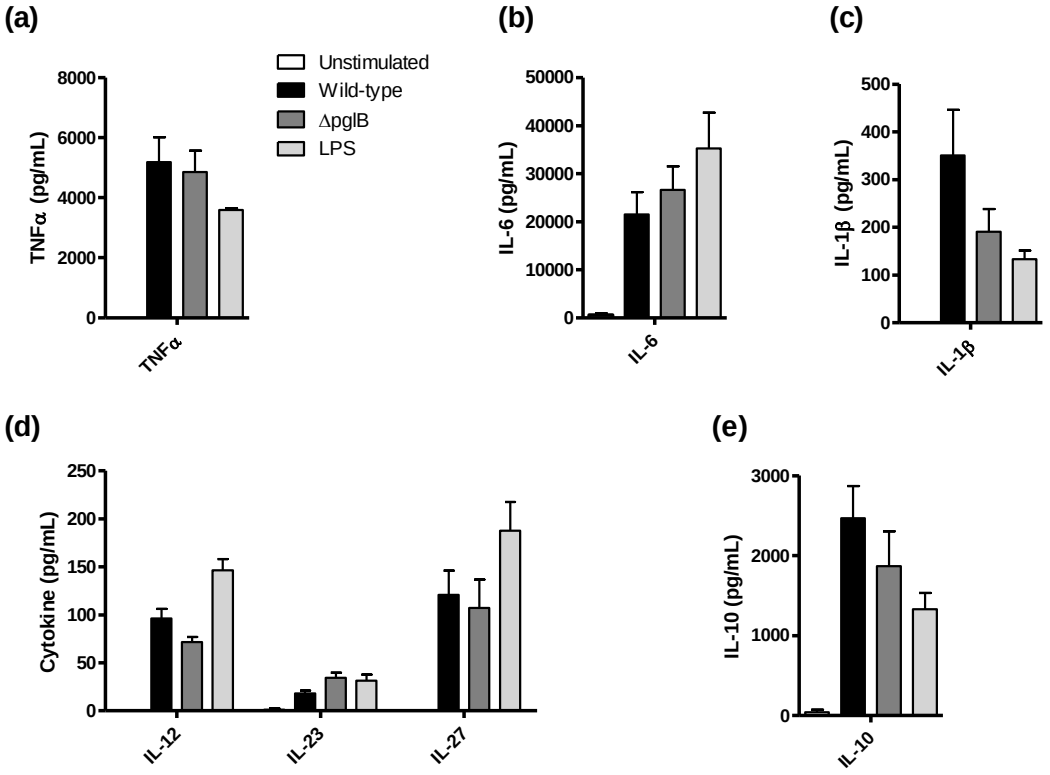


Figure 3-5: *C. jejuni* N-linked glycosylation system does not alter cytokine induction in BMDCs

1 x10^6 BMDCs were co-cultured with WT *C. jejuni* and N-linked glycosylation mutant *ΔpglB* at an MOI 100 for 24h. Cytokine analysis was subsequently performed by ELISA. (a) TNF-α and IL-6 (b) IL-1β (c) IL-12 family cytokines: IL-12, IL-23, and IL-27 (d) IL-10. Values are means ± SEM from a minimum of three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant

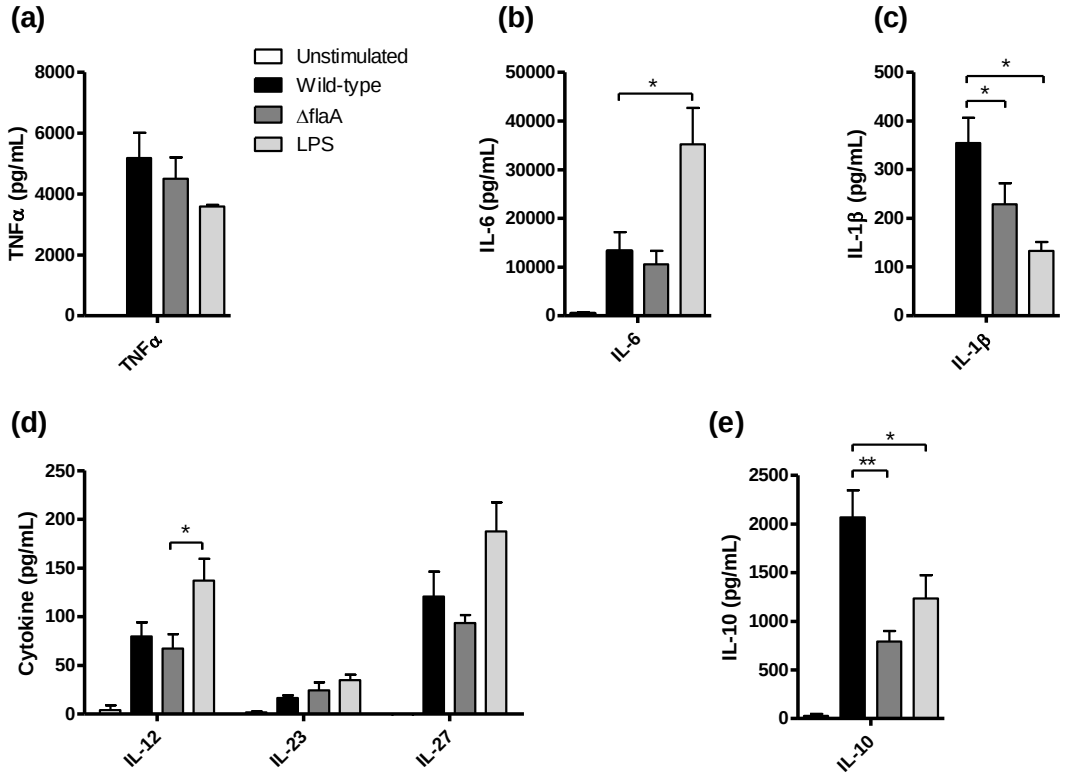


Figure 3-6: *C. jejuni* flagellum modulates BMDC IL-10 production

1 x10^6 BMDCs were co-cultured with WT *C. jejuni* and flagella mutant *ΔflaA* at an MOI 100 for 24h. Cytokine analysis was subsequently performed by ELISA. (a) TNF-α and IL-6 (b) IL-1β (c) IL-12 family cytokines: IL-12, IL-23, and IL-27 (d) IL-10. Values are means ± SEM from a minimum of three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

IL-1β ($p < 0.05$) by *ΔflaA* strain. The contribution of *C. jejuni* flagella in mediating high IL-10 was a novel finding and therefore deserved further attention.

3.3 *Campylobacter jejuni* flagella induces early transcription and secretion of BMDC IL-10

The above study identified the flagella moiety to be a major candidate involved in modulating *C. jejuni*-mediated BMDC responses. The absence of the flagella had an impact on CD40 expression as well as IL-1β and IL-10 induction. We next wished to assess if *C. jejuni* flagella mediated effects were operating on cytokine transcriptional and/or translational levels (Figure 3-7). Both WT and *ΔflaA* induced IL-6 gene expression as early as 1h post-infection, followed by a steady increase with maximal expression noted 4-6h post-infection (Figure 3-7a). Similarly, IL-1β mRNA was detected at early time-points with peak levels seen 2-4h post-infection (Figure 3-7b). p35 and p40 form the two subunits of IL-12 p70. p40 mRNA was induced at 1h post-infection and peaked at 6h, however p35 levels were only detectable at 2h post-infection peaking at 6h with ~25 times lower number of transcripts compared to p40 at 4h post-infection (Figure 3-7c & d). IL-10 mRNA was detectable at 1h post-infection and peaked at 6h. Interestingly, the transcripts for all the cytokines analysed declined after 4-6h post-infection suggesting early signalling events play an important role in *C. jejuni*-mediated cytokine responses. However, transcripts were still detectable 24h post-infection for all the cytokines analysed albeit at low levels.

The *ΔflaA* mutant induced lower levels of IL-6 and p40 than WT *C. jejuni* however this was not statistically significant (Figure 3-7a and c). The induction of IL-1β and p35 were similar between the mutant and WT throughout the duration of the time-course (Figure 3-7b and d). Interestingly, IL-10 transcription levels were markedly reduced in response to infection with the *ΔflaA* mutant (Figure 3-7e). The difference in IL-10 transcript levels between the WT and *ΔflaA* were noted as early as 1h post-infection, however significant difference was not achieved until 6 and 8h post-infection. Whereas IL-10 mRNA levels from BMDCs stimulated with WT strain peaked at 6h post-infection, levels induced by the *ΔflaA* plateaued between 2-6h at approximately half the level detected in response to WT infection. No differences were observed between the conditions at the 24h time point. This data indicated that the difference in IL-10 protein levels seen in response to the WT and *ΔflaA* may be due to effects on gene transcription. In contrast, differences in transcription did not account for the effect on IL-1β protein levels seen in response to the two infections.

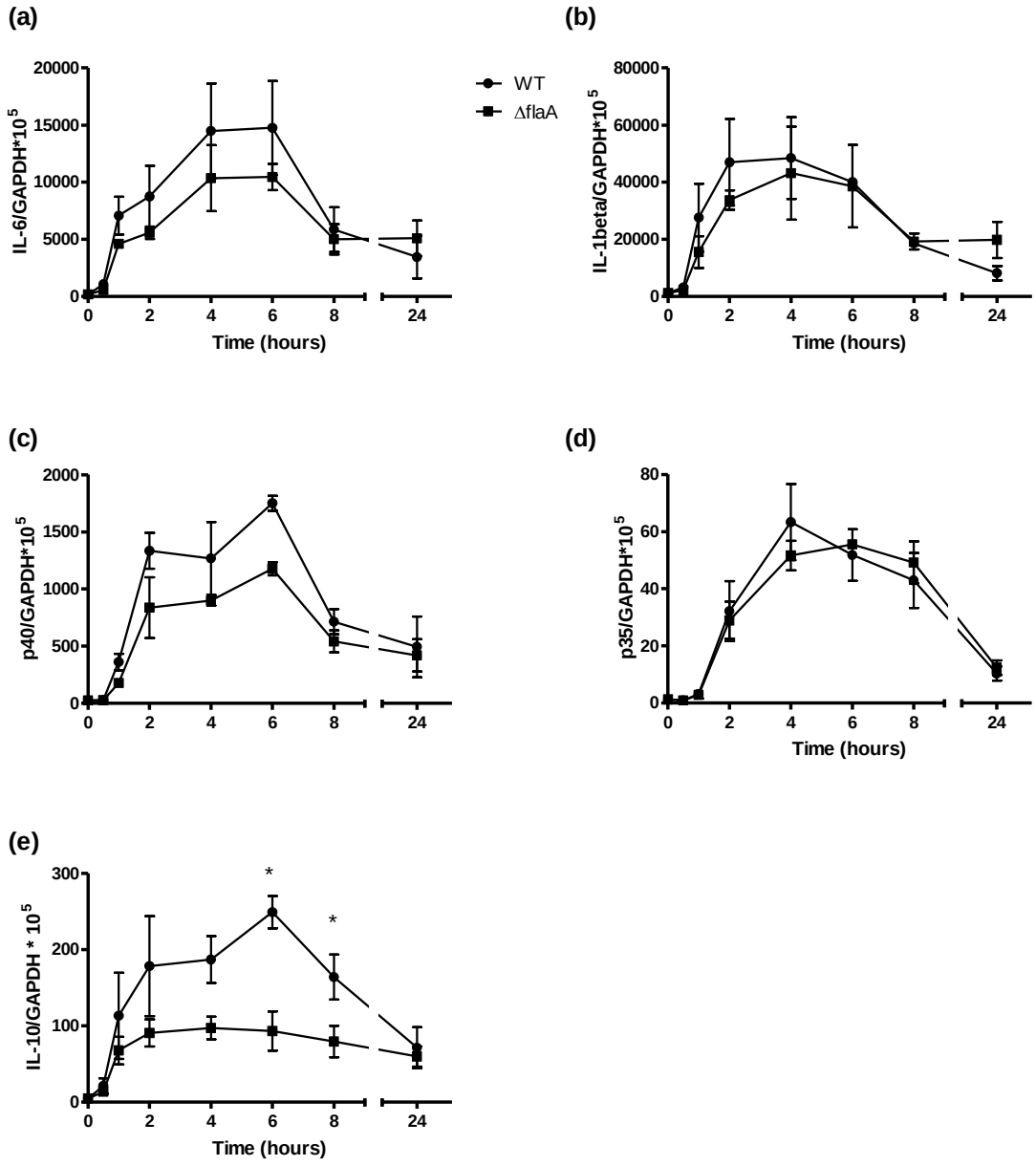


Figure 3-7: IL-10 transcription is reduced in response to infection with *ΔflaA C. jejuni*

1 x 10^6 BMDCs were co-cultured with WT and *ΔflaA C. jejuni* at an MOI 100 for the indicated time points. Quantitative real-time PCR analysis was performed and relative levels of transcripts calculated using the ΔΔct method, utilising the expression of a house-keeping gene, GAPDH, as a control. Values are expressed at means ± SEM from at least three independent experiments. Paired t-test statistical analysis performed. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

Time-dependent changes observed in IL-10 transcription were also followed at the protein level (Figure 3-8). The differential induction of IL-10 by the WT and *ΔflaA* strains was observed at 2h post-infection; however, the difference did not become statistically significant until 6h post-infection, a profile similar to its transcription regulation.

As modulation of IL-10 levels by the *ΔflaA* mutant were seen early in infection, we speculated that direct bacterial mediated signalling events were the likely drivers rather than subsequent secondary effects such as autocrine cytokine mediation affecting cytokine production. We next wished to identify which BMDC signalling events may be involved in regulating IL-10 in response to *C. jejuni*.

3.4 *C. jejuni* flagella modulates p38 but does not alter NF-κB activation

NF-κB signalling is a central regulator of inflammatory responses in APCs. To assess the activation of NF-κB, an NF-κB reporter construct driving the expression of luciferase was transduced at an MOI 10 into BMDCs on day 3 of differentiation. Luciferase activity induced by *C. jejuni* was analysed 6h post-infection. No luciferase activity was detected in untransduced cells either when unstimulated or stimulated with *C. jejuni* (Figure 3-9a). Unstimulated transduced cells showed minimal luciferase activity [less than 1000 arbitrary fluorescent units (AFU)]. A dose-dependent increase in luciferase activity was observed with WT *C. jejuni* at an MOI 10 and 100 confirming the ability of the assay to detect differential activation of NF-κB.

IkBα is an inhibitory molecule that sequestors NF-κB in the cytoplasm. Upon upstream signalling IkBα is phosphorylated and subsequently degraded, this process releases NF-κB to translocate to the nucleus. IkBα is degraded rapidly, within 30mins, in response to *C. jejuni* (Figure 3-9b). New protein synthesis allowed IkBα levels to return to basal levels by 2h post-infection. This highlighted that NF-κB signalling is rapidly activated in BMDCs in response to *C. jejuni* stimulation.

The activation of NF-κB in response to the *ΔflaA* strain was also assessed (Figure 3-9c). Similar levels of luciferase activity were observed between the WT and *ΔflaA* mutant suggesting the flagella may not be important for NF-κB activation.

MAPK signalling is often triggered in addition to NF-κB after the engagement of PRRs. To assess the potential activation of MAPK pathways by *C. jejuni,* time-course experiments were performed. All three MAPK pathways, p38, ERK, and JNK were activated (as measured

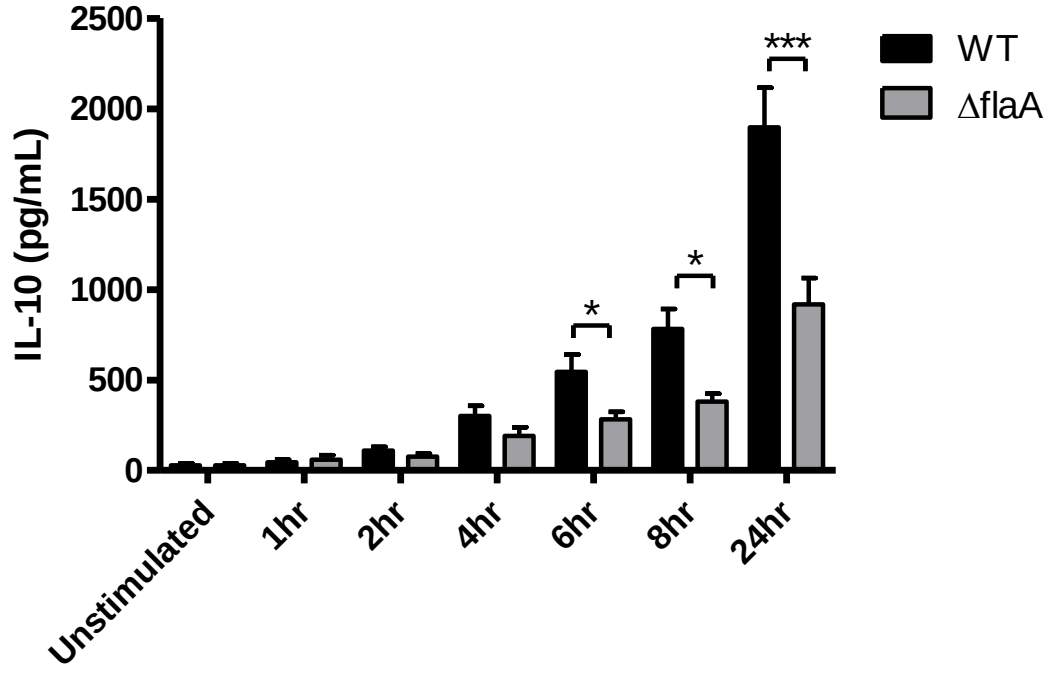


Figure 3-8 Differential IL-10 protein induction in response to infection with WT and *ΔflaA C. jejuni*

1 x10^6 BMDCs were co-cultured with WT *C. jejuni* and flagella mutant *ΔflaA* at an MOI 100 for the indicated time-points. IL-10 cytokine analysis was subsequently performed by ELISA. Values are means ± SEM from a minimum of three independent experiments. Paired t-test statistical analysis performed. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

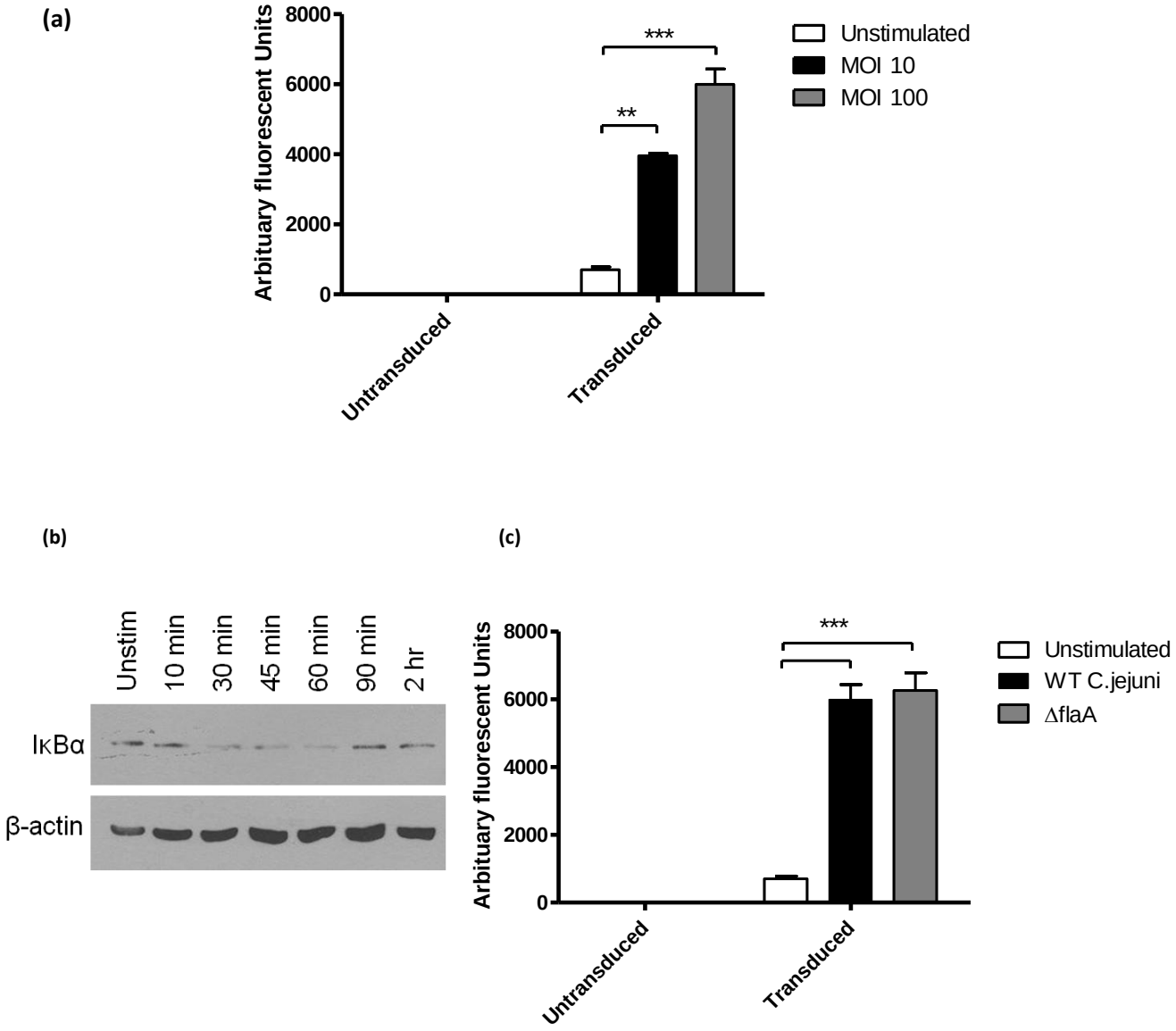


Figure 3-9: *C. jejuni* mediated BMDC NF-κB activation is not influenced by the flagella

(a) and (c) BMDCs were transduced with an NF-κB reporter lentivector on day 3 of differentiation at an MOI 10. On day 8, 1 x 10^5 untransduced or transduced BMDCs were infected with (a) WT *C. jejuni* at an MOI 10 or 100 or (c) WT or *ΔflaA C. jejuni* MOI 100 for 6h. Bright-glo luciferase reagent was subsequently added and luminescence measured. Values are means ± SEM from two independent experiments performed in duplicate. (b) 1 x10^6 BMDCs were infected with WT *C. jejuni* MOI 100 for the indicated time-points. Cell lysates were subjected to SDS-PAGE followed by Western blotting with an IκBα antibody. Membrane were stripped and re-probed for β-actin as a loading control. Western blots are representative of at least three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

by degree of phosphorylation) in response to WT *C. jejuni* (Figure 3-10a and b). p38 activation peaked within 30-60mins post-infection, and declined after 2h although phosphorylated p38 was detectable 6-8h post-infection. Phospho-ERK was seen within 30mins post-infection, levels peaking between 60mins-2h, again phospho-ERK was present 6-8h post-infection. Kinetics of JNK phosphorylation was slow compared to p38 and ERK, as phospho-JNK was detected only between 60-90mins post-infection. In addition, JNK activation diminished more rapidly, with minimal levels seen at 2h with none detectable at 4h.

p38 showed lower magnitude and slower kinetics of activation in response to the *ΔflaA* isogenic mutant when compared to the WT *C. jejuni* (Figure 3-10b). Strong activation was not observed with the mutant until 90mins post-infection compared to 60mins with the WT counterpart. ERK and JNK activation were similarly activated by the *ΔflaA* and WT strain. This suggested a possible role for p38 signalling in the differential cytokine response observed between the WT and the *ΔflaA* strain.

3.5 Inhibition of MAPK signalling pathways alters cytokine production with minimal effect on maturation markers

Once the activation of BMDC MAPK signalling pathways by *C. jejuni* was established, the potential role of MAPK in the induction of maturation was investigated. Small chemical inhibitors of MAPK have been used successfully in both *in vitro* and *in vivo* analysis of the role of MAPK in health and disease. U0126 is a potent MEK1/2 inhibitor, the upstream kinase of ERK1; PD90859 is a MEK1 inhibitor (Alessi et al. 1995;Favata et al. 1998). U0126 has a ~ IC_{50} value of 72nM for MEK1 and 58nM for MEK2 *in vitro*, whereas PD90859 has a ~ IC_{50} value of 2μM and is therefore used at higher concentrations compared to other MAPK inhibitors. SP600125 is a competitive inhibitor of ATP binding for JNK1, 2, 3 and has IC_{50} values of 40nM for JNK1 and 2 and 90nM for JNK3 (Bennett et al. 2001). SB203580 and SB239063 inhibit the ATP binding site of p38 α and β but not γ or δ isoforms, or ERK and JNK (Cuenda et al. 1995;Underwood et al. 2000). Both SB203580 and SB239063 have IC_{50} values of ~50nM. As all the inhibitors were solubilised in DMSO, 1% DMSO was added to control media. Cells were pre-treated with the inhibitors or DMSO control for 1h prior to stimuli.

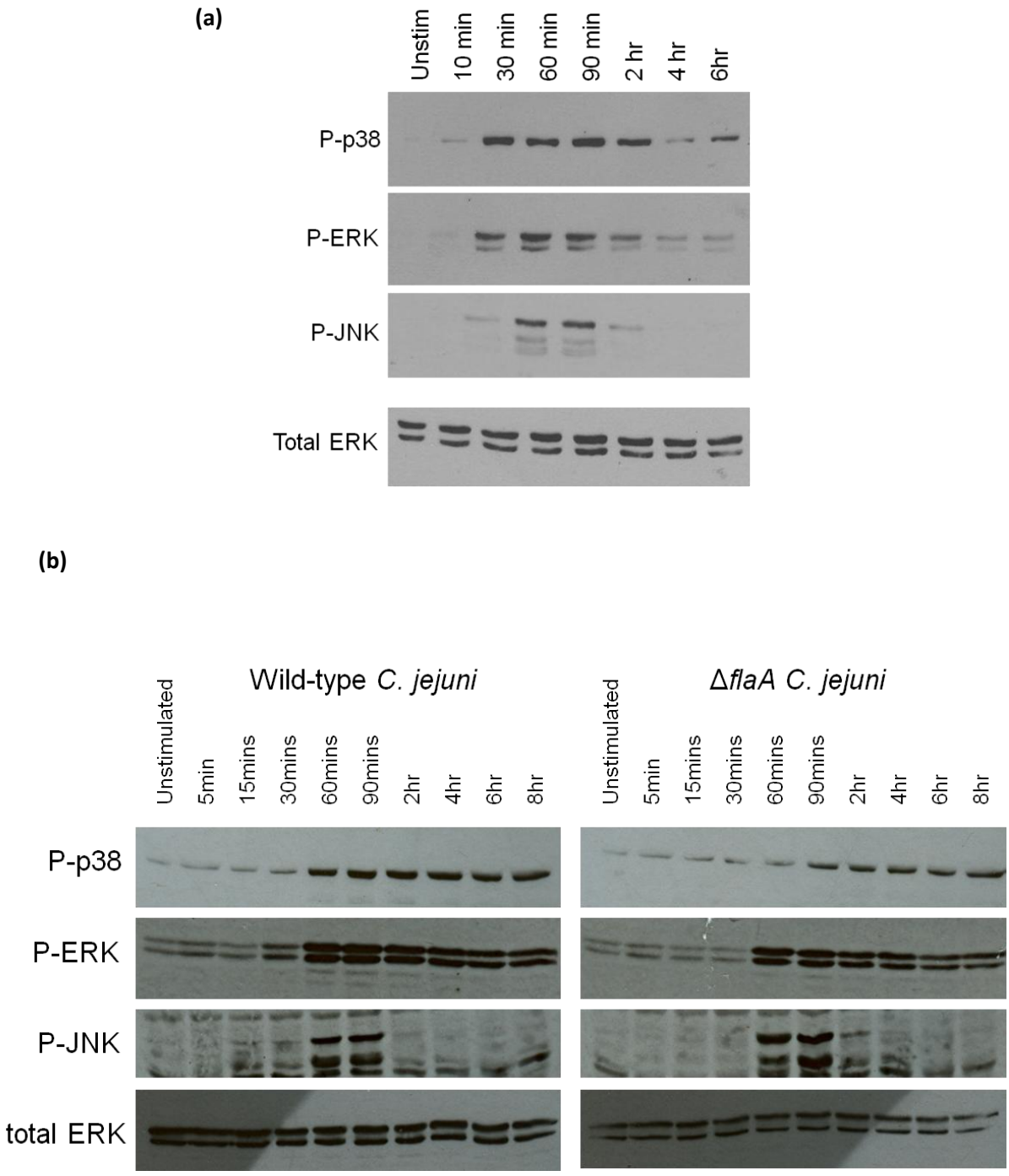


Figure 3-10: *C. jejuni ΔflaA* mutant strain mediated delayed p38 activation with minimal impact on ERK or JNK pathways

1 x 10^6 BMDCs were co-cultured with (a) WT (b) WT and *ΔflaA C. jejuni* MOI 100 for the indicated time-points. Lysates were subjected to SDS-PAGE followed by Western blot analysis for the phosphorylated forms of p38, ERK, and JNK. Blots were stripped and probed for total ERK as a loading control. Blots are representative of at least three independent experiments.

Prior to investigation, it was important to establish if the inhibitors had any effect on the rate of bacterial phagocytosis, as this cellular process is critical for multiple downstream events. In the presence of a standard concentration of 10µM for p38 and ERK inhibitors SB203580 and U0126 rates of bacterial phagocytosis were assessed as described above (Figure 3.1). The presence of the inhibitors had minimal effect on the rates of phagocytosis measured 4h post-infection (Figure 3-11). Next, effect of MAPK inhibition on *C. jejuni*-mediated BMDC maturation marker and cytokine responses was investigated. Two concentrations (1 and 10µM) of inhibitors were tested in this series of experiments to elucidate if a dose-dependent effect was noted. Overall inhibition of MAPKs had little effect on the expression of maturation markers (Figure 3-12). DMSO treated control cells stimulated with *C. jejuni* showed similar upregulation of CD40, CD80, CD86 and MHC class II as seen in the absence of DMSO (Figure 3-12 and Figure 3-3). Inhibition of ERK by U0126 and PD98059 increased expression of CD40 without the addition of *C. jejuni,* suggesting a possible regulatory role for ERK in the constitutive expression of CD40 (Figure 3-12a). No increase in CD40 expression was observed with the addition of *C. jejuni* under these conditions suggesting the CD40 expression had plateaued with the addition of the inhibitor alone. The presence of ERK inhibitors had minimal impact on either unstimulated or *C. jejuni*-mediated CD80, CD86, and MHC class II expression (Figure 3-12b, c, and d). Inhibition of JNK with SP600125 at either 1µM or 10µM had no effect on CD40, CD80, CD86, and MHC class II expression either when unstimulated or stimulated with *C. jejuni* (Figure 3-12). Inhibition of p38 similarly had no effect on the expression of CD40 (Figure 3-12a). Infection in the presence of SB203580 and SB239023 marginally increased CD80, CD86, and MHC class II although this increase was not statistically significant (Figure 3-12 b, c and d).

The importance of MAPK signalling in the induction of both pro- and anti-inflammatory cytokines has been well documented (Escors et al. 2008). Next, MAPK inhibitors were utilised to decipher the importance of MAPK signalling in *C. jejuni*-mediated cytokine induction (Figure 3-13). Inhibition of ERK affected TNF-α secretion at 10µM of both U1026 and PD90859 (Figure 3-13a). Reduction in IL-6 was also observed with ERK inhibitors but a dose-response was not seen and this effect was not statistically significant (Figure 3-13b). ERK inhibition had minimal effect on the IL-12 family members (Figure 3-13d, e, and f). However, both PD90859 and U0126 caused a significant reduction in IL-10 protein levels (Figure 3-13g). PD90859 only affected secretion at 10µM ($p<0.01$), whereas U0126 significantly reduced secretion at both 1µM and 10µM concentration ($p <0.05$, and $p <0.001$ respectively).

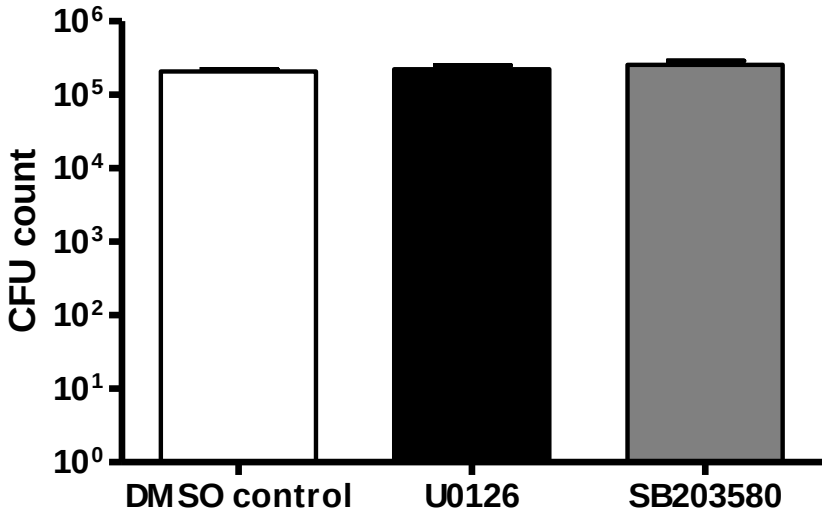


Figure 3-11: Inhibition of p38 and ERK MAPKs does not alter *C. jejuni* phagocytosis

2 x 10^5 BMDCs were pre-treated with 10µM MEK1/2 (U0126) or p38 (SB203580) inhibitors or 1% DMSO as a vehicle control for 1h prior to infection with WT *C. jejuni* MOI 100 for 4h. Intracellular bacterial numbers were assessed by gentamicin protection assay. Values are means ± SEM from two independent experiments performed with two biological replicates. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant

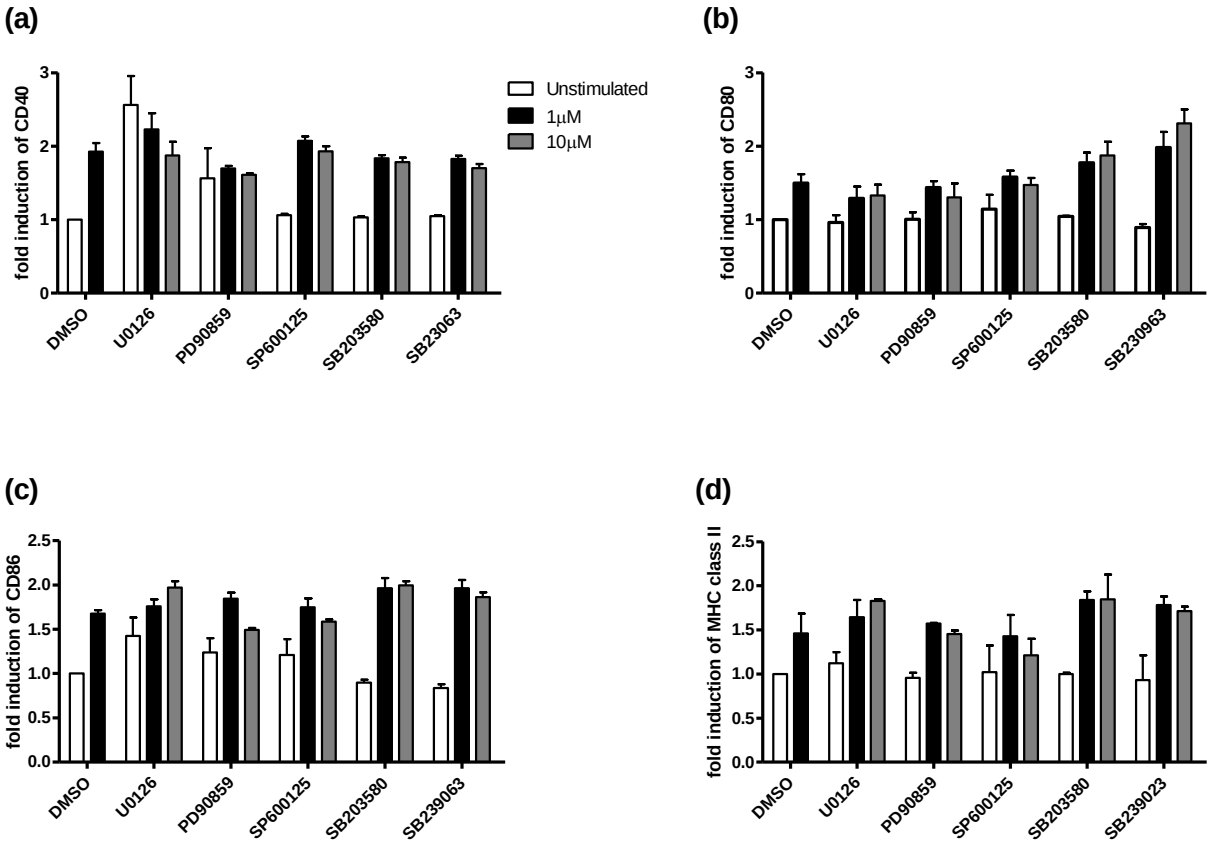


Figure 3-12: Inhibition of MAPKs does not alter *C. jejuni*-mediated maturation marker induction

1 x10^6 BMDCs were co-cultured with WT *C. jejuni* MOI 100 for 24h. The levels of (a) CD40 (b) CD80 (c) CD86 and (d) MHC class II were assessed by flow cytometry. Values are mean fold change in the geometric mean fluorescent intensity (MFI) over the expression in uninfected cells ± SEM from a three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant

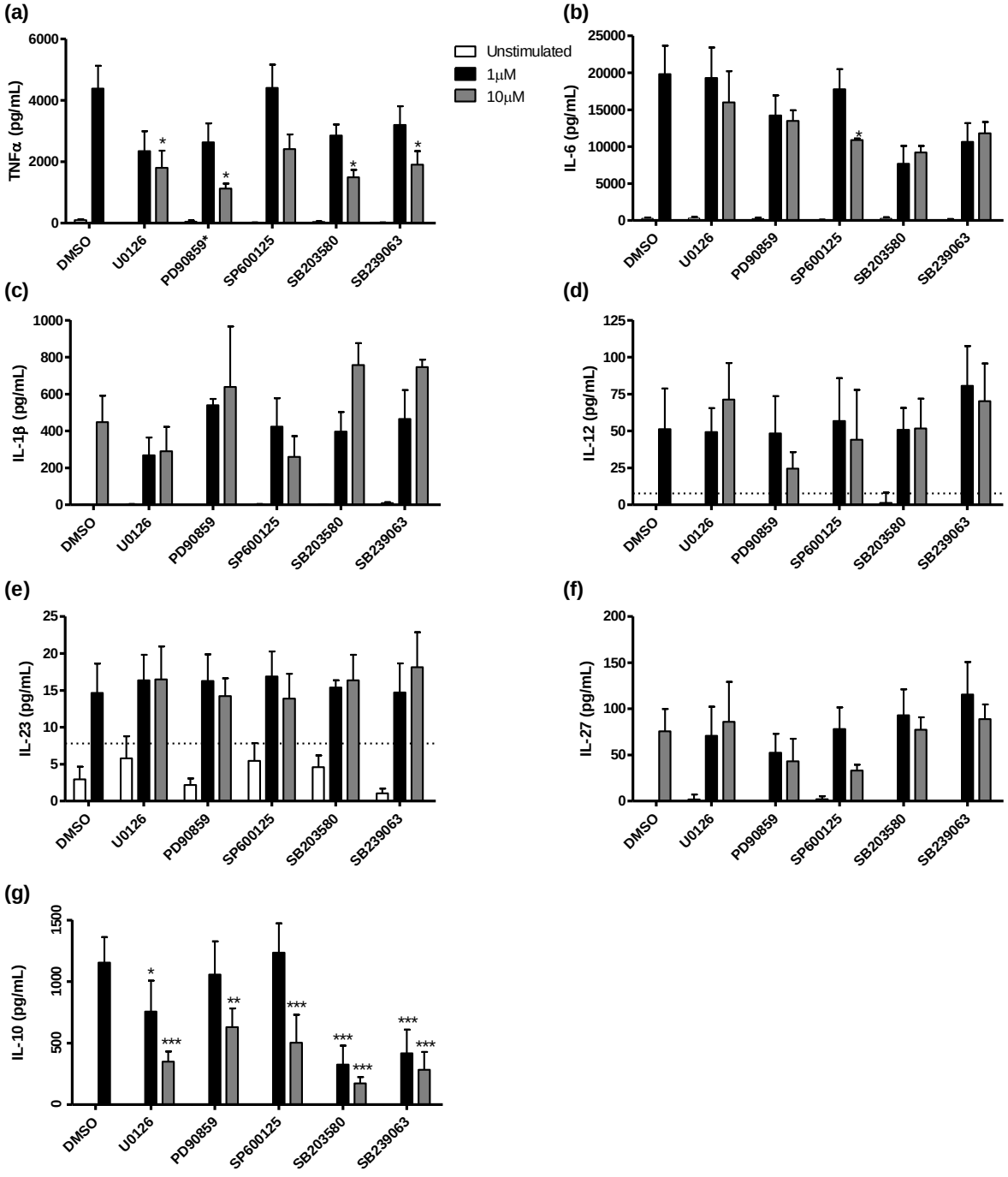


Figure 3-13: Inhibition of MAPKs resulted in reduced IL-10 production with minimal effect on IL-12 family members

1 x 10^6 BMDCs were pre-treated with inhibitors of MAPK pathways, MEK1/2 (U0126 & PD90859), JNK (SP600125), or p38 (SB203580 & SB239063) at a concentration of 1μM or 10μM for 1h prior to infection (PD90859 was used at 5μM and 50μM concentrations). BMDCs were co-cultured with WT *C. jejuni* MOI 100 for 24h in the presence of the inhibitors. Supernatants were assessed for the levels of (a) TNF-α (b) IL-6 (c) IL-1β (d) IL-12 (e) IL-23 (f) IL-27 and (g) IL-10 by ELISA. Values are means ± SEM from three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

Inhibition of JNK signalling had minor effects on the secretion of TNF-α, IL-6, and IL-1β at 10μM concentration, and this was not statistically significant (Figure 3-13 a, b, and c). Secretion of IL-12 family members was largely unaffected by the SP600125 inhibitor apart from IL-27 at 10μM (Figure 3-13d, e and f). IL-10 secretion was reduced at 10μM inhibitor concentration, although this was less than that observed in the presence of the p38 and ERK inhibitors (Figure 3-13g).

At higher inhibitor concentration (10μM) TNF-α and IL-6 production was reduced by ~50% in the presence of SB203580 and SB239023 ($p< 0.05$; Figure 3-13a and b). Interestingly, IL-1β secretion was enhanced by both p38 inhibitors (Figure 3-13c). Similar to the ERK and JNK, p38 inhibition had minimal effect on the IL-12 cytokine family, in contrast this pathway had the most pronounced effect on IL-10 protein levels (Figure 3-13g). A ~60% reduction in IL-10 secretion was observed at 1μM concentration of both inhibitors ($p <0.001$ for both). This reduction increased to 85% in the presence of 10μM SB203580 ($p <0.001$). p38 inhibition had a far greater impact on IL-10 secretion than the other MAPK inhibitors suggesting a greater role for this signalling pathway in IL-10 secretion, although inhibitors of all three MAPK pathways affected IL-10 secretion.

As (a) host MAPK signalling and the flagella of *C. jejuni* modulated IL-10 levels (Figure 3-6, Figure 3-13), and (b) the flagella appeared to be important for p38 signalling in particular (Figure 3-10), collectively the evidence supported the notion that in BMDC, *C. jejuni* flagella by mechanism(s) yet unknown impacts on p38 signalling leading to downstream modulation of IL-10 expression. We next tested this emerging hypothesis. The *ΔflaA* mutant was used to stimulate BMDCs in the presence of MAPK inhibitors (Figure 3-14). As observed previously, the *ΔflaA* mutant induced ~60% less IL-10 than the WT *C. jejuni* strain, and the p38 inhibitors had a potent effect at 1μM. In the presence of 1μM SP600125, no significant change was observed as a similar ~60% reduction was seen in response to infection with the *ΔflaA* mutant (Figure 3-14). This suggested that *C. jejuni* flagella accounts for 60% of the IL-10 induced by the whole bacterium, and this induction is JNK-independent. Interestingly, in the presence of 1μM SB203580 and SB239023 only a 36% and 43% reduction in IL-10 was seen with the *ΔflaA* respectively, and 25% less at the higher 10μM concentration of SB203580. In the presence of 1μM U0126 also only a 45% reduction of IL-10 was observed between the WT and the *ΔflaA* mutant. These results suggested that *C. jejuni* flagella induced IL-10 in BMDCs via a p38 and ERK-dependent mechanism.

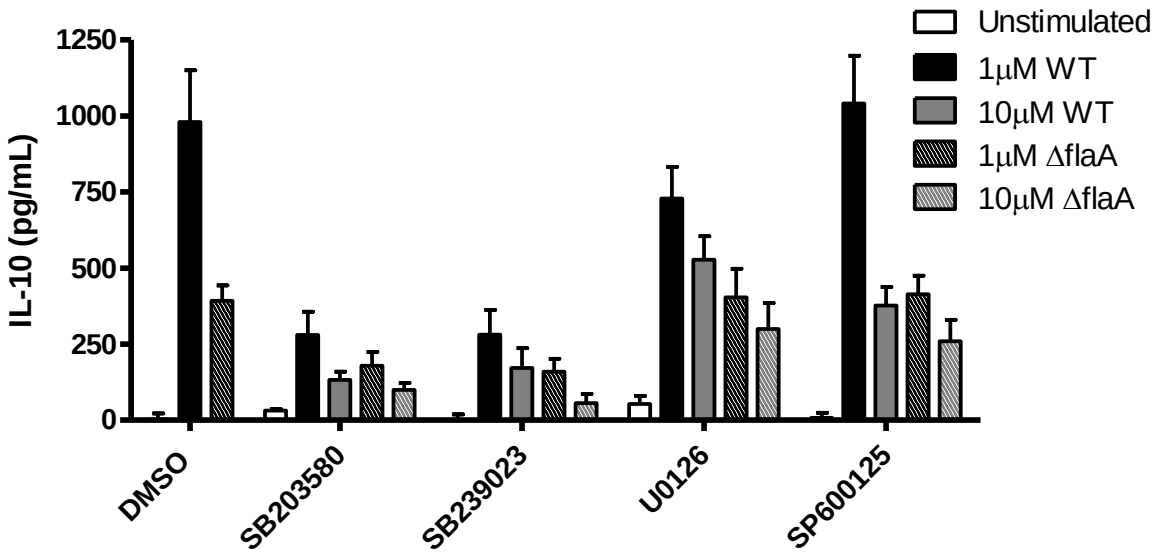


Figure 3-14: *C. jejuni* flagella modulates IL-10 production via p38 and ERK pathways

1 x 10^6 BMDCs were pre-treated with inhibitors of MAPK pathways, MEK1/2 (U0126 & PD90859), JNK (SP600125), or p38 (SB203580 & SB239063) at a concentration of 1μM or 10μM for 1h prior to infection (PD90859 was used at 5μM and 50μM concentrations). BMDCs were co-cultured with WT *C. jejuni* or *ΔflaA* mutant MOI 100 for 24h in the presence of the inhibitors and supernatants were assessed for IL-10 protein by ELISA. Values are means ± SEM from three independent experiments.

3.6 Critical role for the flagella 'glycosylated' moieties in BMDC IL-10 production

During host-microbial crosstalk, bacterial carbohydrate structures and secreted proteins can specifically enhance host anti-inflammatory cytokine response(s), as a means of host immune evasion. (Nagamatsu et al. 2009; van Vliet et al. 2009). The *ΔflaA* mutant utilised above did not allow delineation of the contribution of the flagellin protein *versus* the contribution of the carbohydrate moiety to IL-10 levels. To rectify this, a variety of specific flagella isogenic *C. jejuni* mutants were employed.

Utilising the *ΔrpoN* mutant (which lacks both the extracellular flagella structure and membrane hook complex and is therefore secretion negative), comparable reduction in IL-10 was observed to the *ΔflaA* mutant (Figure 3-15). This finding suggested that active secretion of *C. jejuni* via the flagella plays a minimal role in host IL-10 responses. Different strains of *C. jejuni* bear different sugar structures on their flagella (Champion et al. 2005). 11168H contains both Pse and Leg structures and their derivatives while strain 81-176 encodes only the Pse structures. To assess whether the flagella alters IL-10 secretion from a strain modified with only Pse structures, strain 81-176 was employed. The WT and *ΔflaA* mutant of 81-176 mediated IL-10 response(s) similar to that noted for the 11168H WT and its *ΔflaA* isogenic mutant (Figure 3-16). The *ΔflaA* mutant strains induced less IL-10 than their WT counterparts, although the reduction in IL-10 secretion for the 81-176 *ΔflaA* was marginally less than for the 11168H *ΔflaA*. These observations add weight to the notion that the flagella from multiple *C. jejuni* strains are involved in modulating host IL-10 responses.

To directly assess the role of flagellin glycosylation in IL-10 induction, a series of glycosylation mutants were utilised. Due to the importance of flagellin glycosylation in the assembly of the flagella complex the complete carbohydrate mutant is aflagellated and therefore could not be used in this assay (Goon et al. 2003). The *ΔCj1324* mutant lacks two derivatives of Leg, Leg5AmNMe7Ac and Leg5Am7Ac, and in addition lacks a form of Pse, Pse5Ac7Am. Interestingly the complement restores the expression of the Leg structures but not the Pse structure (Howard et al. 2009). The *ΔCj1316* or *ΔpseA* mutant has been described in strain 81-176 and lacks Pse5Ac7Am modification of the flaA protein (Guerry et al. 2006). The *ΔCj1324* mutant induced less IL-10 than the WT strain ($p < 0.05$), although it induced statistically more IL-10 than the *ΔflaA* mutant ($p < 0.05$; Figure 3-17a). Interestingly, the *ΔCj1324* complement did not restore IL-10 secretion to levels of the WT.

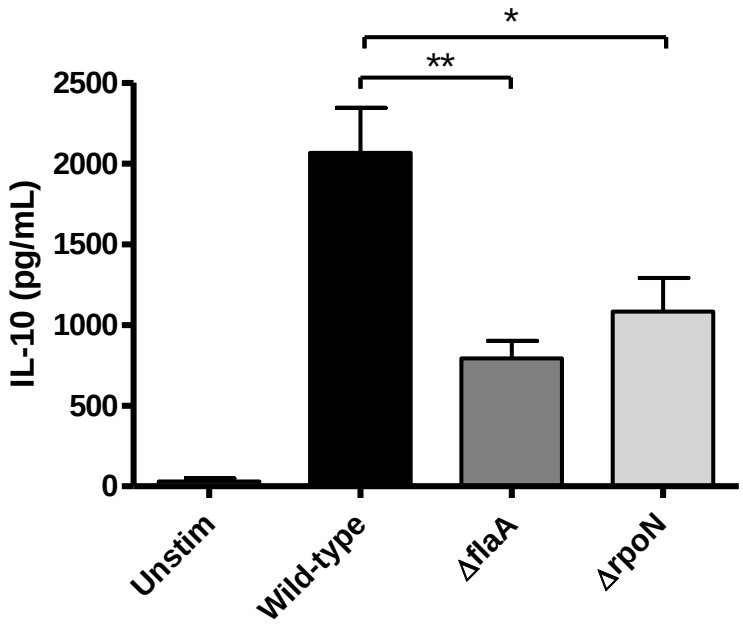


Figure 3-15: *C. jejuni* flagella secreted proteins play a minimal role in IL-10 production

1 x10^6 BMDCs were co-cultured with WT *C. jejuni*, *ΔflaA* (secretion positive) *or ΔrpoN* (secretion negative) isogenic mutants at an MOI 100 for 24h. IL-10 protein levels were analysed by ELISA. Values are means ± SEM from a minimum of three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

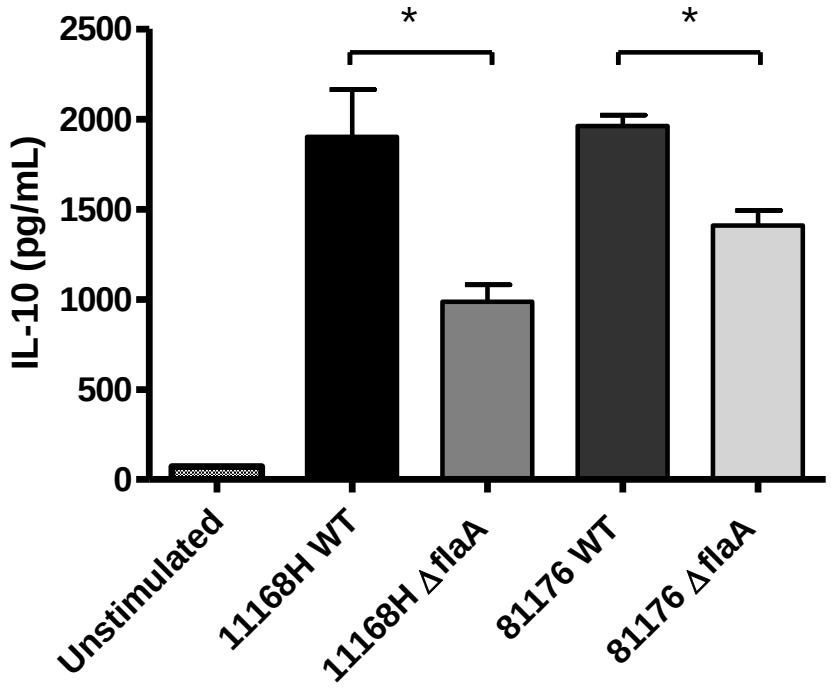


Figure 3-16: The flagella of *C. jejuni* 11168H & 81-176 influences IL-10 production

1 x 10^6 BMDCs were co-cultured with WT 11168H or 81-176 *C. jejuni* or with the corresponding flagella isogenic mutant *ΔflaA* strains at an MOI 100 for 24h. IL-10 protein was analysed by ELISA. Values are means ± SEM from a minimum of three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

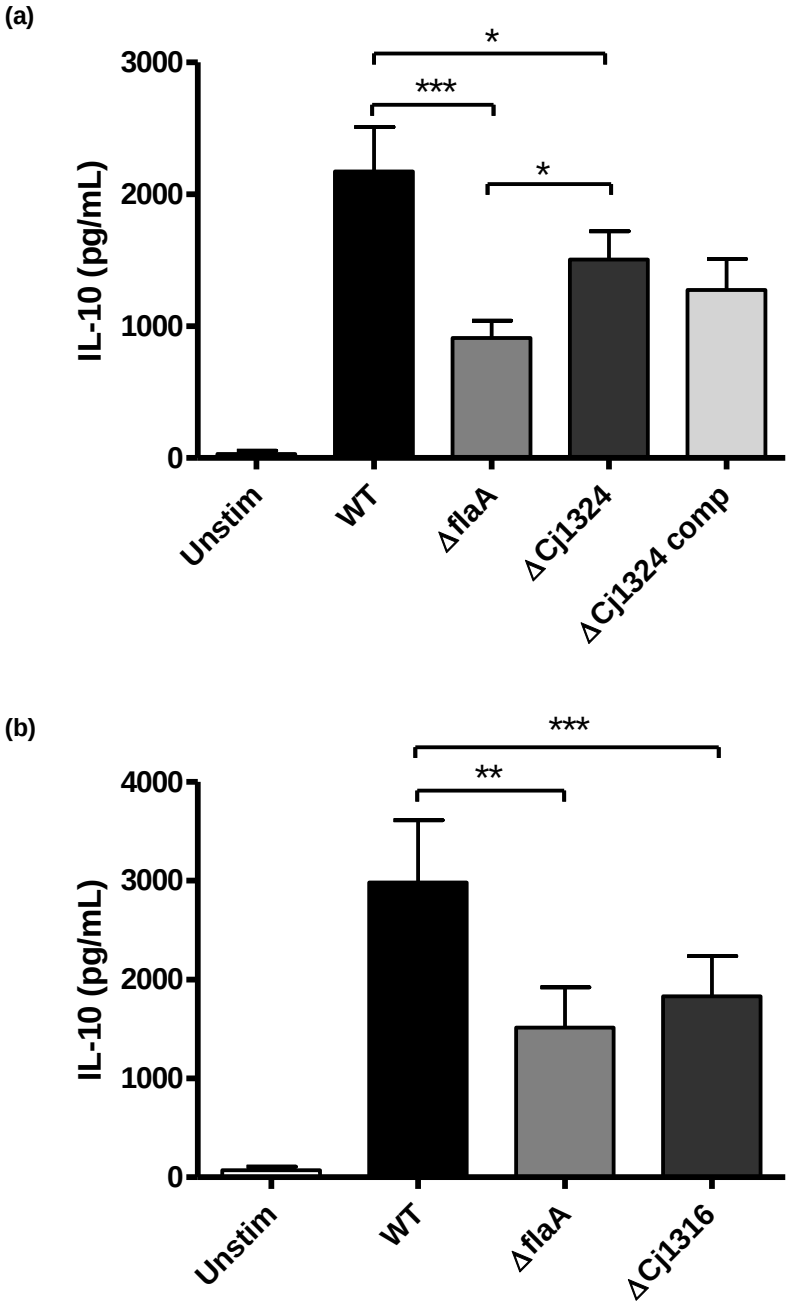


Figure 3-17: The Glycosylation moiety of *C. jejuni* flagella contributes to BMDC derived IL-10 production

1 x 10^6 BMDCs were co-cultured with (a) WT 11168H, *ΔflaA*, or flagella glycosylation mutant *ΔCj1324* or complement (b) WT 11168H, *ΔflaA*, or flagella glycosylation mutant *ΔCj1316* at an MOI 100 for 24h. IL-10 levels were analysed by ELISA. Values are means ± SEM from a minimum of three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

These observations suggested a potential role for the Pse5Ac7Am structure which is not complemented in the strain. This finding was confirmed with the *ΔCj1316* mutant, which also showed reduced IL-10 secretion when compared to the WT strain (Figure 3-17b). As there was no statistical difference between the *ΔCj1316* and *ΔflaA* mutant-mediated IL-10 levels we hypothesised that the Pse5Ac7Am modification is a major component involved in the induction of IL-10.

Reduction in IL-10 secretion was also seen when infections with the *ΔflaA* and *ΔCj1316* mutants were conducted in human monocyte-derived DCs (Figure 3-18). This was an important observation, as it confirmed that findings in murine BMDCs can be extrapolated to human DCs, and therefore the use of BMDCs is an appropriate model to study *C. jejuni* flagella/host interactions.

3.7 Discussion

Various surface determinants of *C. jejuni* are known to modulate adhesion/invasion and/or cytokine induction in IEC and innate immune cells (Bacon et al. 2001; Golden et al. 2004; van Sorge et al. 2009). The aim of the present study was to determine the role of three principal surface structures of *C. jejuni* in the modulation of DC responses.

The ability of BMDCs to phagocytose and kill *C. jejuni* was confirmed in the present study (Figure 3-1). The data obtained was similar to published reports (Hu et al. 2008). This highlighted the inability of *C. jejuni* to replicate within DCs. To quantify intracellular viable bacteria the gentamicin protection assay was used. The seemingly low ratio of total intracellular bacteria/DC obtained by this assay may reflect a change in the culturability of bacteria once exposed to the phagosome/lysosome. However, the ratios obtained in this study were similar to previous reports (Rathinam, Hoag, & Mansfield 2008). Confocal microscopy would be useful to assess the total number of internalised bacteria, in comparison to the viable numbers assessed by the gentamicin protection assay. The reduced phagocytic capability of BMDCs seen at 24h most likely reflects the maturation process of the DCs, as it correlated with the upregulation of maturation markers (Figure 3-3, Figure 3-7). In comparison to neutrophils and macrophages, DCs have reduced capability to acidify their phagolysosomes, which is critical to fulfil their primary role as antigen presenters opposed to pathogen killers (Savina et al. 2006).This likely accounts for why not all internalised bacteria are killed after 4h (Figure 3-1b). In contrast to DCs and macrophages, *C. jejuni* has been reported to survive in IECs for greater than 24h by avoiding delivery to the lysosome (Watson & Galan 2008). This is thought to be important

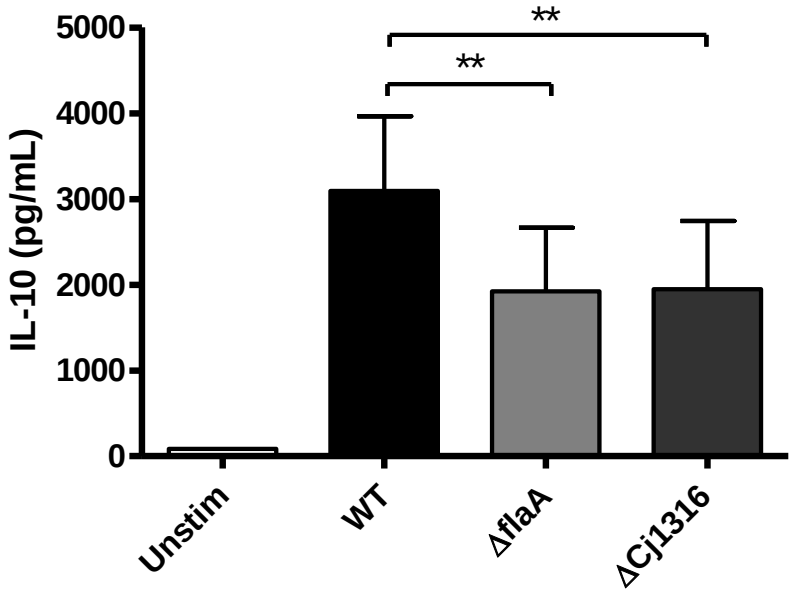


Figure 3-18: The Glycosylation moiety of *C. jejuni* flagella modulates human monocyte-derived DC IL-10 protein

1 x 10^5 human monocyte-derived DCs were stimulated with WT *C. jejuni*, *ΔflaA,* or *ΔCj1316* mutants at an MOI 100 for 24h. Supernatants were assessed for IL-10 protein levels by ELISA. Values are means ± SEM from 3 donors. One-way ANOVA statistical analysis performed with Tukey post-test. * <0.05; ** <0.01.

in disease pathogenesis. The ability of DCs and other APCs to kill *C. jejuni* is likely to be critical in inhibiting dissemination of the bacterium throughout the body. This may aid the eventual clearance of the pathogen negating a chronic infectious state as seen in other pathogenic microbial infections, such as *Mycobacterium tuberculosis,* which is able to inhibit phago-lysosome fusion within APCs (Wolf et al. 2007).

Although the capsule of other pathogenic bacteria are known to aid evasion of phagocytosis, no change in the rate of *C. jejuni* phagocytosis was observed when a capsule isogenic mutant was utilised (Figure 3-2)(Evrard et al. 2010; Unkmeir et al. 2002). The capsule however is important for reduced serum sensitivity and invasion of IECs suggesting it does play a role in interactions with other host cells (Bacon et al. 2001) Professional APCs express a plethora of phagocytic receptors and redundancy between them is an essential part of the host-immune response. This redundancy may explain the lack of difference in phagocytosis observed between the WT and the isogenic mutants of the capsule, N-linked glycosylation system, and the flagella (Figure 3-2).

Despite finding no differences in phagocytosis between the structural mutants and the WT strain, alteration in maturation marker and cytokine expression was observed (Figure 3-3, Figure 3-4, Figure 3-5, Figure 3-6). Figure 3-4 highlights a potential role for the CPS in the inhibition of IL-6 and IL-12 family members. An increase in IL-6, TNF-α, and IL-10 with the capsule mutant has subsequently been reported by Rose and colleagues (Rose et al. 2011) A component of the CPS, O-methyl phospharamidate (MeOPN), is important for *C. jejuni* virulence in the *Galleria mellonella* (wax moth) infection model (Champion et al. 2010). Whether *C. jejuni* CPS engages in specific glycan-binding receptors that suppress immune responses or alternatively masks underlying bacterial components from the host immune system remains to be determined.

On human monocyte-derived DCs, the GalNAc binding receptor, Macrophage-Galactose-type Lectin (MGL) can interact with *C. jejuni* N-linked glycosylated protein(s) leading to a reduction in IL-6 (van Sorge et al. 2009). However in the present study no significant differences were observed between the WT and N-linked glycosylation mutant strain in BMDCs (Figure 3-5). Although BMDCs do express the murine orthologs of human MGL, MGL1 and MGL2, whether these receptors have the same modulatory effects on DC function as the human counterpart remains unknown and may explain the lack of observable difference (Denda-Nagai et al. 2010).

Due to specific mutations in its primary amino acid sequence, *C. jejuni* FlaA protein can no longer be recognised by TLR5 (Andersen-Nissen et al. 2005). It was therefore interesting to find that the flagella mutant, *ΔflaA,* induced ~50% less IL-10 than the WT strain whilst IL-6, TNF-α, and IL-12 family member cytokine levels were unaffected (Figure 3-6). This data clearly indicated that *C. jejuni* flagellin may be interacting with other, as yet unknown receptors to modulate IL-10 expression. IL-10 is a critical cytokine in gut homeostasis, and IL-10 deficient mice develop spontaneous colitis (Kuhn et al. 1993). Due to the potent anti-inflammatory effects of IL-10 it is interesting to speculate that the specific modulation of IL-10 by *C. jejuni* is an immune-evasion strategy. In addition, the *ΔflaA* mutant induced less IL-1β suggesting a potential role for the NLR IPAF which recognises intracellular flagellin, in *C. jejuni*-mediated IL-1β secretion (Miao et al. 2010b). Investigation of the potential effect of *C. jejuni*-BMDC crosstalk in IL-1β secretion is reported in Chapter 4. In agreement with these findings it has subsequently been reported by Rose and colleagues that the *ΔflaA* mutant does indeed induce less IL-10 compared to the WT strain, accompanying only a minor reduction in pro-inflammatory cytokines (Rose et al. 2011).

Although all three MAPKs are linked to the induction of pro-inflammatory cytokines and maturation markers in DCs, p38 and JNK appear to be more critical for these functions (Escors et al. 2008;Miao et al. 2010b). It was therefore surprising to find a trend towards enhanced IL-1β production in the presence of p38 inhibitors (Figure 3-13). This may be explained by the intricate cross-talk that occurs between MAPK pathways. p38 causes the phosphorylation and inhibition of the regulatory subunit of a complex containing the MAP3K TAK-1, a MAP3K involved in the activation of p38, JNK, and NF-κB (Cheung et al. 2003). Therefore, p38 inhibition can lead to an increase in JNK phosphorylation and NF-κB activation, this enhances inflammatory responses. This is just one example that highlights the challenge in dissecting the role of individual MAPKs in DC function. The reduction of *C. jejuni*-induced IL-10 production by p38 inhibition may be linked to another regulatory step in the MAPK signalling web, i.e. activation of the kinases MSK1 and MSK2 (Figure 3-13). Downstream of p38 and ERK, MSK1 and MSK2 activate the transcription of DUSP1 (a regulatory phosphatase of MAPKs) and IL-10, creating a negative feedback loop on the activation of these two MAPKs (Ananieva et al. 2008). This may also explain the reduction in IL-10 observed under MEK1/2 inhibition (Figure 3-13). Both ERK and p38 signalling pathways have been implicated in the induction of IL-10 (Ma et al. 2001; Saraiva and O'Garra 2010). Interestingly, previous reports show TLR stimulation in the presence of ERK and p38 inhibitors almost completely abolishes IL-10 secretion suggesting these pathways

combine to induce IL-10 in DCs and macrophages (Saraiva & O'Garra 2010). A dependence on ERK and p38 for IL-10 induction may also explain why the *ΔflaA* mutant affects p38 signalling, and IL-10 induction (Figure 3-9; Figure 3-10; Figure 3-6). A minimal effect on other signalling pathways, such as NF-κB, may explain why the *ΔflaA* and MAPK inhibitors failed to impact on IL-12 family members (Figure 3-6; Figure 3-13).

The use of a secretion positive (*ΔflaA*) and secretion negative (*ΔrpoN*) flagella mutant strains revealed that proteins secreted from *C. jejuni* flagella play no role in IL-10 induction (Figure 3-16). Use of *C. jejuni* flagella glycosylation mutants implied that the carbohydrate modification of *C. jejuni* flagellin protein, FlaA, is involved in modulating BMDCs and human monocyte-derived DC IL-10 levels (Figure 3-17; Figure 3-18). In particular, the Pse5Ac7Am moiety appeared to be critical. Pse and Leg are structurally related to sialic-acid. Siglecs are receptors that engage sialic acid both on host cells and microorganisms and are generally regarded as immunomodulatory (Crocker, Paulson, & Varki 2007; Crocker & Redelinghuys 2008). It has been suggested that interaction between pathogens and host Siglecs receptors may promote mechanisms involved in immune evasion, including the induction of IL-10 (Ando et al. 2008). We next wished to delineate any potential cross-talk between Pse and Leg-linked C. jejuni flagella and Siglec receptors, interactions that remain largely unexplored in *C. jejuni*-mediated disease pathogenesis.

Chapter 4.

Recognition of *C. jejuni* by innate immune cells is both TLR-dependent and TLR-independent

4.1 Background

The activation of PRRs leads to a wide plethora of cellular phenotypic changes; changes that depend on the PRR engaged, and in addition the specificity of the ligand involved. PRRs can both activate and modulate immune signalling within DCs. The most widely studied PRRs are the TLRs, which are critical for stimulating inflammatory responses via the activation of MAPK, NF-κB, and IRF3 signalling pathways. In comparison the role of other PRRs such as glycan binding receptors in pathogen recognition and host defence is just starting to be appreciated. Additionally, the role of intracellular PRRs such as the NLR family in activation of the inflammasome which aids pathogen clearance has recently gained attention in innate immune research.

TLR2 and TLR4 are important for the recognition of *C. jejuni* by BMDCs (Rathinam et al. 2009). TLR4 signalling is particularly crucial for the induction of IL-12, IL-6, and TNF-α; however the role of TLR signalling in IL-10 responses has not been reported. Our observations (Chapter 3) suggested that the *C. jejuni* flagellin protein, FlaA, and in particular the pse5Ac7Am modification on the flagellin monomers may be important for the induction of IL-10. Mutations in the *C. jejuni* FlaA protein (also seen in other ε-proteobacteria FlaA proteins) results in a PAMP that is unable to bind to its cognate TLR; TLR5 (Andersen-Nissen et al. 2005). The only other known PRR for flagellin protein is the NLR member, IPAF (NLRC4). IPAF is also capable of detecting the type three secretion system (TTSS) of multiple pathogens (Miao et al. 2010b). The mechanism for this multi-specificity involves the detection of either flagellin or TTSS rod proteins by different NLR family Apoptosis Inhibitory Protein (NAIP) members upstream of IPAF which upon ligand binding induce interaction between NAIPs and IPAF causing the activation of the inflammasome (Zhao et al. 2011). NAIP5 is the intracellular receptor for flagellin proteins for *Salmonella* and *Legionella* and most likely may interact with other bacterial species. Mutational studies have identified specific amino acid residues in the carboxyl-terminal of flagellin protein that are involved in mediating IPAF-dependent inflammasome activation (Miao et al. 2010b). No studies to date have analysed if and how *Campylobacter sp.* activates the inflammasome.

Candidate receptors for the engagement of these sialic-acid like structures include the Siglec family of glycan receptors. In a previous study the ability of *C. jejuni* to bind to Siglec-10 was reported; this interaction was considered to be independent of sialylated LOS as the *C. jejuni/*Siglec-10 interaction was not sensitive to sialidase treatment and purified LOS did

not bind the receptor (Avril et al. 2006). However, as the LOS is the only known sialylated structure on *C. jejuni* it was concluded that this interaction was likely to be non-specific (Professor Paul Crocker, University of Dundee; personal communication). The ability of sialic-acid like structures on the flagella to engage Siglec-10 remains unclear. Although sialic acids are the best characterised ligands for Siglecs, recent emerging data suggests that multiple ligands may bind and activate Siglec receptors (Carlin et al. 2009a; Kivi et al. 2009). The 9-carbon pseudaminic acid (Pse) and legionaminic acid (Leg) share many of the reported critical residues required for binding to the Siglec sialic-acid binding pocket (May et al. 1998). Recognition of structures similar to Neu5Ac by Siglecs has not been extensively studied. A single report found discrepancy between human and chimpanzee Siglec-9, which have greater affinities for Neu5Ac and Neu5Gc respectively, as during evolution of higher primates humans have lost the ability to produce Neu5Gc (Sonnenburg et al. 2004).

4.2 TLR signalling is essential for the activation of BMDCs in response to *C. jejuni*

TLR signalling is essential for the activation of APCs in response not only to a plethora of microbes but also to endogenous danger ligands, DAMPs. At the start of this study, little was known of the role of TLR engagement in *C. jejuni*-mediated DC activation; although the ability of *C. jejuni* CPS to stimulate IEC TLR2 leading to IL-6 production was known (Friis, Keelan, & Taylor 2009). MyD88 and TRIF are two essential adaptor molecules for TLRs 1, 2, 4, 5, 6, 7, 8, 9, and TLR 3, 4 respectively (Takeda et al. 2003). TLR4 is the only TLR known to utilise both MyD88 and TRIF adaptor molecules. To investigate a potential role for TLR signalling in DC responses to *C. jejuni*, BMDCs were generated from singular and combined double knock-outs (DKO) in the two adapter molecules ($MyD88^{-/-}$, $TRIF^{-/-}$, $MyD88^{-/-}$ $TRIF^{-/-}$ (DKO) and WT). Co-cultures studies were performed and DC cellular processes including phagocytosis, the regulation of maturation markers, cytokine secretion, and signalling pathways were analysed.

TLR signalling increases rate of macropinocytosis (West et al. 2004). It was therefore essential to assess the rate of *C. jejuni* phagocytosis in the knock-out cells prior to commencing detailed analysis. Figure 4-1 shows abolishing TLR signalling did not alter the rate of uptake of *C. jejuni* as measured 4h post-infection. This data indicated that TLR signalling may not be essential for early internalisation of *C. jejuni*.

The upregulation of maturation markers, CD40 and CD80, in WT BMDCs in response to *C. jejuni* infection was similar to results reported in Chapter 3 (Figure 3-3); a ~2-fold increase

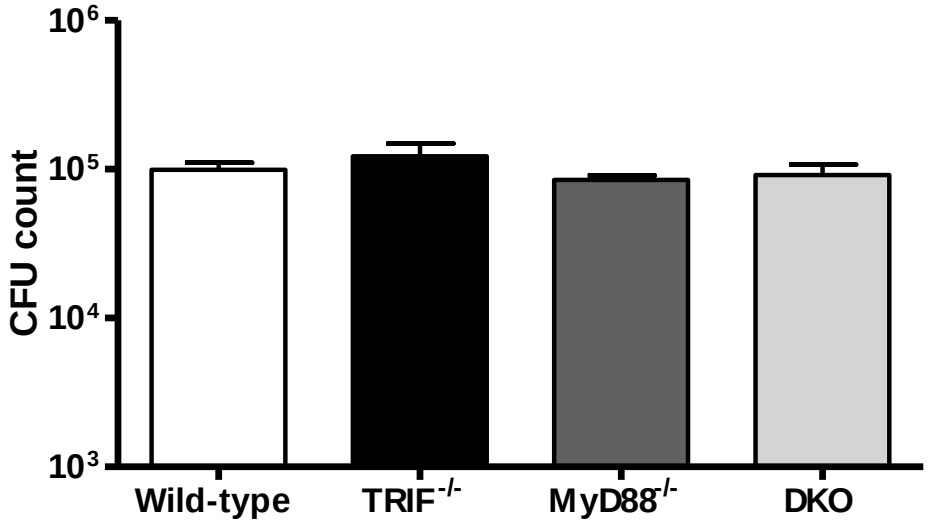


Figure 4-1: TLR signalling does not alter *C. jejuni* phagocytosis by BMDC

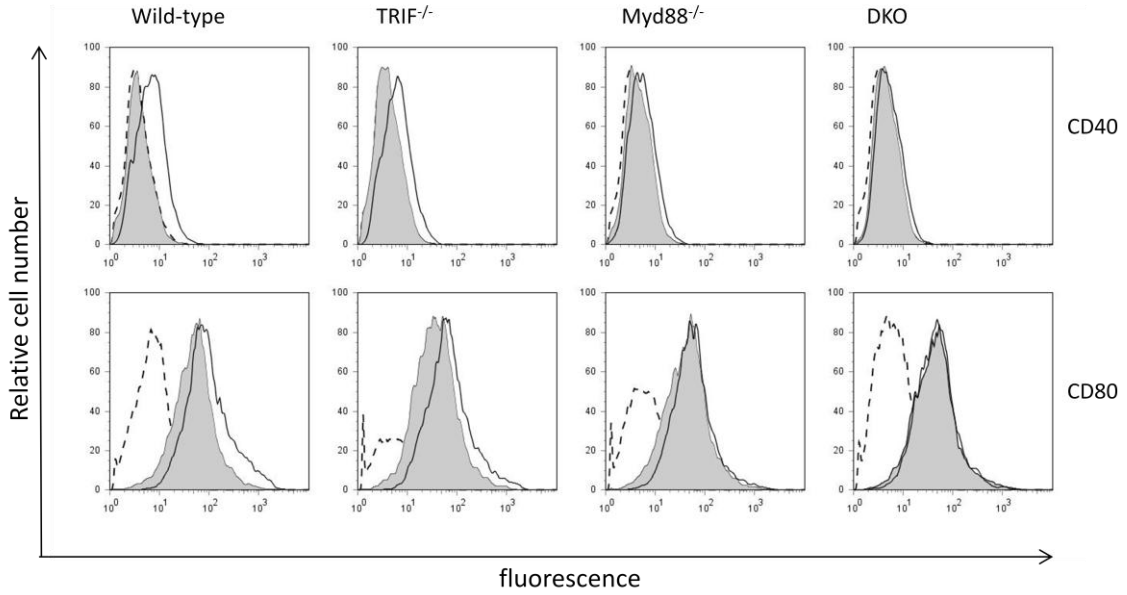
2 x10 ^{5} BMDCs from WT (WT), TRIF$^{-/-}$, MyD88$^{-/-}$, and TRIF$^{-/-}$MyD88$^{-/-}$ double knock-out (DKO) were co-cultured with *C. jejuni* 11168H MOI 100 for 4h. Viable intracellular bacterial numbers were enumerated by the gentamicin protection assay. Values are means ± SEM. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant.

in the MFI of both markers was observed (Figure 4-2). The upregulation of CD40 in both MyD88$^{-/-}$ and TRIF$^{-/-}$ DC was ~ 50% of the response to that seen in WT DCs. Co-culture with the DKO resulted in no upregulation of CD40 (p=0.03). A similar phenotype was also observed for CD80, both single mutations approximately halved the induction of CD80 whereas the DKO completely abolished upregulation. Upregulation of CD40 and CD80 in response to the LPS control was very similar to live *C. jejuni.* This pattern suggested a combined impact of both MyD88- and TRIF-dependant TLR signalling pathways in the induction of CD40 and CD80, and that these signalling adaptors may act in an additive manner in mediating regulation of CD40 and CD80.

The activation of TLRs is known to drive differential cytokine expression (Sing et al. 2002;Takeda, Kaisho, & Akira 2003). It was therefore important to study the cytokine expression profiles induced by *C. jejuni* in the different KO cells (Figure 4-3). LPS mediates TLR signalling via TLR4 which utilises both TRIF and MyD88 adaptor molecules and was therefore included as a control. Marked increase in IL-6 was observed in WT BMDCs upon infection, similar to those reported in Chapter 3 (Figure 4-3a; Figure 3-4). A trend for decreased IL-6 induction was noted in TRIF$^{-/-}$ BMDCs but this gained greater significance in the MyD88$^{-/-}$ BMDCs. Complete abrogation of IL-6 was seen in DKO BMDCs. LPS-mediated IL-6 was more markedly reduced in TRIF$^{-/-}$ BMDCs than for *C. jejuni*. However, similar to *C. jejuni*-stimulation, a greater reduction in LPS-mediated IL-6 was noted in MyD88$^{-/-}$ compared to TRIF$^{-/-}$ BMDCs. Together data suggested greater dependence on MyD88 signalling for the induction of IL-6 by *C. jejuni* whereas LPS induced IL-6 required both signalling pathways.

Conversely, IL-1β secretion depended on the signalling of both TRIF and MyD88 pathways (Figure 4-3b). A similar reduction of IL-1β levels was observed in both single and double KO cells for both *C. jejuni* and LPS stimulation. ~50% reduction in IL-12 levels was observed in TRIF$^{-/-}$ and MyD88$^{-/-}$ BMDCs, the cytokine was undetectable in DKO BMDCs (Figure 4-3c). IL-23 levels were below the detection threshold for both *C. jejuni* stimulated single and double KO BMDCs (Figure 4-3d). Interestingly, *C. jejuni*-mediated IL-10 levels were not significantly reduced in the TRIF$^{-/-}$ BMDCs, whereas it was completely abolished in the MyD88$^{-/-}$ and DKO cells (Figure 4-3e). In contrast LPS-mediated IL-10 secretion was affected by both TRIF and MyD88 signalling pathways. These observations suggested a cumulative effect of MyD88 and TRIF signalling in IL-12 release by *C. jejuni* in contrast, IL-10 secretion was solely MyD88-dependent.

(a)



(b)

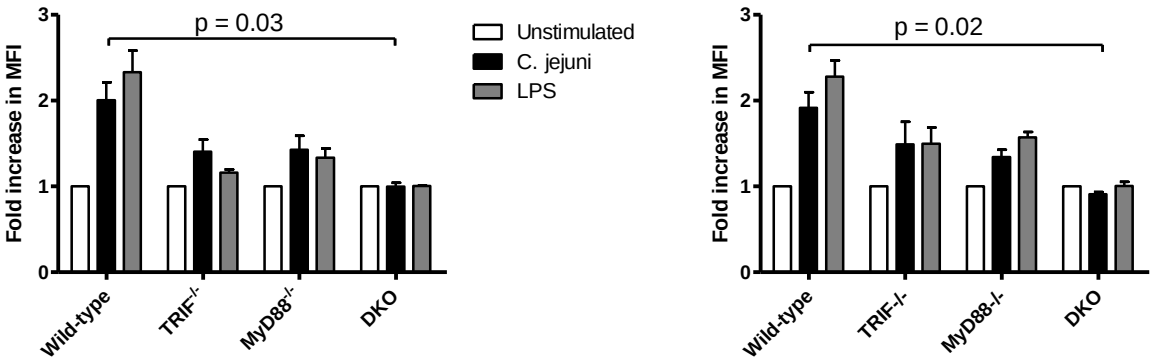


Figure 4-2: TLR signalling is essential for *C. jejuni*-mediated induction of BMDC maturation markers

1 x 10^6 BMDCs generated from WT (WT), $TRIF^{-/-}$, $MyD88^{-/-}$, and $TRIF^{-/-}MyD88^{-/-}$ double knock-out (DKO) mice were co-cultured with *C. jejuni* 11168H MOI for 24h. CD40 and CD80 expression levels were assessed by flow cytometry. (a) Representative histograms from three independent experiments are shown. Isotype control (dashed line), unstimulated cells (solid grey), and *C. jejuni* stimulated levels (back line). (b) Fold increases in MFI from the unstimulated cells. Values are means ± SEM. Paired t-test statistical analysis performed to compare specific columns.

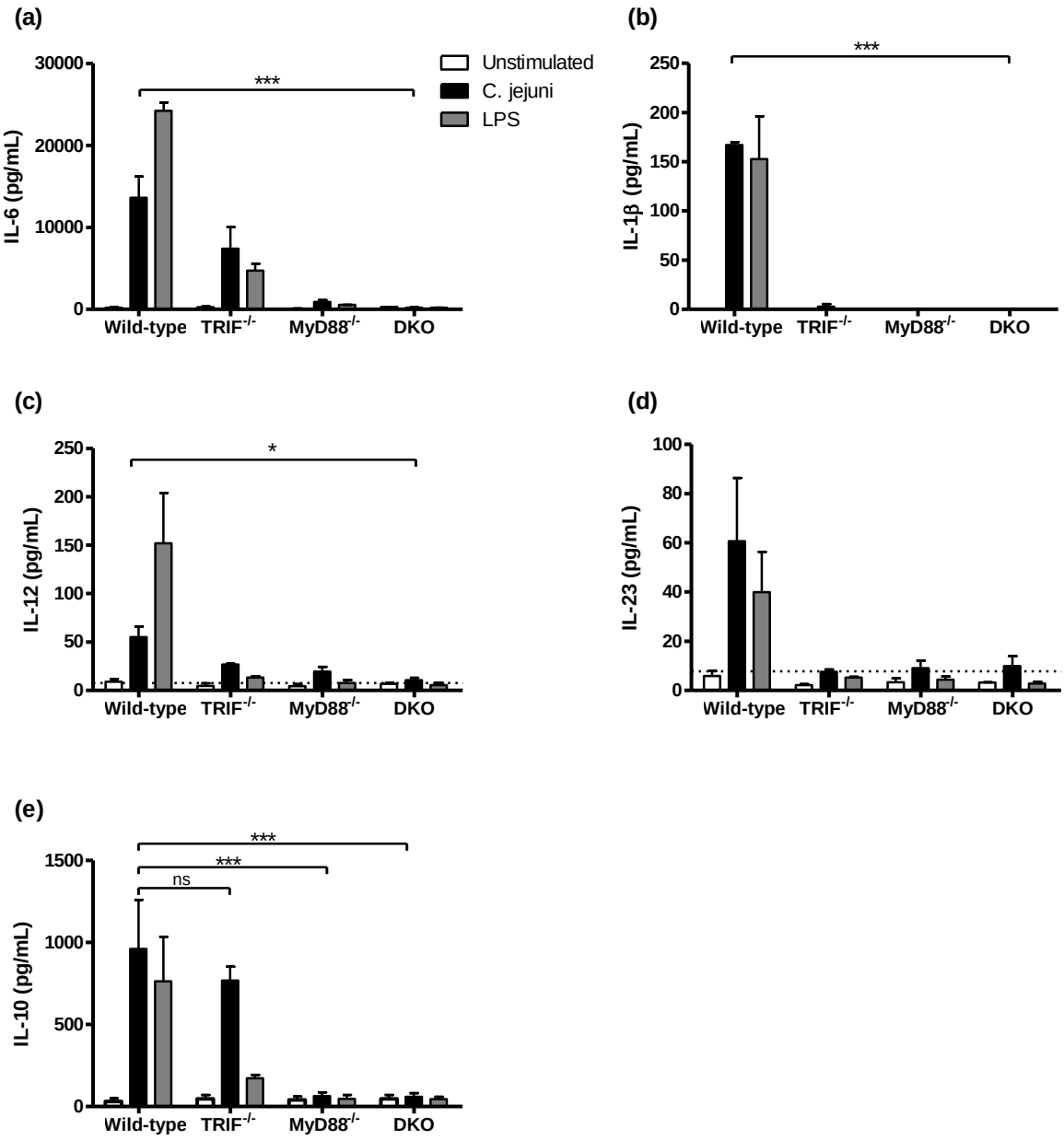


Figure 4-3: TLR signalling is essential for *C. jejuni*-mediated BMDC pro- and anti-inflammatory cytokine responses

1 x10^6 WT, TRIF$^{-/-}$, MyD88$^{-/-}$, and DKO BMDCs were co-cultured with *C. jejuni* 11168H at an MOI 100 for 24h. Cytokine analyses was performed by ELISA for IL-6 (b) IL-1β (c) IL-12 (d) IL-23 (e) IL-10. Values are means ± SEM from a minimum of three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

As MAPK activation is important in the induction of certain cytokines by *C. jejuni* (Figure 3-13), and is downstream of TLRs (Takeda, Kaisho, & Akira 2003), we next wished to establish the impact of the absence of TLR signalling on *C. jejuni*-mediated MAPK activation (Figure 4-4). Interestingly, early activation of ERK was unaffected in the KO cells, however strong ERK signalling at 2h post-infection was only observed in the WT and TRIF$^{-/-}$ BMDCs. The DKO and MyD88$^{-/-}$ cells showed a similar pattern of early weak activation to *C. jejuni* but a lack of subsequent strong ERK activation at 2h. JNK activation was observed at 1h post-infection, with strong signalling at 2h. JNK phosphorylation was completely abolished in the DKO cells highlighting the necessity of TLR signalling for JNK activation. In TRIF$^{-/-}$ BMDCs JNK activation followed the same kinetics as the WT cells, however in MyD88$^{-/-}$ cells JNK activation was delayed being only detectable at 2h post-infection. In WT cells p38 phosphorylation was detectable at 1h and 2h post-infection. Similarly to JNK signalling, p38 phosphorylation was completely abolished in the DKO cells. Mirroring JNK activation, in TRIF$^{-/-}$ BMDCs kinetics of p38 phosphorylation was similar to that seen in WT cells. In contrast, p38 activation was completely abrogated 2h post-infection in MyD88 KO DCs. It is worth noting that the MyD88-dependent signalling appeared to alter all three MAPK pathways to a greater extent than TRIF-dependent signalling, and correspondingly MyD88 seemed to have a greater potential role in the induction of specific cytokines, in particular IL-6 and IL-10.

Taken together our observations suggested that *C. jejuni*-mediated BMDC cytokine responses were TLR-dependant. This raised a conundrum as to how *C. jejuni* flagella mediates IL-10 production and is yet not recognised by TLR5 (Andersen-Nissen, Smith, Strobe, Barrett, Cookson, Logan, & Aderem 2005). There have been multiple examples of pathogens interacting with carbohydrate binding receptors that modulate TLR signalling, often for the pathogens advantage (Gringhuis et al. 2007; Gringhuis et al. 2009). We hypothesised that glycosylated moiety of *C. jejuni* flagella interacts with as yet unidentified host receptors leading to modulation of IL-10 responses. We therefore assessed the ability of *C. jejuni* glycosylated flagella to interact with carbohydrate receptors independent of TLRs.

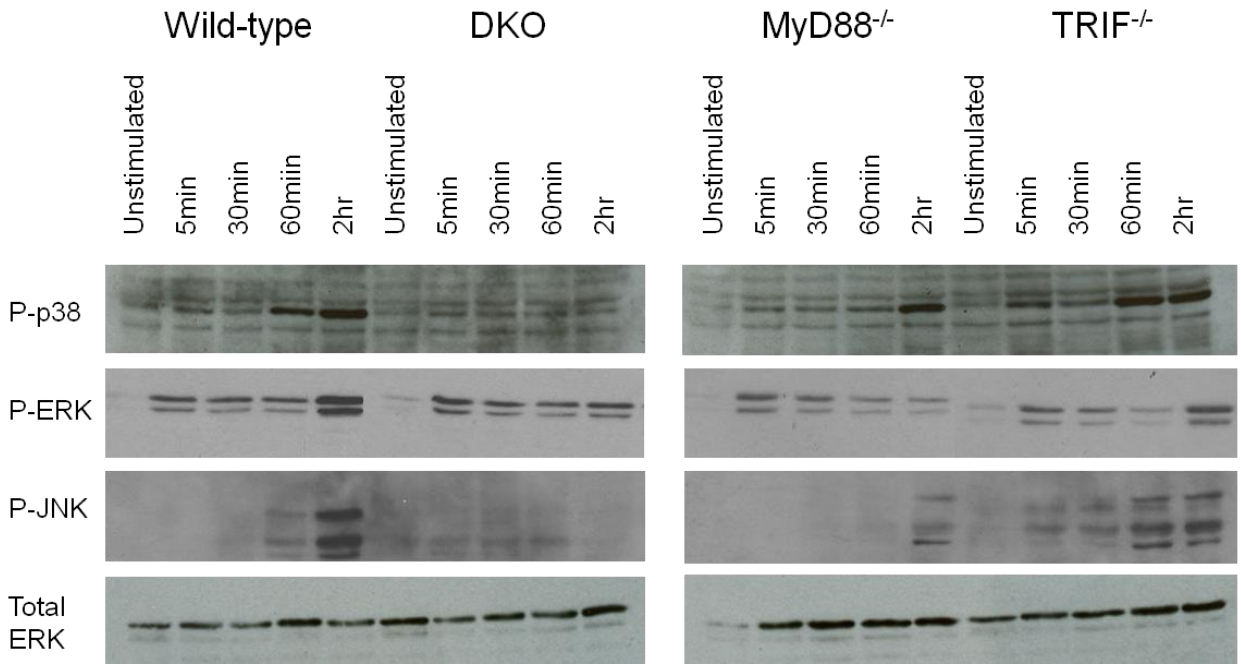


Figure 4-4: TLR signalling is essential for *C. jejuni*-mediated MAPK activation

1 x 10^6 WT, TRIF$^{-/-}$, MyD88$^{-/-}$, and DKO BMDCs were co-cultured with *C. jejuni* 11168H MOI 100 for the indicated time-points. Lysates were subjected to SDS-PAGE followed by Western blot analysis for the phosphorylated forms of p38, ERK, and JNK. Blots were stripped and probed for total ERK as a loading control. Blots are representative of two independent experiments.

4.3 Siglec-10 binds derivatives of Pseudaminic acid, components of *C. jejuni* flagella

In 2006 a report indicated that *C. jejuni* binds to Siglec-7 via α2,8- linked sialic acid residues found on *C. jejuni* LOS (Avril et al. 2006). The authors also found two *C. jejuni* strains were able to bind to Siglec 10-Fc chimeras. As the interaction was not sialidase-sensitive the authors speculated that the interaction between the *C. jejuni* strains and Siglec-10 was non-specific and therefore did not investigate this phenomenon any further. Here, the possible role of Siglec-10 in the binding of derivative of pseudaminic acid (Pse) structures present on *C. jejuni* flagella was explored. Siglec-10 is the human ortholog of Siglec-G which is expressed on murine BMDCs (Chen et al. 2009; Ding et al. 2007). Siglec-10 and Siglec-G share high sequence identity and conserved protein structure (Angata et al. 2001).

Before undertaking binding assays the expression of Siglec-10 on human monocyte-derived DCs was firstly confirmed by flow cytometry (Figure 4-5) (Li et al. 2001). To investigate potential *C. jejuni*-Siglec-10 interactions, CHO cells overexpressing Siglec receptors were utilised. Siglec-7 is known to bind to *C. jejuni* bearing α2,8-linked sialylated LOS. 99% of Siglec-7 overexpressing cells expressed the receptor (Figure 4-6a) and ~80% of Siglec-10 CHO cells expressed Siglec-10 (Figure 4-6b). WT CHO cells expressed neither Siglec-7 nor Siglec-10. To perform binding assays, *C. jejuni* strains were labelled with fluorescein isothiocyanate (FITC) for 1h at 37^{o}C. *C. jejuni* strains WT, *ΔflaA* and *ΔCj1316* were all labelled with FITC to the same extent (Figure 4-7). Sialidase treatment of Siglec overexpressing CHO cells is routinely performed in binding assays to eliminate cis-interactions between Siglecs and sialic acid found on the cell surface as this interaction reduces binding of Siglecs to ligands in trans (Avril et al. 2006). Optimisation assays were initially performed with Siglec-7 overexpressing CHO cells with *C. jejuni* strains GB11 and GB19, strains known to express α2,3-linked and α2,8-linked sialic acid on their LOS respectively. Siglec-7 CHO cells were pre-treated with 0.1U/mL *C. perfringens* sialidase for 1h to remove sialic acid prior to exposure to C. jejuni GB11 and GB19 at an MOI 100 for 2h at 4^{o}C. Differential binding pattern to siglec-7 CHO cells was observed between the two strains (Figure 4-8). Incubation at 4^{o}C prevents endocytosis and bacterial uptake, therefore the data depicted Siglec-ligand binding alone.

WT 11168H *C. jejuni* bound specifically to sialidase-treated Siglec-10 CHO cells compared to WT CHO cells (Figure 4-9a). Bacterial binding index (BBI) is a value derived from multiplying the percentage of CHO cells bound with FITC labelled bacteria by the geometric mean

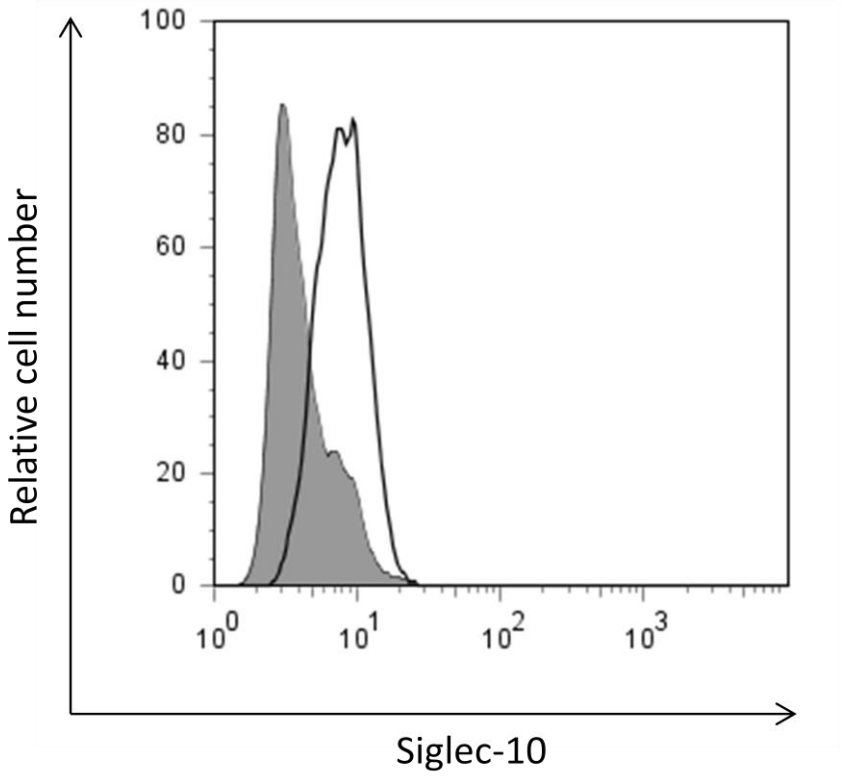


Figure 4-5: Siglec-10 is expressed on human monocyte-derived DCs

A representative histogram of human monocyte-derived DCs were stained with primary mouse monoclonal antibody, 5G6, and PE-conjugated donkey anti-mouse secondary antibody (black line), or secondary antibody alone (solid grey line). Histograms are representative of three independent experiments.

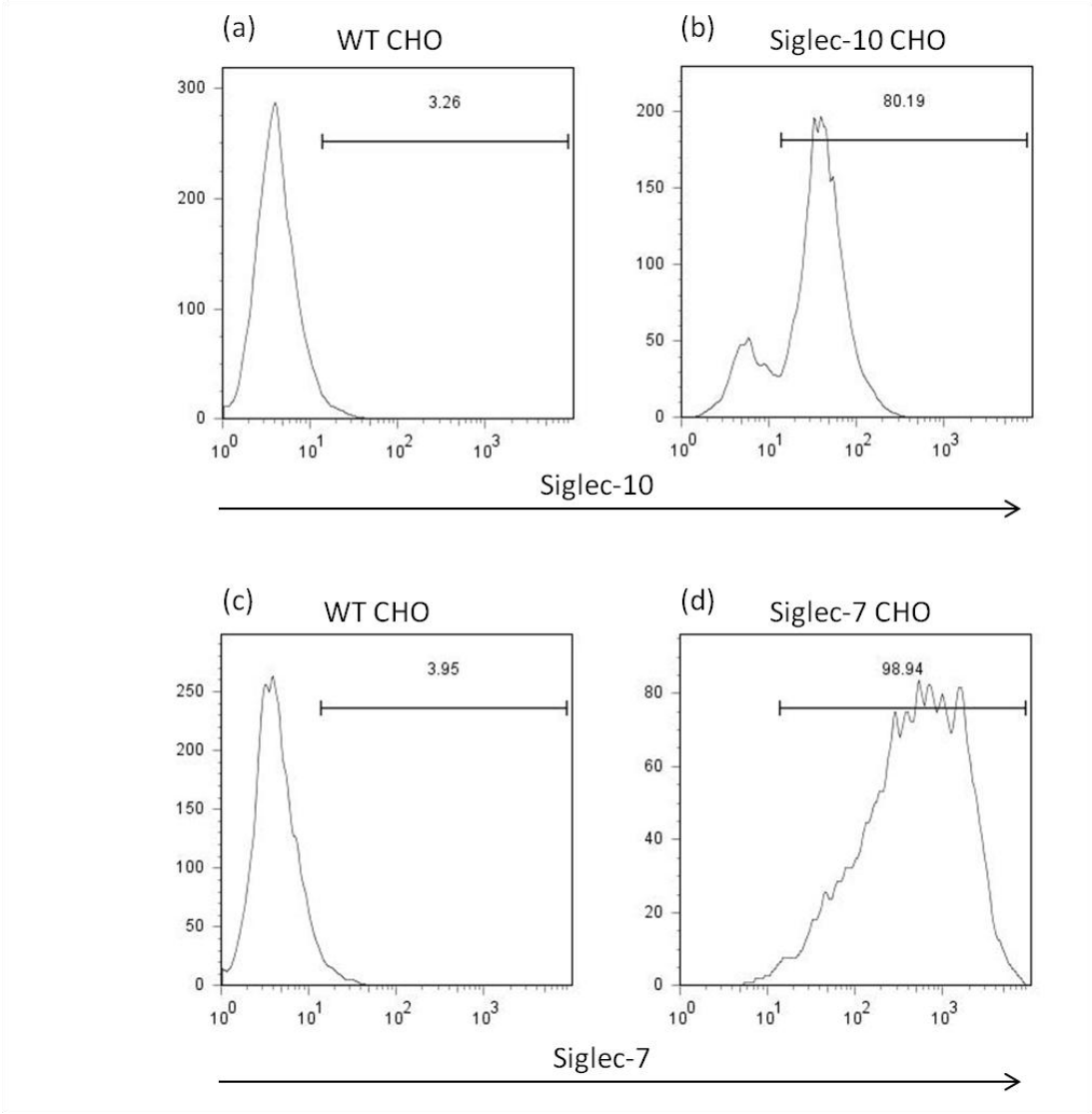


Figure 4-6: Siglec-CHO overexpressing cells specifically express Siglec-10 and Siglec-7

WT and Siglec-overexpressing CHO were stained for Siglec expression and analysed by flow cytometry (a) WT CHO cells stained with anti-siglec-10 (b) siglec-10 CHO cells stained with anti-siglec-10 (c) WT CHO cells stained with anti-siglec-7 (d) siglec-7 CHO cells stained with anti-siglec-7. Data are representative histograms from two independent experiments.

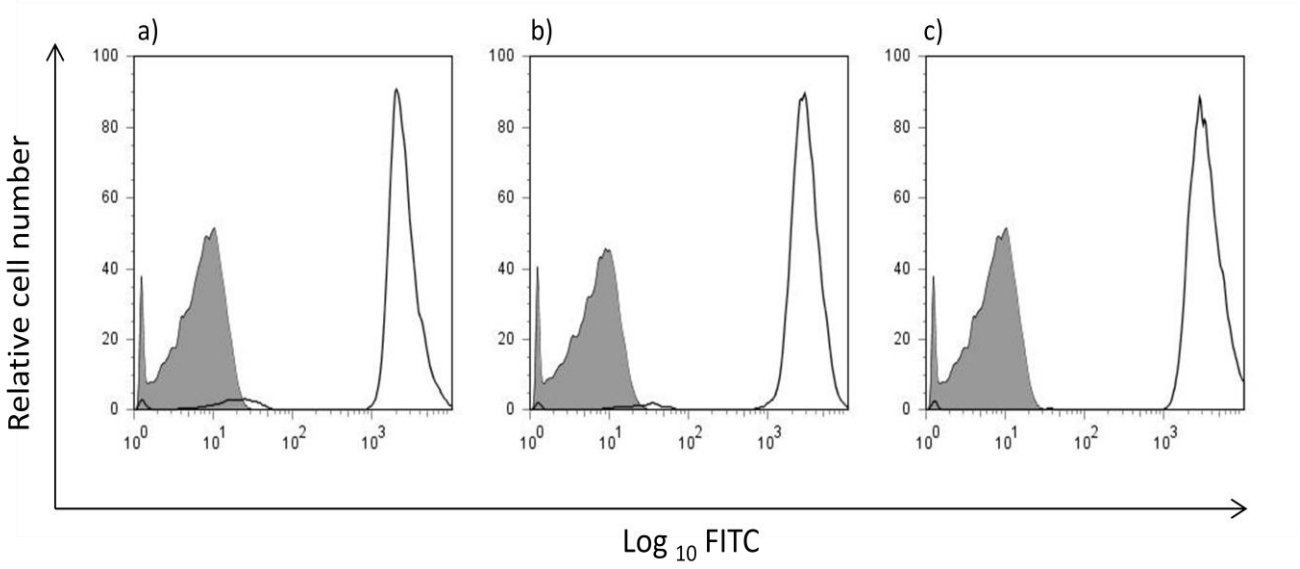


Figure 4-7: FITC labelling of *C. jejuni*

3 x 10^9 CFU/mL [(a) WT (b) *ΔflaA* (c) *ΔCj1316*] *C. jejuni* were mixed 1:1 with a saturated FITC solution for 1h at 37^{o}C. Labelling of bacteria was subsequently assessed by flow cytometry.

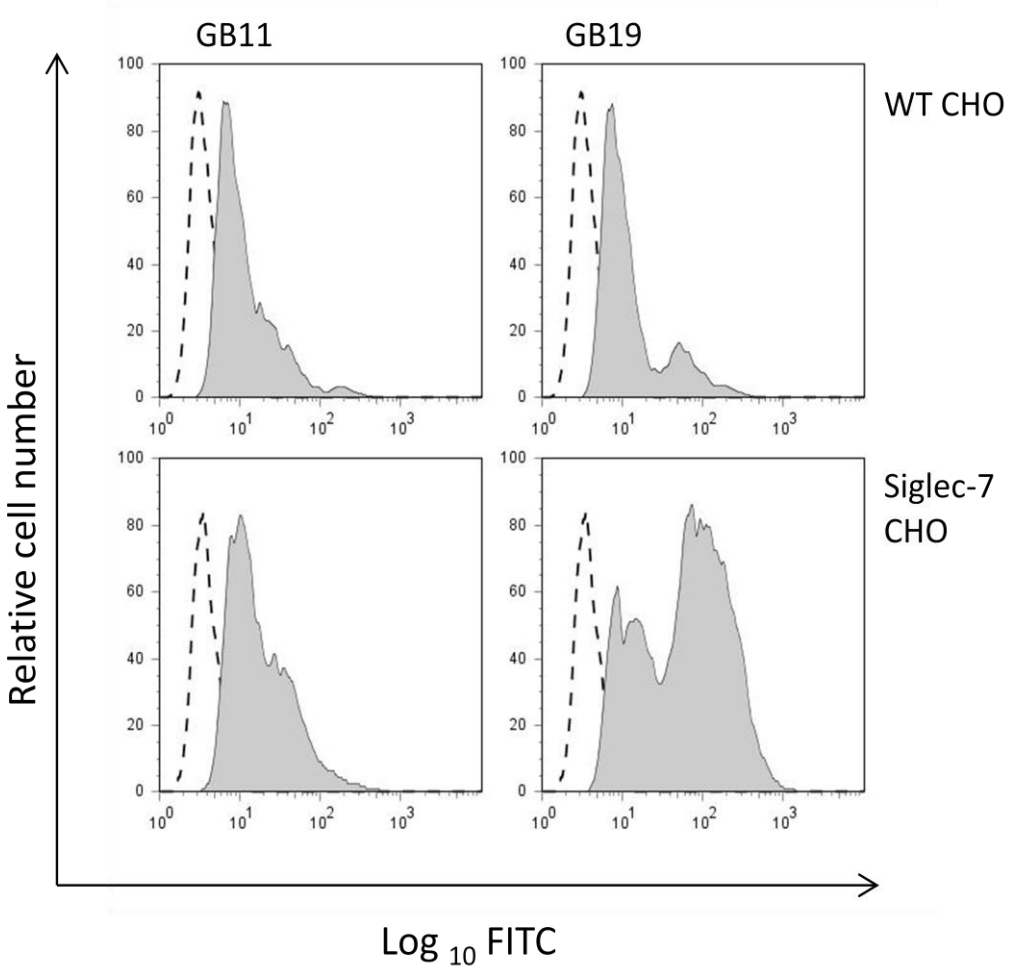


Figure 4-8: *C. jejuni* strain GB19 binds specifically to Siglec-7

Potential Siglec-CHO cells and *C. jejuni* interactions. Optimisation studies; 3 x 10^5 sialidase-treated WT or siglec-7 expressing CHO cells were incubated with FITC-labelled *C. jejuni* strains GB11 or GB19 at an MOI 100 for 2h at 4^{o}C. Binding was analysed by flow cytometry. Histograms are representative of two individual experiments.

fluorescence intensity (MFI) of the positive population. BBI gives an indication of the level of bacterial binding to the CHO cells (Avril et al. 2006). The BBI used in this study is a slight revision from previous studies as it uses the geometric mean rather than the mean fluorescence intensity because the population was normally distributed on a logarithmic scale.

Figure 4-9b shows a trend towards an increase in BBI value with sialidase treatment of the siglec-10 CHO cells. As only 80% of Siglec-10 CHO cells express the receptor this enabled a more detailed analysis of which cells specifically bound *C. jejuni*. Siglec-10 CHO cells were counter stained with Siglec-10 after a 2h co-culture. Figure 4-10 shows a doubling in the BBI value with the Siglec-10 positive cells over Siglec-10 negative cells. This value is less than the binding seen in Figure 4-9. One possible explanation is the additional wash steps in the counterstaining removes many of the bound bacteria. The binding of 11168H to Siglec-10 CHO cells could be specifically inhibited with the addition of a polyclonal anti-siglec-10 antibody (Figure 4-11), whereas the irrelevant polyclonal anti-Siglec-5 antibody made no impact on bacterial binding.

C. jejuni strain 11168H contains only internal α2,3-linked sialic acid residues on its LOS, and is therefore not predicted to bind to any known Siglec. Binding assays were performed with various sialic-acid family mutants of *C. jejuni* 11168H to determine the critical moieties involved in interacting with Siglec-10. Figure 4-12a shows representative histograms of *C. jejuni* WT and isogenic mutant strain interactions with Siglec-10 CHO cells. The top four histograms show comparable binding of the WT and isogenic mutants to WT CHO cells. The lower four histograms show WT *C. jejuni* can bind to Siglec-10 cells as seen in Figure 4-9. In addition the *ΔwaaF* mutant (which expresses a truncated LOS structure and therefore lacks the sialic acid residue on the LOS) bound to Siglec-10 CHO cells signifying that the LOS sialic acid is not critical for Siglec-10 engagement. Conversely both the flagella mutant, *ΔflaA,* and flagella Pse5Ac7Am mutant, *ΔCj1316,* showed comparable binding to Siglec-10 and WT CHO cells. Differences in binding of the WT 11168H and Pse5Ac7Am mutant strain to Siglec-10 CHO cells was found to be statistically significant ($p < 0.01$; Figure 0.12b). These observations suggest potential novel interaction(s) between the Pse5Ac7Am modifications (or derivatives of) on *C. jejuni* flagella and host Siglec-10 receptors.

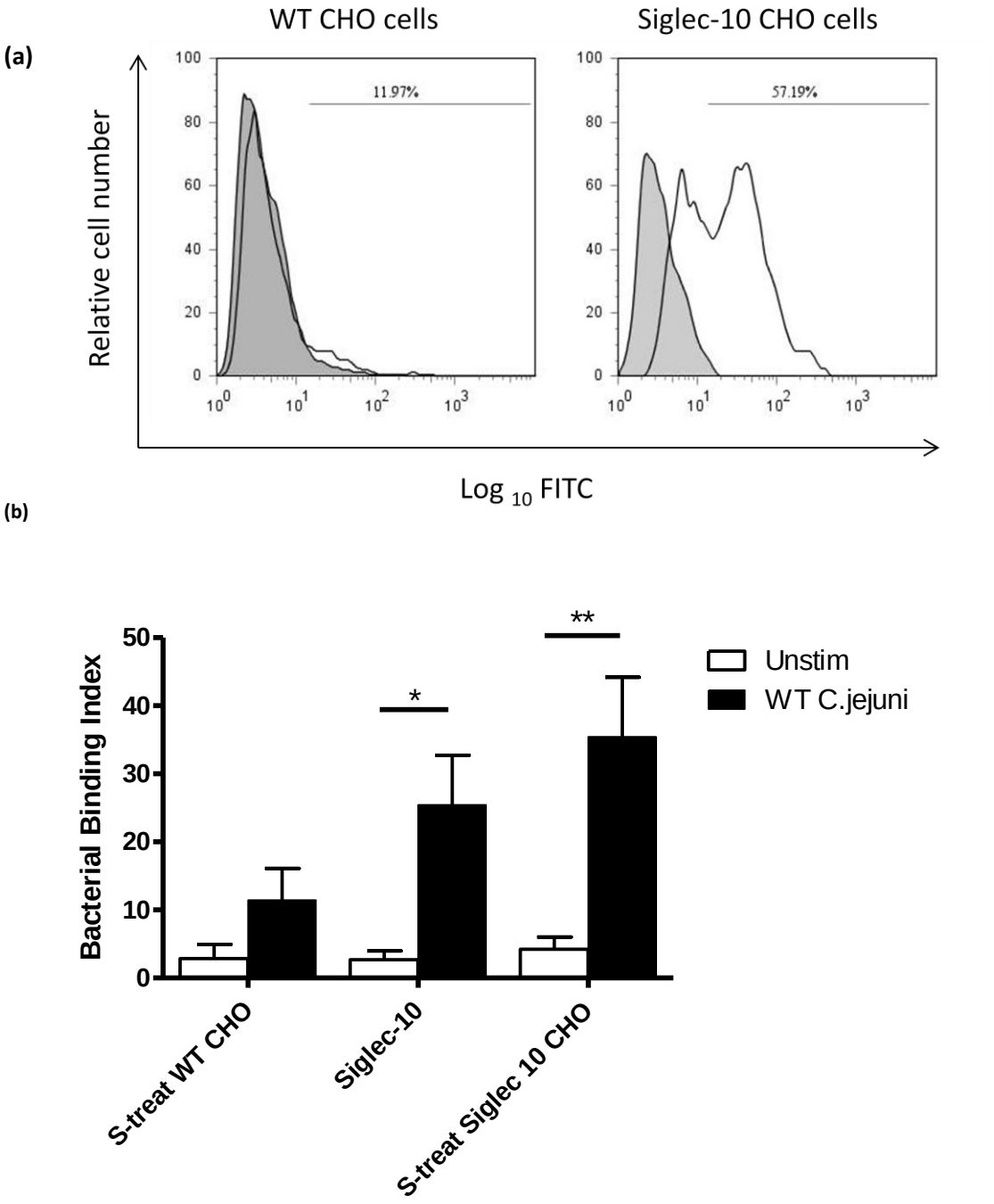
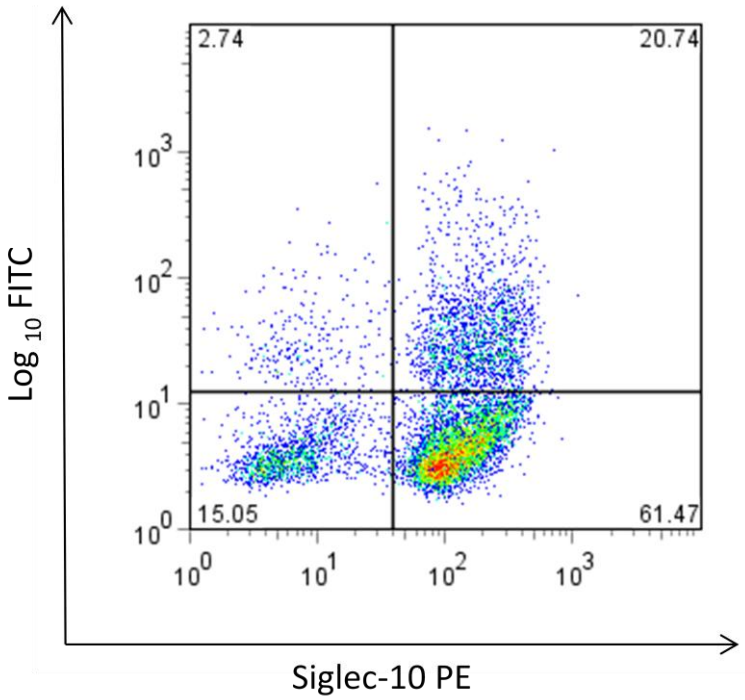


Figure 4-9: *C. jejuni* 11168H binds to Siglec-10 overexpressing CHO cells

3 x 10^5 sialidase-treated (S-treat) WT, un-treated siglec-10 overexpressing, or sialidase-treated siglec-10 overexpressing CHO cells were mixed with FITC-labelled *C. jejuni* strain 11168H MOI 100 for 2h at 4^{o}C. Binding was analysed by flow cytometry. (a) Both WT and Siglec-10 cells were sialidase treated. Histograms are representative of six independent experiments. (b) Bacterial binding index was calculated by the percentage of CHO cells bound with FITC labelled bacteria by the geometric MFI of this population. Values are means ± SEM from a minimum of three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

(a)



(b)

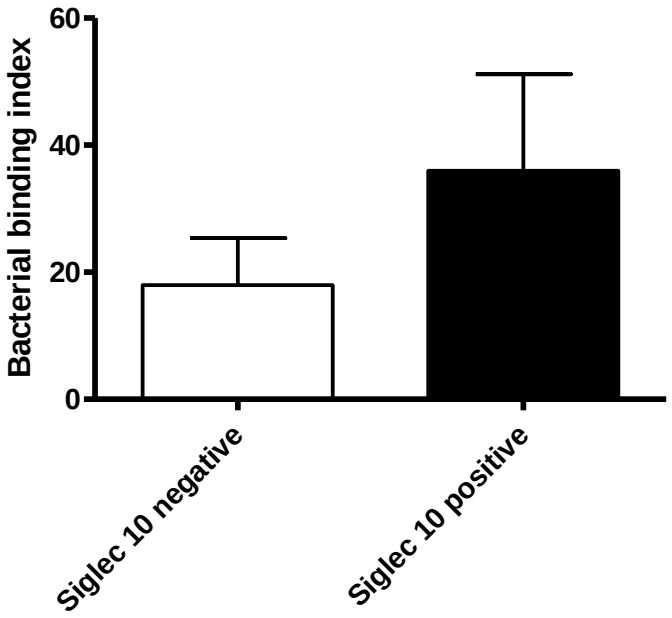
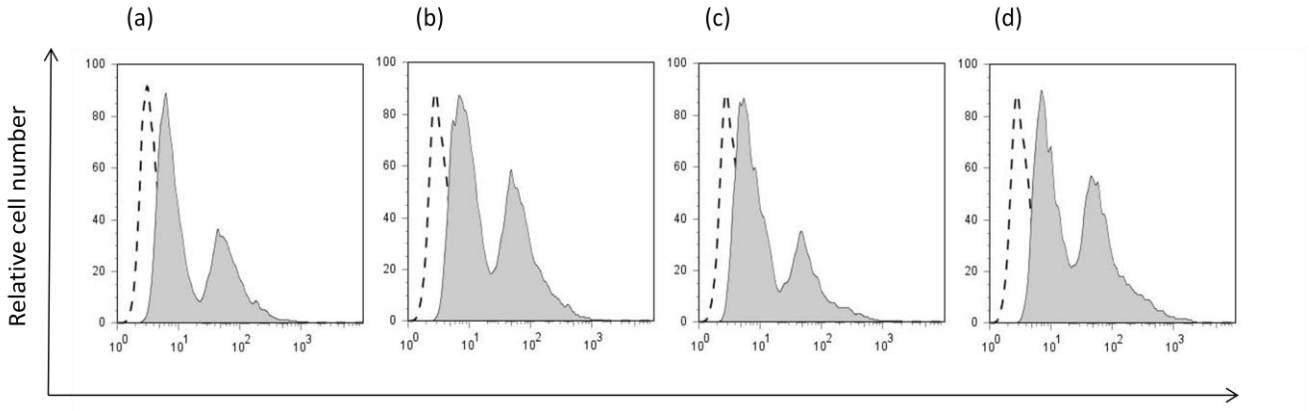


Figure 4-10: *C. jejuni* binds specifically to Siglec-10 overexpressing CHO cells

3 x 10^5 sialidase-treated Siglec-10 overexpressing CHO cells were mixed with FITC-labelled *C. jejuni* strain 11168H MOI 100 for 2h at 4°C. Cells were counter-stained with monoclonal 5G6 anti-siglec-10 primary antibody and PE-secondary antibody. Cells were analysed by flow cytometry. (a) Representative dot plot of four individual experiments. Values are percentages within the individual quadrants (b) Mean values ± SEM for the bacterial binding index of the siglec-10 negative and positive populations.



(e)

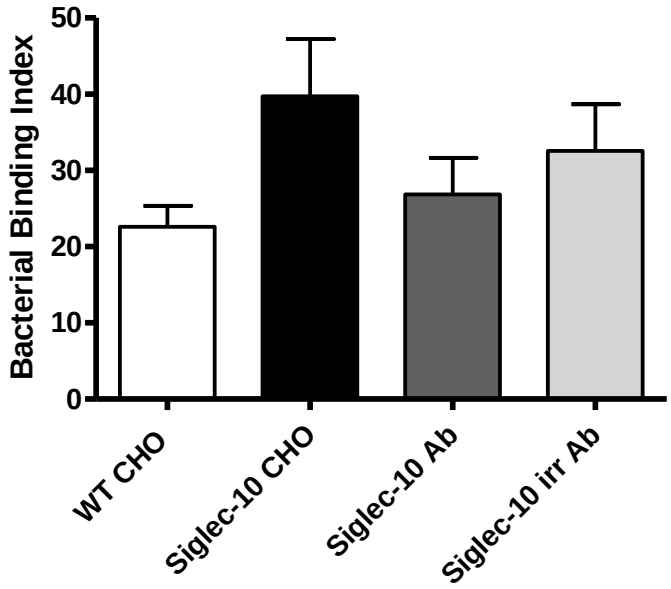


Figure 4-11: Polyclonal antibodies specifically inhibit *C. jejuni* binding to Siglec-10 cells

3 x 10^5 sialidase-treated (a) WT CHO cells (b) sialidase-treated siglec-10 CHO cells (c) sialidase-treated siglec-10 CHO cells in the presence of 10µM polyclonal antibody anti-Siglec 10 (Ab) (d) sialidase-treated siglec-10 CHO cells in the presence of 10µM irrelevant polyclonal anti-Siglec 5 Ab mixed with FITC-labelled *C. jejuni* strain 11168H MOI 100 for 2h at 4^{o}C. Binding was analysed by flow cytometry. Histograms are representative of two independent experiments. (e) Mean bacterial binding index values ± SEM from two independent experiments.

(a)

(b)

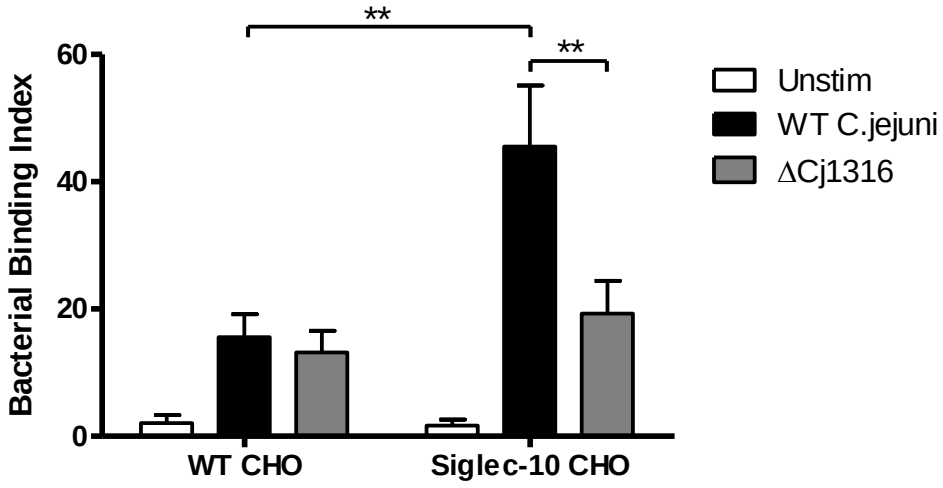


Figure 4-12: Pseudaminic acid structures on *C. jejuni* flagella mediate Siglec-10 binding

3 x 10^5 sialidase-treated WT or Siglec-10 CHO cells with FITC-labelled WT *C. jejuni* strain 11168H, or isogenic mutant strains *ΔflaA, ΔCj1316, ΔwaaF* MOI 100 for 2h at 4^{o}C. Binding was analysed by flow cytometry. (a) Histograms are representative of three independent experiments. (b) Mean bacterial binding index values ± SEM from six independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

4.4 *C. jejuni*-mediated inflammasome activation is IPAF-independent

The *ΔflaA* flagellin mutant of *C. jejuni* 11168H strain induces significantly less IL-1β secretion than the WT strain (Figure 3-6). IPAF (NLRC4) is an intracellular NLR that recognises the flagellin protein and the type III secretion apparatus of bacteria (*e.g. Salmonella typhimurium*), this interaction initiates a cascade culminating in caspase-1 activation and secretion of bioactive IL-1β and IL-18 (Franchi et al. 2006; Miao et al. 2010b). Amino acid alignment revealed that the *C. jejuni* FlaA protein contains critical amino-acids required for the activation of IPAF (Figure 4-13a) (Miao et al. 2010b), we therefore tested the ability of *C. jejuni* to activate the inflammasome (caspase-1 cleavage) via IPAF and other NLR proteins.

Activation of the inflammasome can also induce pyroptosis, an alternative form of cell death to apoptosis and necrosis (Miao et al. 2010a). Trypan blue exclusion revealed minimal cell death in BMDCs as a consequence of co-culture at an MOI 100 for 24h suggesting pyroptosis is unlikely being induced by *C. jejuni* (data not shown). To decipher the potential crosstalk between *C. jejuni* and the inflammasome machinery, infections were conducted in BMDCs lacking individual NLRs, this included IPAF, NALP3, and ASC KO BMDCs. Phagocytosis rates after a standard 2h co-culture revealed comparable levels of uptake between the inflammasome mutants and WT BMDCs (Figure 4-13a). The bactericidal activities of the different mutants were also comparable to the WT cells; all internalised bacteria were killed by 24h post-infection (Figure 4-13b). This data showed efficient killing of internalised *C. jejuni* was independent of IPAF-, NALP3- and ASC-dependent inflammasome activation.

The ability of *C. jejuni* to induce IL-1β secretion was almost completely abrogated in the NALP3- and ASC-deficient BMDCs (Figure 4-14). In IPAF$^{-/-}$ BMDCs IL-1β secretion levels were comparable to WT in response to both *C. jejuni*- or LPS-stimulation, suggesting that IPAF plays a minimal role in IL-1β responses during *C. jejuni* infection (Figure 4-14a). Interestingly, LPS stimulation alone induced ~50% of IL-1β levels as *C. jejuni* despite being an inflammasome-independent PAMP. Western blot analysis revealed the 31KDa pro-IL-1β was induced to comparable levels between the different inflammasome KO cells (Figure 4-14b). A faint 17KDa band (corresponding to the bioactive cleaved IL-1β) was visible at 4h and 8h post-infection in WT and IPAF$^{-/-}$ but not in NALP3$^{-/-}$ and ASC$^{-/-}$ BMDCs, which

(a)

532 RDVDFAAESANYSKANILAQSGSYAMAQANSVQQN**V**LR**L**LQ 572
. | . | : | . | . : | . | : | . | | . | : | : . . : | | | | . | . | | | | . | | :
332 EDSDYATEVSNMSRAQILQQAGTSVLAQANQVPQN**V**LS**L**LR 372

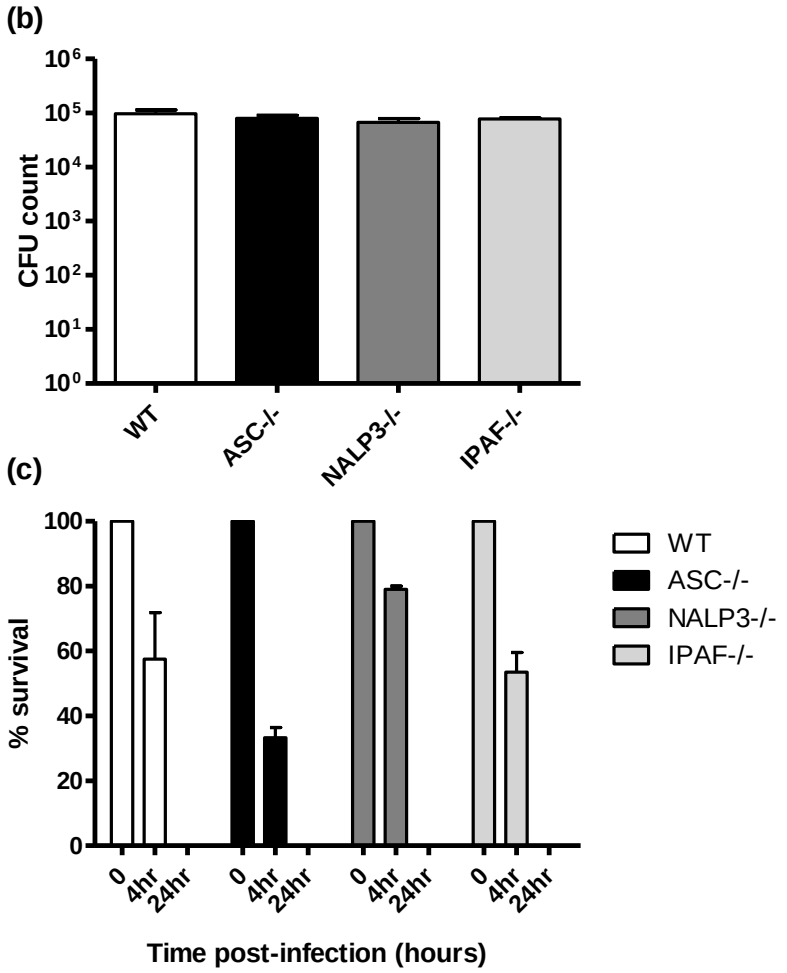
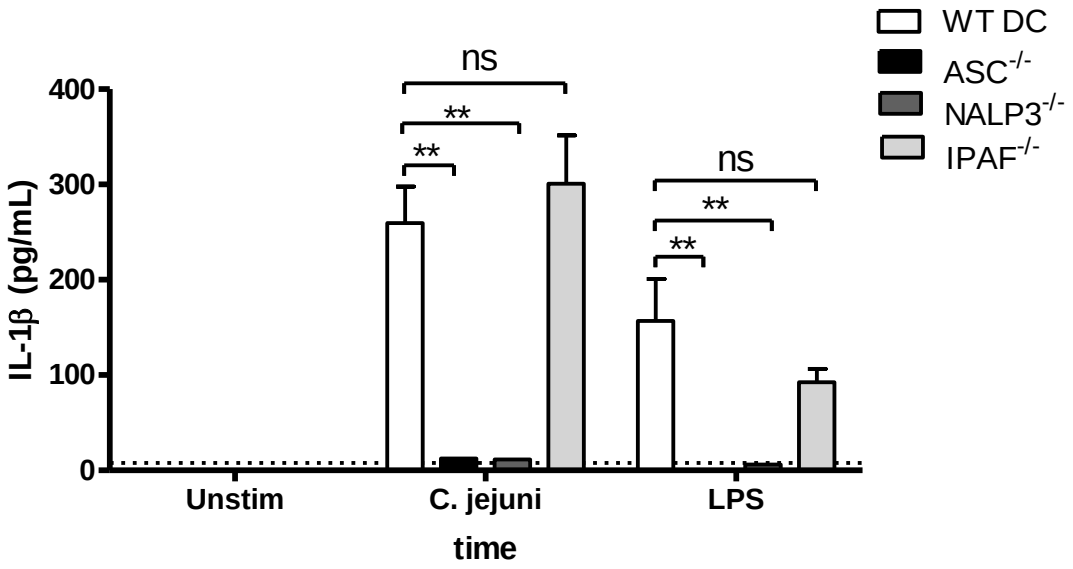


Figure 4-13: ASC, NALP3, and IPAF do not alter bactericidal activity of BMDCs

(a) Sequence alignment for C-terminal *C. jejuni* FlaA (top line) and *S. typhimurium* FliC (bottom line). Known critical residues for IPAF recognition are underlined (Miao et al. 2010b) (b) 2 x10 ^{5} WT (WT), ASC$^{-/-}$, NALP3$^{-/-}$, and IPAF$^{-/-}$ BMDCs were co-cultured with *C. jejuni* 11168H MOI 100 for 4h. Viable intracellular bacterial numbers were enumerated by the gentamicin protection assay. Values are means ± SEM. (c) 2 x10 ^{5} WT (WT), ASC$^{-/-}$, NALP3$^{-/-}$, and IPAF$^{-/-}$ BMDCs were co-cultured with *C. jejuni* 11168H MOI 100 for 2h and incubated in media containing gentamicin for the indicated time-points. Viable intracellular bacterial numbers were enumerated by the gentamicin protection assay. Values are means ± SEM. Survival values are given as a percentage of the bacterial numbers enumerated at the 0h time point.

(a)



(b)

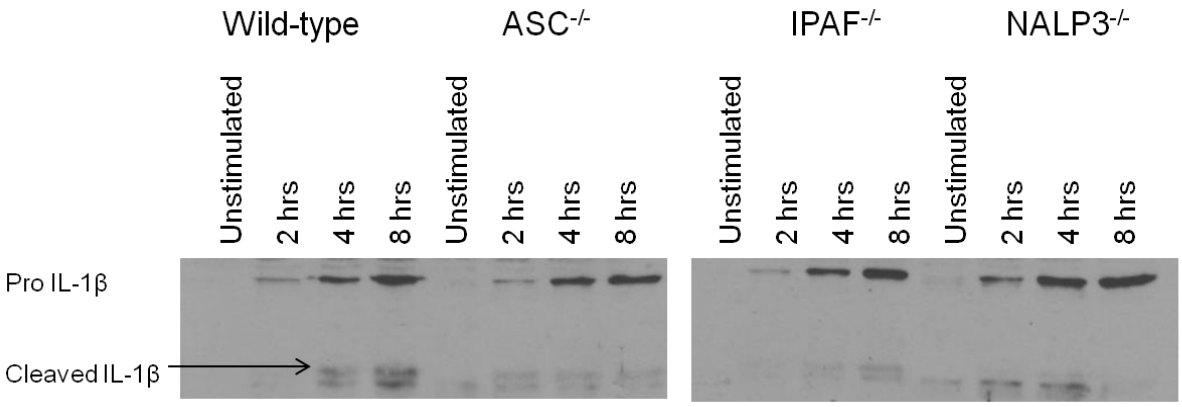


Figure 4-14: ASC and NALP3 but not IPAF are essential for the IL-1β secretion in response to *C. jejuni*

1 x10^6 WT, ASC$^{-/-}$, IPAF$^{-/-}$, and NALP3$^{-/-}$ BMDCs were co-cultured with *C. jejuni* 11168H at an MOI 100. (a) IL-1β secretion was analysed by ELISA 24h post-infection. Values are means ± SEM from four independent experiments. (b) Lysates were subjected to SDS-PAGE followed by Western blot analysis using an antibody that recognizes both the pro- and cleaved- form of IL-1β. Western blots are representative of three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

confirmed the ELISA data (Figure 4-14). As a control, the supernatants from stimulated inflammasome KO cells were analysed for the secretion of IL-10. Figure 4-15 shows that the inflammasome KO cells were capable of secreting IL-10 in response to *C. jejuni* to comparable levels as those seen with WT BMDCs. Collectively this data indicated that *C. jejuni* flagella did not engage with IPAF in mediating inflammasome activation. In contrast, a dependence on NALP3 and ASC was documented.

4.5 *C. jejuni* activated BMDCs promote Th1 mediated immunity

We and others have previously shown that *C. jejuni* promotes Th1 and Th17 responses in human and mouse DC/T cell co-culture systems (Edwards et al. 2010; Rathinam, Hoag, & Mansfield 2008). In this study the role and contribution of the flagella in the activation of T cell responses was elucidated (Figure 4-16). BMDCs were infected for 24h with WT, *ΔflaA*, or 100ng/mL *C. jejuni* LOS. After gentamicin treatment the BMDCs were co-cultured for 4 days with freshly isolated naïve $CD4^+$ T-cells from the spleen in the presence of activating anti-CD3 beads. Supernatants were subsequently analysed for the presence for the Th1 cytokine, IFNγ, the Th2 cytokine, IL-4, and the Th-17 cytokine, IL-17A. In control experiments neither BMDCs nor T cells alone secreted significant levels of these cytokines (data not shown). Naïve T cells cultured with uninfected BMDCs secreted detectable levels of IFNγ, IL-4, and IL-17A (Figure 4-16). Interestingly, WT *C. jejuni* induced higher levels of IFNγ above the unstimulated control although this was not statistically significant. The flagella mutant *ΔflaA* induced similar levels of IFNγ as the WT strain, showing the induction of Th1 responses was independent of the flagella. This was largely expected as the levels of the Th1 polarising cytokine, IL-12 was similar between the WT and mutant strain (Figure 3-6) although minor differences in the level of the maturation marker CD40 had been observed. Both WT and *ΔflaA* strains failed to induce IL-4 and IL-17A levels above the levels of the unstimulated control. IL-17A in the unstimulated control were high (>1000pg/mL) which may explain why the bacterium failed to induce IL-17A unlike the reported induction in the human co-culture system (Edwards et al. 2010). This data showed a propensity of *C. jejuni*-stimulated BMDCs to induce Th1 polarisation independent of the flagella.

4.6 Discussion

At the start of this study little was known as to how *Campylobacter jejuni* triggers TLRs to drive inflammatory responses in innate immune cells. It was interesting to find a complete dependence in BMDCs on TLR signalling for the induction of maturation markers and cytokine secretion in response to *C. jejuni* (Figure 4-3, Figure 4-2). The importance of TLR4

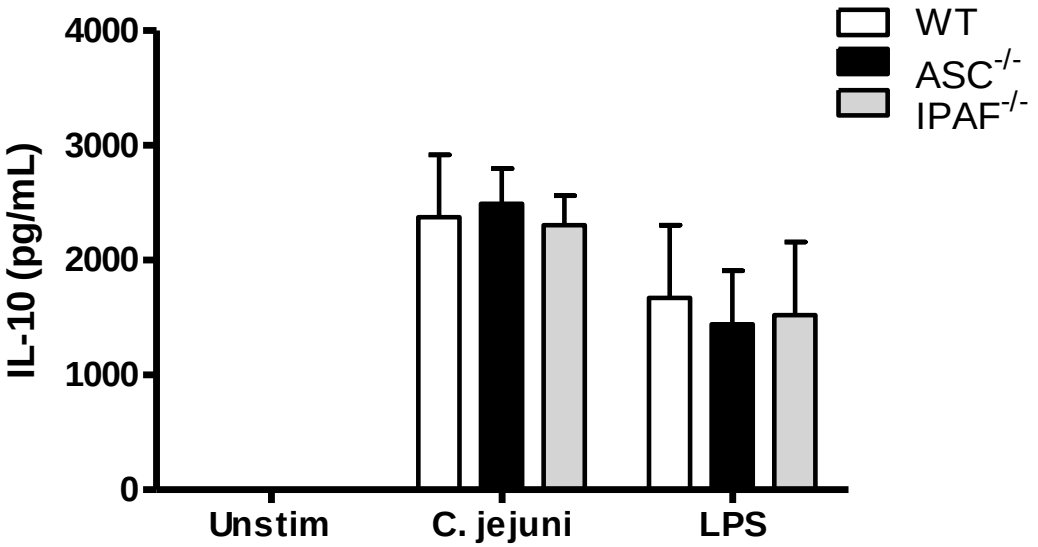


Figure 4-15: IPAF plays no role in the induction of IL-10 by *C. jejuni* flagella

1 x10^6 WT, $ASC^{-/-}$, and $IPAF^{-/-}$ BMDCs were co-cultured with *C. jejuni* 11168H at an MOI 100 or LPS (1µg/mL) for 24h. IL-10 levels were assessed by ELISA. Values are means ± SEM from three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant.

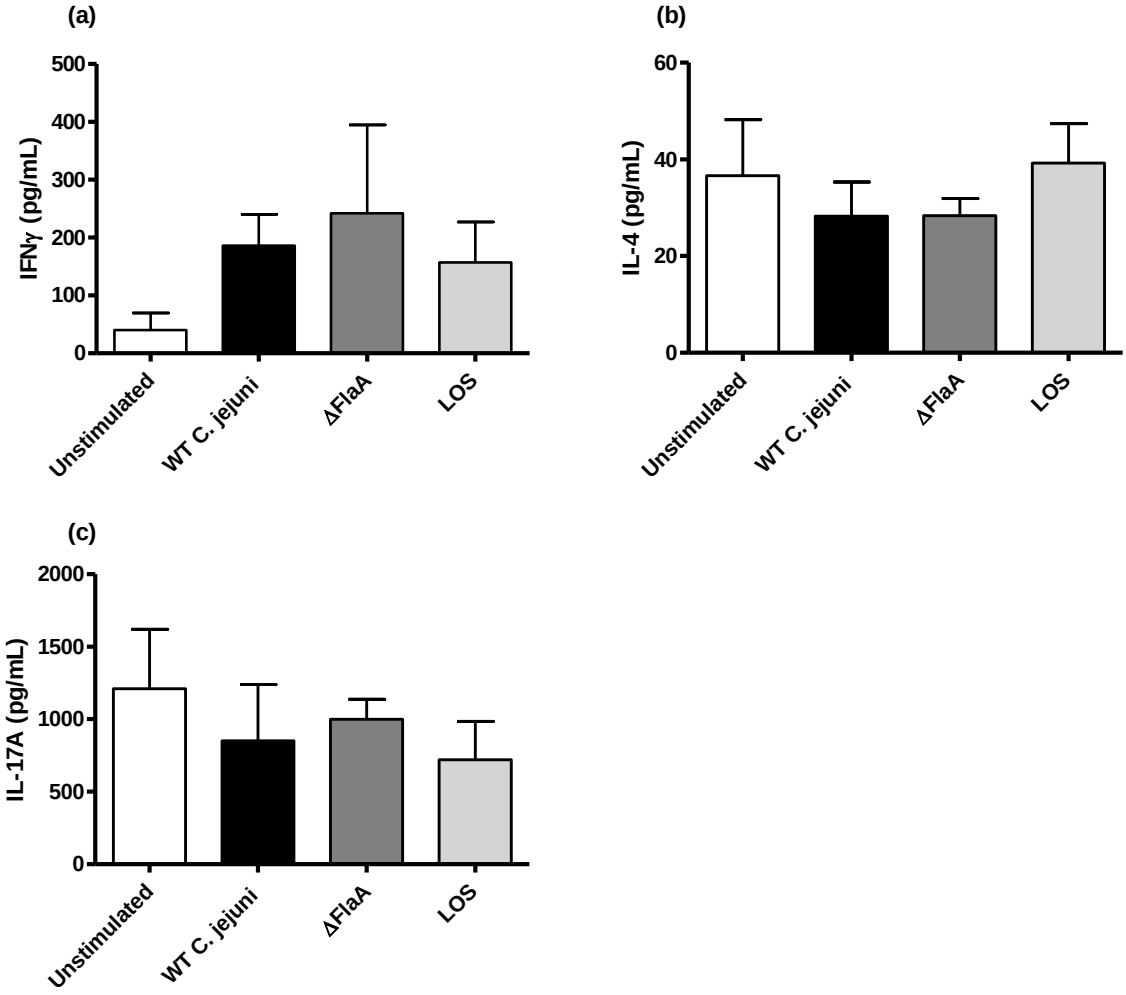


Figure 4-16: *C. jejuni* drives Th1 responses independent of the flagella

$CD4^+$ naïve T-cells were co-cultured for 4 days with BMDCs pre-stimulated with WT *C. jejuni, ΔflaA,* or *C. jejuni* LOS (100ng/mL). (a) IFNγ (b) IL-4 (c) IL-17A levels were analysed by ELISA. Values are means ± SEM from four independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant.

(MyD88- and TRIF-dependent) and TLR2 (MyD88-dependent) in the recognition of *C. jejuni* by BMDCs has since been reported (Rathinam et al. 2009). A dependency for both TLR4 and TLR2 signalling was required for the induction of both maturation markers and pro-inflammatory cytokines, although TLR4 signalling was shown to have a greater impact than TLR2 in these pro-inflammatory responses. Interestingly, live *C .jejuni* is a poor stimulator of TLRs whereas as lysed bacteria are potent stimulators of TLR4 and TLR1/2/6 (de Zoete et al. 2010). Differential dependency for either MyD88- or TRIF- signalling was observed for the secretion of various cytokines (Figure 4-3). The necessity of TLR signalling for the induction of IL-10 was previously unknown, and it was interesting to find a complete dependence on MyD88 signalling for its induction (Figure 4-3e). The ability of TLR4 to trigger both MyD88 and TRIF signalling whereas other potential TLRs in the recognition of *C. jejuni* trigger only MyD88 signalling suggested that IL-10 induction is most likely TLR4-independent or solely driven by the TLR4-MyD88 axis. TLR2 signalling is known to be important for IL-10 secretion triggered by other pathogens or microbial stimuli (Manicassamy et al. 2009; Sing et al. 2002). *C. jejuni* CPS has been implicated in the secretion of IL-6 by IECs via TLR2, although the purity of the CPS maybe questionable as complete removal of other microbial products such as LOS and lipoproteins during CPS purification remains challenging (Friis, Keelan, & Taylor 2009). The CPS isogenic mutant *ΔkpsM* did not influence IL-10 secretion negating a role for this bacterial structure in its regulation (Figure 3-4). The surface lipoprotein, JlpA, binds to IECs and triggers pro-inflammatory cytokine induction (Jin et al. 2003), and has been proposed to be the ligand for TLR2 recognition of *C. jejuni* (Rathinam et al. 2009).The shared dependency on TRIF- and MyD88-signalling for the induction of maturation markers and IL-12 family cytokines suggests TLR4 signalling may be important for their induction (Figure 4-2, Figure 4-3). This is supported by previous reports that $TLR4^{-/-}$ BMDCs have marked reduction in IL-12 secretion in response to *C. jejuni* and that the purified LOS drives IL-12 secretion from human monocyte-derived DCs (Hu et al. 2006; Rathinam et al. 2009). The use of BMDCs from specific TLR KO mice would elucidate specifically which TLR(s) are critical in the induction of IL-10 and whether they differ for IL-12 induction. It is interesting to hypothesise that *C. jejuni* has evolved to induce high IL-10 levels through the triggering of MyD88-dependent TLR signalling to dampen the immune system. Similar mechanisms are at play during *Yesinia sp.* and *Helicobacter pylori*/DC interactions (Hu et al. 2006;Sing et al. 2002; Wang et al. 2010). Although the acute inflammation seen in the majority of people infected with high doses of *C. jejuni* suggests this anti-inflammatory mechanism can be over-ridden by other pro-inflammatory stimuli (Hu et al. 2006; Tribble et al. 2010).

The ability of early (within 10mins post-infection) ERK signalling to occur in the absence of TLR-signals suggests alternative PRRs may be triggered in response to *C. jejuni* (Figure 4-4). However, this early ERK response varied between experiments and was not always observed (Figure 3-10). The strong activation of all three MAPK pathways between 1-2h post-infection was completely dependent on TLR signalling (Figure 4-4). The kinetics of activation suggested that the bacteria may need to be phagocytosed prior to initiation of TLR signals. This is supported by reports showing the inhibition of *C. jejuni* phagocytosis by chemical inhibitors blocks the secretion of pro-inflammatory cytokines (Rathinam et al. 2008). Additionally, TLR4 and TLR2 are expressed in the endosomal compartments of human DCs and not on the cell surface (Uronen-Hansson et al. 2004). MyD88-dependent signalling appears to have a greater role in MAPK activation than TRIF-dependent signalling (Figure 4-4). For IL-10 induction there is an interesting correlation between the necessities of MyD88 signalling, MAPK signalling, and the ability of MyD88-dependent events to drive MAPK activation (Figure 3-13, Figure 4-3, Figure 4-4). Conversely, IL-12 induction is not influenced by MAPK inhibition, is both TRIF- and MyD88-dependent, and TRIF signalling does not influence MAPK activation. It is possible that a particular TLR, independent of TRIF, triggers MAPK activation and subsequently IL-10; whereas as TLR4 activation triggers NF-κB activation and subsequent production of IL-12 related cytokines. Interestingly, TLR2 and TLR4 mediated MyD88-dependent signalling requires the adaptor molecule Mal, which via p38 activates the transcription factor CREB leading to transcription and subsequent expression of IL-10 (Mellett et al. 2011).

The finding that the modification of *C. jejuni* flagellin protein, FlaA, with a derivative of Pse is important for the induction of high levels of IL-10 suggested a possible role of carbohydrate recognition receptors in *C. jejuni*-BMDC interactions (Figure 3-17). In this study we found the ability of WT *C. jejuni* strain 11168H to bind to Siglec-10 to be specific, which was not previously thought (Avril et al. 2006). Siglec-10 bound to *C. jejuni* flagella Pse5Ac7Am structures in a manner independent of the sialic acid structures on the LOS (Figure 4-12). It is tempting to hypothesise that the interaction between Pse5Ac7Am structures on the flagella is a pathogen driven strategy to manipulate host antimicrobial responses, including effects on anti-inflammatory cytokines such as IL-10. This interaction allows novel means to target and modulate TLR signalling, a signalling pathway critical for IL-10 expression (Figure 4-3). Previous reports have implicated Siglecs in host IL-10 immunity. Overexpression of Siglec-9 and Siglec-5 in the macrophage cell-lines, RAW264 and THP-1, followed by TLR stimulation results in increased IL-10 production with a

parallel decrease in TNF-α (Ando et al. 2008). Tyrosine residues in the intracellular domain of Siglec-9 are critical for the recruitment of downstream signalling molecules SHP-1 and SHP-2, events involved in immune modulation. Siglecs are largely immunomodulatory and have been hypothesised to be important in distinguishing "danger-associated" *versus* "pathogen-associated" signals (Chen et al. 2009). The ability of pathogens to express sialic acid and engage Siglecs to dampen immune responses via molecular mimicry can be considered an evolutionary advantage, as demonstrated by the ability of group B *streptococcus* to engage Siglec-9 on neutrophils and reduce their oxidative burst capabilities (Carlin et al. 2009b). It has been hypothesised, to counterbalance the ability of particular pathogens to engage Siglecs and dampen immune responses, paired receptors have evolved which are composed of the same extracellular domain but have altered the intracellular domain and are therefore able to activate signalling pathways (Cao and Crocker 2011a).

As yet there are no reports investigating the influence of Siglec engagement on MAPK activation, therefore whether the altered activation of p38 by the flagella mutant was caused by its inability to bind to Siglec-10 requires clarification. There is however precedence in the literature as ligation of the C-type lectin receptor, Dendritic-cell-associated C-type lectin-2 (DCAL2; which contains an ITIM) modulates MAPK activation and IL-10 production (Chen et al. 2006). Analysis of MAPK activation in either specific Siglec KO BMDCs or Siglec overexpressing DCs would aid in determining whether Siglec engagement can alter these signalling pathways. The ability of Siglec-E to dampen TLR-induced pro-inflammatory cytokine induction in antibody cross-linking experiments occurs via dampening of NF-κB (Boyd et al. 2009). In addition, engagement of CD24 and Siglec-G/siglec-10 to the DAMP high mobility group box 1 (HMGB1) leads to a decrease in pro-inflammatory responses, again via NF-κB regulation (Chen et al. 2009). How Siglec and other carbohydrate receptors intercept NF-κB and MAPKs in fine tuning host immunity is an emerging area of host-pathogen research.

In addition to the binding of sialic acid in the sialic acid binding pocket, Siglecs are able to bind other ligands on different parts of the receptor (Carlin et al. 2009a; Kivi et al. 2009). In the present study we did not undertake experiments to define if the Pse5Ac7Am structure of *C. jejuni* flagella binds directly to the sialic acid binding site of Siglec-10. The first partial crystal structure of a Siglec-family member, the N-terminal domain Sialoadhesin, revealed critical interactions in the carbohydrate binding domain with its ligand, 3-sialylactose (May

et al. 1998). Hydrogen bonds are predicted to form between the hydroxyl groups on the 8th and 9th carbon as well as the 4th carbon of the pyranose ring and amide group of the N-acetyl group. The carboxylate group is also predicted to form salt bridges. The structure of pseudaminic acid is very similar to N-acetyl neuraminic acid (sialic acid) (Figure 1-4). The carboxylate group, hydroxyl groups on the 4th and 8th carbon, and amide group on the 5th carbon are all conserved. Siglecs have varying specificities for sialic acid structures, with linkage conformation and underlying carbohydrate backbone structure playing critical roles in their ligand specificity. As yet, the structure of Siglec-G and Siglec-10 is unknown; therefore the potential binding of Pse5Ac7Am to these receptors are a matter for future research.

In the monocyte/macrophage cell line, THP-1, *C. jejuni* is able to induce high levels of IL-1β in the range of 2000pg/mL 24h post-infection (Siegesmund et al. 2004). In contrast, levels of IL-1β induced by BMDCs in responses to *C. jejuni* 24h post-infection were far lower, in the range of 200-400pg/mL (Figure 4-14a). The disparity between the levels of IL-1β in the different cell types may be due to differential regulation of caspase-1. Monocytes have been shown to constitutively activate capase-1 and therefore only require a TLR-stimulus to induce high levels of IL-1β (Netea et al. 2009). Conversely, macrophages and DCs do not express constitutively active caspase-1, therefore in addition to a TLR-stimulus which drives the production of pro-IL-1β, a second signal such as adenosine triphosphate (ATP) or muramyl dipeptide (MDP) is required to activate the inflammasome which results in caspase-1 cleavage and the eventual processing of IL-1β (Mariathasan et al. 2006). The low level of IL-1β in *C. jejuni* stimulated BMDCs despite the use of high MOIs suggests that *C. jejuni* is a poor inducer of inflammasome function. LPS stimulation reportedly does not induce IL-1β secretion in DCs due to a lack of constitutively activated caspase-1, therefore it is interesting to find LPS stimulation induces 50% of the levels of IL-1β that *C. jejuni* induces (Netea et al. 2009). One explanation is the possible contamination of the LPS with other microbial products that could act as the second signal for caspase-1 cleavage, such as MDP which activates NALP3. Alternatively, there may be minimal constitutive activation of capase-1 creating a "leaky system" that allows the activation of TLRs alone to drive secretion of minimal levels of mature IL-1β. Interestingly, both NALP3 and the more broadly used adaptor protein ASC were necessary for the induction of IL-1β from both the *C. jejuni*- and LPS-stimulated BMDCs. The necessity of this receptor and adaptor molecule has already been reported in the constitutive activation of caspase-1 in monocytes,

therefore neither of the possible explanation for the induction of IL-1β by LPS can be eliminated (Netea et al. 2009).

Despite the flagellin protein, FlaA, sharing many "critical" amino acids that have been identified in the activation of IPAF, *C. jejuni* did not require IPAF in the induction of IL-1β (Figure 4-14a and b). The activation of IPAF by the flagellin proteins of other pathogens requires the translocation of the flagellin monomers directly into the cytosol, which is achieved via a type III secretion apparatus or artificially *in vitro* by transfection of the monomers (Sun et al. 2007). As IPAF is dispensable for the secretion of IL-1β by *C. jejuni* it is likely that the bacterium does not actively secrete flagellin monomers into the BMDC cytosol. There are two possible explanations for the reduced levels of IL-1β seen with the flagellin mutant, *ΔflaA*. Firstly, differential induction of pro-IL-1β between the WT and flagellin mutant may occur. Alternatively, the ability of the motility-deficient bacterium to enter the cytosol triggering ASC- and NALP3-dependent inflammasome activation may be altered; although there is currently no evidence which proves *C. jejuni* is able to enter the cytosol in DCs.

C. jejuni-stimulated BMDCs were able to induce a strong IFNγ responses in T-cells (Edwards et al. 2010;Rathinam, Hoag, & Mansfield 2008), independent of the flagella (Figure 4-16a), but did not induce significant levels of IL-4. Strong IFNγ responses that are specific to *C. jejuni* antigens correlate with protection against re-infection, which suggests Th1 responses are critical in the clearance of the pathogen and in limiting infection to an acute disease (Tribble et al. 2010). Interestingly, the ability of *H. pylori*-stimulated DCs to induce regulatory T cells (Treg) skewing is via its ability to induce high levels of IL-10 and TGF-β (Kao et al. 2010), therefore it would be interesting to analyse the ability of *C. jejuni* to induce Tregs in the future.

Chapter 5.

Structural Analyses of *C.* jejuni Lipooligosaccharide from Different Phylogenetic Clades

5.1 Background

There are multiple reservoirs of *C. jejuni* that include not only livestock but also environmental or non-livestock sources such as contaminated water supplies and crops (Gardner et al. 2011; Kemp et al. 2005). Despite the microaerophillic nature favoured by *C. jejuni*, the bacteria can survive and grow in water in laboratory tests which suggests that *C. jejuni* may be able to adapt to ecological niches outside a mammalian or avian host (Tatchou-Nyamsi-Konig et al. 2007). The genetic adaptation to specific ecological niches by *C. jejuni* is beginning to be appreciated. Wren and colleagues investigated the phylogenetic relationship of 111 different *C. jejuni* strains isolated from either asymptomatic human carriers, human clinical isolates, livestock (chicken, bovine, and ovine) and environmental sources and the authors found a phylogenetic divide between livestock and environmental sources (Champion et al. 2005). Interestingly, the human clinical isolates are spread across the two phylogenetic clades suggesting that different genetic necessities promote livestock colonisation *versus* those that cause enteritis in humans. A key genetic locus that distinguishes the two phylogenetic clades includes the genes involved in the legionaminic acid (Leg) biosynthesis pathway. The deletion of this genetic cluster leads to a reduction in *C. jejuni* colonisation in chickens (Howard et al. 2009). However, certain chicken isolates lack this genetic cluster suggesting that multiple genetic loci are likely to be important in livestock colonisation.

The LOS structure varies widely amongst *C. jejuni* strains. The major variation lies in the outer core region of the oligosaccharide (OS) (Figure 1-2) (Dorrell et al. 2001), although diversity in the phosphorylation and number of amide linkages of the lipid A also exist (Figure 5-1) (Szymanski et al. 2003). Further, the level of phosphorylation and the abundance of amide linkages can vary between growths of the same *C. jejuni* strain suggesting that LOS structure may actively respond to the bacteria's immediate environment (*e.g.* laboratory culture broth *versus* host nutritional status plus additional impact of the host microbial composition); such dynamic changes may promote virulence. In addition to environment-driven LOS modification(s), genetic variation in the *C. jejuni* LOS locus is also documented. In the present study we wished to test the hypothesis that structural variation in the LOS not only lends to differential cross-talk with the host innate immune system but this moiety may also contribute to defining the phylogenetic separation of *C. jejuni* strains.

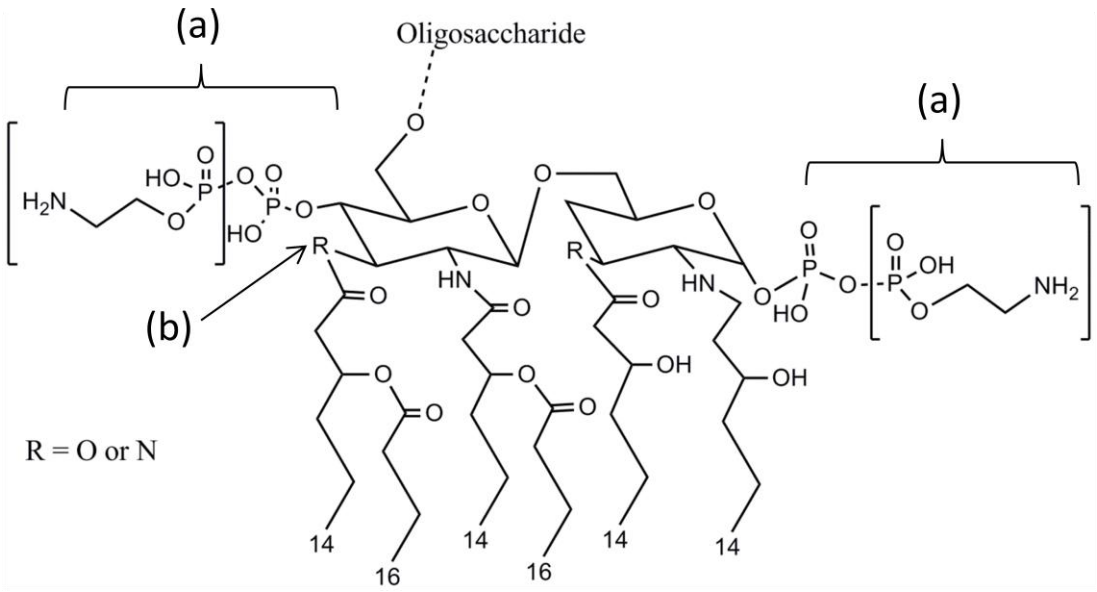


Figure 5-1 *C. jejuni* Lipid A structure

Hexacylated lipid A bearing (a) two phosphate and two phosphoethanolamine residues. Acyl chains are linked by either (b) ester (R = O) or amide (R = N) bonds.

5.2 Isolation of the lipooligosaccharide (LOS) moiety from *C. jejuni* strains

To assess whether the LOS structure is important in the distinction of the two phylogenetic clades, the LOS moiety from 15 *C. jejuni* strains (7 and 8 strains from livestock (including reference strain 11168H) and non-livestock (environmental) clades respectively) were purified (Table 5-1). Of the 7 livestock-associated strains, 3 were human clinical isolates (patients presenting with diarrhoea or bloody diarrhoea), 2 were from asymptomatic carriers, and 2 were from colonised livestock. Of the 8 environment-associated strains 4 were human clinical isolates (patients presenting with diarrhoea or bloody diarrhoea) and 4 were from asymptomatic carriers.

To perform LOS structure and functional analyses, 1L broth cultures were grown for each strain under micro-aerophillic conditions for 24-48h. LOS was isolated using the hot-phenol method of extraction (Chapter 2.9.1). Yields of between 2-15mg were achieved and were quantified on a microbalance. Purity was assessed by SDS-PAGE and subsequent silver staining (Figure 5-2). High purity was achieved as no other bands were detected. The size of the LOS differed between the various preparations, suggesting that structural differences may occur between the strains. To assess these possible differences, Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis was

performed. The high purity suggested that the extracted LOS could be used directly without any further purification for structure (this chapter) and functional studies (Chapter 6).

5.3 MALDI-TOF analysis of O-Deacylated LOS

MALDI-TOF MS analysis allowed structural determination of the 15 LOSs' isolated. MALDI-TOF MS was performed on O-deacylated LOS samples to analyse lipid A amide linkages and determine the OS composition (described here). MALDI-TOF MS was also performed on the intact LOS to analyse lipid A phosphorylation status (Chapter 5.5). Sample preparation and ~80% of the MALDI-TOF spectra acquisitions' were conducted by or under the direct guidance of Dr. C. John (UCSF, USA). The remaining data was obtained by Dr. John. Removal of the O-linked acyl chains (O-deacylation) of the lipid A backbone aided MALDI-TOF analysis, in particular for the acquisition of detailed spectra for the full length species (lipid A + OS). *C. jejuni* is known to have varying numbers of amide and ester bonds linking the acyl chains to the lipid A backbone (Figure 5-1) (Van Mourik et al. 2010). The sugar components of the disaccharide lipid A backbone are either 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N; 2 amide linkages) or D-glucosamine (GlcN; 1 amide linkage). Varying amounts of GlcN3N-GlcN3N, GlcN-GlcN3N, and GlcN-GlcN lipid A backbones have been observed amongst *C. jejuni* strains, although a comparison of abundance of these linkages from different strains has not been performed (Szymanski et al. 2003). As the O-deacylation treatment removes only the acyl chains that are linked via ester bonds (not amide bonds), it was possible to quantify the relative abundance of each disaccharide composition (GlcN3N-GlcN3N, GlcN-GlcN3N, and GlcN-GlcN). However, O-deacylation treatment also removed all O-linked amino acids and O-linked acyl modifications of the OS in addition to removing PEA residues on the lipid A core; therefore MALDI-TOF analysis of the native LOS was also performed.

Strain	Phylogenetic Clade	Clinical Presentation
43205	E	Bloody Diarrhoea
32787	E	Asymptomatic
31481	E	Asymptomatic
56519	E	Bloody Diarrhoea
64555	E	Bloody Diarrhoea
33106	E	Asymptomatic
33084	E	Asymptomatic
40917	E	Bloody Diarrhoea
31485	L	Asymptomatic
11168H	L	Diarrhoea
45557	L	Bloody Diarrhoea
KJShpSm4	L	Colonised Livestock
56282	L	Diarrhoea
32799	L	Asymptomatic
KJCattle8	L	Colonised Livestock

Table 5-1: *C. jejuni* strains used in this study

Strain numbers, phylogenetic clade, and clinical presentation for each strain

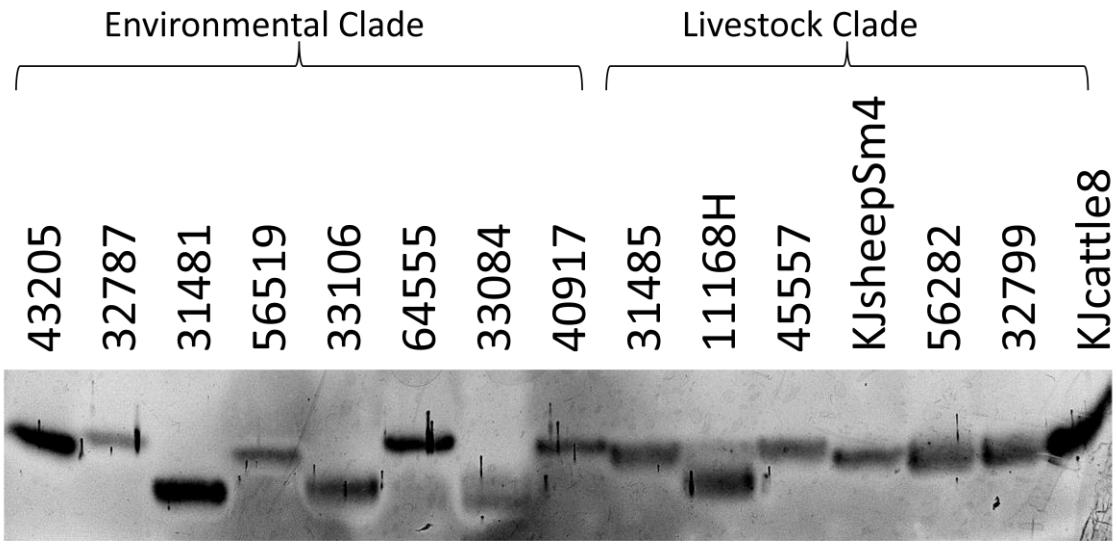


Figure 5-2: Silver stain of purified LOS from 15 different *Campylobacter jejuni* strains

1µg of purified LOS was separated by SDS-PAGE and subsequently silver-stained using silver nitrate.

To O-deacylate the LOS, ~300µg of purified LOS was treated with anhydrous hydrazine. Prior to addition to the matrix, O-deacylated LOS were desalted and added to a 2, 5-dihydroxybenzoic acid (DHB) matrix and subsequently analysed by MALDI-TOF in the negative ion mode (Chapter 2.9.3). Figure 5-3 and Figure 5-4 show a representative spectra for the O-deacylated LOS from each strain of the environmental and livestock clade respectively. Table 5-2 shows the calculated masses for the different lipid A species observed. The presence of GlcN3N-GlcN3N (four amide linkages; *m/z* 1402.6 Da) and GlcN3N-GlcN (three amide linkages; *m/z* 1177.2 Da) lipid A backbone containing two phosphate groups is seen for all of the LOSs' analysed. The presence of lipid A containing only two amide linkages GlcN-GlcN (*m/z* 951.8 Da) was detected in LOS from environmental strains 64555 and 40917 (Figure 5-3f and h) and livestock strain KJcattle8 (Figure 5-4f) and at far lower abundance (Table 5-3). Despite increasing the length of incubation of the LOS with anhydrous hydrazine from 20mins to 2h not all of the O-linked fatty acid chains were removed (under O-deacylated; *m/z* 1641.0 and 1415.6 Da of the GlcN3N-GlcN3N and GlcN3N-GlcN species respectively). Peaks were detected for masses corresponding to the loss of a phosphate group and one water molecule (-98Da; *m/z* 1304.6 and 1079.2; under O-deacylated *m/z* 1543.0 and 1317.6). The loss of the water in conjunction with the phosphate, and the lack of peaks showing loss of water alone, suggested these species were generated by hydrolysis of a phosphate group during the MALDI-TOF analysis and did not correspond to the presence of a monophosphorylated lipid A backbone. The lack of observable peaks for monophosphorylated species (*m/z* 1322.6, 1097.2, and 871.8 Da) agreed with this hypothesis. Peaks at *m/z* 1374.5 and 1149.2 Da corresponded to GlcN3N-GlcN3N and GlcN3N-GlcN backbones containing a 14 carbon (C14) instead of a 16 carbon (C16) acyl chain respectively (under O-deacylated *m/z* 1612.9, 1387.6, and 1162.2).

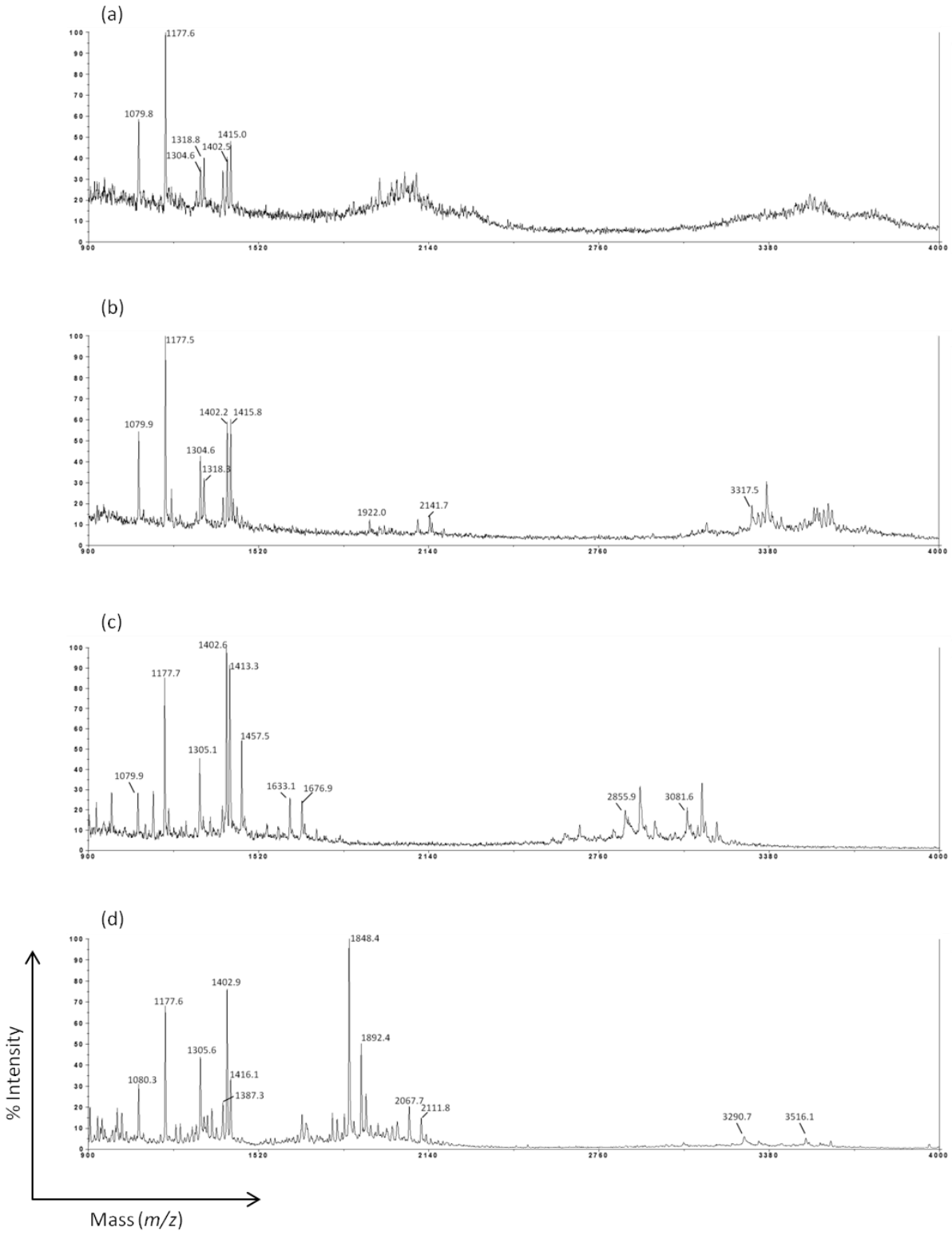


Figure 5-3: MALDI-TOF analysis of O-deacylated environmental phylogenetic clade LOS

Representative spectra from at least two independently acquired spectra for O-deacylated LOS from environmental strains (a) 43205 (b) 32787 (c) 31481 (d) 56519

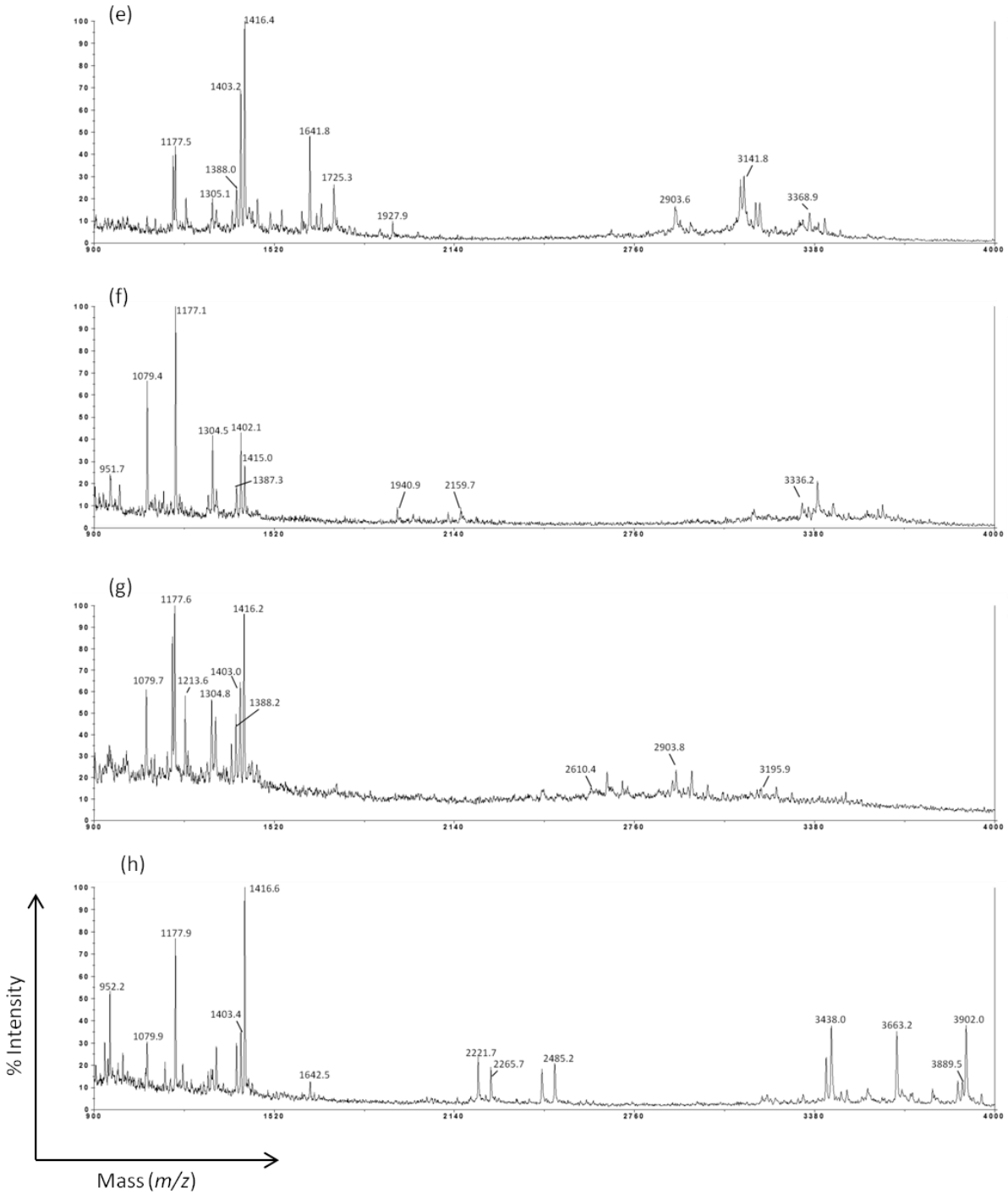


Figure 5-3: MALDI-TOF analysis of O-deacylated environmental phylogenetic clade LOS

Representative spectra from at least two independently acquired spectra for O-deacylated LOS from environmental strains (e) 33106 (f) 64555 (g) 33084 (h) 40917.

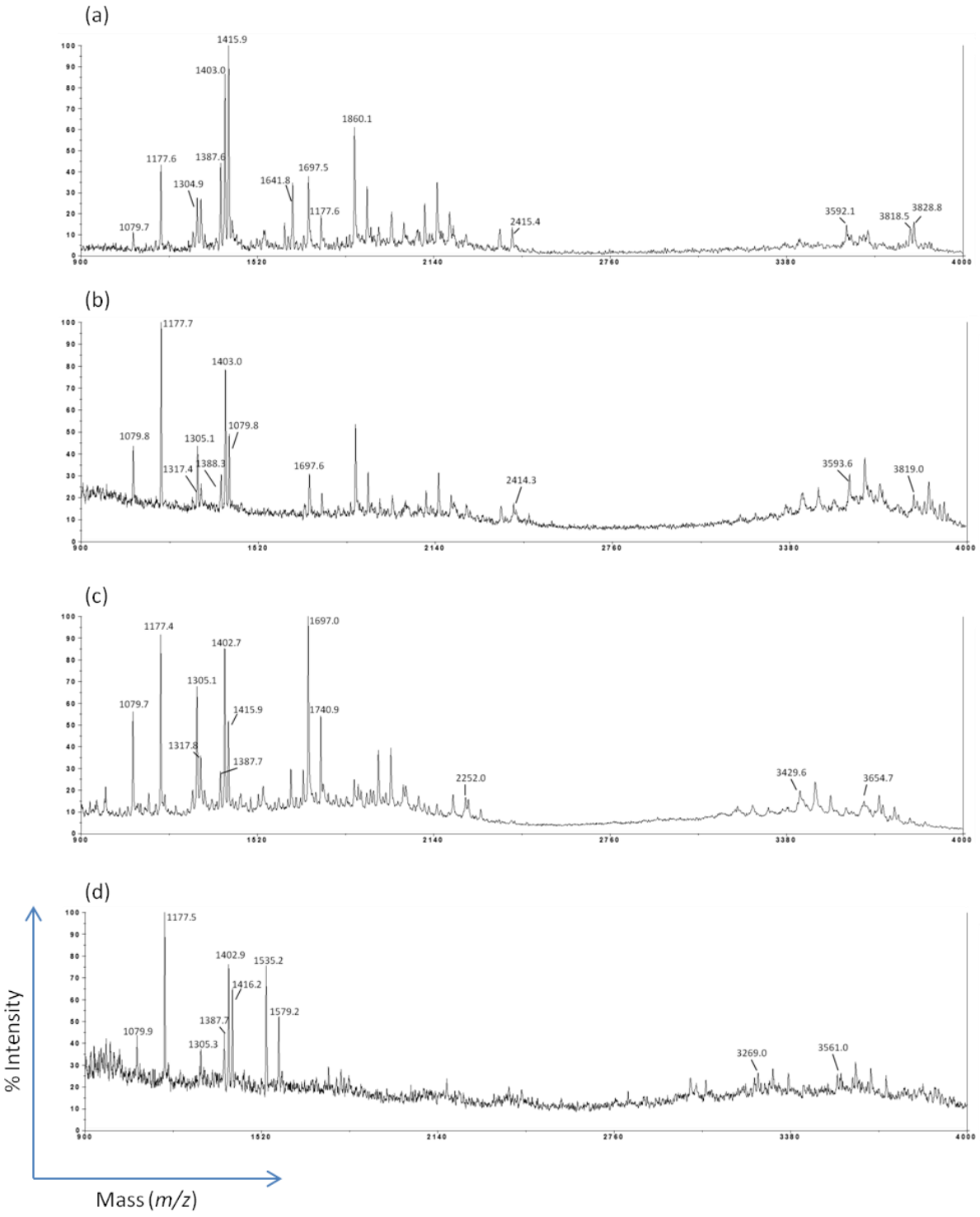


Figure 5-4: MALDI-TOF analysis of O-deacylated livestock phylogenetic clade LOS

Representative spectra from at least two independently acquired spectra for O-deacylated LOS from livestock strains (a) 31485 (b) 45557 (c) KJShpSm4 (d) 56282 (e) 32799 (f) KJcattle8 (g) 11168H

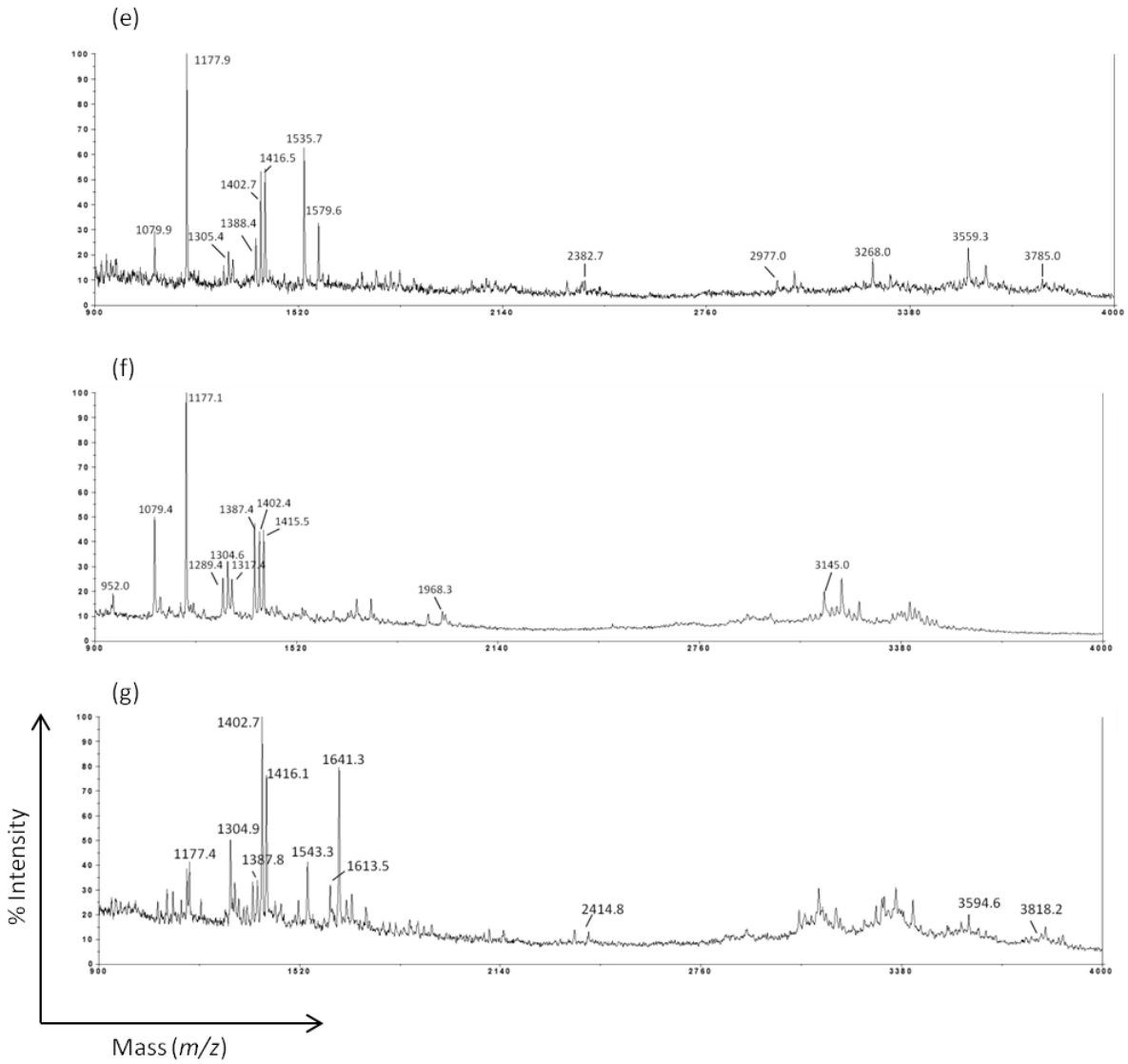


Figure 5-4: MALDI-TOF analysis of O-deacylated livestock phylogenetic clade LOS

Representative spectra from at least two independently acquired spectra for O-deacylated LOS from livestock strains (e) 32799 (f) KJcattle8 (g) 11168H

O-Deacylated lipid A Structure	Calculated mass $(M-H)^-$ (Da)
GlcN3N-GlcN3N-2P	1402.6
GlcN-GlcN3N-2P	1177.2
GlcN-GlcN-2P	951.8
GlcN3N-GlcN3N-2P (C14)	1374.5
GlcN-GlcN3N-2P (C14)	1149.2
GlcN-GlcN-2P (C14)	923.8
GlcN3N-GlcN3N-2P (minus P)	1304.6
GlcN-GlcN3N-2P (minus P)	1079.2
GlcN-GlcN-2P (minus P)	853.8
GlcN3N-GlcN3N-2P (under O-deacylated)	1641.0
GlcN-GlcN3N-2P (under O-deacylated)	1415.6
GlcN-GlcN-2P (under O-deacylated)	1191.2

Table 5-2: MALDI-TOF lipid A species for O-deacylated *C. jejuni* LOS

Calculated mass for the described O-deacylated structures. Lipid A composed of two 2,3-diamino-2,3-dideoxy-D-glucose sugars (GlcN3N-GlcN3N; 4 amide linkages), two D-glucosamine sugars (GlcN-GlcN; 2 amide linkage); or a combination of one D-glucosamine and one 2,3-diamino-2,3-dideoxy-D-glucose sugar (GlcN-GlcN3N; 3 amide linkages). P = phosphate group; C14 = 14 carbon long acyl chain instead of one 16 carbon long acyl chain.

The proportion of the GlcN3N-GlcN3N, GlcN3N-GlcN, and GlcN-GlcN backbone in the LOS for each species was calculated by combining the relative abundance of all the peaks relating to number of amide linkages from two independent spectra (Table 5-3). For the majority of the strains analysed, GlcN3N-GlcN was the most abundant disaccharide backbone observed. Although there was a wide variation in the abundance of four amide linkages (GlcN3N-GlcN3N; range 26.1 – 73.7%, mean 33.5%, standard deviation 14.7%), this did not correlate with the phylogenetic clade or clinical presentation of each strain (Figure 5-5). This data highlighted that variation in the relative abundance of amide and ester linkages of the acyl chains in the lipid A backbone of *C. jejuni* LOS was independent of the ecological niche of the corresponding strain. Additionally, the relative abundance of amide linkages in the lipid A backbone did not account for asymptomatic carriage of *C. jejuni.*

In addition to the acyl linkage analysis, the composition of the OS for each strain was calculated from the O-deacylated spectra as the resolution for the larger masses was higher than for the intact spectra (compare Figure 5-3 and Figure 5-4 to Figure 5-10 and Figure 5-11). The spectra for environmental strain, 31481, showed a peak at *m/z* 1679.9 Da which is consistent with an OS composed of $KDO_2PEAHep_2Hex_2HexNAc_2$; peaks at 1633.3 and 1475.5 corresponded to the loss of CO_2 (-44Da) and KDO (-220Da) from the OS respectively (Figure 5-3c). Full length species at *m/z* 2855.9Da and 3081.6Da corresponded to the OS plus the GlcN3N-GlcN and GlcN3N-GlcN3N backbone respectively. Strain 64555 had a peak at *m/z* 2159.7 Da corresponding to an OS structure of $KDO_2Hep_2Hex_7HexNAc_1$ (Figure 5-3f). Strain 33106 had a peak at *m/z* 1725.3 Da corresponding to an OS structure of $KDO_2PEAHep_2Hex_3$; an additional peak at 1927.9 corresponded to an additional $HexNAc_1$ residue on the OS (Figure 5-3e). Peaks at *m/z* 2903.6, 3141.8 and 3368.9 Da corresponded to the $KDO_2PEAHep_2Hex_3$ OS with the addition of GlcN3N-GlcN, under O-deacylated GlcN3N-GlcN, and under O-deacylated GlcN3N-GlcN3N lipid A backbone respectively. No peaks for the OS alone were observed for strain 33084 (Figure 5-3g). Peaks at *m/z* 3195.9 Da corresponded to a GlcN3N-GlcN lipid A backbone with the addition of a $KDO_2PEAHep_2Hex_6NeuAc_2$ OS. Peaks at *m/z* 2903.8 and 2610.4 Da corresponded to the loss of one and two sialic acid residues respectively. The OS peak for strain 40917 is *m/z* 2485.2 Da corresponding to $KDO_2PEAHep_2Hex_5HexNAc_2NeuAc_1$, and loss of KDO at *m/z* 2265.7 Da Figure 5-3h). Full length peaks at *m/z* 3438.0, 3663.2, 3889.5, and 3902.0 Da corresponded to the OS plus GlcN-GlcN, GlcN3N-GlcN, GlcN3N-GlcN3N, and under O-deacylated GlcN3N-GlcN lipid A backbone respectively. The peaks observed in the OS and full-length range for strains 43205, 32787, and 56519 did not correspond to any predicted carbohydrate

Strain	Phylogenetic	Clinical	GlcN3N-GlcN3N	GlcN3N-GlcN	GlcN-GlcN
43205	E	BD	19.6	77.8	2.7
32787	E	A	35.0	65.0	0.0
31481	E	A	39.5	60.5	0.0
56519	E	BD	48.4	51.6	0.0
64555	E	BD	27.8	70.8	1.5
33106	E	A	38.7	61.3	0.0
33084	E	A	26.2	72.6	1.2
40917	E	BD	13.7	77.1	9.2
31485	L	A	39.6	60.4	0.0
11168H	L	D	73.7	26.3	0.0
45557	L	BD	32.8	67.2	0.0
KJShpSm4	L	CL	37.7	58.7	3.5
56282	L	D	31.3	68.2	0.5
32799	L	A	20.3	79.2	0.5
KJCattle8	L	CL	18.3	74.5	7.2

Table 5-3: Relative abundance of disaccharide backbone composition (amide linkages) for each strain

The relative abundance of GlcN3N-GlcN3N, GlcN3N-GlcN, and GlcN-GlcN lipid A backbone was calculated from two individual spectra for each strain based on the area under the peaks of the masses from Table 0-2. E = environmental strain; L = livestock strain; BD = bloody diarrhoea; D = diarrhoea; A = asymptomatic; CL = colonised livestock.

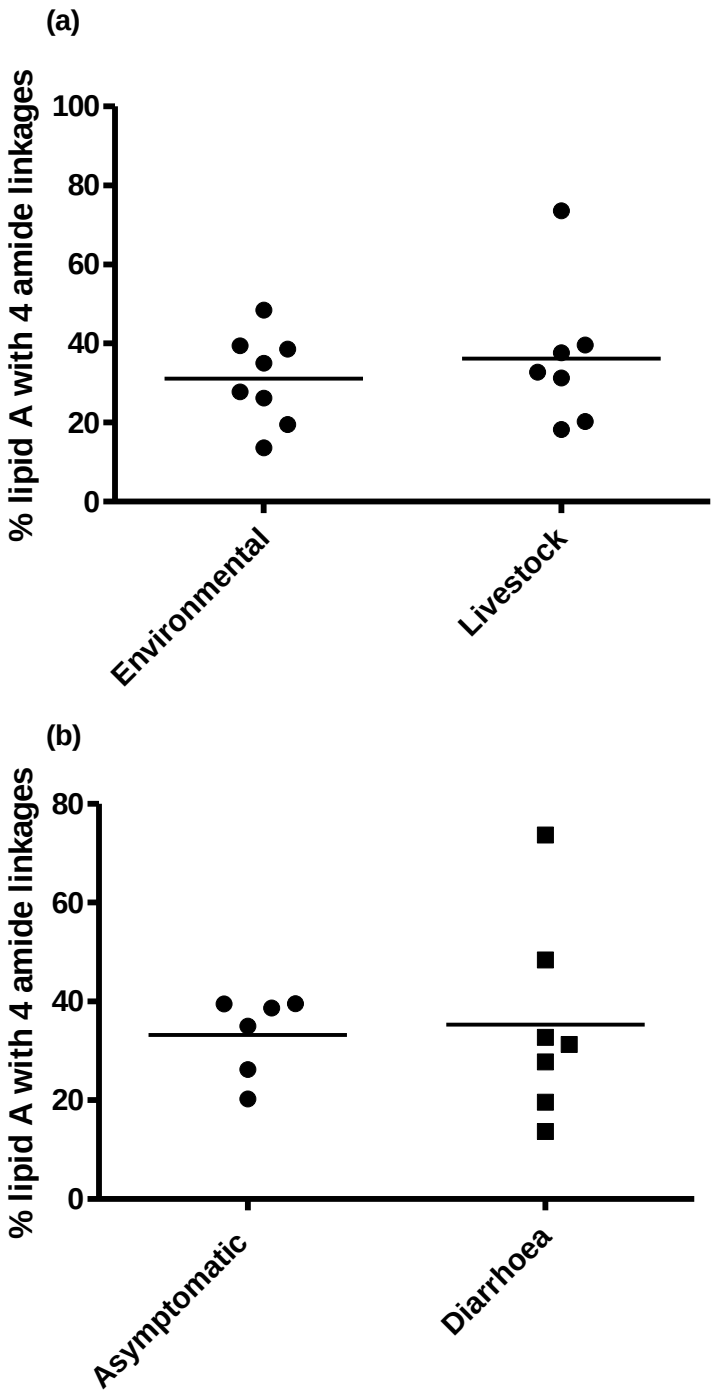


Figure 5-5: No correlation between the phylogenetic clade or clinical presentation of *C. jejuni* strains and the abundance of four amide linkages on the LOS

The relative abundance of GlcN3N-GlcN3N (four amide linkages) lipid A backbone was calculated from two individual spectra for each strain based on the area under the peaks of the masses from Table 0-2. The relative abundances were plotted against (a) the phylogenetic clade of each strain (b) the clinical presentation of each strain.

composition, therefore higher resolution MALDI-TOF analysis was performed by Dr C. John at UCSF on intact LOS to obtain OS structural details and are described in Section 5.5.

Livestock strain, 31485, had a peak at *m/z* 2415.4 correlating with an OS composed of $KDO_2PEAHep_2Hex_6HexNAc_1NeuAc_1$ (Figure 5-4a). Peaks at *m/z* 3592.1, 3818.5, and 3828.8 corresponded to the OS joined to a lipid A backbone GlcN3N-GlcN, GlcN3N-GlcN3N, and under O-deacylated GlcN3N-GlcN respectively. Strains 45557 and 11168H had the same OS composition as 31485, with peaks observed at *m/z* 2414.3 and 2414.8 Da respectively (Figure 5-4b and g). Peaks at *m/z* 3593.6 and 3819.0 Da for strain 45557, and *m/z* 3594.6 and 3818.2 Da for strain 11168H, corresponded to the full length O-deacylated structures with a GlcN3N-GlcN and GlcN3N-GlcN3N respectively. The OS peak for strain KJShpSm4 was *m/z* 2252.0 Da, which matched $KDO_2PEAHep_2Hex_5HexNAc_1NeuAc_1$ (Figure 5-4c), with corresponding full length peaks at *m/z* 3429.6 and 3654.7 Da for the OS plus a lipid A backbone composed of GlcN3N-GlcN and GlcN3N-GlcN3N respectively. Both strains 56282 and 32799 have an OS composed of $KDO_2PEAHep_2Hex_4HexNAc_1NeuAc_2$ shown at peaks *m/z* 2382.8 and 2382.7 Da respectively (Figure 5-4d and e). Full length structures composing the OS with GlcN3N-GlcN lipid A backbone were seen at peaks *m/z* 3561.0 Da for strain 56282 and 3559.3 Da for strain 32799, with loss of one sialic residues at *m/z* 3269.0 Da and 3268.0 Da respectively. In addition, strain 32799 had peaks at *m/z* 2977.0 and 3785.0 Da corresponding to the loss of two sialic acid residues and the full length OS with a GlcN3N-GlcN3N backbone respectively. Strain, KJCattle8 had an OS composed of $KDO_2PEAHep_2Hex_2HexNAc_2NeuAc_1$ observed *m/z* 1968.3 Da, and full length *m/z* 3145.0 Da with GlcN3N-GlcN lipid A backbone (Figure 5-4f).

MALDI-TOF analysis highlighted a wide variation in the OS structure of the 15 different strains (Table 5-4). Most strains were predicted to contain the inner core OS KDO_2Hep_2PEA. All strains bar 33084, 32787, 56519 contained a HexNAc residue. The OS structure of all 15 strains was predicted to contain between 2 and 6 hexose residues. The presence of sialic acid in the OS was predicted for 10 out of the 15 strains based on the MS data. Comparison of the LOS MALDI-TOF data highlighted the presence of sialic acid as a possible distinguishing feature between the clades as at least one sialic acid residue was present in all 7 livestock strains tested; in contrast only 3 out 8 environmental strains were sialic acid positive. We next confirmed the presence of sialic acid synthesis genes (sialic acid synthase (*neuB1*), sialic acid transferase (*cst*) and sialic-acid O-acetyl transferase (*SOAT*) genes).

Strain	Clade	Predicted carbohydrate structure	Predicted mass $(M-H)^-$ (Da)	Observed mass $(M-H)^-$ (Da)
11168H	L	KDO_2 PEA Hep_2 Hex_6 $HexNAc_1NeuAc_1$	2414.1	2414.8
45557	L	KDO_2 PEA Hep_2 Hex_6 $HexNAc_1NeuAc_1$	2414.1	2414.3
31485	L	KDO_2 PEA Hep_2 Hex_6 $HexNAc_1NeuAc_1$	2414.1	2414.6
32799	L	KDO_2 PEA Hep_2 Hex_4 $HexNAc_1NeuAc_2$	2381.0	2382.5
KJShpsm4	L	KDO_2 PEA Hep_2 Hex_5 $HexNAc_1NeuAc_1$	2251.9	2252.0
KJCattle8	L	KDO_2 PEA Hep_2 Hex_2 $HexNAc_2NeuAc_1$	1968.7	1968.3
56282	L	KDO_2 PEA Hep_2 Hex_4 $HexNAc_1NeuAc_2$	2381.0	2382.8
33106	E	KDO_2 PEA Hep_2 Hex_3 $HexNAc_1NeuAc_1$	1926.9	1927.9
33084	E	KDO_2 PEA Hep_2 Hex_6 $NeuAc_2$	2015.7	2017.5
40917	E	KDO_2 PEA Hep_2 Hex_5 $HexNAc_2NeuAc_1$	2485.1	2485.5
31481	E	KDO_2 PEA Hep_2 Hex_2 $HexNAc_2$	1677.4	1676.9
56519	E	KDO_2 PEA Hep_2 Hex_7 P_2 OAc	2282.6*	2282.1*
32787	E	KDO_2 PEA Hep_2 Hex_7 P_2 OAc	2282.6*	2282.8*
64555	E	KDO_2 Hep_2 Hex_7 $HexNAc_1$	2161.9	2159.7
43205	E	KDO_2 P Hep_2 Hex_8 $HexNAc_1$	2402.7*	2402.7*

Table 5-4: *C. jejuni* LOS OS structures predicted from O-deacylated spectra

Calculated and observed average masses for the subsequent predicted structures for the OS portions of the 15 strains, based on peaks in the OS and full length section of a minimum of two individual O-deacylated spectra for each strain. Hex = hexose; PEA = phosphoethanolamine; Hep = heptose; KDO = 3-Deoxy-D-manno-oct-2-ulosonic acid ; HexNAc = N-acetyl hexosamine ; NeuAc = N-acetyl neuraminic acid (sialic acid). * denotes exact masses (performed by high resolution MALDI-TOF on intact species

5.4 Genetic analysis of sialic acid-synthesis genes

The sequence of the sialic acid synthase gene in the LOS locus, *neuB1*, varies widely between LOS classes A and B compared to C (Parker et al. 2005). The identity between 11168H *neuB1* (class C) and 81-176 (class B) is 76.7% (EMBOSS Needle: Pairwise Sequence Alignment Software). A previous microarray study utilised primers based on the genomic sequence of strain 11168H, therefore the lack of identity between the difference LOS classes most likely accounts for why only 4 of the 15 strains were reported to be positive for the *neuB1* gene (Champion et al. 2005) (Dr O Gundogdu, LSHTM, personal communication). Using primers designed to anneal to the homologous regions shared between the *neuB1* genes from the various LOS classes, PCR analysis was performed on whole genomic DNA extracted from *C. jejuni* strains. 10 strains predicted to have sialic acid in the LOS by MS were also found to be positive for the presence of the *neuB1* gene (Figure 5-6, top band). In addition, primers designed by Parker and colleagues (Parker et al. 2005) were also used to distinguish strains on the basis of the presence of the *neuB1* and sialic acid transferase (*cst*) genes from classes A and B from class C (Figure 5-6). Out of the 10 strains positive for the *neuB1* gene, 5 were positive for both class A & B *neuB1* and *cst* genes, and the others were positive for class C *neuB1* and *cst* genes. The four strains that were positive according to Champion et al. all belonged to class C, confirming that the observed low degree of identity in the *neuB1* gene may be responsible for the false negative data for sialic acid synthase genes in some of the strains. Interestingly, the 5 strains that were negative for sialic synthesis genes (for all sets of primers utilised) were from the environmental clade (5/8). Primers annealing to a gene present in the LOS locus of all *C. jejuni* strains, *htrB*, was used as a positive control. Taken together, gene analysis and the MS data indicated that compared to the environmental strains, a higher proportion of the livestock strains LOS contained the sialic acid moiety.

To add greater weight to the observations noted above, the presence of sialic acid synthesis genes was sought in a further 12 strains from the environmental clade and 14 strains from the livestock clade. Of the 12 environmental strains analysed, 6 were positive for the *neuB1* gene (Figure 5-7). Of the 14 livestock strains analysed all 14 were positive for the *neuB1* gene (Figure 5-8). Therefore, overall 45% (9/20) of all environmental-associated clade strains contained the *neuB1* gene, compared to 100% (21/21) for the livestock-associated clade. Of the sialic acid positive strains, 78% for the environmental strains belonged to class A or B compared to 43% for the livestock clade. All strains analysed from the environmental clade were human isolates. 10 of the 21 livestock strains were human

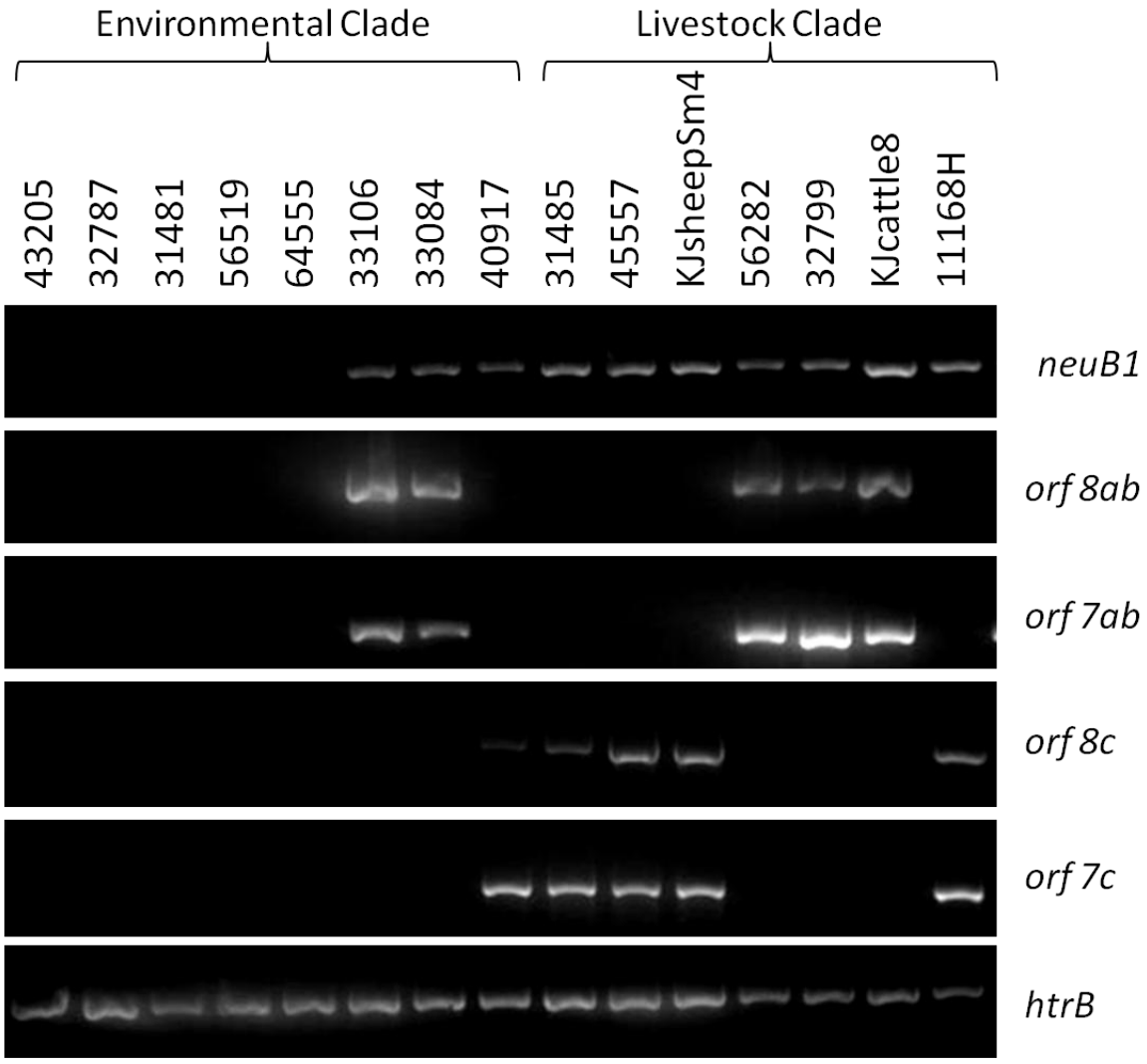


Figure 5-6: Detection of sialic acid synthesis genes in *Campylobacter jejuni* environmental and livestock strains

Sialic acid synthesis genes were amplified by PCR and visualised by agarose gel electrophoresis with gel-red staining. Primers utilised are described in the text. 30 PCR cycles were performed at an annealing temperature of 52^{o}C for orf 7ab (*neuB1 ab*), orf 7c (*neuB1c*), orf 8ab (*cst ab*), and orf 8c (*cst c*); *neuB1* and *htrB* at 55^{o}C.

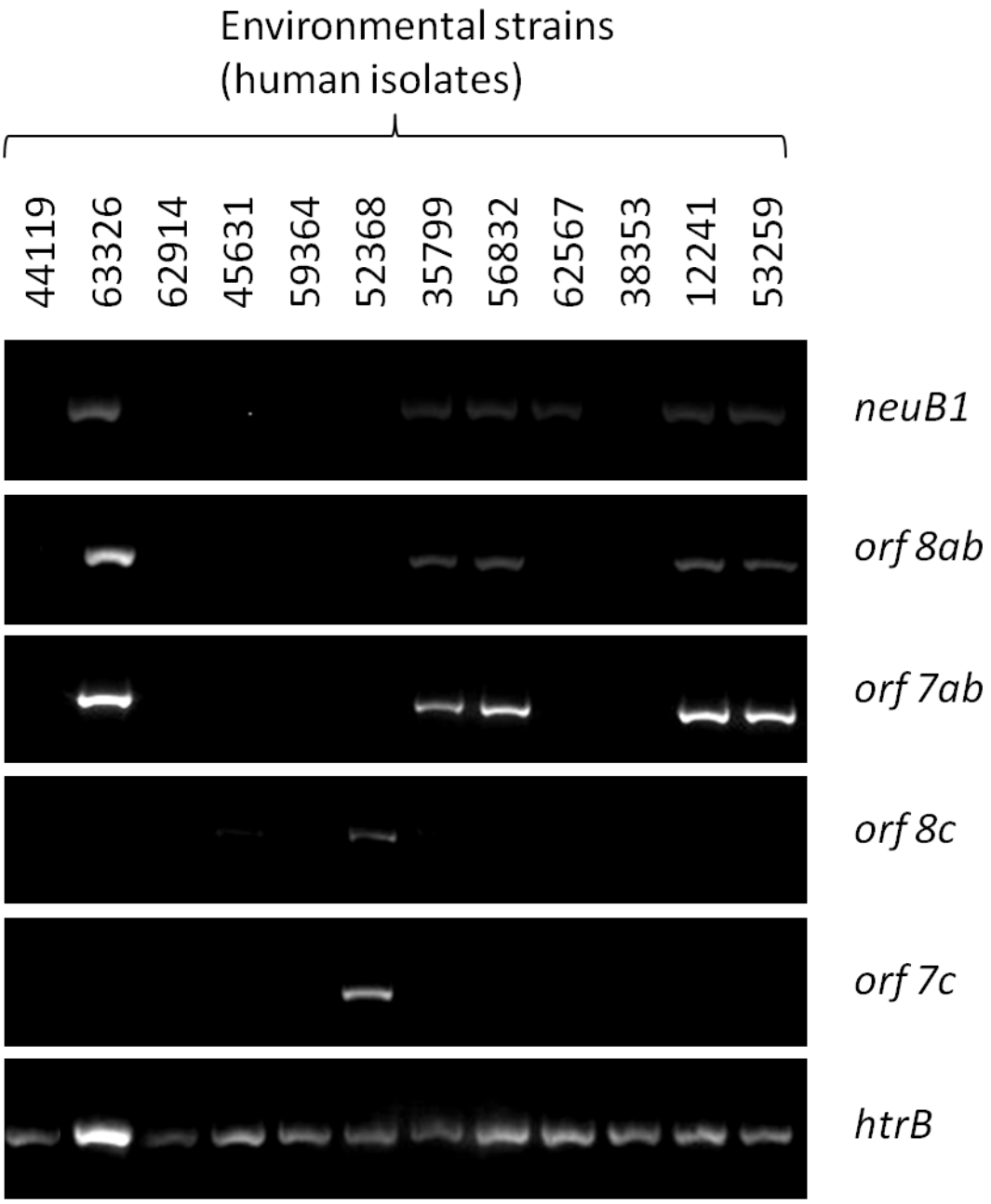


Figure 5-7: Detection of Sialic acid synthesis genes in environmental *Campylobacter jejuni* strains

Sialic acid synthesis genes were amplified by PCR and visualised by agarose gel electrophoresis with gel-red staining. 30 PCR cycles were performed at an annealing temperature of 52^{o}C for orf 7ab (*neuB1 ab*), orf 7c (*neuB1c*), orf 8ab (*cst ab*), and orf 8c (*cst c*); *neuB1* and *htrB* at 55^{o}C.

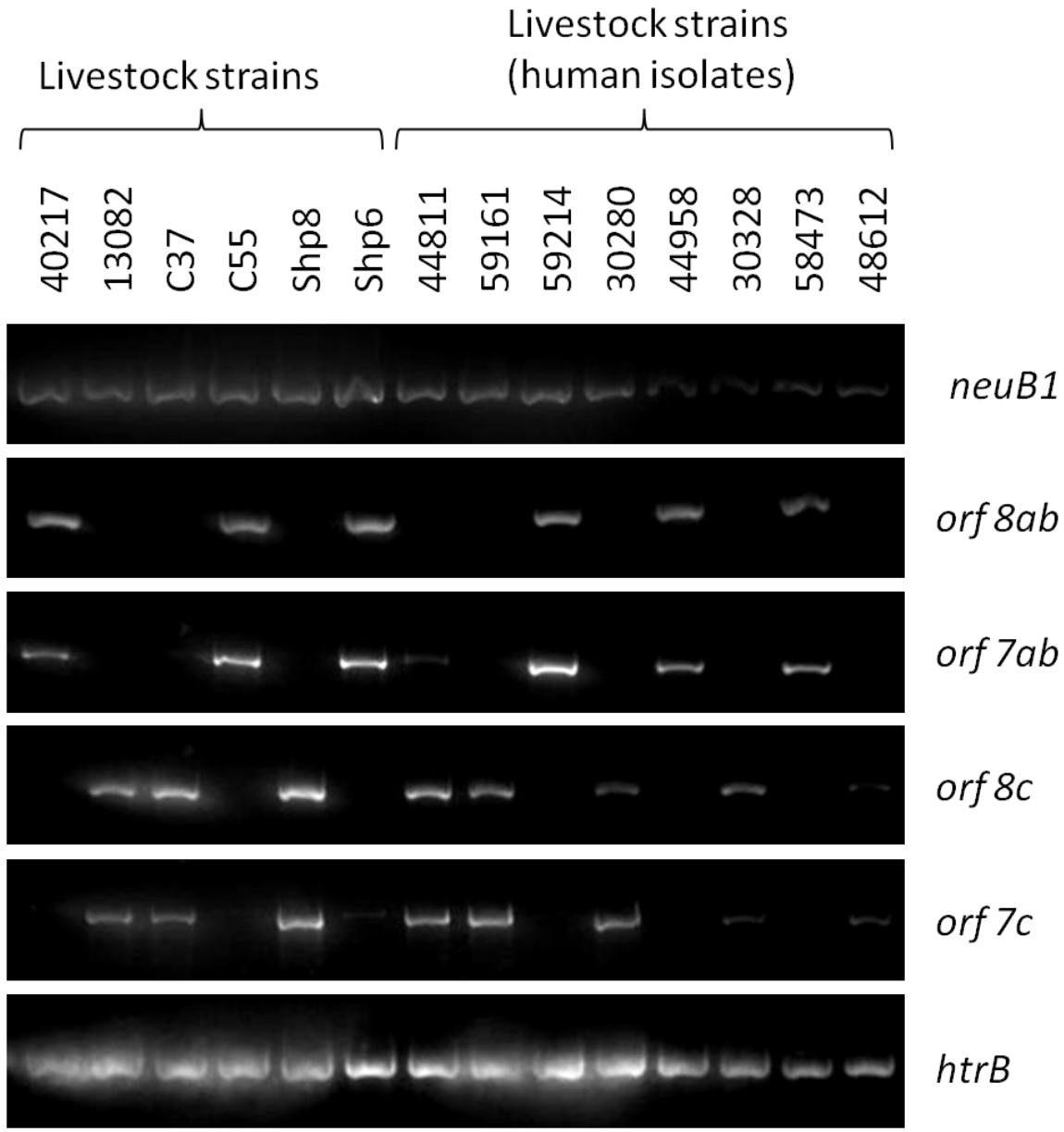


Figure 5-8: Detection of sialic acid synthesis genes in livestock *Campylobacter jejuni* strains

Sialic acid synthesis genes were amplified by PCR and visualised by agarose gel electrophoresis with gel-red staining. 30 PCR cycles were performed at an annealing temperature of 52^{o}C for orf 7ab (*neuB1 ab*), orf 7c (*neuB1c*), orf 8ab (*cst ab*), and orf 8c (*cst c*); *neuB1* and *htrB* at 55^{o}C.

isolates, for which 40% were classes A or B; the other 11 were from colonised livestock for which 45% were classes A or B. Of the 6 strains isolated from asymptomatic carriers 33% did not contain sialic-acid synthesis genes, 50% were positive for class A or B, and 17% were positive for class C. No correlation between the asymptomatic strains and the presence/absence of sialic acid synthesis genes was found. The expanded PCR analysis confirmed the presence of sialic acid synthesis genes in the LOS locus as a distinguishing feature between the two phylogenetic clades, but not for asymptomatic carriage of *C. jejuni*.

Champion and colleagues suggested a possible correlation between strains that contain the sialic acid synthesis genes and the presence of the two-domain containing *Cj1135 (lgtF)*, a gene predicted to function in the addition of two glucose residues to the two heptose residues in the core OS (Champion et al. 2005; Parker et al. 2005). Strains that lack the two-domain *lgtF* carry an *lgtF* gene that encodes a one-domain protein which is predicted to only add one glucose residue to the heptose residue proximal to the KDO residue. To confirm the correlation between strains containing sialic acid synthesis genes and the presence of the two-domain *lgtF* gene PCR analysis was performed. PCR analysis using the same forward primer (*lgtF* fwd 1) and a reverse primer that binds to a region in the first domain (*lgtF* rev 1) or a reverse primer that binds to a region in the second domain (*lgtF* rev 2) was used to distinguish the one-domain and two-domain genes (top two lanes Figure 5-9). 11 of the 15 strains contained the two-domain *lgtF* gene. Two of the strains yielded a PCR product that corresponded to the one-domain *lgtF,* with no product indicative for the presence of a second domain. Similar to the *neuB1* gene, sequence variation between strains occurs for *lgtF.* A third set of primers for *lgtF* were designed on the basis of the sequence of the one-domain *lgtF* gene from *C. jejuni* strain 81116 which has low sequence homology to 11168H *lgtF* (*lgtF* fwd2 and *lgtF* rev3). The genome sequence for strain 81116 is known and the strain lacks sialic acid synthesis genes in its LOS locus. The two strains that were negative for the primers based on 11168H *lgtF* were positive for the one-domain 81116 based *lgtF* primers. Only strain 64555 from the environmental clade was negative for the sialic acid synthesis genes but positive for the two domain *lgtF.* The other 4 strains that lacked sialic acid synthesis genes contained only a one-domain *lgtF* gene. This data suggested a strong correlation between the presence of the two-domain *lgtF* gene and the presence of sialic-acid synthesis genes.

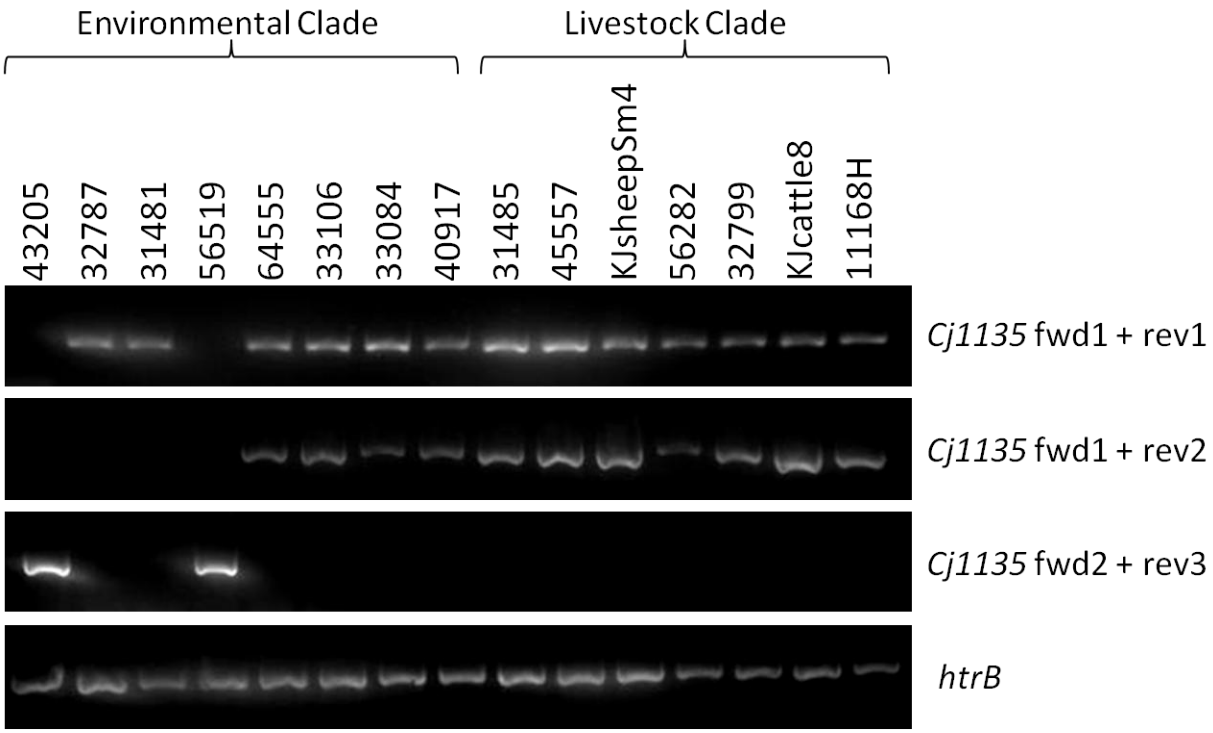


Figure 5-9: Detection of the *Cj1135* gene in environmental and livestock *Campylobacter jejuni* strains

Cj1135 gene was amplified by PCR and visualised by agarose gel electrophoresis with gel-red staining. 30 PCR cycles were performed at an annealing temperature of 55^{o}C for all primers sets.

5.5 MALDI-TOF analysis of native LOS

Phosphorylation of the lipid A moiety can modulate the degree of TLR4 activation. As the LOS phosphorylation status can vary between strains of the same species it was of interest to correlate phosphorylation levels and the biological activity of the LOSs' under investigation (Liu et al. 2010). As the O-deacylation of LOS removes PEA residues from the lipid A backbone MALDI-TOF analysis was performed on the native LOS to quantify the relative abundance of phosphorylation of the lipid A backbone (Figure 5-10 and Figure 5-11). Native LOS was first desalted before being loaded onto a thin layer of 2,4,6-trihydroxyacetophenone/nitrocellulose matrix (Chapter 2.9.4.1) MALDI-TOF analysis was performed in the negative ion mode.

Figure 5-10 and Figure 5-11 show representative spectra for native LOS of the 8 environment- and 7 livestock-associated strains respectively. Table 5-5 shows the predicted lipid A masses for native *C. jejuni* LOS. The masses are calculated for lipid A backbones containing 2 amide and 2 ester linkages (GlcN-GlcN) for the fatty acid chains. A decrease of 2Da and 4Da from these values would be observed for GlcN3N-GlcN and GlcN3N-GlcN3N respectively. In general, the peaks were broader than those seen for the O-deacylated data which is likely a reflection of the composition of the linkages in the backbone differing within the same species, resulting in a wider mass range. The most abundant peaks observed for the majority of the species corresponded to the addition of two phosphates (P) and one phosphoethanolamine (PEA) residues on the lipid A backbone (*m/z* 2004.4 Da) and the corresponding peak for the hydrolysis of a phosphate group during the MALDI-TOF analysis (loss of -98Da; *m/z* 1906.4 Da). For other strains the most abundant peak was for 2P modified lipid A backbone (*m/z* 1881.4; corresponding loss of phosphate *m/z* 1783.4 Da). In addition minor peaks were seen for 2P2PEA modified lipid A, *m/z* 2127.5 Da (loss of phosphate, *m/z* 2029.5 Da), 2P3PEA *m/z* 2250.5 (loss of phosphate, *m/z* 2152.5Da). Only strain 45557 showed a peak correlating to the presence of lipid A modified with one phosphate only (*m/z* 1801.4; Figure 5-11b). Peaks were also observed for masses corresponding to one acyl chain being 14 carbons long instead of 16; masses for these peaks are detailed in Table 5-2. Peaks at *m/z* 1878.0 corresponded to 2PPEA (C14) with loss of one phosphate; due to the similarity in mass between this peak and *m/z* 1881.4 Da 2P caution was taken when assigning the peaks of around this mass to either lipid A structure. Calculations were done to assess whether peaks were likely to correspond to the 1906.4 Da peak (loss of 28.1Da; C14 instead of C16) or 1783.4 Da peak (mass difference 98Da; 1783.4 corresponds to the loss of phosphate for 2P modified lipid A).

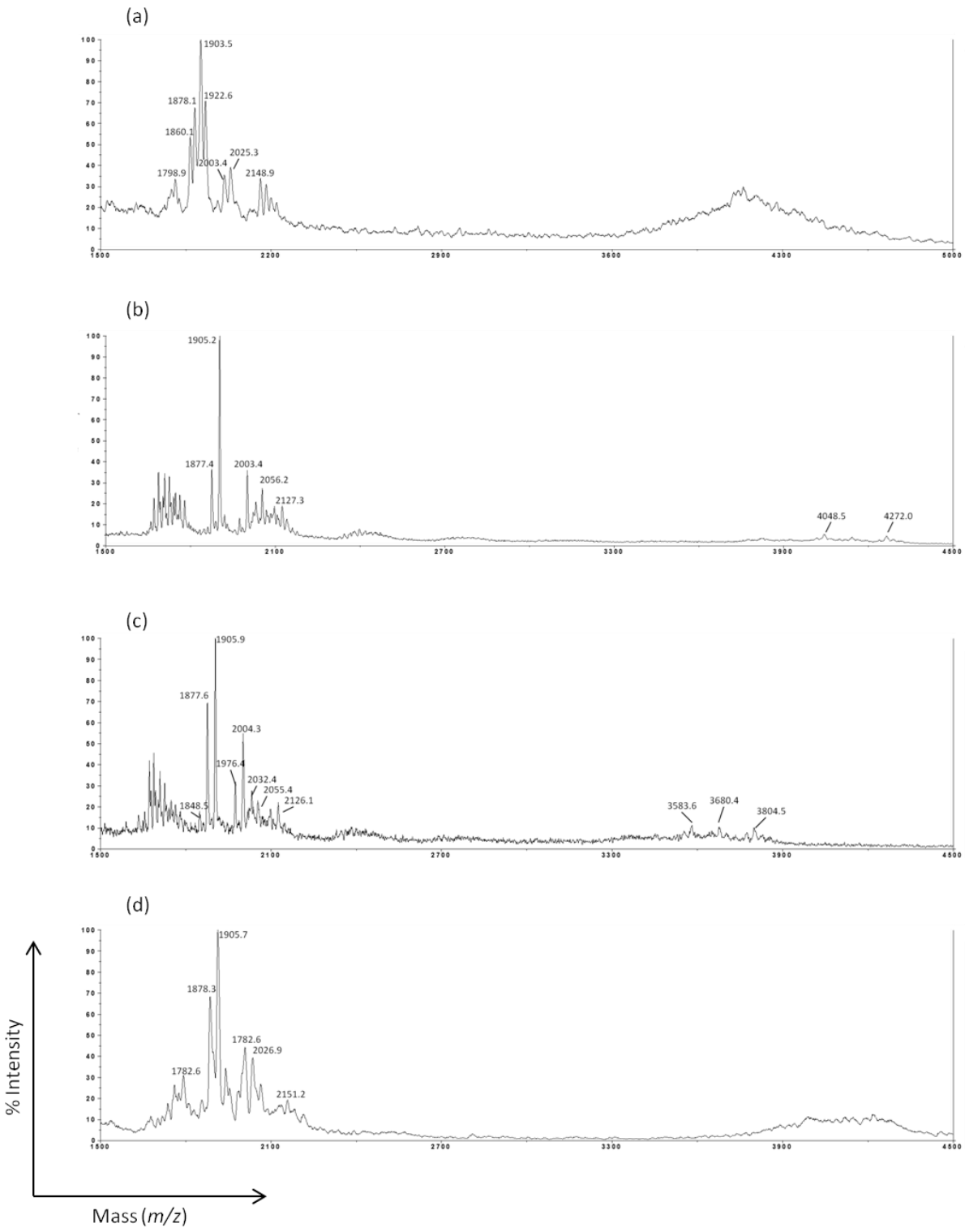


Figure 5-10: MALDI-TOF analysis of intact environmental phylogenetic clade LOSs

Representative spectra from at least two independently acquired spectra for the intact LOS from environmental strains (a) 43205 (b) 32787 (c) 31481 (d) 56519

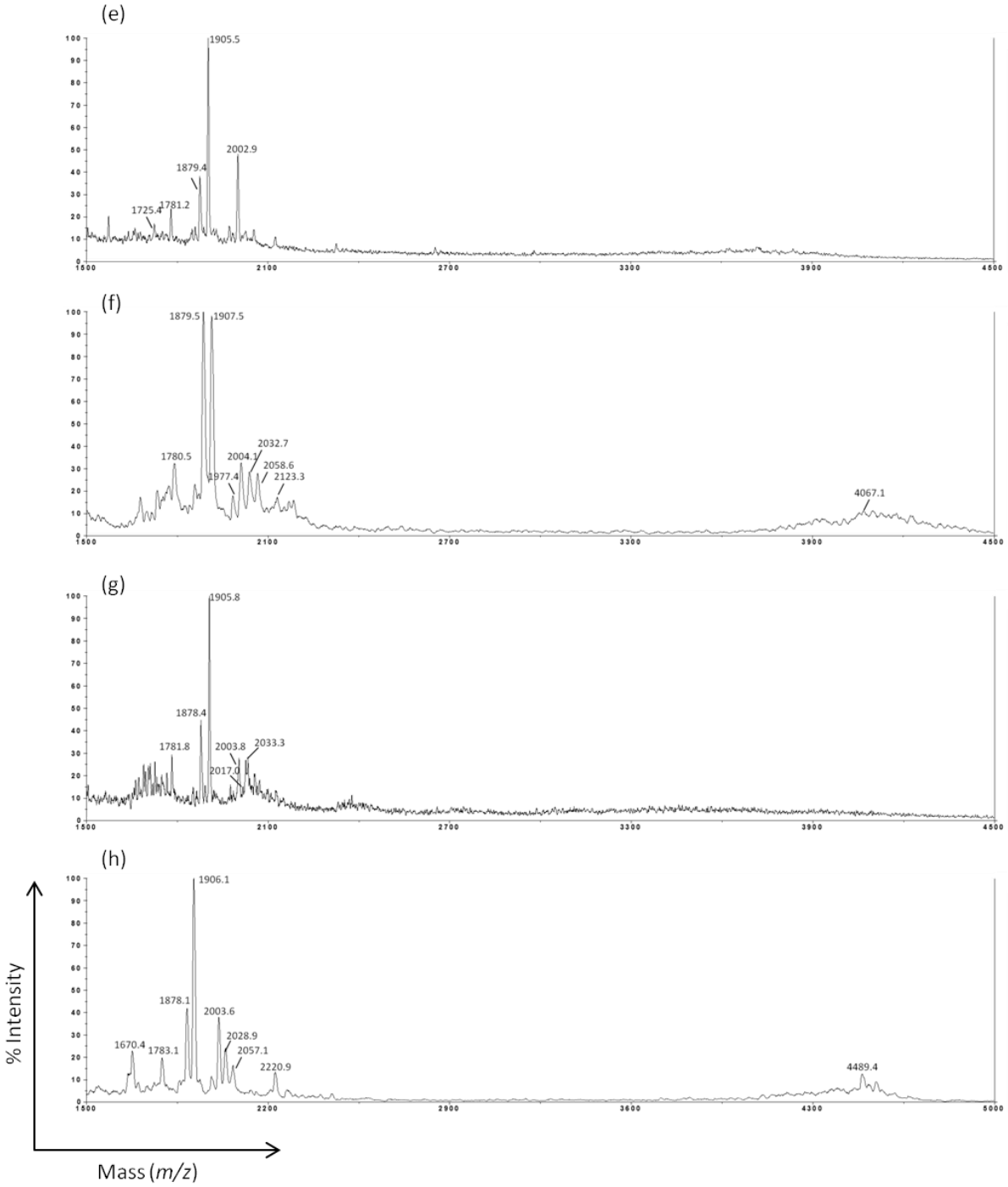


Figure 5-10: MALDI-TOF analysis of intact environmental phylogenetic clade LOSs

Representative spectra from at least two independently acquired spectra for the intact LOS from environmental strains (e) 33106 (f) 64555 (g) 33084 (h) 40917.

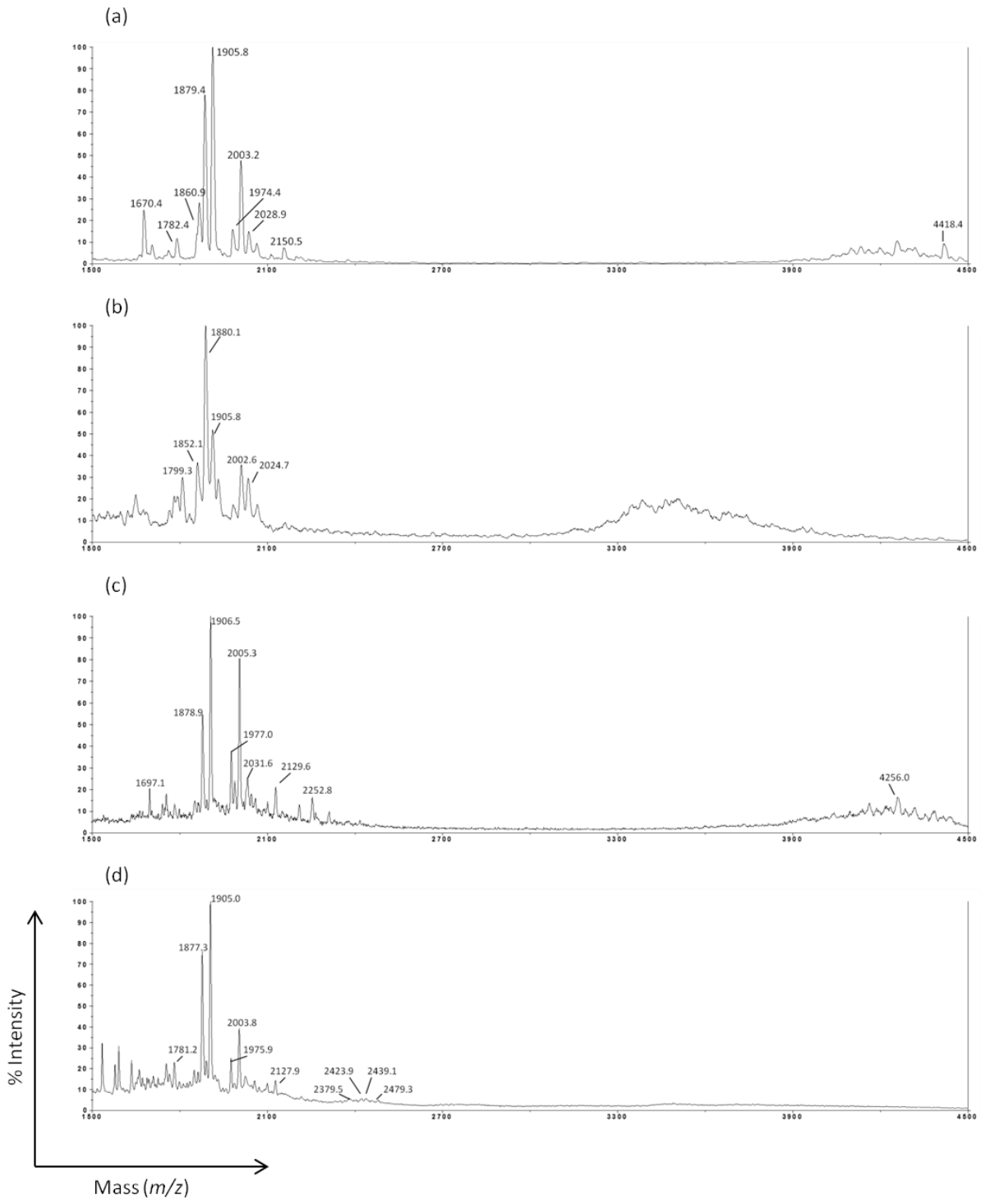


Figure 5-11: MALDI-TOF analysis of intact livestock phylogenetic clade LOSs

Representative spectra from at least two independently acquired spectra for the intact LOS from livestock strains (a) 31485 (b) 45557 (c) KJShpSm4 (d) 56282

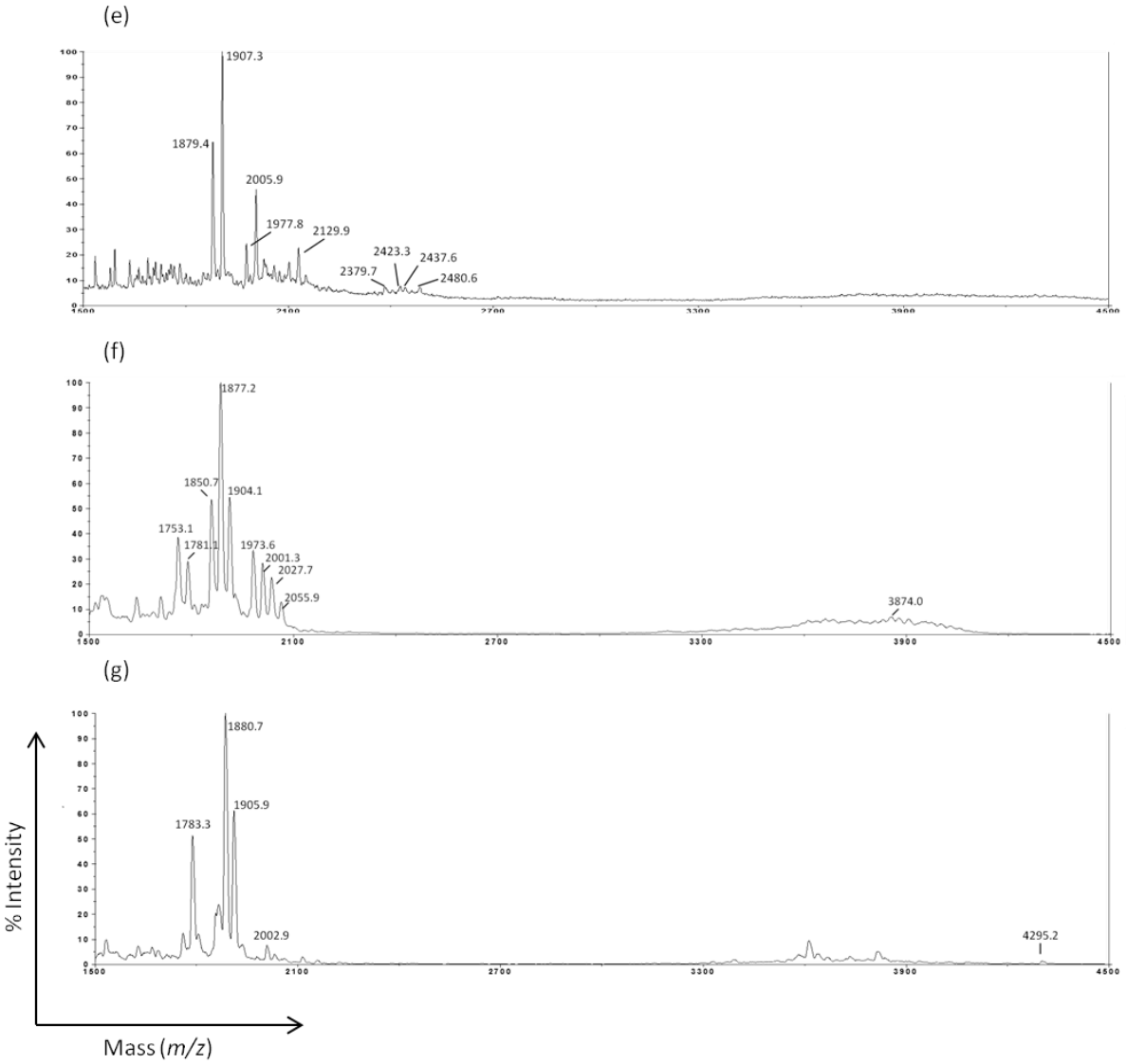


Figure 5-11: MALDI-TOF analysis of intact livestock phylogenetic clade LOSs

Representative spectra from at least two independently acquired spectra for the intact LOS from livestock strains (e) 32799 (f) KJcattle8 (g) 11168H

Native Lipid A Structure	Calculated mass $(M-H)^-$ (Da)
GlcN-GlcN-P	1801.4
GlcN-GlcN-2P	1881.4
GlcN-GlcN-PPEA	1924.5
GlcN-GlcN-2PPEA	2004.4
GlcN-GlcN-2P2PEA	2127.5
GlcN-GlcN-2P3PEA	2250.5
GlcN-GlcN-P (C14)	1769.0
GlcN-GlcN-2P (C14)	1853.0
GlcN-GlcN-PPEA (C14)	1892.0
GlcN-GlcN-2PPEA (C14)	1976.0
GlcN-GlcN-2P2PEA (C14)	2099.0
GlcN-GlcN-2P3PEA (C14)	2223.0
GlcN-GlcN-P (minus P)	1703.4
GlcN-GlcN-2P (minus P)	1783.4
GlcN-GlcN-PPEA (minus P)	1826.5
GlcN-GlcN-2PPEA (minus P)	1906.4
GlcN-GlcN-2P2PEA (minus P)	2029.5
GlcN-GlcN-2P3PEA (minus P)	2152.5

Table 5-5 : Native LOS lipid A masses from MALDI-TOF

Calculated masses for the described intact lipid A species. Lipid A composed of two D-glucosamine sugars (GlcN-GlcN; 2 amide linkage) is used for the calculations. P = phosphate group; PEA = phosphoethanolamine; C14 = 14 carbon long acyl chain instead of one 16 carbon long acyl chain.

To compare the level of phosphorylation between the different strains, the relative abundance of the tri- (2PPEA) or tetra-phosphorylated (2P2PEA) lipid A was compared to the relative abundance of the un- mono- (P) or di-phosphorylated (2P or PPEA) lipid A. There was a wide diversity between the strains in the level of phosphorylation. The range for the percentage of the tri- or tetra-phosphorylated lipid A was 26.3-87.5% (mean 65.4%, standard deviation 19.03%). Despite this diversity no one particular clade had a statistically higher level of phosphorylation than the other (Figure 5-12a). Similarly, the clinical outcome of each strain did not correlate with the level of phosphorylation (Figure 5-12b). There also was no correlation between the relative abundance of amide linkages and the relative abundance of tri- and tetra-phosphorylation of the lipid A (Figure 5-13). This data suggested the regulation of phosphorylation did not contribute to phylogenetic division and also did not account for the clinical presentation of each *C. jejuni* strain.

The resolution of the full length intact LOS species was far lower than that observed for the O-deacylated spectra, however conformation of some of the OS structures predicted from the O-deacylated data was possible. Peaks at *m/z* 3680.4, 3583.6, and 3804.5 Da for strain 31481 corresponded to the OS, $KDO_2PEAHep_2Hex_2HexNAc_1$, with lipid A modified with 2PPEA, 2PPEA with loss of one phosphate, and 2P2PEA respectively (Figure 5-10c). The peak at *m/z* 4489.4 Da for strain 40917 corresponded to the OS, $KDO_2PEAHep_2Hex_5HexNAc_2NeuAc_1$, with 2PPEA lipid A (Figure 5-10h). Livestock strain, 31485, had a peak at *m/z* 4418.4 Da, corresponding to the OS, $KDO_2PEAHep_2Hex_6HexNAc_1NeuAc_1$, with 2PPEA lipid A (Figure 5-11a). However, for strain 11168H predicted to have the same OS composition as 31485, a peak at *m/z* 4256.0 Da corresponding to a lipid A modified with only 2P was observed (Figure 5-11g). For strain KJShpSm4, a peak at *m/z* 4256.0 Da corresponded to $KDO_2PEAHep_2Hex_5HexNAc_1NeuAc_1$ OS plus a 2PPEA lipid A (Figure 5-11c). Strains 56282 and 32799 showed peaks corresponding to the $KDO_2PEAHep_2Hex_4HexNAc_1NeuAc_2$ OS at *m/z* 2379.5 and 2379.7 Da respectively (Figure 5-11d and e). The peak seen at *m/z* 3874.0 Da for KJcattle8 corresponded to the $KDO_2PEAHep_2Hex_2HexNAc_2NeuAc_1$ OS plus a lipid A modified with 2PPEA (with loss of one phosphate) (Figure 5-11f).

To obtain structural composition of environmental strains 43205, 32787, and 56519 high resolution MALDI-TOF analyses were performed on the native LOS structures. These analyses revealed peaks that corresponded to exact masses of structures opposed to the average masses seen with lower resolution MALDI-TOF. Strains 32787 and 56519 yielded

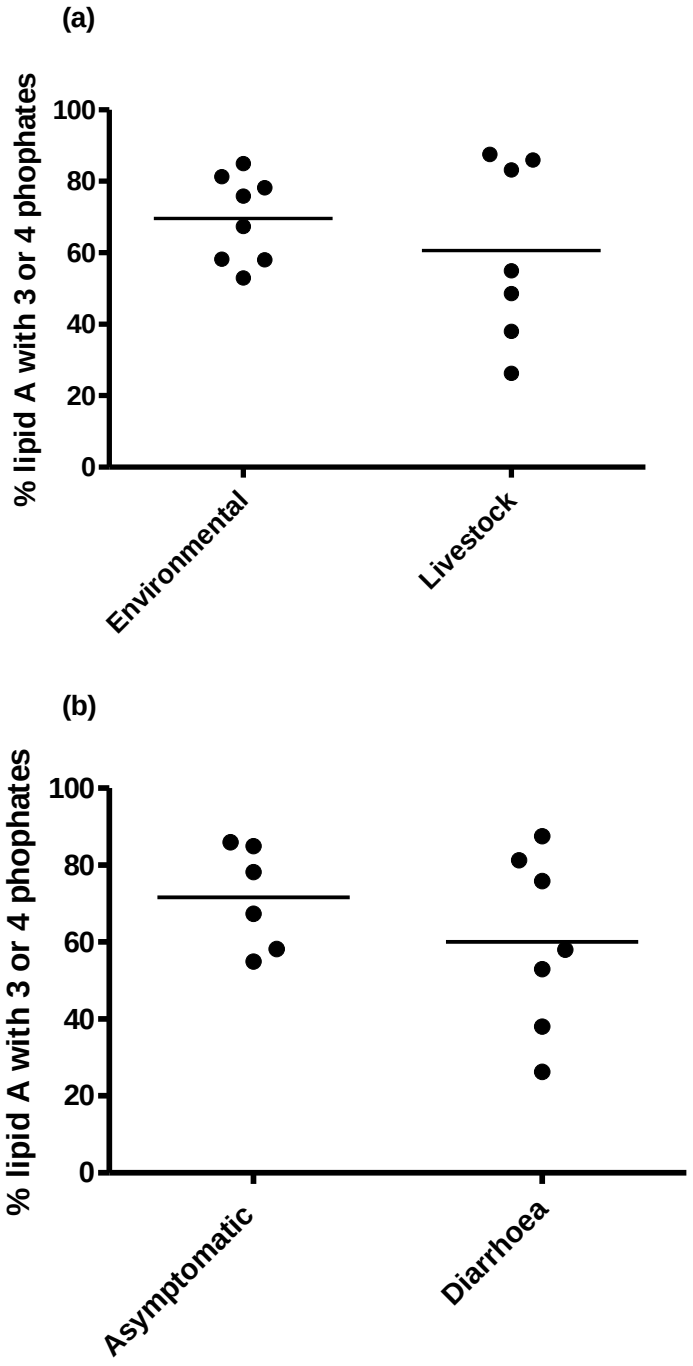


Figure 5-12: No correlation between the phylogenetic clade of *C. jejuni* strains and the presence of LOS phosphate groups

The relative abundance of lipid A species modified with either 3 or 4 phosphoryl groups (phosphate or phosphoethanolamine) was calculated from two individual spectra for each strain based on the area under the peaks of the masses from Table 0-5. The relative abundances were plotted against (a) the phylogenetic clade of each strain (b) the clinical presentation of each strain.

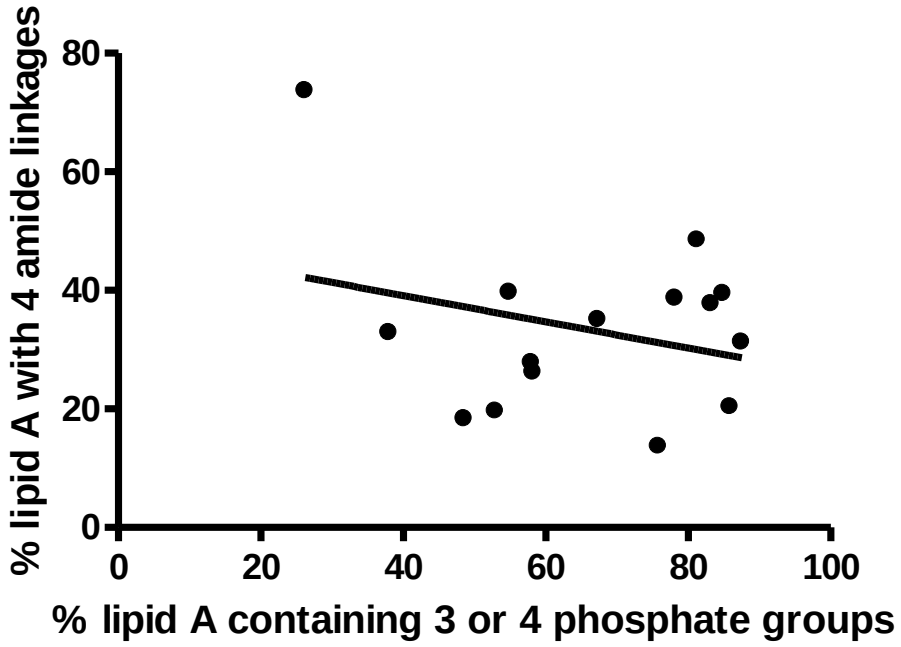


Figure 5-13: No correlation between the percentage of amide linkages and the phosphorylation status of the lipid A

The relative abundance of GlcN3N-GlcN3N lipid A backbone was plotted against the relative abundance of lipid A backbone modified with 3 or 4 phosphoryl groups for each strain.

peaks at *m/z* 2282.1 and 2282.8 respectively corresponding to an OS composed of $KDO_2PEAHep_2Hex_7P_2Ac$. Strain 43205 had an OS peak at *m/z* 2345.7 of unknown composition. Currently these are provisional structures for these strains and await further confirmation by MS.

The sialic acid residues of *C. jejuni* LOS from particular strains can be modified with the addition of O-linked acetyl groups (Dzieciatkowska et al. 2007). The gene responsible encodes an O-acetyl transferase; *SOAT* (sialylate O-acetyltransferase). Using primers for the *SOAT* gene which has high identity between strains, only 5 of the 15 strains under investigation were found to encode this gene (Figure 5-14). Of these 5 positive strains all encoded sialic acid synthesis genes from LOS class A or B, two were from the environmental clade and 3 were from the livestock clade. The addition of acetyl groups could be seen in the spectra for two strains, 56282 and 32799 (Figure 5-11d and e). Peaks at *m/z* 2423.9 and 2423.3 Da corresponded to the addition of an acetyl group to the OS of 56282 and 32799 respectively. In addition peaks at *m/z* 2439.1 and 2437.6, and 2479.3 and 2480.6 Da correlated with the addition of a glycine residue and both glycine and an acetyl group on the 56282 and 32799 OS respectively.

5.6 Discussion

Mass spectroscopy combined with gene analysis revealed differences in three structural components of *C. jejuni* LOS, these included variation in the number of amide *versus* ester linkages, lipid A phosphorylation and the carbohydrate moiety. These observations confirm previous reports on the diversity of *C. jejuni* LOS (Moran et al. 1991; Szymanski et al. 2003).It was found that only the OS structure variations correlated with the phylogenetic clade, this was a novel finding (Table 5-4, Figure 5-6, Figure 5-7, Figure 5-8, Figure 5-9). The level of phosphorylation and abundance of amide linkages was not distinguished by clade (Figure 5-5, Figure 5-12). The level of phosphorylation and abundance of amide linkages has previously been reported to vary between growths of one particular strain of *C. jejuni* as well as between strains, whereas the expression of the OS does not vary (unless OS genes are under phase variable regulation (Szymanski et al. 2003)). This may explain the lack of correlation, despite the wide range in the expression of these modifications between strains. The variability of these modification in the lipid A backbone has been suggested as a potential virulence factor, as both the chemical linkages of the acyl groups and lipid A phosphorylation status have been shown to modulate the immune response to *C. jejuni* and other pathogens (Liu, John, & Jarvis 2010; Van Mourik et al. 2010). The ability of the

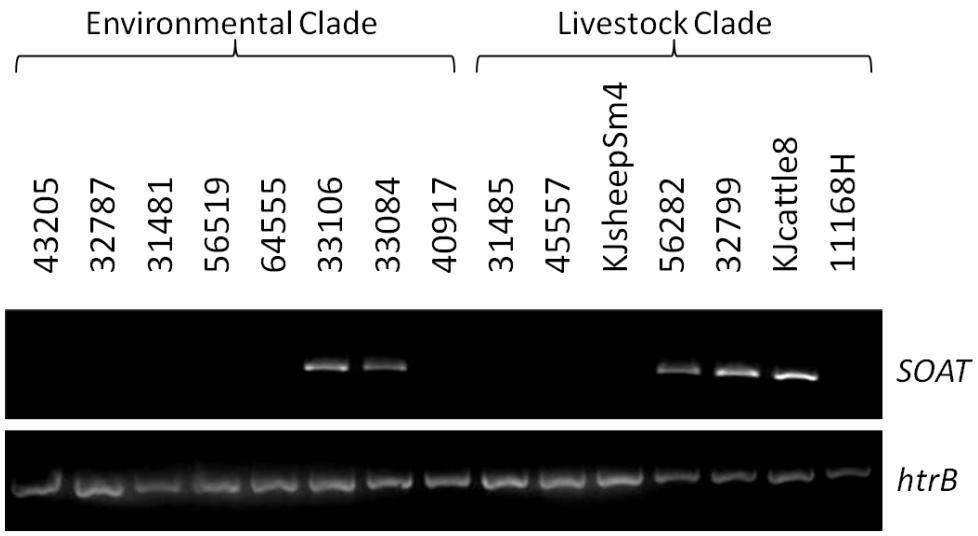


Figure 5-14: Detection of *SOAT* genes in *Campylobacter jejuni*

SOAT gene was amplified by PCR in 15 strains under investigation and visualised by agarose gel electrophoresis followed with gel-red staining. 30 PCR cycles were performed at an annealing temperature of 55^{o}C for *SOAT* and *htrB* primers.

LOS isolated from these different strains to activate innate immune cells is explored further in Chapter 6.

Interestingly, no correlation between the *C. jejuni* strains isolated from either humans presenting with clinical symptoms of disease or asymptomatic carriers was observed for the abundance of amide linkages, the phosphorylation of the lipid A, or the OS structure. This is in keeping with previous findings showing no phylogenetic link between *C. jejuni* strains with similar clinical outcomes (Champion et al. 2005). This data suggests that that the host rather than bacterial factors may play a greater role in defining asymptomatic carriage of *C. jejuni*.

A range in the relative abundance of the GlcN-GlcN, GlcN3N-GlcN, and GlcN3N-GlcN3N was observed, although for the majority of the strains the GlcN3N-GlcN backbone was the dominant species (Figure 5-3, Figure 5-4, Figure 5-5). GlcN-GlcN lipid A structures were seen at a low abundance in a few strains. Recently, the genes responsible for the production of the GlcN3N precursor, UDP-GlcNAc3N, have been identified as *gnnA* and *gnnB* (Van Mourik et al. 2010). These genes are present in the genomes of all strains of *C. jejuni* studied to date. Mutation of either of the genes results in the production of only GlcN-GlcN disaccharide lipid A backbone, suggesting that there is an evolutionary advantage for *C. jejuni* in the production of GlcN3N. Indeed, the mutation of these genes increased the ability of the *C. jejuni* LOS to activate a TLR4-reporter cell line, and increased the susceptibility of the bacterium to the antimicrobial peptides, polymixin B, colistin, and cathelicidin-1 (Van Mourik et al. 2010). This may explain the relative lack of GlcN-GlcN lipid A structures for the strains.

Wide variation in the level of lipid A phosphorylation (addition of phosphate or PEA groups) was observed in the intact LOS structures (Figure 5-10, Figure 5-11, Figure 5-12). A diverse mix of lipid A structures modified by one or two phosphate groups and one or two PEA groups were observed. The spread of the strains in abundance of 3 or 4 phosphoryl modifications was greater than seen for the abundance of 4 amide linkages. The modification of *C. jejuni* lipid A with PEA has recently been attributed to the gene, *Cj0256* (Cullen & Trent 2010). Interestingly, mutation of this gene also abolishes modification of the flagellar rod protein, FlgG, with PEA residues. An increase of 20-fold in susceptibility to polymixin B was observed for the *Cj0256* mutant. The modification of *Salmonella enterica* lipid A with PEA groups has also been shown to decrease their susceptibility to polymixin B (Lee et al. 2004). The effects of this *Cj0256* mutation on TLR4 activation are unknown. The

abundance of phosphoryl-modification of the lipid A for *Neisseria menigitidis* is critical for TLR4 activation which suggests phosphorylation of *C. jejuni* may also alter TLR signalling (Liu, John, & Jarvis 2010). The effects of *C. jejuni* phosphorylation on TLR4 activation is described in Chapter 6.

The OS portion is the most variable region of *C. jejuni* LOS, and has been studied extensively; this is due to its association with the onset of GBS (Table 5-4 ; (Godschalk et al. 2004)). The number of genetic classes of the LOS locus has increased to 19 in recent years (Parker et al. 2008). The genes that distinguish these classes largely encode proteins that modify the outer core OS. In the present study, use of MALDI-TOF MS has allowed prediction of the OS composition for 15 strains of *C. jejuni,* only one of which was previously known. The inner core OS, composed of two heptose residues linked by a KDO residue to the lipid A is predicted for all of the strains studied (Table 5-4). Additional modification of the inner core with another KDO residue and PEA is also predicted for most of the strains. The modification of the two heptose residue in the inner core is performed by the *lgtF* gene product. 11 of the 15 strains encode a two-domain lgtF protein that is predicted to add a glucose residue to both heptose residues in the inner core (Figure 5-9; (Naito et al. 2010)). The other four strains contained a one-domain *lgtF* gene that is predicted to add only one glucose residue to the heptose residue proximal to the KDO linking the OS to the lipid A. Interestingly, the four strains that encoded the one-domain *lgtF* gene were all from the environmental-clade. This correlation is more likely to be a reflection of the correlation between the two-domain protein and LOS classes containing sialic acid synthesis genes although the possibility of the second glucose being important in the colonisation of livestock cannot be ruled out as all ABC LOS classes contain the two domain protein (Parker et al. 2008). The outer core OS structure is known to vary widely between strains, and it is this portion that contains the antigenic determinants responsible for development of auto-reactive antibodies and GBS. Only one study to date has analysed the OS structure of non-ABC *C. jejuni* strains and therefore the present study greatly expands on our current understanding of non-ABC OS structures (Houliston et al. 2011).

The *SOAT* gene was present in 5 strains containing LOS classes A or B (Figure 5-14), and yet MS masses corresponding to O-linked acetyl groups were only found in two strains. The acetylation of *C. jejuni* sialic acid is known to require an α2,8-linked sialic acid, which only arises when it is attached to an proximal sialic acid residue (Dzieciatkowska et al. 2007). Therefore strains that contain only one sialic acid residue, or strains that contain only α2,3-

linked sialic acid cannot be modified by the addition of an acetyl group. Only 3 of the 5 strains from LOS classes A or B contained two sialic acid residues, and two of these strains indeed showed peaks by MS for acetyl modification. A most likely explanation for the observed data suggested that the third strain with two sialic acid residues may contain only α2,3-linked sialic acid or alternatively may have the *SOAT* gene under phase-variable genetic control.

The flagellin glycosylation *Cj1321-26* island is an important genetic marker for the distinction of the two *C. jejuni* phylogenetic clades (Champion et al. 2005). However, even if these genes were removed from the analysis the strains still divide into the two distinct clades, suggesting a distinct evolutionary separation irrespective of this genetic island. Here, all 21/21 strains from the livestock-clade were positive for genes encoding sialic synthesis genes; in comparison only 9/20 environment-associated strains were positive. The PCR data confirmed MALDI-TOF MS data for all 15 strains. This genetic distinction was not noticed during the original microarray study, this is most likely due to a lack of identity between the sialic acid synthesis gene, *neuB1*, amongst strains. Of the 15 strains, 11 were also positive for the *Cj1324* legionaminic acid synthesis gene, a gene identified as critical in distinguishing the phylogenetic clades in the 2005 study (data not shown). Similar to the sialic acid synthesis genes, all of the livestock strains tested were positive for *Cj1324*.

Multi-locus sequence typing (MLST) is used to group different *C. jejuni* strains based on specific sequences within particular house-keeping genes. In agreement with our findings, a separate study showed a greater propensity for *C. jejuni* poultry and bovine/lamb sources to contain sialic acid on the LOS (Hotter et al. 2010). However, another study of 335 *C. jejuni* strains, sampling multiple STs, found ~85% of *C. jejuni* strains from bovine sources contained LOS sialic-acid; this value dropped to ~40% from poultry sources (Revez and Hanninen 2012). The smaller sample population of our study (n = 41) may mean that sampling bias may have resulted in a greater skew towards sialic acid positive strains from the livestock strains. The LOS classes of *C. jejuni* strains have recently been reported to correlate strongly with the ST type of each strain (Revez & Hanninen 2012). As the ST types of the strains used in our study are unknown, potentially a sampling bias towards one particular ST type may have introduced LOS class bias into the study. However, our data agrees with previous reports suggesting an increased association between livestock strains and the presence of sialic-acid synthesis genes, even if they do not show as strong a correlation as seen in our study.

It is interesting to hypothesise that similar to legionaminic acid on the flagellin, sialic acid expression on the LOS is required for the long term colonisation of livestock, potentially due to the binding to specific receptors. Potentially redundancy between these structurally related carbohydrates may account for why not all livestock-associated strains contain both sialic-acid and legionaminic acid. In contrast, *C. jejuni* strains that both lack sialic acid or contain it are both able to cause acute disease in humans. Similarly, in the aforementioned study only ~45% of human clinical isolates contained sialic acid biosynthesis genes (Revez & Hanninen 2012). This data highlights sialic acid as a critical determinant involved in *C. jejuni* interactions with a particular host i.e. livestock *versus* human.

Chapter 6.

Phosphorylation and Sialylation of *C. jejuni* Lipooligosaccharide Alters Pro-inflammatory Cytokine Response(s)

6.1 Background

The detection of LPS/LOS moiety of Gram-negative bacteria by the host is essential for the elimination and resolution of infection. This has been best demonstrated by the ability of the causative agent of plague, *Yersinia pestis,* to produce tetra-acylated lipid A which is a potent antagonist of human TLR4 when grown at 37^{o}C (Montminy et al. 2006). The failure of TLR4 to detect *Y. pestis* LPS allows the rapid systemic dissemination of the bacterium often resulting in fatality before an effective immune response can be mounted. Murine mouse models also show that removal of MD2, a critical component of the TLR4 recognition complex, results in increased susceptibility to *S. typhimurium* infection (Nagai et al. 2002). Conversely to the protective role of TLR4 signalling in host defense, overactivation of TLR4 can in itself have pathological consequences. For example, LPS/LOS is the cause of endotoxic shock during infections; this is mainly due to the induction of high levels of pro-inflammatory cytokines and acute-phase proteins (Poltorak et al. 1998). Individuals with polymorphisms in TLR4 that render them hypo-responsive to inhaled LPS have increased likelihood of suffering from endotoxic shock (Lorenz et al. 2002b). This suggests that a muted TLR4 response can also lead to endotoxic shock as a consequence of increased bacterial load.

The recognition of LPS/LOS by TLR4 involves a multiple protein complex. First, the serum protein LPS-binding-protein (LBP) extracts LPS/LOS from the bacterial outer membrane, LPS/LOS bound LBP is then recognised by CD14 (which can be present in soluble or membranous form (Wright et al. 1990). CD14 serves to increase affinity of LPS/LOS for the TLR4 complex. Two LPS/LOS moieties are subsequently recognised by the tetrameric TLR4 complex, composed of two TLR4 and two MD2 molecules (Poltorak et al. 1999; Park et al. 2009). Engagement of TLR4 leads to the activation of both MyD88-dependant and –independent (TRIF-dependant) signalling cascades which results in the transcriptional activation of a plethora of inflammatory genes (Figure 1-5)

Structural variation in LPS/LOS between bacterial species results in variable activation of human TLR4. Typically, hexacylated lipid A, as seen in *Escherichia coli* and also *C. jejuni,* is a potent activator of TLR4. Both tetra-acylated lipid A, such as lipid IVa (an intermediary in *E. coli* lipid A biosynthesis) or from *Y. pestis*, and penta-acylated lipid A, from *Rhodobacter sphaeroides*, are antagonists of human TLR4 (Lien et al. 2000; Lohmann et al. 2003; Montminy et al. 2006). Additionally phosphate modifications of the lipid A backbone from *S. enterica* and *Neisseria meningitidis* induce higher levels of TLR4 activation (Liu, John, &

Jarvis 2010; Mata-Haro et al. 2007). In the case of *C. jejuni* LOS, the amide linkage of the acyl chains reduces to human TLR4 activation when compared to the presence of an ester linkage (Van Mourik et al. 2010). At present, the contribution of the phosphorylation status of the lipid A moiety to TLR4 activation is unknown. An added complication to dissecting these interactions is that the same strain can vary many of these modifications as described in Chapter 5.

The elucidation of the three-dimensional structure of hexacylated *E. coli* LPS in complex with TLR4/MD2 has greatly aided in our current understanding of TLR4 activation (Park et al. 2009). The accommodation of hexacylated lipid A into the hydrophobic β-sheet folds of MD-2 leaves one acyl chain partially exposed creating the dimerisation interface for TLR4. Alteration in the lipid A conformation upon dimerisation allows interaction between phosphate groups on the lipid A backbone and positively charged residues in TLR4 and MD2 which aids formation of the complex. TLR4 undergoes a conformational change upon dimerisation; a conformation that allows the docking of adaptor molecules to the intracellular TIR domain resulting in the activation of downstream signalling.

Interestingly, human TLR4 is far less promiscuous than murine TLR4. Tetra-acylated lipid A is an agonist of murine TLR4 whilst an antagonist of human TLR4. It has been suggested that the differences in affinity for LPS structures may account for the variations in disease outcomes between mice and humans (Miller et al. 2005). The differences between human and mice TLR4 highlight the necessity to use human cells during studies assessing LPS/LOS activation.

6.2 Sialic acid on *C. jejuni* LOS impacts on TNF-α production from THP-1 cells

In chapter 5, interesting distinctions in the LOS structure from *C. jejuni* strains of the different phylogenetic clades were described. We next wished to investigate if these structural differences translated into differential TLR4-mediated immune response(s). For this purpose, the release of TNF-α in response to *C. jejuni* LOSs' was investigated. The monocytic cell-line THP-1 was employed for these studies. Prior to stimulation, THP-1 cells were treated overnight with phorbol 12-myristate 13-acetate (PMA) to induce differentiation of pro-monocytic cells to cells with phenotypic similarities to monocytes/macrophages (Daigneault et al. 2010). Cells were stimulated with either 10ng or 100ng of isolated LOS and TNF-α protein levels were quantified 20h post stimulation. At 10ng concentration, LOS from the livestock strains induced approximately twice the

amount of TNF-α than that observed in response to LOS from the environmental strains, interestingly this difference rose to 50% at 100ng concentration (Figure 6-1). These observations suggested that structural differences found in the LOS from the two phylogenetic clades may indeed impact on TLR4 function as noted by variation in TNF-α levels.

We next wished to know if the whole live bacteria from the different clades showed a similar pattern of TLR4 activation as noted in the presence of their respective purified LOS. For this series of experiments optimisation assays to assess bacterial growth rates were first performed (Figure 6-2). This was necessary as differential growth rates may of *C. jejuni* strains may alter cytokine induction. The CFU counts at an OD of 1 (equivalent to 3 x 10^9/mL CFU of strain 11168H) were comparable between the strains; with the exception of the environmental strain, 40917 (Figure 6-2 white bars). The CFU counts after 24h in culture media (RPMI containing 10% FCS, 5% CO_2, 37^oC without shaking) increased ~10-fold with the exception of environmental strains 43205 and 31481 which remained similar to the initial inoculum (Figure 6-2 black bars). The strain with the lower initial CFU count, 40917, had similar final CFU counts to the other strains. This data suggested that *C. jejuni* strains under investigation contained similar CFU counts in the initial inoculum and also no significant difference was noted in the growth rates of the various strains.

To assess the ability of live bacteria to induce TNF-α, co-cultures studies utilising *C. jejuni* at an MOI of 10 or 100 were conducted (Figure 6-3). There was a statistically significant increase in TNF-α production by the livestock-associated strains over the environmental strains upon stimulation with live bacteria (Figure 6-3; $p = 0.04$). At an MOI 100, the mean secretion of TNF-α induced by the livestock strains was approximately 50% higher than the environmental strains, however the spread of the data was far larger and the difference was no longer statistically significant. Similarity between the live infection and the corresponding purified LOS effects on TNF-α production suggested that the distinctions in the LOS structures are most likely to impact on the immunity and maybe a critical determinant defining disease outcome.

LOS sialylation is known to contribute to higher levels of pro-inflammatory cytokines during *C. jejuni*–mediated DC activation (Kuijf et al. 2010). As only 45% of the environmental clade-associated strains contained sialic acid *versus* 100% for the livestock clade-associated strains the correlation between LOS sialylation and TNF-α production was assessed. Figure 6-4 shows the data presented in Figure 6-1 but in this instance the data was re-derived to

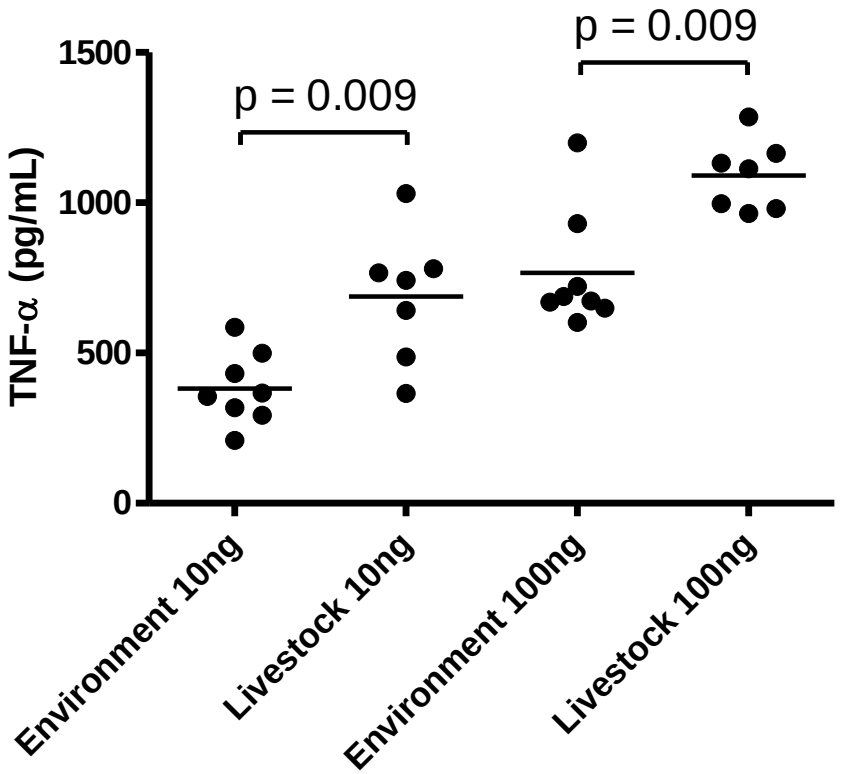


Figure 6-1: Differential TNF-α production in response to stimulation with LOS moieties from *C. jejuni* strains belonging to the livestock and environmental phylogenetic clades.
1x 10^5 PMA-matured THP-1 cells were stimulated with either 10ng or 100ng of LOS isolated from 8 environment-clade associated strains or 7 livestock-clade associated strains for 20h. TNF-α levels were analysed by ELISA. Data are plotted against the phylogenetic clade of each strain. Each dot represents average TNF-α levels induced by LOS isolated from an individual strain from four independent experiments. Mann-Whitney statistical test performed.

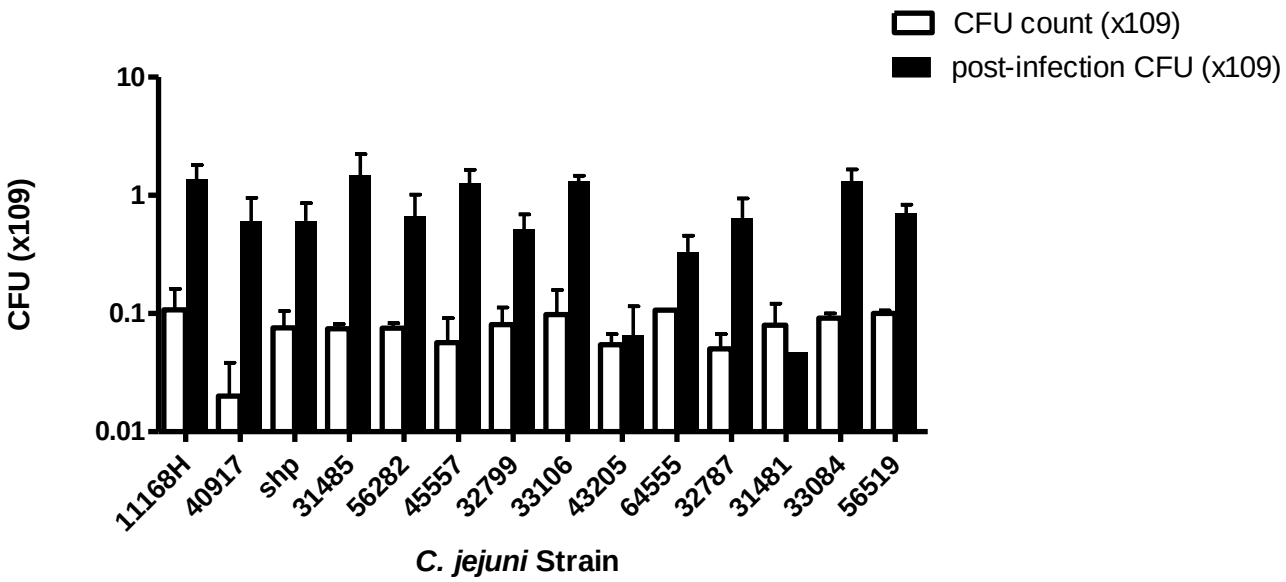


Figure 6-2: Bacteria CFU counts and survival data

CFU counts were performed for individual strains at an OD 0.03 (white bars; calculated to be 1 x10^8 CFU/mL for strain 11168H from previous experiments). Bacteria were left in culture media for 20h at 37^{o}C under aerobic condition without shaking to mimic infection conditions and CFU counts were subsequently performed (black bars).

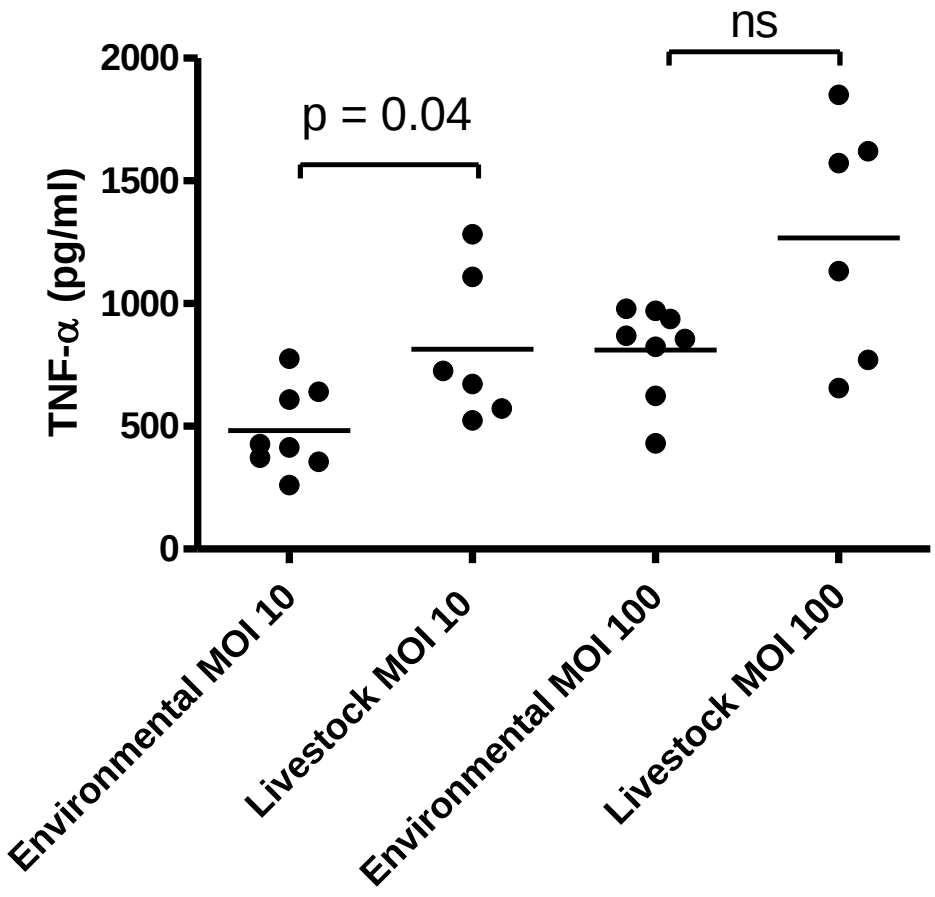


Figure 6-3: Live bacteria from the livestock phylogenetic clade induce higher levels of TNF-alpha from THP-1 cells

1x 10^5 PMA-matured THP-1 cells were stimulated with the 8 environment and 6 livestock-clade associated *C. jejuni* strains at an MOI 10 or 100 for 20h. TNF-α levels were analysed by ELISA. Data are plotted against the phylogenetic clade of each strain. Each dot represents average TNF-α levels induced by live bacteria of an individual strain from four independent experiments. Mann-Whitney statistical test performed.

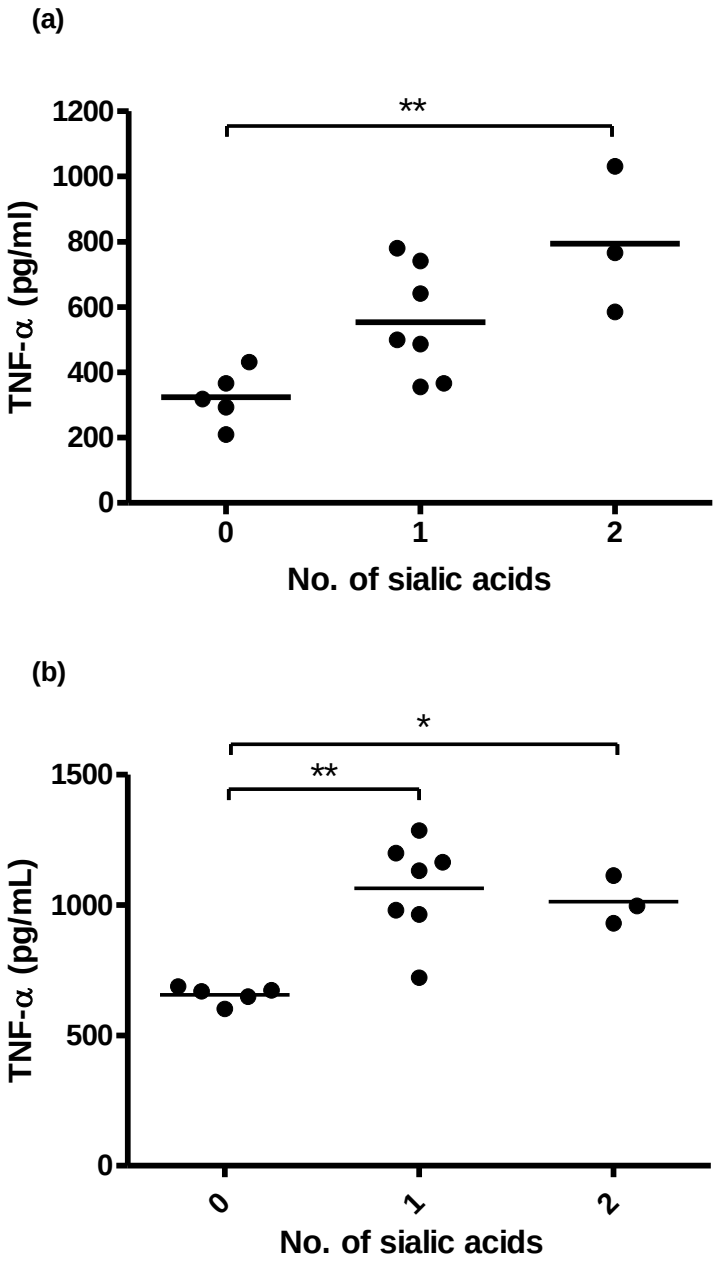


Figure 6-4: Presence of sialic acid on *C. jejuni* LOS increases TNF-α levels in THP-1 cells

1x 10^5 PMA-matured THP-1 cells were stimulated with either (a) 10ng or (b) 100ng of LOS isolated from 8 environment-clade associated strains or 7 livestock-clade associated strains for 20h. TNF-α levels were analysed by ELISA. Data are plotted against the number of sialic acid residues in the LOS of each strain. Each dot represents average TNF-α levels induced by LOS isolated from an individual strain from four independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

separate the strains into groups based on their expression of 0, 1, or 2 sialic acid residues. There was a progressive increase in the levels of TNF-α induced with increasing numbers of sialic acid at 10ng LOS (Figure 6-4a). This increase was statistically significant between no sialic acid containing and 2 sialic-acid containing LOS structures ($p < 0.01$). At 100ng the increase induced by LOS from strains that contain 1 and 2 sialic acid residue was similar (Figure 6-4a). These differences were also observed when THP-1 cells were exposed to live bacteria (Figure 6-5). At an MOI 10 a significant increase in TNF-α production was observed by strains containing 1 sialic acid residue over sialic-acid negative strains (Figure 6-5a). At an MOI 100 there was a progressive increase in TNF-α induction with increasing numbers of sialic acid residues, however due to the spread of the data the difference was only statistically significant between strains that contain 0 and 2 sialic acid residues (Figure 6-5b; $p < 0.05$). Collectively, this data suggested a correlation between the level of LOS sialylation and TNF-α production, in response to both purified LOS and live bacteria; sialylation may therefore account for the observed increase in TNF-α levels noted in response to infection with livestock strains.

6.3 *C. jejuni* lipid A phosphorylation impacts on TNF-α production

The experiments described above were performed during a 3 month visit to Professor Gary Jarvis's laboratory (University of California, San Francisco (UCSF)). Similar experiments were performed on returning to ICH and surprisingly the impact of increasing LOS sialylation had less impact on experiments performed in the UK, although there was still a statistically significant correlation (Figure 6-6; $p < 0.05$). The preparations of the LOS were the same in both sets of experiments; however the THP-1 cells were different. Interestingly, the magnitude of TNF-α induced was also different between the UK and US THP-1 cells. TNF-α levels induced in the US THP-1 cells ranged between 200-1000pg/mL 20h post-infection, compared to 3000-4000pg/mL in the UK THP-1 cells. This data suggested that potential biological differences between US and UK THP-1 cells may impact on responsiveness to *C. jejuni* LOS stimulation.

A previous report indicated that the amide linkage between the acyl chain and the lipid A backbone generates a *C. jejuni* LOS that is less potent in activating TLR4 when compared to an LOS with ester linkages (Van Mourik et al. 2010). Data in Chapter 5 highlighted the potential of different strains to express varying levels of amide and ester linkages, although this did not correlate with the phylogenetic clade of the strain. The potential correlation between amide linkage abundance and the induction of TNF-α was assessed by plotting

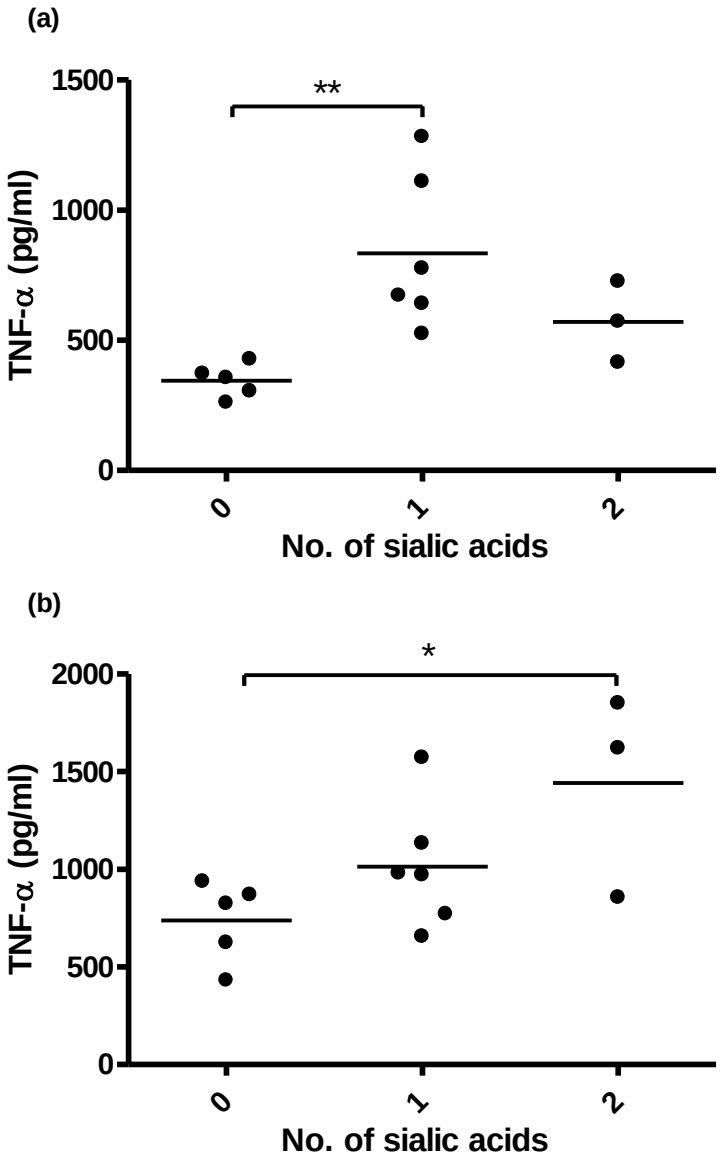


Figure 6-5: Presence of sialic acid on *C. jejuni* LOS increases TNF-α levels in THP-1 cells in response to live bacteria

1x 10^5 PMA-matured THP-1 cells were stimulated with either (a) an MOI 10 or (b) an MOI 100 of 8 environment-clade associated strains or 6 livestock-clade associated *C. jejuni* strains for 20h. TNF-α levels were analysed by ELISA. Data are plotted against the number of sialic acid residues in the LOS of each strain. Each dot represents average TNF-α levels induced by live bacteria from an individual strain from four independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

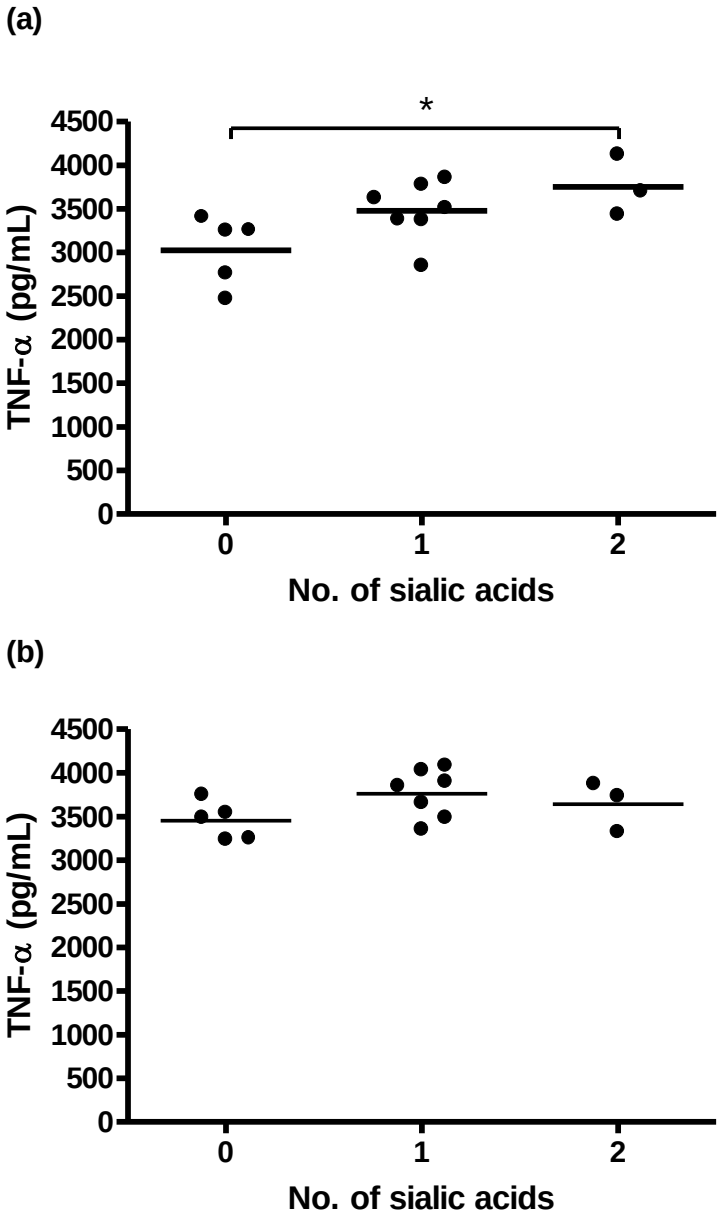


Figure 6-6: Sialic acid on LOS has less impact on TNF-α secretion from UK THP-1 cells

1x 10^5 PMA-matured THP-1 cells were stimulated with either (a) 10ng or (b) 100ng of LOS isolated from 8 environment-clade associated strains or 7 livestock-clade associated strains for 20h. TNF-α levels were analysed by ELISA. Data are plotted against the number of sialic acid residues in the LOS of each strain. Each dot represents average TNF-α levels induced by LOS isolated from an individual strain from five independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

cytokine levels against the percentage of lipid A from each strain that contained 4 amide linkages (Figure 6-7). Only strains containing sialic acid were analysed to eliminate confounding the data. Although there was a trend towards reduced TNF-α production with increasing abundance of the 4 amide linkages in the lipid A backbone this was not statistically significant when data from the US THP-1 cells was analysed (Figure 6-7). However, there was a statistically significant correlation between the abundance of amide linkages and decreasing TNF-α production from the UK THP-1 cells stimulated with 10ng but not 100ng LOS (r^2 = 0.66; Figure 6-7c and d). This data suggested a correlation between the abundance of amide linkages and a reduction in pro-inflammatory responses may occur.

Lipid A phosphorylation status can affect pro-inflammatory responses via an increase in TLR4 stimulation from other bacterial species (Liu, John, & Jarvis 2010). In chapter 5 it was established that the relative abundance lipid A modified with 3 or 4 phosphate compared to 0, 1, or 2 varied between the 15 strains of *C. jejuni* studied, although similar to the amide linkage data this did not correlate with the phylogenetic clades. The effect of lipid A phosphorylation status on TNF-α levels was analysed by plotting TNF-α levels against abundance of lipid A modified with 3 or 4 phosphate groups. Figure 6-8a and b shows a lack of correlation between the relative abundance of 3 or 4 phosphate groups and TNF-α from the US THP-1 cells at 10ng and 100ng concentration respectively. In contrast, a clear correlation between the level of phosphorylation and the induction of TNF-α was observed at the 10ng LOS concentration for the UK THP-1 cells (r^2 =0.81, p = 0.0003), and 100ng concentration (r^2 = 0.41, p = 0.045) (Figure 6-8c and d). This data suggested a strong correlation between the level of phosphorylation of the lipid A and the induction of pro-inflammatory responses, however this was only observed in the UK THP-1 cells.

In chapter 5, no correlation between the levels of phosphorylation and the number of amide linkages was found, however the strain with the highest abundance for amide linkages also expressed the lowest levels of phosphate groups on the lipid A (strain 11168H). These two factors were both predicted to reduce levels of TNF-α therefore it was important to re-analyse the data without this one strain. Removing 11168H from the amide linkage correlation graph (Figure 6-7) meant the correlation was no longer statistically significant, therefore this correlation relied heavily on this one data point. For the phosphorylation correlation, the removal of the one point decreased the r^2 value from 0.83 (p = 0.0003) to 0.73 (p = 0.003) for 10ng and from 0.41 (p = 0.045) to 0.38 for 100ng (p =

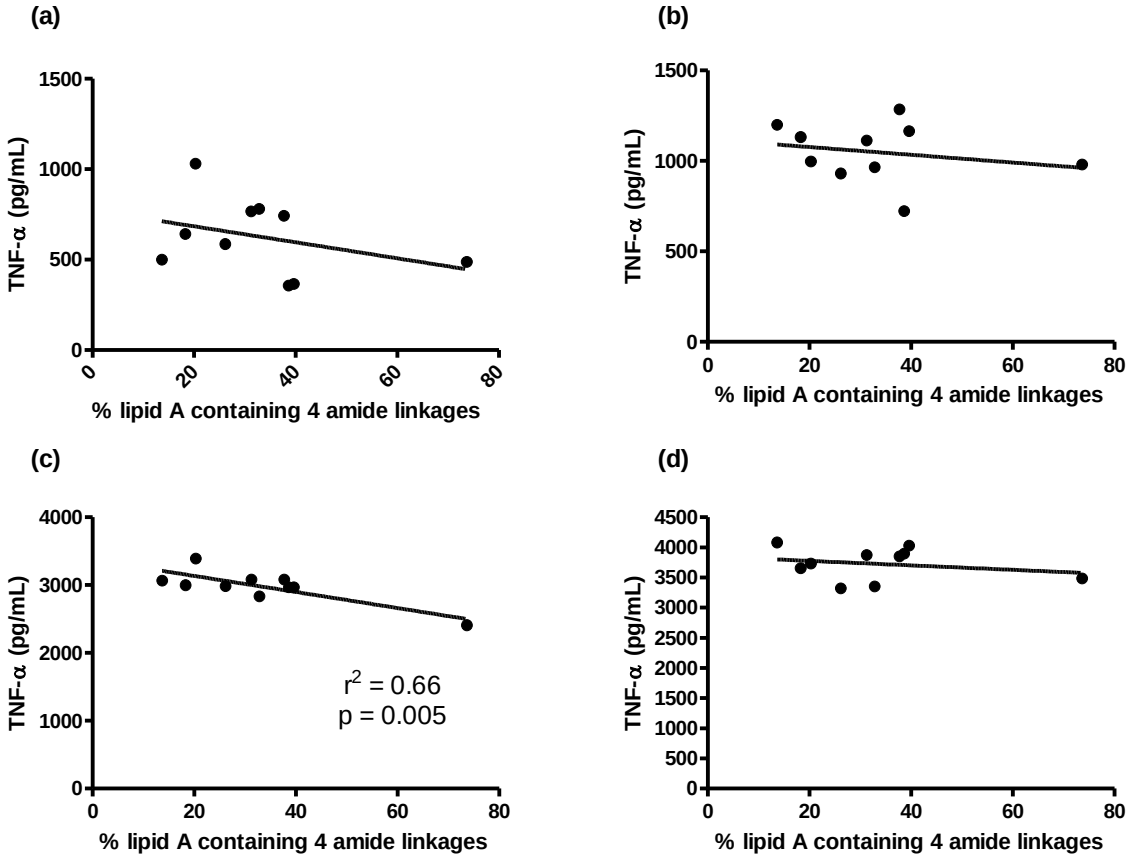


Figure 6-7: Amide linkages in *C. jejuni* LOS influence TNF-α secretion in UK THP-1 cells

1x 10^5 PMA-matured THP-1 cells were stimulated with either (a) and (c) 10ng or (b) and (d) 100ng of LOS isolated from 10 sialic-acid containing *C. jejuni* strains for 20h. TNF-α levels were analysed by ELISA. Data are plotted against percentage abundance of lipid A containing four amide linkages. (a) and (b) data from US THP-1 cells. (c) and (d) data from UK THP-1 cells. Each dot represents average TNF-α levels induced by LOS isolated from an individual strain from at least four independent experiments. Linear regression statistical analysis performed.

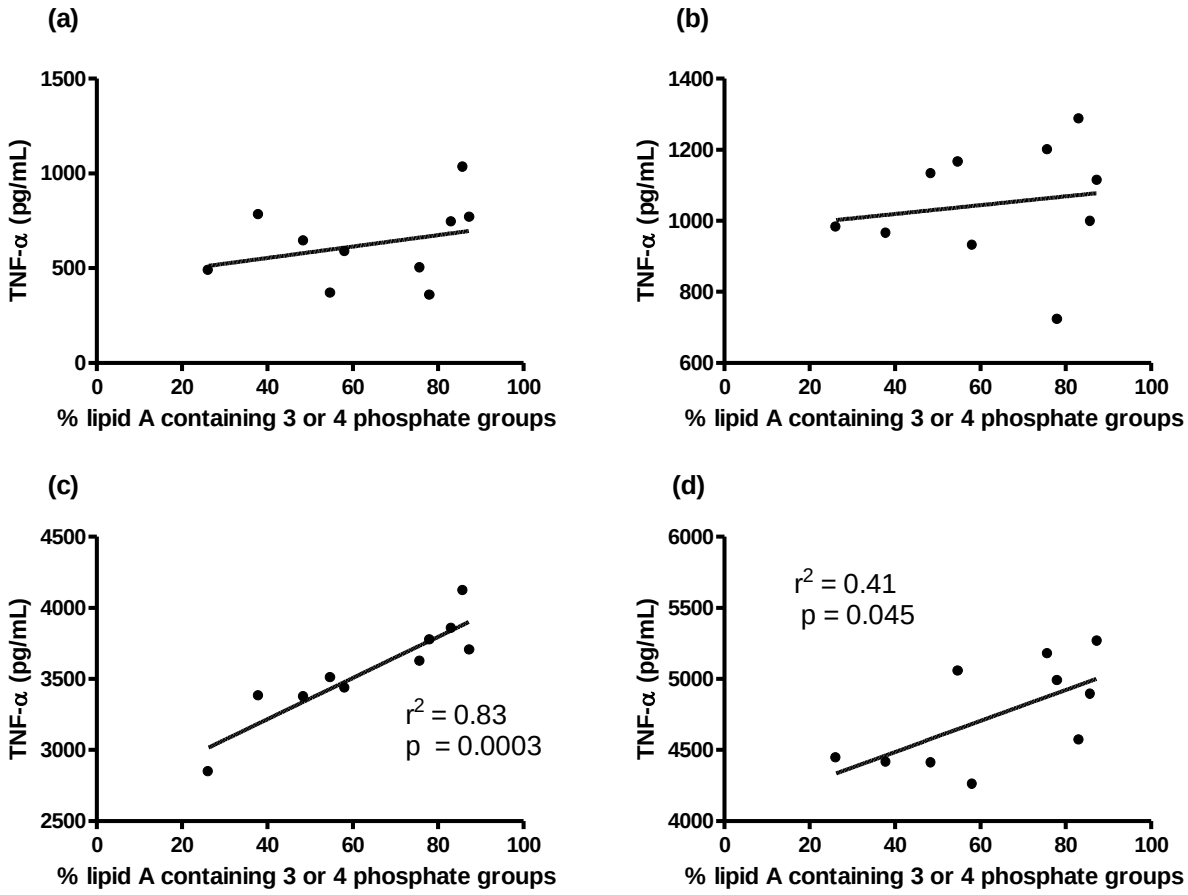


Figure 6-8: Phosphate groups on the lipid A on *C. jejuni* LOS increase TNF-α production

1x 10^5 PMA-matured THP-1 cells were stimulated with either (a) and (c) 10ng or (b) and (d) 100ng of LOS isolated from 10 sialic-acid containing *C. jejuni* strains for 20h. TNF-α levels were analysed by ELISA. Data are plotted against percentage abundance of lipid A modified with either 3 or 4 phosphate groups. (a) and (b) data from US THP-1 cells. (c) and (d) data from UK THP-1 cells. Each dot represents average TNF-α levels induced by LOS isolated from an individual strain from at least four independent experiments. Linear regression statistical analysis performed.

0.08). This showed that the correlation between the phosphorylation and induction of TNF-α did not rely heavily on this one strain. This data suggested the presence of the 11168H data point in the amide linkage correlation may confound the analysis as it has low levels of phosphorylation in addition to high amide linkage abundance.

6.4 Potential role of LOS sialic acid and phosphorylation in modulating primary human monocyte response(s)

We next wished to investigate the impact of LOS modifications on human peripheral blood monocytes. Interestingly, of 6 donors studied 3 showed a progressive increase in TNF-α induction with increasing numbers of sialic acid residues on the LOS (Figure 6-9a; $p < 0.01$). 5 of the 6 donors showed a correlation between the degree of lipid A phosphorylation and TNF-α protein levels (Figure 6-9b; $r^2 = 0.61$ $p = 0.01$). This data confirmed our previous observations obtained in the THP-1 cell line. The data also highlighted that differences in responsiveness to different structural elements of the LOS was donor- dependent.

As induction of pro- and anti-inflammatory cytokines can be differentially regulated, it was imperative to investigate the impact of *C. jejuni* LOS modifications on IL-10 production. The same donors that induced increased levels of TNF-α in response to LOS sialylation also showed increased levels of IL-10. Figure 6-10a shows IL-10 data from these three donors. In contrast, none of the donors showed a correlation between the induction of IL-10 and the phosphorylation of the lipid A (Figure 6-10b). This data suggested LOS sialylation impacts both on pro- and anti-inflammatory TNF-α and IL-10 responses, in contrast lipid A phosphorylation only influenced TNF-α responses.

In Chapter 5, a correlation between the presence of the one-domain *lgtF* gene and a lack of sialic acid synthesis genes in the *C. jejuni* LOS locus was observed. To confirm that the cytokine responses observed in response to purified LOS was influenced by sialic acid residues and not the glucose modifications of the inner core OS (encoded by *lgtF*) biological assays were repeated using sialidase treated-LOS. LOS was treated with sialidase from *Athrobacter ureafaciens,* which is the only known sialidase to remove internally branched sialic acid residues (Uchida et al. 1979). Removal of the sialic acid from the LOS reduced the mean level of TNF-α in THP-1 cells; this decrease was observed only in the LOS that contained sialic acid (Figure 6-11). Comparable levels of TNF-α secretion were observed between the strains that lacked sialic acid and the sialidase-treated LOSs'. In addition, the sialidase treated LOS also caused less potent TNF-α production in monocytes from 2 donors (Figure 6-11b). Although the present study was relatively small, the data suggested that

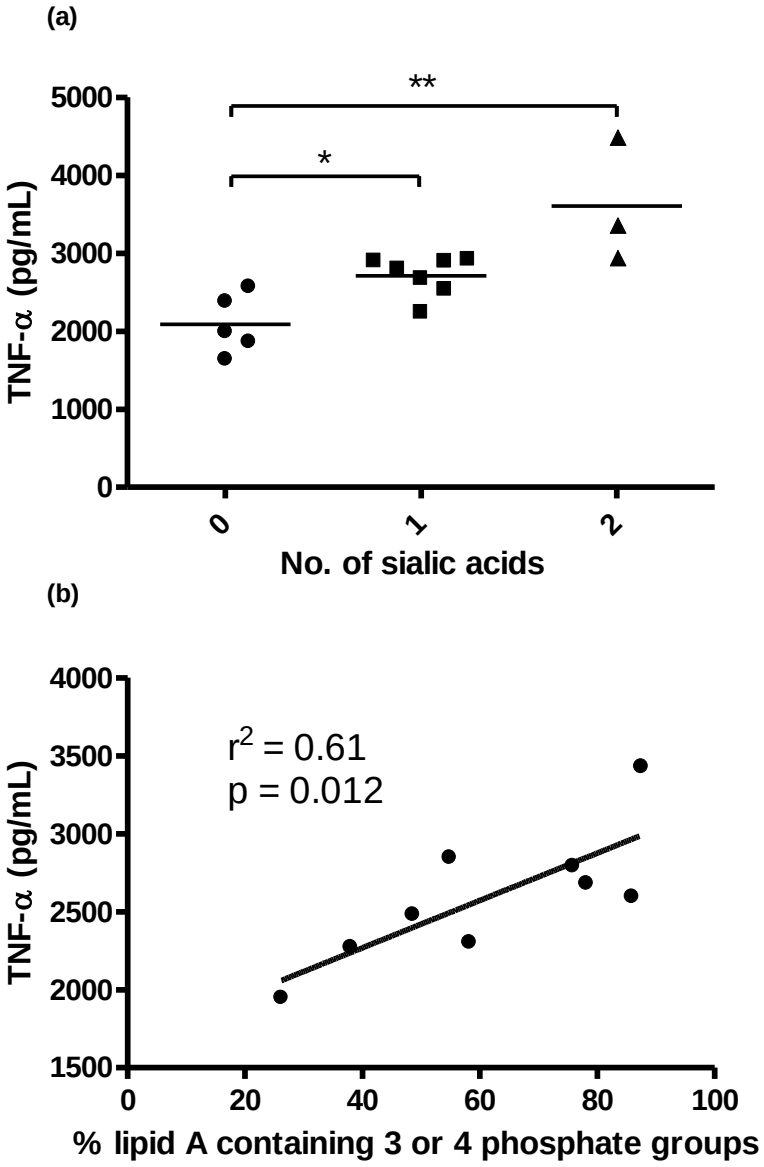
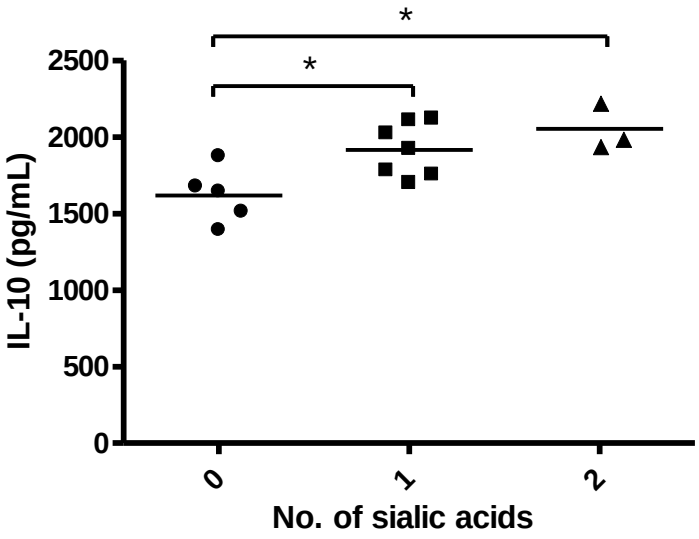


Figure 6-9: Sialic acid residues and phosphorylation on the lipid A influence TNF-α production from primary human monocytes

1 x 10^5 human peripheral blood monocytes were stimulated with either 10ng of LOS isolated from (a) 8 environment-clade associated strains and 7 livestock-clade associated strains (b) 9 sialic-acid containing *C. jejuni* strains for 20h. TNF-α levels were analysed by ELISA. Data were plotted against (a) number of sialic acid residues (b) percentage abundance of lipid A modified with either 3 or 4 phosphate groups. Each dot represents average TNF-α levels induced by LOS isolated from an individual strain from at least four independent experiments. (a) One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001. (b) Linear regression statistical analysis performed.

(a)



(b)

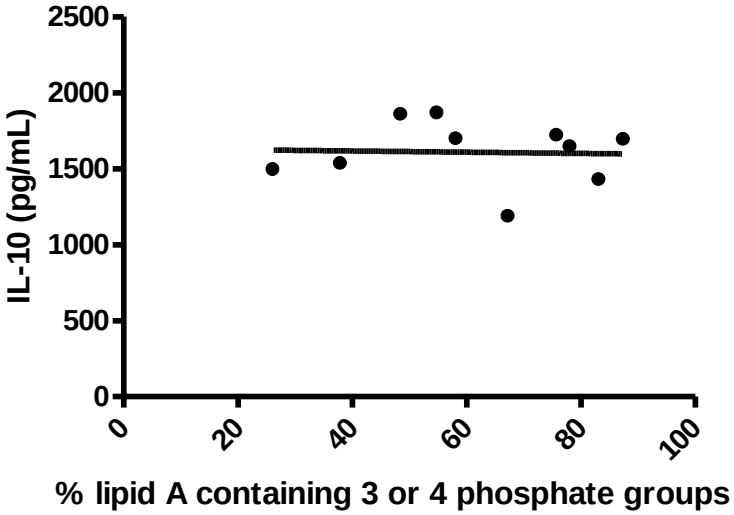


Figure 6-10: Sialic acid but not phosphorylation on *C. jejuni* LOS influences IL-10 production by primary human monocytes

1x 10^5 human peripheral blood monocytes were stimulated with either 10ng of LOS isolated from (a) 8 environment-clade associated strains and 7 livestock-clade associated strains (b) 10 sialic-acid containing *C. jejuni* strains for 20h. IL-10 levels were analysed by ELISA. Data were plotted against (a) number of sialic acid residues (b) percentage abundance of lipid A modified with either 3 or 4 phosphate groups. Each dot represents average IL-10 levels induced by LOS isolated from an individual strain from at least four independent experiments. (a) One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001. (b) Linear regression statistical analysis performed.

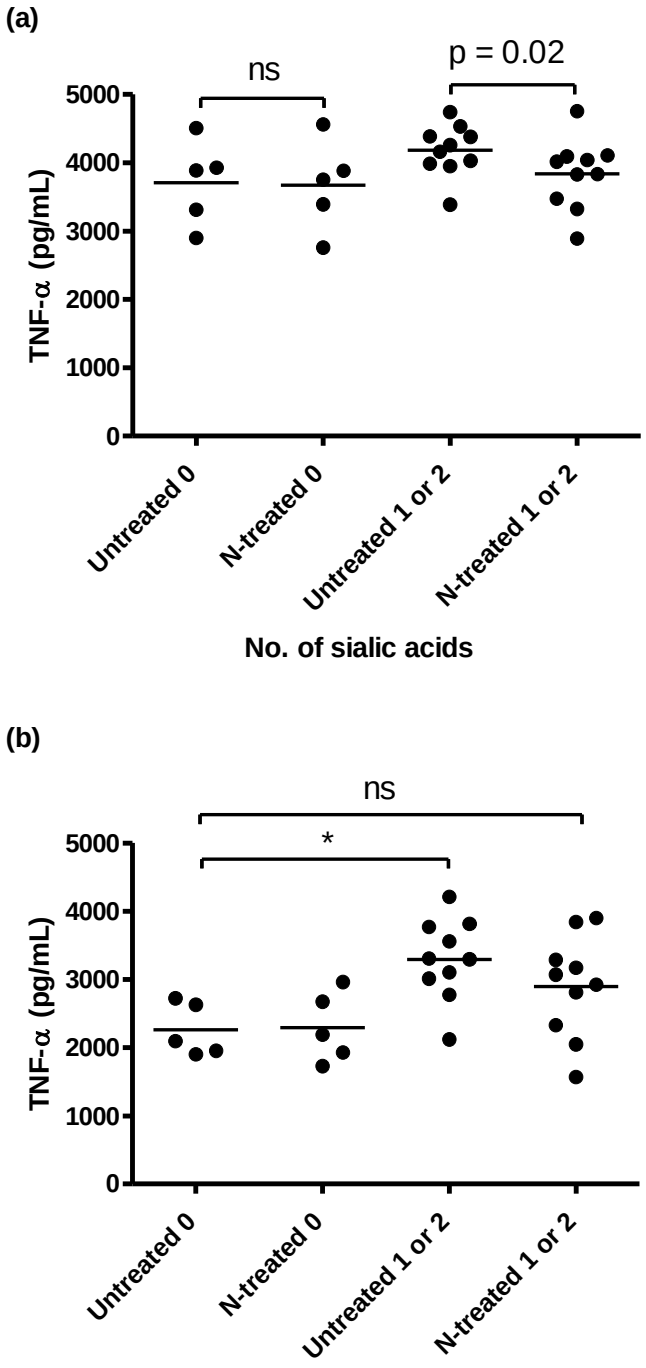


Figure 6-11: Sialidase treatment of sialic-acid containing LOS reduces TNF-α production in primary monocytes

1 x 10^5 (a) UK THP-1 cells (b) human monocytes were stimulated with 10ng LOS either treated with 0.8U/mL sialidase from *Arthrobacter ureafaciens* (N-treated) or untreated for 20h. TNF-α levels were analysed by ELISA. Each dot represents average TNF-α levels induced by LOS isolated from an individual strain from (a) five independent experiments (b) two individual donors. (a) Mann-Whitney statistical analysis performed (b) One-way ANOVA statistical analysis performed with Tukey post-test. No stars = not significant; * <0.05; ** <0.01; ***<0.001.

LOS sialic acid residue mediated an increase in TNF-α, and different structural feature on the LOS such as the glucose residues on the inner core OS were less critical.

Lipid IVa, an antagonist of human TLR4, was used to assess the importance of TLR4 on the induction of TNF-α levels (Lien et al. 2000). THP-1 cells were pre-treated with either 50 or 500ng/mL Lipid IVa for 1h prior to stimulation with 10ng/mL LOS in the presence of the inhibitor for a further 20h. In the presence of 50ng/mL of the inhibitor there was a marked reduction in TNF-α levels in response to both sialylated and non-sialylated LOS; this reduction was even more prominent (>90% reduction) at the 500ng/mL concentration (Figure 6-12). No statistical difference in TNF-α levels were observed between sialylated and non-sialylated LOS at both 50ng/mL and 500ng/mL which suggested that sialylation mediated its pro-inflammatory effects via TLR4. The almost complete inhibition in the LOS-stimulated TNF-α levels in the presence of the inhibitor also indicated that the LOS preparations utilised were of high purity with minimal lipoprotein contamination (TLR2 agonists) which is a common problem with LOS/LPS preparations. For control experiments, peptidoglycan (PGN), a known TLR2 agonist, was used to stimulate THP-1 cells in the presence of Lipid IVa. 1μg/mL PGN stimulation is the absence of lipid IVa induced and average of ~1700pg/mL TNF-α after 20h, compared to ~1500pg/mL in the presence of 500ng/mL lipid IVa showing that there was non-specific effect of treating the cells with the Lipid IVa inhibitor.

A TLR4 reporter cell-line was utilised to assess the effects of structural differences in *C. jejuni* LOS specifically during TLR4 engagement. Human embryonic kidney (HEK) cells express no or minimal basal levels of TLRs which therefore makes it a good reporter cell-line to genetically over-express and analyse TLR function. HEK293 cells stably transfected with human TLR4, CD14, and MD-2 co-receptors and in addition a secreted embryonic alkaline phosphatase (SEAP) reporter gene were employed (Invivogen). The SEAP gene was under the control of a minimal IL-12p40 promoter with additional transcription binding sites for NF-κB and AP-1 which render the SEAP responsive upon TLR4 engagement. The level of SEAP activity was assessed by the addition of a substrate which underwent colour change in the presence of alkaline phosphatase. An increasing trend towards higher TLR4 activity with increasing LOS sialylation was observed (Figure 6-13a). There was a large spread of the data from LOS that contained one sialic acid but statistical significance was

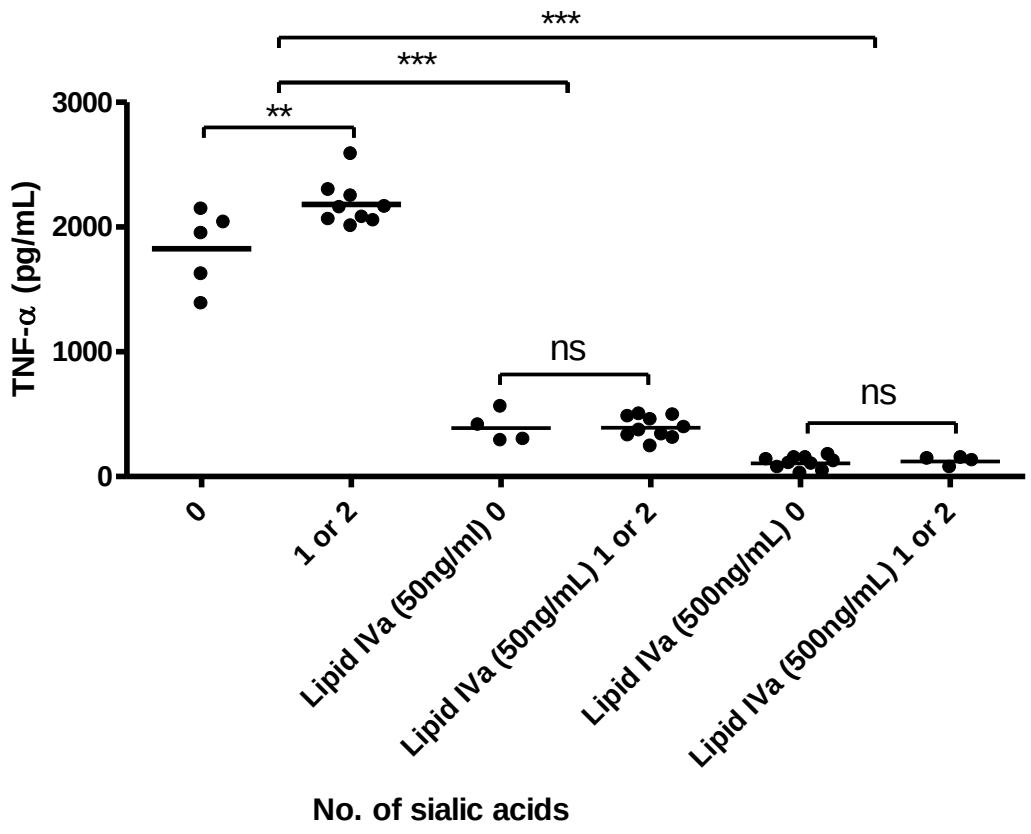


Figure 6-12: TLR4 signalling is essential for *C. jejuni* LOS-mediated TNF-α induction in THP-1 cells

1x 10^5 PMA-treated UK THP-1 cells were pre-treated for 1h with Lipid IVa at 50ng/mL or 500ng/mL, or left untreated. 10ng LOS isolated from 8 environment-clade associated strains or 7 livestock-clade associated strains were used to stimulate the cells for 20h. TNF-α levels were analysed by ELISA. Each dot represents average TNF-α levels induced by LOS isolated from an individual strain from three independent experiments. One-way ANOVA statistical analysis performed with Tukey post-test. ns = not significant; * <0.05; ** <0.01; ***<0.001.

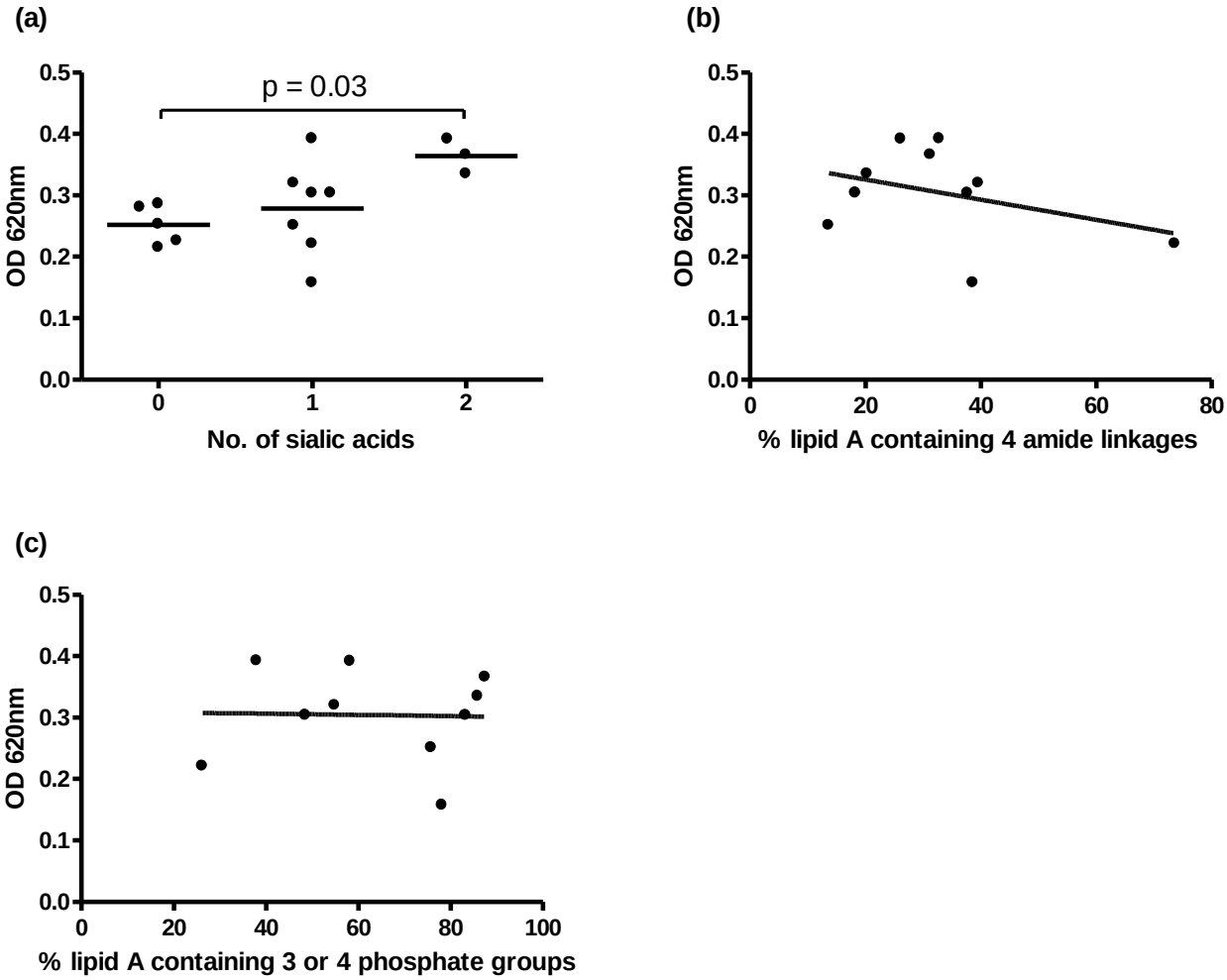


Figure 6-13: Sialic acid on the LOS influences TLR4 activation in a TLR4-reporter cell line

HEK/TLR4 SEAP reporter cells were stimulated with 1ng/mL purified LOS from (a) 8 environment-clade associated strains and 7 livestock-clade associated strains (b) & (c) 10 sialic-acid containing *C. jejuni* strains for 20h. SEAP activity was measured with the addition of an alkaline phosphatase sensitive substrate that turned blue/purple upon phosphatase activity. Data are from three independent experiments and are plotted against (a) the number of sialic acid residues in the LOS of each strain (b) the percentage abundance of four amide linkages in the lipid A backbone (c) percentage abundance of 3 or 4 phosphate groups on the lipid A. (a) Mann-Whitney statistical analysis performed on specific columns. (b) and (c) Linear regression statistical analysis performed.

noted between the non-sialylated *versus* LOS containing 2 sialic acid residues ($p < 0.05$). There was no statistically significant correlation between TLR4 function and the level of LOS phosphorylation nor with the extent of amide linkage (Figure 6-13b and c). This data clearly suggested that LOS sialylation directly impacted on TLR4 engagement.

6.5 Discussion

The present study highlighted a significant difference in monocytic/macrophage derived TNF-α production in response to *C. jejuni* LOS from the two different phylogenetic clades (Figure 6-1). LOS sialylation was found to be the main structural feature contributing to this biological response. In Chapter 5, 7/7 of the livestock strains were found to contain sialic acid compared to only 3/5 of the environmental strains. Comparing cytokine response(s) with the expression of sialic acid clearly showed that THP-1 cell-derived TNF-α levels were dependent upon the presence of LOS sialic acid; in primary human monocytes both TNF-α and IL-10 production showed this dependence (Figure 6-4; Figure 6-9; Figure 6-10). Evidence for these findings were obtained by exposing cells to sialidase-treated LOS. Co-cultures in the presence of treated LOS led to reduced TNF-α levels confirming the importance of sialylation (Figure 6-11). A previous study reported the importance of sialic acid on *C. jejuni* LOS in modulating human monocyte-derived DC cytokine responses (Kuijf et al. 2010). The induction of cytokines was significantly reduced in DCs stimulated with either sialidase-treated LOS or LOS isolated from the sialic acid transferase mutant, *ΔcstII*. By analysing the effects of natural variants of *C. jejuni* LOS sialylation, the current study extends our current knowledge of *C. jejuni* LOS sialylation function.

The effects of LOS sialylation were found to be TLR4 dependent. The inhibition of TLR4 using the antagonist, lipid IVa caused marked reduction (~90%) in TNF-α levels and this effect abolished the observed differences between the sialylated and non-sialylated strains (Figure 6-12). In addition, the level of sialylation impacted on the degree of TLR4 activation (Figure 6-13). The ability of *C. jejuni* LOS sialylation to alter TLR4 responses has previously been reported (Kuijf et al. 2010). LOS isolated from a *ΔcstII* mutant induced <50% activity when compared to its WT counterpart. The sialic-acid modification of *C. jejuni* LOS has been identified as important in binding to two receptors: Sialoadhesin and Siglec-7 (Avril et al. 2006; Heikema et al. 2010). Siglec-7 is able to bind to terminal α2,8-linked sialic acid, whereas Sialoadhesin binds terminal linked α2,3-linked sialic acid. Interestingly, DC-mediated cytokine responses and downstream polarisation of naïve T cells differs depending on linkage conformation of the sialic-acid on the LOS (Bax et al. 2011). In the

current study the potential differences in sialic acid linkage between various LOS species was not investigated and at present remains unknown. As differences in sialylation impacted on TLR4 signalling in a reporter cell line that lacks expression of Siglecs, the potential importance of Siglecs or other co-receptors in the differential responses between non-sialylated and sialylated LOS requires further investigation. . The ability of most Siglecs to signal via intracellular inhibitory ITIM motifs to dampen inflammation also raises mechanistic concerns over the ability of Siglecs to modulate pro-inflammatory responses (Cao and Crocker 2011b). A more plausible mechanism for the differential responses is a direct impact of sialylation on the recognition LOS by the TLR4-MD2 complex. However, no known structural studies have elucidated how sialylation may impact on TLR4 recognition and engagement; to date only the inner core OS residues have been shown to interact with the TLR4-MD2 complex and any potential interactions between the outer core of the OS and the host TLR complex remain ill-defined (Park et al. 2009).

The effects of LOS sialylation on DC TNF-α production were reproducible in cells stimulated by whole live bacteria, signifying the importance of structural modification of *C. jejuni* LOS in the recognition of the whole bacterium by the innate immune system (Figure 6-3). Interestingly, LOS sialylation of *C. jejuni* human clinical isolates is associated with severity of gastro-enteritis (Mortensen et al. 2009). Patients infected with *C. jejuni* bearing sialic-acid on the LOS are more likely to suffer from bloody diarrhoea and have longer duration of symptoms. Sialylation of *C. jejuni* LOS has also been shown to increase *C. jejuni* invasion of IECs (Louwen et al. 2008). It is possible that both the increased invasion of IECs and elevated pro-inflammatory responses contributes to the severity of gastro-enteritis. In patients, elevated antibody responses are also associated with sialylated *C. jejuni* strains (Mortensen et al. 2009). *In vitro* studies indicate that sialylated *C. jejuni* strain-mediated DC activation lead to enhanced B-cell responses (Kuijf et al. 2010). In summary, sialylation of *C. jejuni* LOS affects many aspects of the host-pathogen crosstalk; this spectrum ranges from initial epithelial/innate immune response to later adaptive immune outcome.

Pathogenic bacteria have adapted sialylation as a successful immune evasion strategy. This moiety can be employed via a number of different mechanisms. Sialic acid on *Neisseria menigitidis* LOS inhibits DC phagocytosis and it impairs complement activation for *Neisseria gonorrhoea* (Ngampasutadol et al. 2008; Unkmeir et al. 2002). Interestingly, the sialylation of *Neisseria mengitidis* LOS has no impact on cytokine induction, suggesting that the influence of LOS structural modifications is pathogen specific (Pridmore et al. 2003).

Sialylation of Group B *Streptococcus* capsular polysaccharide engages the inhibitory receptor, Siglec-9, which reduces neutrophil oxidative burst (Carlin et al. 2009b). Complex interactions have evolved between pathogens and the host; sialic acid on microbes clearly interacts with the host innate immune system; in the case of *C. jejuni* our data suggested that sialic acid can elevate pro-inflammatory responses via increased activation of PRRs. It is interesting to speculate that the nature of these interactions may be critical for the outcome of *C. jejuni* depending on the host. As 100% of all livestock strains analysed contain genes required to sialylate the LOS, this may be critical for a potential receptor interaction or immune evasion strategy that allows long-term colonisation of livestock. Conversely, LOS sialylation is not essential for acute pathogenic infection of the human host, although it increases disease severity. This suggests that the interaction between the sialylated LOS and the human immune system is very different to that seen in livestock.

Phosphate modification (addition of phosphate or phosphoethanolamine groups) of lipid A in other pathogens is known to be critical in increasing cytokine induction in innate immune cells (Liu, John, & Jarvis 2010). Variation in the level of lipid A phosphorylation between *C. jejuni* strains has previously been observed but not quantified (Szymanski et al. 2003). Here, the level of phosphorylation of different *C. jejuni* strains was found to strongly correlate with the induction of TNF-α from UK THP-1 cells and human primary monocytes (Figure 6-8; Figure 6-9). The recently elucidated crystal structure of the TLR4-MD2 complex with hexacylated *Escherichia coli* LPS highlighted the critical importance of lipid A phosphorylation in the formation of the TLR4-MD2 complex (Park et al. 2009). The two phosphate groups form interactions with a cluster of positively charged residues on both MD-2 and TLR4, and a hydrogen bond forms with a residue in MD-2, aiding the formation of the heterodimer. It is interesting to speculate that additional phosphate groups on *C. jejuni* LOS may form additional interactions in the TLR4-MD2 complex increasing the signalling capacity of the receptor complex and therefore increasing downstream cytokine induction. Disappointingly, phosphorylation levels did not alter the induction of TLR4 in the HEK/TLR4 reporter cell line (Figure 6-13). This may have been a problem with saturation of TLR4 activation, lower concentrations of LOS were needed to activate the HEK/TLR4 cells in comparison to THP-1 cells. Interestingly, the level of phosphorylation varies between growths of the same *C. jejuni* strain (Szymanski et al. 2003), and although this is a potential virulence factor nothing is known about the regulation of the genes involved in controlling phosphorylation levels *in vivo.*

Alteration of *C. jejuni* lipid A from ester to amide bonds linking the acyl chains to the backbone has been reported to reduce TLR4 activation (Van Mourik et al. 2010). Although the correlation between the abundance of four amide linkages and a reduction in TNF-α production from UK THP-1 cells was statistically significant, the correlation relied heavily on data from one strain that coincidently had both high levels of amide linkages and low levels of phosphorylation, both factors predicted to induce low TNF-α levels (Figure 6-7). It was therefore not possible to conclude whether the natural variation in the abundance of amide linkages influenced the levels of TNF-α secretion.

Interestingly, different THP-1 cells appeared to have differing sensitivity to structural modifications of *C. jejuni* LOS. US THP-1 cells were responsive to sialylation but not phosphorylation of the LOS (Figure 6-4; Figure 6-8). Conversely, sialylation has less impact on UK THP-1 cells but phosphorylation greatly increased the induction of TNF-α (Figure 6-6; Figure 6-8). It is interesting to speculate that point mutations may have occurred in TLR4 or MD2 during the continual passage of the THP-1 cells, resulting in diverging specificities for LOS structural features. Interestingly, monocytes from different donors also showed differing responsiveness to LOS modifications (Figure 6-9). Only 3/6 donors showed a correlation between sialylation and TNF-α production. 5/6 donors showed a trend towards a correlation between phosphorylation and TNF-α production, which only became statistically significant when the data from the donors was combined. Polymorphisms in the human TLR4 are well characterised and alter susceptibility to a range of diseases such as septic shock, ulcerative colitis, and pre-term labour (Lorenz et al. 2002a; Lorenz et al. 2002b; Torok et al. 2004). TLR4 polymorphisms with reduced responsiveness to LPS conversely increased susceptibility of the host to septic shock due to increased burdens of Gram negative infection (Lorenz et al. 2002b). It would be interesting to assess the impact of TLR4 polymorphisms on human *C. jejuni* infection as it remains unknown whether TLR4 signalling protects the host by reducing bacterial load or exacerbates disease by inducing pro-inflammatory responses.

Chapter 7.

Discussion

The immune system has evolved in parallel with the microbes that it encounters. This has allowed the evolution of interactions between host Pattern Recognition Receptors (PRRs) and conserved Pathogen- or Microbe-Associated Molecular Patterns (PAMPs/MAMPs). The detection of invading pathogens by PRRs provides a robust immediate immune response that limits pathogen spread before a specific adaptive immune response can be generated. The ability to detect MAMPs is most likely an absolute requirement for species survival. Although TLRs were first discovered in Drosophila, it is now appreciated that plants also express transmembrane receptor kinases bearing LRR domains that are involved in microbial recognition (Gomez-Gomez and Boller 2000; Song et al. 1995). The plant PRR for flagellin, FLS2, recognises a conserved domain in flagellin which is distinct from the domain involved in TLR5 interactions, suggesting that plants and animals have evolved MAMP recognition convergently which highlights its importance in host defense (Gomez-Gomez & Boller 2000). The idea that these PRRs can also detect host-related components when out of their usual context (DAMPs) suggests that PRRs also function to detect deviations from normal homeostatic conditions. During homeostasis it is likely that a large proportion of the gut microbiota do not interact directly with the GI mucosa due to the thick mucus layer covering the epithelium. However, it is also likely that many “commensal” bacteria are able to survive within the mucus layer and interact with cells of the mucosa via MAMPs/PRR interactions, generating a tolerogenic immune response with the overall effect of allowing commensalism and/or symbiosis to flourish. One method for generating tolerogenic immunological status is the regulation of TLR expression and signalling within the lamina propria (LP) . For example, TLR4 is expressed in lower levels on intestinal immune cells and TLR signalling pathways are dampened in intestinal macrophages (Smythies et al. 2010). The invasive properties of enteric pathogens however overcome the dampened intestinal immune status by engaging PRRs in an overt manner promoting an inflammatory status.

In vitro studies show *C. jejuni* is able to invade intestinal epithelial cells (IECs) and disrupt tight barrier junctions, this is likely to aid passage into the LP whereupon *C. jejuni* will encounter innate immune cells (Bras & Ketley 1999; Wine, Chan, & Sherman 2008). ~1.6% of LP cells are $CD11c^{+}$ DCs (in mice) which function both in intestinal homeostasis and present antigen during infection (Denning et al. 2007). In the present study we investigated *C. jejuni*-mediated effects on BMDC and human monocyte derived DCs. *C. jejuni* induced DC maturation resulting in the secretion of a wide-range of inflammatory cytokines and infection also led to the upregulation of co-stimulatory molecules and MHC class II (Figure 3-3 and Figure 3-4).

The pro-inflammatory cytokines IL-6 and TNF--α were induced in high levels by *C. jejuni* in BMDCs (Figure 3-4). The induction of the cytokines upon DC/*C. jejuni* interaction may aid understanding of *C. jejuni*-mediated pathology in the intestine. IL-6 and TNF--α both induce the acute phase response causing infiltration and activation of neutrophils and other leukocytes into the LP (Andus et al. 1988). Collectively the induction of high levels of pro-inflammatory cytokines likely aids in the clearance of *C. jejuni* but at the cost of inducing pathology. Regulation of IL-1β however presented a more complex scenario. *C. jejuni* induced fairly low levels of IL-1β from BMDCs (Figure 3-4).The physiological relevance of this IL-1β induction during early *C. jejuni* infection is debateable as few reports suggest high levels of IL-1β during *C. jejuni* infection in *in vitro* DC- or *ex vivo* biopsy-co-culture studies (Edwards et al. 2010; Hu et al. 2006). Parallel studies in our laboratory investigating other enteric pathogens found IL-1β levels of >2000 pg/mL in response to *EPEC, C. difficile,* and *S. typhimurium* at low MOI of between 1 and 10, compared to ~300pg/mL by *C. jejuni* at an MOI 100. We have previously reported low levels of IL-1β induction (<20pg/mL) in human intestinal biopsies co-cultured with *C. jejuni* 8h post-infection in an *ex-vivo* model of infection (Edwards et al. 2010). In contrast, *C. difficile* induces levels of ~500pg/ml in a similar *ex-vivo* model (Jafari N, unpublished data). Although this may be a result of the conditions of our *in vitro* assays not inducing expression of specific virulence factors in *C. jejuni* that may induce the inflammasome. No reports so far have shown the activation of the inflammasome by ε-proteobacteria, a factor that may aid long-term colonisation with the host. The lower activation levels of IL-1β by *C. jejuni* may explain why other enteric pathogens have shorter incubation periods before inducing overt intestinal inflammation.

Interestingly, *C. jejuni* induced higher levels of IL-12 compared to IL-23 from BMDCs (Figure 3-4). This correlated with induction of IFNγ but not IL-17A in naive T-cells stimulated with *C. jejuni*-activated DCs and is in agreement with other studies highlighting Th1 skewing by *C. jejuni* (Figure 4-16) (Edwards et al. 2010;Rathinam, Hoag, & Mansfield 2008). The driving of Th1 in the mucosa is linked to the activation of innate immune cells, including PMNs which is likely to contribute to the inflammatory pathology associated with campylobacteriosis. However, in humans with prior exposure to infection *C. jejuni*-antigen dependent induction of IFNγ is associated with protection from pathology but has limited impact on colonisation (Tribble et al. 2010). This suggests that the production of IL-12 in DCs in responses to *C. jejuni* is beneficial to the host via the induction of protective Th1 responses which may limit invasion of *C. jejuni* into the mucosa and minimise the onset of inflammatory diarrhoea. However, *C. jejuni* may still be able to inhabit the mucus layer as Th1 responses afford

minimal protection from colonisation. This highlights a potential dual role of Th1 responses in driving pathology in immune naïve individuals whilst affording protection in humans with prior exposure by limiting *C. jejuni* contact with LP immune cells.

A recent review of the literature suggested that as less than 1/100 human exposures to *C. jejuni* results in pathology (Havelaar et al. 2009). It is also known that repeated exposure to *C. jejuni* limits pathology but not colonisation upon re-infection. It is interesting to speculate that *C. jejuni* may manipulate the adaptive immune response in order to favour colonisation in the human intestine. Despite symptomatic disease lasting on average less than week the mean carriage of *C. jejuni* after infection is 37 days (Kapperud et al. 1992). This suggests that the bacterium is able to persist within the mucosa without causing further pathology and adds weight to the hypothesis of bacterial-mediated immune manipulation. The ability of the murine pathobiont *Helicobacter hepaticus* and human pathogen *Helicobacter pylori* to drive Treg induction in *in vivo* and *in vitro* highlights the notion that bacteria other than "true" commensals do manipulate the adaptive immune system as an immune evasion strategy (Kao et al. 2010; Kullberg et al. 2002).

It was interesting to observe the high levels of IL-10 induction by *C. jejuni* in BMDCs (Figure 3-4). Other enteric pathogens such as *EPEC, C. difficile,* and *S.typhimurium* show markedly lower IL-10 induction when compared to *C. jejuni* (data not shown). IL-10 plays a critical role in intestinal immune regulation which is highlighted by the spontaneous development of colitis in IL-10 KO mice (Kuhn et al. 1993). The induction of IL-10 by *C. jejuni* may be advantageous to the bacterium as it would limit inflammation and potentially reduce clearance by the immune system. It is interesting to note that only IL-$10^{-/-}$ mice develop pathology to *C. jejuni* despite similar levels of colonisation to wild-type mice (Mansfield et al. 2007). This suggests colonisation can occur without pathology, and only the dysregulation of the immune system generates pathology. High IL-10 induction is important for Treg skewing by *H. pylori,* and is implicated in the establishment of long-term infection as IL-10 KO mice show markedly lower colonisation rates than WT mice (Kao et al. 2010; Panthel, Faller, & Haas 2003). Differential induction of cytokines such as IL-10 by *C. jejuni* in humans may explain the multiple outcomes observed upon infection.

Engagement of PRRs by microbes tailors the innate immune response which dictates the overall immune outcome to infection. PRRs detect specific MAMPs because they are largely conserved on microbes which suggest they are structurally necessary for cell viability; mutations that may aid in reduced PRR detection may also not support survival raising a

conundrum for microbial fitness. Some bacteria have however evolved to manipulate these interactions by altering surface structures; exemplified by the ability of α- and ε-proteobacteria to evade TLR5 recognition via specific mutation in the flagellin protein monomers (Andersen-Nissen et al. 2005). Due to the absolute requirement of MyD88- and TRIF-dependent TLR signalling for *C. jejuni*-mediated cytokine production (Figure 4-3) and the lack of interaction between *C. jejuni* FlaA and TLR5, it was interesting to observe a marked reduction in IL-10 and IL-1β production with the *ΔflaA* mutant (Figure 3-6) These observations led to the hypothesis that *C. jejuni* flagellin may modulate immune responses via TLR5-independent mechanism(s). The identity of these interactions was further sought (Chapter 4).

In recent years, questions have arisen as to whether Janeway's original paradigm of microbial detection by engagement of PAMPs/PRRs is sufficient to explain the differential immune response seen between the gut microbota during homeostasis and/or during infection. This has led researchers to favour Matzinger's danger theory, whereby the host does not elicit an overt inflammatory immune response unless "danger" is sensed (Matzinger 2001). This is exemplified by the detection of intracellular bacteria by the inflammasome. *C. jejuni* induces IL-1β secretion from BMDCs (Figure 3-6) which is reduced when the FlaA protein is mutated. Interestingly, *C. jejuni*-dependent IL-1β secretion was independent of the intracellular flagellin receptor, IPAF but dependent on NLRP3 and ASC (Figure 4-14). This suggests an as yet unknown ligand of *C. jejuni* induces the inflammasome and IL-1β in BMDCs. There are two possible hypothesises for the lower induction of IL-1β by the *ΔflaA* isogenic mutant. Firstly, the loss of motility may alter subcellular localisation of the bacterium once phagocytosed. It is possible that low numbers of *C. jejuni* may escape the phagosome resulting in NALP3- and ASC-dependent activation of the inflammasome, and this may be altered with the mutation of the flagella. Secondly, glycosylation moieties on the flagellin may directly interact with other lectin-receptors that may activate the non-canonical inflammasome pathways as was recently reported for dectin-1 (Gringhuis et al. 2012).

Signalling *via* the Pse5Ac7Am structural modification (or derivatives of) of *C. jejuni* flagellin accounted for ~a third of *C. jejuni*-induced IL-10 production (Figure 3-17). In contrast, the carbohydrate moiety of the flagella did not significantly alter the induction pro-inflammatory cytokines. This suggested that particular carbohydrate modifications of *C. jejuni* surface structures may be beneficial to the bacterium by manipulating the host

innate immune response. Interestingly, many of the surface structures of *C. jejuni* are glycosylated. Many of these structural modifications vary between different *C. jejuni* strains including the LOS, capsule, and flagella. Glycosylation can be a means of immune evasion as it allows masking of antigenic surface proteins from immune recognition. In addition, it is becoming increasing clear that these carbohydrate structures themselves are important for interaction with the host via PRR engagement. Genes encoding the *Pse* (pseudaminic acid) pathway have been found in most *C. jejuni* strains studied to date whereas a significant proportion of strains lack the *Ptm* (legionaminic acid) pathway (Champion et al. 2005). The *Pse* pathway is also found in *H. pylori* (Schirm et al. 2003). However, the flagella of *H. pylori* are coated in a sheath which protects it from immune detection; this corresponds to a lack of diversity in the pseudaminic structures found between *H. pylori* compared to the diversity observed between strains of *C. jejuni*.

The ability of *C. jejuni* to bind Siglec-10 via the glycosylated flagella was elucidated in this study (Figure 4-9). We are currently undertaking experiments to decipher whether direct engagement of Siglec-10 by *C. jejuni* induces IL-10. The engagement of Siglec-9 leads to the specific induction of IL-10 suggesting the engagement of Siglec-10 by *C. jejuni* could induce IL-10 (Ando et al. 2008). Although Siglec-10 is known to be expressed in the small intestine, the specific cellular expression is unknown (Li et al. 2001). It is interesting to speculate that the expression of modulating immune receptors such as Siglecs may vary between humans and chickens and may in part account for the difference in disease outcomes between the two hosts. Indeed, one report has highlighted a link between the underexpression of Siglecs and the over-reactivity of human B- and T-cells compared to chimpanzee cells to a number of different stimuli (Soto et al. 2010). The engagement of the CLR, SIGNR1, on LP DCs has also been shown to specifically induce IL-10 promoting the generation of regulatory T cells and inducing oral tolerance (Zhou et al. 2010). This suggests engagement of inhibitory receptors on DCs in the mucosa could be a target for enteric pathogens. Potentially, differential expression of inhibitory receptors between individuals may also account for why only a small percentage of people who are exposed to *C. jejuni* develop pathology (Havelaar et al. 2009). Interestingly, chickens do not express a functional homolog of Siglec-10 which could lead to differential immune interactions between *C. jejuni* with the mucosal immune cells of the chicken and human intestine.

In Chapters 3 and 4 we observed that *C. jejuni* activated MAPK signalling in a TLR-dependent manner. IL-10 in particular was suppressed during pharmalogical inhibition of

the MAPK signalling cascades (Figure 3-13). Additionally, the *ΔflaA* isogenic mutant showed reduced activation of p38 (Figure 3-10). Analysing the ability for Siglec-10 engagement to modulate MAPK signalling is critical to taking the current observations further. The presence of a grb2-binding domain on the intracellular portion of Siglec-10/G suggests receptor engagement could potentially link to the activation of MAPK signalling (Zhang et al. 2003). This however would be the first report of Siglec-mediated MAPK activation.

We studied *C. jejuni* LOS-mediated innate immune interactions in addition to responses to the flagella (Chapter 6). Mutation of genes involved in *C. jejuni* lipid A biosynthesis, the ligand for TLR4, is lethal suggesting that the LOS is critical for membrane integrity (Phongsisay, Perera, & Fry 2007). Although the lipid A acyl chains form the major structural mass in the dimerisation of the TLR4 complex, phosphorylation of the lipid A disaccharide backbone is also involved in charge interactions with the TLR4 complex (Park et al. 2009). Different strains of *C. jejuni* contained varying levels of phosphorylation on the lipid A backbone (Chapter 5). Further, abundance of phosphorylation correlated with TNF--α levels in monocytes and THP-1 cells; a process that was TLR4-dependent (Chapter 6). Mutation of *C. jejuni* lipid A PEA modifications causes a 20-fold increase in susceptibility to cationic AMPs suggesting that the phosphorylation of lipid A is beneficial to the bacterium despite inducing higher levels of TNF--α (Cullen & Trent 2010). Variation in the abundance of amide *versus* ester linkage of the acyl chains to the lipid A backbone between different *C. jejuni* strains was also noted (Chapter 5). The alteration of ester linkages to amide linkages has previously been reported to both reduce TLR4 activation and also decrease susceptibility to AMPs (Van Mourik et al. 2010). We observed a trend towards this correlation between the strains under investigation, however due to lack of spread of the strains the data did not prove this statistically. Collectively the data indicated that different *C. jejuni* strains possess various chemical modifications on their lipid A, which can alter TLR4 engagement and the induction of pro-inflammatory cytokines.

C. jejuni strains from the "livestock" clade all contained genes involved in the biosynthesis of LOS sialic acid, whereas less than 50% of strains from the "environmental" clade contained these genes (Chapter 5). This data suggested that LOS sialylation is similar to the modification of *C. jejuni* flagella with legionaminic acid and its derivatives, with a greater propensity for livestock strains to contain these modifications. The glycosylation of many *C. jejuni* surface structures is hypervariable therefore it was interesting to find a critical component of *C. jejuni* in the colonisation of livestock.

There are many reasons why bacteria sialylate their surface. These include enhancing factor H binding which limits complement deposition (Ram et al. 1998); molecular mimicry of host glycoconjugates which reduces bacteria-specific antibody responses (Vimr and Lichtensteiger 2002); to reduce neutrophil oxidative burst by binding the inhibitory receptor Siglec-9 (Carlin et al. 2009b); and to aid binding to epithelial cells (Louwen et al. 2008). Any of these cellular events may explain the greater propensity for the presence of sialic acid biosynthesis genes in livestock *C. jejuni* strains. Interestingly, in our study human clinical isolates did not show as strong a correlation with sialic acid biosynthesis genes as livestock strains. Potentially, overlapping function between the sialic acid on the LOS and sialic acid-like structures on the flagella in the human intestine could account for why fewer human clinical isolates contain sialylated LOS.

Contrary to its role in immune evasion, increasing levels of sialylation correlated with an increase in TNF-α from monocytes (Chapter 6). This correlation was abolished upon sialidase treatment. Additionally, inhibition of TLR4 with the agonist lipid IVa inhibited TNF-α secretion which implicated TLR4 signalling. This data agreed with a previous report which showed a sialic acid-deficient isogenic mutant of *C. jejuni* causes reduced activation of TLR4 compared to the WT strain (Kuijf et al. 2010). The crystal structure of TLR4 bound to *E. coli* hexacylated LPS does not show a definitive position of the OS moiety in the receptor complex (Park et al. 2009). However this does not negate potential contribution of OS in receptor ligation. Interestingly, the KDO residue which links the OS to the lipid A is found in most bacterial LPS/LOS moieties. The KDO is similar to sialic acid in that it is negatively charged due to the presence of a carboxyl group on the 5-carbon ring structure. Potentially this negative charge could aid in a similar manner as suggested for the lipid A phosphate in TLR4 complex formation. The charge on sialic acid could potentially modify the strength of receptor ligation. Interestingly, the sialic acid on LOS from other bacterial species, such as *Histophilis somni*, has been shown to reduce TLR4 signalling in murine macrophages (Howard et al. 2011). This suggests that the impact of LPS/LOS sialylation may be dependent on the host species, which raises interesting questions as to the possible differences between TLR4 signalling between chickens and humans. The increased activation of human TLR4 by sialylated LOS may have evolved to counter the other immune evasion benefits of sialylation. It is interesting that other surface structures of *C. jejuni* contain sialic acid-like structures, which potentially confer similar advantages of immune evasion seen with sialic-acid whilst not increasing activation of TLRs.

A recent phylogenomic study showed that ~20% of microbial genomes examined contained genes encoding sialic acid biosynthesis pathways (Lewis et al. 2009). Interestingly, sialic-acid biosynthesis pathways are most commonly found in ε-proteobacteria, a class of bacteria that often associate with animals. Until recently it was believed that *de novo* sialic acid-synthesis was limited only to bacterial strains that associate with animals and that this genetic information was inherited by horizontal gene transfer from animal cells. It is now appreciated that the genetic loci for sialic acid biosynthesis is more ancient and that bacteria may have evolved this capability from mutation of the Leg biosynthesis pathway.

Other bacterial carbohydrate structures have been shown to be important in manipulating intestinal immune responses. In germ-free mice *H. hepaticus* drives colitis due to impaired development of $CD4^+$ T cells. These mice are protected from colitis if treated with *B. fragilis* polysaccharide A (PSA) due to the inhibition of Th17 cells and expansion of Tregs (Mazmanian et al. 2008). Interestingly, the PSA does not alter numbers of *H. hepaticus* in the intestine. Protection from *H. hepaticus*—mediated colitis by PSA is dependent on the production of IL-10 from $CD4^+$ T cells. This suggests that manipulation of IL-10 signalling can alter intestinal pathology generated by particular bacteria.

In conclusion, this study has shown how the carbohydrate moieties of two critical *C. jejuni* surface structures, the flagella and LOS, modulate host innate immune responses. Firstly, the modification of the flagella with derivatives of pseudaminic acid drives high IL-10 responses by the manipulation of the TLR-activated MAPK pathway. This potentially is a down-stream effect of Siglec-10 engagement however a direct connection between receptor ligation and IL-10 remains to be proven. Secondly, sialylation of the LOS drives higher pro-inflammatory cytokine responses and is dependent on the phylogenetic source of the *C. jejuni.* This may be an evolutionary feature of human TLR4 to counter the immune dampening effects of bacterial sialylation. Many more questions remain as to how the carbohydrate surface structure of *C. jejuni* modulates immune responses.

Chapter 8.

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