EARLY IMMUNITY TO THE *CAMPYLOBACTER* GENUS –

INSIGHTS INTO HOST AND BACTERIAL FACTORS INVOLVED IN

HEALTH AND DISEASE

By

Katja Brunner

A thesis submitted for the degree of Doctor of Philosophy

UCL

London, February 2016

Infection, Immunity, Inflammation Programme

Institute of Child Health

DECLARATION

I, Katja Brunner 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

Human *Campylobacter jejuni* infection and disease manifestations include severe enterocolitis which is associated with a marked neutrophil influx. Disease severity varies between individuals and an increased risk of chronic gastrointestinal conditions is seen in the susceptible. Molecular events causative to neutrophil infiltration, subsequent bacterial interaction(s) and underlying host risk factors remain poorly understood.

In this study we investigated neutrophil-mediated phagocytosis of *C. jejuni.* We found that bacterial internalisation by neutrophils was serum-dependent and identified complement-opsonins and complement receptor CR1 as a major driver of bacterial uptake. Phagocytosis was accompanied by a modest generation of neutrophil reactive oxygen species (ROS) but interestingly, we failed to observe bacterial clearance.

Activation of the complement cascade by *C. jejuni* was recognised to be multifactorial. Evidence is provided that implicated the classical and/or alternative pathway whilst usage of the lectin-mediated complement activation may be limited. *C. jejuni* surface glycosylation may modulate complement activation as enhanced uptake and serum-mediated bactericidal activity was observed in a mutant strain lacking the polysaccharide capsule.

Finally, we identified in parts the lipooligosaccharide (LOS) structure of *C. concisus,* a potential pathogenic member of the *Campylobacter* genus. Mass spectrometry indicated no evidence for the presence of sialic acid and phosphoethanolamine (PEA) in *C. concisus* LOS; this data is novel and is in contrast with the *C. jejuni* LOS structure where both, sialyation and phosphorylation, are considered important in promoting the TLR4 pro-inflammatory axis. In line with this, we observed lower levels of cytokine secretion by PBMCs in response to *C. concisus* when compared to *C. jejuni* and observed reduced virulence in a *Galleria mellonella* infection model.

Taken together, the present study suggests a novel role for the complement system in bacterial-neutrophil interaction and highlights the importance of various bacterial and host factors in immunity to the *Campylobacter* genus.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my primary supervisor, Dr. Mona Bajaj-Elliott, for all her help and guidance throughout the past three years. I am especially grateful for her continual dedication in seeking local, national and international scientific advice and collaborations which have been an incredible learning experience and an essential part of my PhD. Thank you, Mona, for your support, encouragement and trust.

I would like to thank my secondary supervisor Professor Nigel Klein for his help and advice especially in the part of the Complement story. I wish to thank my supervisor and dear friend Dr. Hannah Jones, who has been an invaluable source of advice, help and suggestions and has been a calming anchor of encouragement in good times and the bad. I would also like to thank Dr. Holly Stephenson, who introduced me to the *Campylobacter* work and was incredibly helpful especially at the start of my PhD, as well as Dr. Dagmar Alber for her inestimable support towards the end.

I wish to thank Professor Gary Jarvis and his colleagues Dr. Constance John and Dr. Nancy Philips for the invaluable opportunity to work with them at their laboratory at the University of California, San Francisco. This three month long visit was a most joyful enterprise and both, a scientific and personal delight. I cannot thank you enough for welcoming me so warmly into your group, and I am deeply grateful for your generosity in sharing your lab facilities, knowledge and time.

I would also like to thank Professor Wilhelm Schwaeble and his team around Dr. Nick Lynch for inviting me to visit their laboratory at the University of Leicester. Thank you for sharing your unlimited knowledge on the Complement field, which has permitted me to undertake my own studies in this area of research. I would have been hopelessly lost without.

Many thanks to so many wonderful people at the Institute of Child Health: Mario, David, Alastair, Ariane, James, Nayani, Hannah, Ronan, Anne and many, many more. Thanks for being fantastic colleagues and all the joyful good times post-work. And of course, my biggest thanks and respect to Vania, Jude and John; nothing would work without them.

Last but not least, I would like to thank my family, my parents Gudrun and Berthold and my sisters Nicole, Tina and Carina. Thank you for your constant support and for always believing in me.

PUBLICATIONS & ABSTRACTS

Publications:

Jowiya W, Brunner K, Abouelhadid S, Hussain HA, Nair SP, Sadiq S, Williams LK, Trantham EK, Stephenson H, Wren BW, Bajaj-Elliott M, Cogan TA, Laws AP, Wade J, Dorrell N, Allan E: Pancreatic Amylase Is an Environmental Signal for Regulation of Biofilm Formation and Host Interaction in *Campylobacter jejuni*. *Infection and Immunity* 2015 Dec; 83(12): 4884-95

Oral abstracts:

Brunner K, Jones HE, Klein N, Bajaj-Elliott M: Complement promotes neutrophil-mediated phagocytosis of *Campylobacter jejuni.* CampyUK, September 2014, Liverpool, UK

Poster abstracts:

Brunner K, Jones HE, Klein N, Bajaj-Elliott M: *Campylobacter jejuni* induces cleavage and secretion of interleukin-1β (IL-1β) in human peripheral blood neutrophils. International Symposium - Neutrophil, May 2014, Montréal, Canada

Brunner K, Jones HE, Stephenson H, Klein N, Bajaj-Elliott M: The impact of butyrate on the NLRP6-inflammasome in humans. International Congress of Immunology (ICI), July 2013, Milan, Italy

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

12-LOX	12-lipoxygenase
AP	Alkaline phosphatase
APC	Antigen presenting cell
APS	Ammonium persulfate
ASC	Apoptosis associated speck-like protein containing a CARD
ATP	Adenosin
BA	Blood agar
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CatG	Cathepsin G
CCV	Campylobacter-containing vacuoles
CD	Crohn's disease
CDT	Cytolethal distending toxin
CFU	Colony forming unit
CHO	Chinese Hamster Ovary
Cia	*Campylobacter* invasion antigen
CRP	C-reactive proteins
cytD	Cytochalasin D
DAMP	Danger associated molecular patterns
DC	Dendritic cell
dHex	Deoxyhexose
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
Efb	Extra fibrinogen binding protein
EFSA	European Food Safety Authority
EGTA	Ethylene glycol tetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
fB	Factor B
Fcn	Fi*coli*n
FcR	Fc receptor
fD	Factor D
fH	Factor H
fI	Factor I
FITC	Fluorescein isothiocyanate
fMPL	N-formyl-Met-Leu-Phe
FSA	Food Standard Agency
Gal	Galactose
GalNAc	Galactosamine
GBS	Guillain-Barré Syndrome
GC-MS	Gas chromatography-mass spectrometry
gh	Globular head
GI	Gastrointestinal
Glc	Glucose
GlcA	Glucuronic acid
GlcN	Glucosamine
GlcN	Glucosamine
H_2	Molecular hydrogen
H_2O_2	Hydrogenperoxide
hBD2	Human β-defensin 2
HBSS	Hank's balanced salt solution
HEK	Human embryonic kidney
Hep	Heptose
Hex	Hexose
HexN	Hexosamine

HexNA	Hexaminuronic acid
HexNAc	N-acetylhexosamine
HF	Hydrogen fluoride
HIS	Heat-inactivated serum
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HRP	Horse radish peroxidase
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IEC	Intestinal epithelial cell
Ig	Immunoglobolin
IL	Interleukin
IMS	Ion-mobility separation
INF	Interferon
IRF3	Interferon-regulatory transcription factor 3
ITAM	Tyrosine-based activation motif
KDO	2-keto-3-deoxyoctulonosonic acid
KO	Knock-out
LAD-1	Leukocyte adhesion deficiency-1
LBP	LPS binding protein
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
M	Molar
MAC	Membrane attack complex
MAG	Myelin-associated glycoprotein
MALDI-TOF	Matrix assisted laser desorption/ionisation-time of flight
MAMP	Microbial associated molecular patterns
Man	Mannose

MAPK	Mitogen-activated protein kinases
MASP	MBL-associated serine proteases
MBL	Mannose binding lectins
MBP	Maltose-binding protein
MCP-1	Monocytes chemottractant protein-1
MDP	Muramyl dipeptide
MeOPN	O-methyl phosphoramidate
MFI	Mean fluorescent index
Mio	Million
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MWCO	Molecular weight cut-off
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NeuAc	N-acetyl neuraminic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	Normal human serum
NK	Natural killer
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerisation domain
O_2	Molecular oxygen
OD	Optical density
OS	Oligosaccharide
P	Phosphate
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEA	Phosphoethanolamine
Pen	Pentose

PFA	Paraformaldehyde
PGN	Peptidoglycan
PI3K-γ	Phosphatidylinositol 3-kinase-γ
PMA	Phorbol 12-myristate 13-acetate
pNPP	P-nitrophenylphosphate
PP	Pyrophosphate
ppm	Parts per million
PPR	Pathogen recognition receptors
PYD	Pyrin domain
qPCR	Quantitative polymerase chain reaction
RC	Baby rabbit complement
rec	Recombinant
Rib	Ribose
RIP-2	Receptor-interacting serine/threonine kinase-2
ROS	Reactive oxygen species
RT	Room temperature
SabA	Sialic acid binding adhesion
SAP	Serum amyloid P
SEM	Standard error of the mean
Siglec	Sialic-acid binding Ig-like receptors
T3SS	Type 3 secretion system
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
Th	T helper
THAP	2,4,6-trihydroxyacetophenone
TIR	Toll/IL-1 Receptor
TLR	Toll-like receptor
TMS	Tetramethylbenzidine

TNF-α	Tumor necrosis factor alpha
TRIF	Toll-interleukin-1 receptor domain-containing adaptor inducing interferon-β
UC	Ulcerative *coli*tis
WAS	Wiskott-Aldrich syndrome
WHO	World Health Organisation
wt	Wild-type

CHAPTER I

INTRODUCTION

1.1 *Campylobacter* genus

1.1.1 Taxonomy

The genus *Campylobacter* belongs to the *Campylobacteraceae* family of the class of the *Epsilonproteobacteria* (Vandamme et al., 1991). The genus was established in 1963 following the re-classification of *Vibrio fetus* to *Campylobacter fetus* after the discovery of distinct biochemical properties from other *Vibrio* species (Sebald and Veron, 1963). The family of *Campylobacteraceae* includes, next to *Campylobacter*, the genera *Sulfurospirillum* and *Acrobacter*, the latter known to cause gastrointestinal (GI) symptoms. *Helicobacter* and *Wolinella* of the *Helicobacteraceae* family are genetically close relatives of *Campylobacter* (Vandamme et al., 2000). The *Campylobacter* genus consists of more than 20 species of which some are important human or animal pathogens and cause of GI infection (Fernandez et al., 2008). *Campylobacter jejuni* and *Campylobacter coli* cause gastroenteritis in humans whilst *C. fetus* can promote bacteraemia in immunocompromised patients (Monno et al., 2004, Skarp et al., 2015). Other, non-*jejuni/coli* species including *Campylobacter concisus*, *Campylobacter showae* and *Campylobacter curvus* have been proposed to cause GI disease, however their role as potential human pathogen(s) is currently unclear (Kaakoush et al., 2015).

1.1.2 General characteristics

Campylobacter are gram-negative bacteria ~0.5 to 6µm long and 0.2 to 0.5µm wide with either a single- or bi-polar flagella or a-flagellated, depending on the species (Penner, 1988). The name *Campylobacter* was derived from the Greek word for curved rod due to the characteristically curved- or spiral-shaped appearance. However, under environmental stresses some species, including *C. jejuni*, can change morphology to a spherical or coccoid form (Figure 1.1; Ng et al., 1985). *Campylobacter* are microaerophilic in nature and require 5 to 10% oxygen (O_2) and 3 to 5% carbondioxide (CO_2) for optimal growth. Some species, including *C. concisus* and *C. showae*, also require 5 to 10% hydrogen (H_2) as an electron donor for microaerobic growth (Lee et al., 2014). *Campylobacter* grow at a temperature between 30°C to 42°C but can survive in sub-optimal conditions (De Cesare et al., 2003). Optimum growth is at 42°C which represents the body temperature of the chicken and other birds contributing to its preferential colonisation of the avian host (Penner, 1988, Skarp et al., 2015).

Graph removed due to copyright restrictions.

Figure 1.1 Transmission electron micrograph of flagellated *C. jejuni* showing spiral and coccoid shaped cells. Adapted from (Ng et al., 1985)

C. jejuni surface is heavily glycosylated (Figure 1.2); over 8% of the genome encodes proteins involved in surface carbohydrate structures most of which undergo frequent phase variations (Dorrell et al., 2001, Parkhill et al., 2000). Analysis of the *C. jejuni* NCTC1168 genome revealed a great insight into *C. jejuni* lipooligosaccharide (LOS), the O-linked glycosylation system that coats the flagellum and a novel polysaccharide capsule. Furthermore it provided the first evidence for an N-linked glycosylation pathway which had never been identified on bacterial surfaces before (Karlyshev et al., 2005b).

The *C. jejuni* genome is rich in hypervariable sequences primarily in genes associated with biosynthesis or modifications of surface structures including LOS, polysaccharide capsule and flagella (Parkhill et al., 2000). In contrast, the genetic locus encoding the N-linked glycosylation system is highly conserved with the key gene product being the oligosaccharyltransferase PglB (Szymanski and Wren, 2005). The wide range of carbohydrate structures found on *C. jejuni* surfaces facilitate invasion of eukaryotic cells, colonisation and evasion of host immune recognition and defence system(s) thus contributing to bacterial survival and pathogenicity (Karlyshev et al., 2005b, Young et al., 2007). One may hypothesise that the frequent phase variations observed in LOS, capsule and flagellin encoding gene loci may stand for a special role for the bacterium in adapting to the environment.

Graph removed due to copyright restrictions.

Figure 1.2 Overview of *C. jejuni* surface glygolipids and glycoproteins. Adapted from (Karlyshev et al., 2005b)

1.1.3 Epidemiology

Infection with *Campylobacter* is highly prevalent in both the developed and less developed world and is recognised as the leading cause of foodborne disease worldwide (Silva et al., 2011). Of all campylobacteriosis cases 90% are attributed to *C. jejuni* while *C. coli* accounts for 5% to 10% of infections (Sheppard and Maiden, 2015, Siemer et al., 2005). The primary source of infection is associated with poultry in developed countries and contaminated drinking water in less developed countries (Young et al., 2007). In the past decade, the global incidence of campylobacteriosis has significantly increased and infection control has become an important topic for global and national health organisations including the World Health Organisation (WHO), the European Food Safety Authority (EFSA) and the Food Standard Agency (FSA). In the European Union, the incidence of *Campylobacter* infections is estimated to 9.2 Mio cases per year, ahead of *Salmonella* infection (6.2 Mio per year). While the numbers of reported *Salmonella* cases have decreased over the last decade, campylobacteriosis is on the rise and accounts for an estimated health burden of €2.4 billion in the EU per year (EFSA, 2010). In the United States, the annual incidence is estimated to be 13.5 cases per 100.000 population as based on the outbreak surveillance survey conducted by the Foodborne Diseases Active Surveillance Network (FoodNet) (Crim et al., 2015). The annual costs of illness caused by *Campylobacter* are estimated to be $1.7 billion in the US (Hoffmann et al., 2012). Importantly, in 2014, FoodNet records a 13% increase in campylobacteriosis compared to 2006-2008 (Crim et al., 2015).

Incidence rates per population vary greatly between EU member states (30 to 13 500 cases per 100.000 population) and are directly correlated with the prevalence of *Campylobacter* found in poultry products (Havelaar et al., 2013). In an EU-wide survey from 2009, broiler carcasses were found to be highly colonised and contaminated with *Campylobacter* as reported by the caecal content (71.2% *Campylobacter* positive) and surface samples of broiler carcasses (75.8% *Campylobacter* positive), respectively. The EFSA Panel on Biological Hazards estimates that poultry accounts for 50% to 80% of *Campylobacter* cases primarily due to the consumption of undercooked meat or cross-contamination of other foodstuffs during meal preparation (EFSA, 2010).

Epidemiological data on the burden of *Campylobacter* infection in non-western countries is scarce (Kaakoush et al., 2015). Most investigations report high incidence rates of campylobacteriosis suggesting an important role in gastrointestinal disease. The prevalence of *Campylobacter* positive stool cultures assessed in hospitalised diarrhoea patients range from 7% detected in a hospital in Kolkata, India to 14.9% reported in a study conducted in Beijing, China (Chen et al.,

2011, Mukherjee et al., 2013). In a ten year study (1997-2007) from Blantyre, Malawai, Mason et al. detected *C. jejuni*/*coli* in 21% of hospitalised children presenting with gastroenteritis. Interestingly, 14% of non-diarrheic children were also positive for these strains suggesting asymptomatic carriage of *Campylobacter* in this paediatric population (Mason et al., 2013). In contrast to industrialised countries, *Campylobacter* disease is primarily seen in children and the source of infection is associated with environmental factors particularly drinking water in the less developed world (Coker et al., 2002, Lengerh et al., 2013). The high incidence and the emerging recognition of an association between campylobacteriosis and malnutrition highlight the urgent need for additional research especially in these parts of the world (Platts-Mills and Kosek, 2014).

1.1.4 Acute gastroenteritis

C. jejuni is highly pathogenic in humans where a bacterial load of less than 500 to 800 organisms can cause disease in susceptible individuals (Mentzing, 1981, Black et al., 1988). Most common symptoms of *C. jejuni* infection are severe diarrhoea, fever, nausea and vomiting which usually occur 24 to 48 hours after first contact and can persist for up to one week (Wassenaar and Blaser, 1999). In the developed world, campylobacteriosis typically manifests as bloody diarrhoea while watery diarrhoea is predominantly observed in less developed countries where children are most commonly affected (Young et al., 2007). The reason for this disparity is not fully understood but it has been speculated that the difference in symptoms might be due to the early exposure to the pathogen in less developed countries which might induce immune competence and prevent later infections (Young et al., 2007).

C. jejuni infected tissue shows characteristics of acute inflammation with a vast infiltration of neutrophils into the epithelium and lamina propria leading to abscess formation in the crypts (Wassenaar and Blaser, 1999). The infection usually begins in the jejunum and ileum and progresses distally to the caecum and colon when leukocytes and high calprotectin levels are typically detected in stool of infected individuals (Blaser, 1997). The clinical presentation and localisation of *C. jejuni*-induced *coli*tis can make it difficult to distinguish it from other inflammatory GI conditions including ulcerative *coli*tis (Siegal et al., 2005).

Infection with *C. coli* is clinically indistinguishable from *C. jejuni* but occurs less frequently (5% to 10% of campylobacteriosis cases) (Siemer et al., 2005). Recent research has focused on the role of other *Campylobacter* species as potential pathogens including *C. concisus*, *C. showae* and *C. curvus* (Man et al., 2010a). It has been proposed that these species have an underreported contribution to the disease aetiology related to the *Campylobacter* genus as they require hydrogen for growth and therefore fail to be detected by conventional screening tools in clinic

(Man, 2011). *C. concisus* has been reported to induce a milder form of gastroenteritis with lower calprotectin levels detected in stool of affected individuals. Symptoms however, were found to be more persistent with 80% of patients presenting with diarrhoea for 14 days or more in comparison to only 32% for *C. jejuni*/*coli* infected patients (Nielsen et al., 2013).

1.1.5 *Campylobacter*-associated sequelae

While *Campylobacter* induced gastroenteritis is self-limiting in most cases and symptoms typically resolve within two weeks, infection is associated with a number of chronic post-infectious conditions within the GI tract and other parts of the body (Kaakoush et al., 2015). Some of them are described in more detail below.

Irritable Bowel Syndrome (IBS)

Post-infectious irritable bowel syndrome (IBS) is a common phenomenon following bacterial gastroenteritis (Wang et al., 2004, James et al., 2004, Spiller and Campbell, 2006, Spiller and Garsed, 2009). *C. jejuni* is associated with an IBS risk of 9% to 13%. However, in a severe case following a water-borne outbreak of acute *C. jejuni* and *E. coli* enteritis, 36% of the subjects reported persistent GI symptoms 24 months after infection despite confirmed bacterial clearance (Marshall et al., 2006). IBS pathogenesis is still poorly understood but has been proposed to be due depletion of the commensal intestinal flora allowing for the overgrowth of other unfavourable organisms that are usually inhibited (Pham and Law, 2014). This, so called dysbiosis, has been observed following bacterial enteritis including *C. jejuni* and is thought to exacerbate GI symptoms (Spiller and Garsed, 2009, Lone et al., 2013b). Others have observed increased levels of immune cells and gut permeability during acute *Campylobacter* enteritis which were found to persist for over a year and may also contribute to the development of IBS (Spiller et al., 2000).

Inflammatory Bowel Diseases (IBD)

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative *coli*tis (UC), are chronic inflammatory conditions of the GI tract of yet unknown aetiology. Changes to the gut barrier function and a dysregulated immune response are thought to be causative to recurrent inflammation of the intestinal mucosa. This is probably triggered by a combination of genetic predisposition and environmental factors (Thompson-Chagoyan et al., 2005). Bacterial GI infections, including campylobacteriosis, have been proposed as risk factor(s) in the development of IBD. In a population-based cohort study, Gradel *et al.* found a 2.9 fold increased risk for post-infectious IBD in patients diagnosed with *Campylobacter* or *Salmonella* infection compared to unexposed individuals (Gradel et al., 2009). It has been proposed that *C. jejuni*-induced epithelial damage might allow for non-invasive commensal bacteria to pass the gut-barrier and facilitate an

exacerbated immune response and chronic inflammation as seen in IBD (Kalischuk et al., 2009). Infection with *C. jejuni* has also been shown to exacerbate IBD symptoms and to play a role in disease relapse (Goodman et al., 1980, Newman and Lambert, 1980, Boyanova et al., 2004).

More recently, focus has turned to understanding the potential role of non-*jejuni*/*coli* *Campylobacter* species in IBD (Man et al., 2010b). Some studies identified a greater prevalence of *C. concisus* in paediatric and adult CD patients when compared to healthy controls (Man et al., 2010b, Zhang et al., 2009, Mahendran et al., 2011). Another study reports *C. concisus* on colonic biopsies in 33% of adult UC patients compared to 11% in controls (Mukhopadhya et al., 2011). Other investigators however have found equal high prevalence of *C. concisus* in ill and in healthy individuals questioning a specific role of these species in IBD pathogenesis (Zhang et al., 2010, Van Etterijck et al., 1996).

Guillain-Barré Syndrome (GBS)

Guillain-Barré Syndrome (GBS) is a severe immune-mediated polyneuropathy characterised by acute ascending paralysis that is associated with bacterial infection (Nyati and Nyati, 2013). The association between bacterial LOS and GBS is mainly due to molecular mimicry between surface LOS structures of some *C. jejuni* strains and human nerve gangliosides (Islam et al., 2012, Heikema et al., 2013); consequently auto-antibodies cross react with self-cells promoting neurologic damage (Tam et al., 2007). GBS is the most common cause of acute flaccid paralysis worldwide with an incidence of 0.6 to 4 cases per 100.000 people per year (Vucic et al., 2009). *C. jejuni* is recognised as the most common bacterial pathogen associated with the disease (Jacobs et al., 1998). It is estimated that 30% to 40% of GBS patients had suffered from *C. jejuni* infection during the two weeks prior to disease onset (Allos, 1997).

1.1.6 *Campylobacter* in the non-human host

Despite the increasing acknowledgement as an important human pathogen, the aetiology of *Campylobacter*-mediated disease remains poorly understood (Sun et al., 2013). This may be partly explained by the lack of a suitable animal model as *C. jejuni* fails to colonise the murine gut under normal conditions. Recent studies have shown that pre-treatment of mice with antibiotics or reconstitution with a human microbiome allows successful intestinal colonisation thus suggesting an important role of the commensal intestinal bacteria in *C. jejuni* exclusion in mice (Bereswill et al., 2011). Interestingly, in the murine model, colonisation alone does not result in acute entero*coli*tis and bloody diarrhoea usually observed in human disease. A new promising approach might lie in the recently developed gnotobiotic IL-10 knockout (KO) mouse model that present

bacterial colonisation and a severe clinical phenotype mimicking human infection (Haag et al., 2012a).

Poultry is recognised as the primary source of human infection in the developed world, this is mainly due to the high carriage rate of *Campylobacter* within broiler chickens. *Campylobacter* species can be detected in high abundance in broiler flocks and their immediate surrounding environment including soil, water sources, building surfaces and other farm animals (Ellis-Iversen et al., 2012). *C. jejuni* has long been regarded as a commensal in chicken but recent evidence suggests this paradigm may need revision as an upregulated innate immune response, inflamed and damaged gut mucosa and diarrhoea have been recorded in infected birds (Humphrey et al., 2014). Nevertheless, the high rate of colonisation and in most cases milder pathogenesis distinguishes the avian host from the human. It has been postulated that the body temperature of the host (42°C in birds *versus* 37°C in humans) may contribute to the expression of bacterial virulence factors (Semchenko et al., 2010).

1.2 *C. jejuni* virulence factors

1.2.1 Secretion of effector proteins

In contrast to other GI pathogens, are the specific virulence mechanisms of the *Campylobacter* genus are still unclear (Silva et al., 2011). The whole genome sequence of *C. jejuni* NCTC11168 identified only one toxin-associated gene locus, the cytolethal distending toxin (CDT) (Parkhill et al., 2000). The functional studies that followed revealed CDT to be a tripartite toxin composed of the cdtA, cdtB and cdtC subunits which are all essential for functional activity (Asakura et al., 2008). CDT has been shown to be cytotoxic in *in-vitro.* Analysis of *C. jejuni* strains isolated after clinical gastroenteritis failed to show distinct disease severity between toxin positive and negative strains suggesting a minor and as yet undefined role of the CDT in bacterial virulence (Mortensen et al., 2011).

Another surprise of the *C. jejuni* whole genome analysis was the lack of evidence for the existence of a type 3 secretion system (T3SS) typically present in invasive bacterial pathogens (Parkhill et al., 2000, Schroeder and Hilbi, 2008, Ogino et al., 2006). A later publication by Christensen *et al.* identified an additional function of *C. jejuni* flagella as T3SS, facilitating the secretion of non-flagellar proteins (Poly and Guerry, 2008). These secretory agents consist of FlaC and *Campylobacter* invasion antigen (Cia) proteins which aid bacterial invasion of IECs (Christensen et al., 2009). The recently identified effector protein, ciaD is associated with *C. jejuni* invasion of

human IEC *via* p38 and Erk 1/2 and downstream IL-8 secretion (Samuelson et al., 2013, Samuelson and Konkel, 2013) .

1.2.2 Lipooligosaccharide (LOS)

The endotoxin LOS is the best defined virulence factor of *C. jejuni* to date. Lipopolysaccharide (LPS), or -oligosaccharide (LOS) in the case of *Campylobacter*, forms an integral part of the outer membrane of Gram-negative bacteria. LOS is composed of two parts: the hydrophobic lipid A that is embedded into the outer membrane and the extracellular hydrophilic oligosaccharide (OS) lacking the repeating outer O-chain unit of LPS (Klena et al., 2005).

The lipid A moiety of *C. jejuni* species is composed of a disaccharide which is phosphorylated with varying numbers and combinations of phosphate (P), pyrophosphate (PP) and/or phosphoethanolamine (PEA) moieties (Figure 1.3 A). Composition of the disaccharide backbone and the phosphorylation pattern are areas of inter-strain variations that have been associated with bacterial virulence and immune recognition (Moran et al., 1991, Stephenson et al., 2013). *C. jejuni* lipid A is hexaacylated with typically four hydroxylated palmitic acids (C:14) attached to the disaccharide via primary amide/ester linkages and two myristic acids (C:16) linked to the primary fatty acids of the non-reducing sugar end (Moran et al., 1991). The hexaacylated nature of *C. jejuni* LOS facilitates activation of Toll-like receptor 4 (TLR4) which is an important mediator of the host inflammatory response to this pathogen (Maeshima and Fernandez, 2013).

C. jejuni OS consists of a mainly conserved inner core and a highly variable outer core region (Figure 1.3 B). The inner core is located proximal to the lipid A backbone and, in the case of strain NCTC 11168, contains two 2-keto-3-deoxyoctulonosinic acid (KDO) residues, two heptose (Hep) residues with two branched glucose (Glc) residues on each Hep and one PEA/P residue on one Hep (St Michael et al., 2002). The outer core is composed of varying numbers of galactose (Gal), Glu, Acetygalactosamine (GalNAc) and Acetylneuraminic acid (NeuAc, sialic acid) residues. The latter has been the major focus of research as sialyation of *C. jejuni* LOS is linked with ganglioside mimicry and the risk to develop post-infectious GBS (Gilbert et al., 2008).

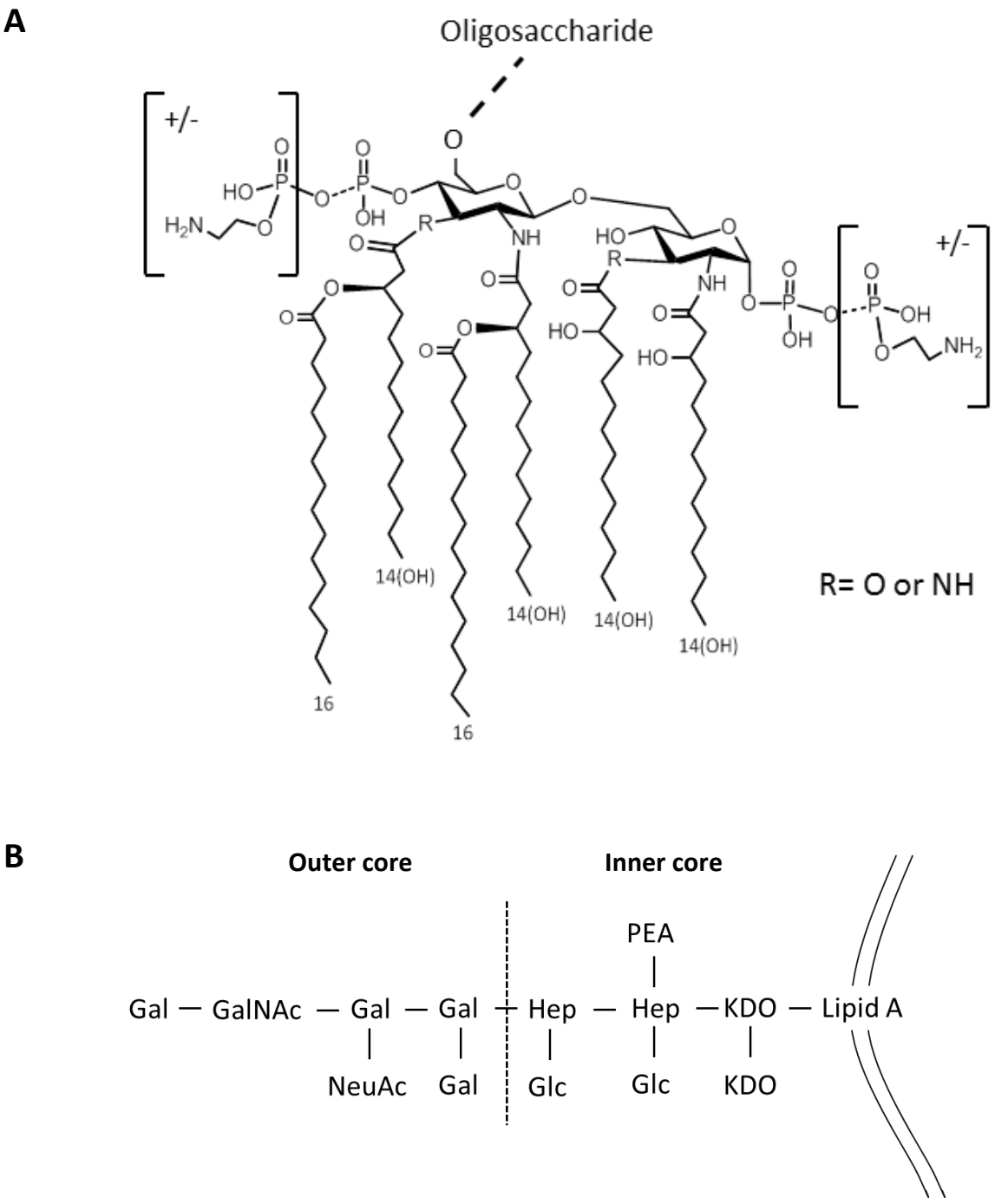


Figure 1.3 Structure of *C.jejuni* lipooligosaccharide (LOS)

(A) Hexaacylated *C. jejuni* lipid A. **(B)** Oligosaccharide structure from *C. jejuni* strain 11168. Galactose (Gal), Glucose (Glc), Heptose (Hep), Acetylgalactosamine (GalNAc), 2-keto-3-deoxyoctulonosinic acid (KDO), Neuraminic acid (NeuAc, sialic acid). Adapted from (Moran et al., 1991, St Michael et al., 2002)

In comparison to non-sialyated *C. jejuni* strains, strains expressing sialic acid in their LOS exhibit significantly increased human IEC invasive capacity and pro-inflammatory cytokine response *in-vitro* (Louwen et al., 2008, Stephenson et al., 2013). There are five genes associated with LOS sialyation; three encoding enzymes involved in the biosynthesis of sialic acid from the N-acetylmannose precursor (neuA, neuB, neuC) and two sialic acid transferases (cstII, cstIII) (Gilbert et al., 2008). Interestingly, LOS is sialyation not only dependent on gene expression but varies under environmental changes. For example, when *C. jejuni* 11168 is grown under conditions mimicking the avian host only half of the LOS is sialyated while 90% sialyation is found when grown at 37°C. These findings suggest a role for LOS sialic acid in human disease pathogenesis (Semchenko et al., 2010).

1.2.3 Polysaccharide capsule

C. jejuni is unique amongst GI pathogens as it expresses a polysaccharide capsule that covers its surface; this structure is associated with serum resistance, cellular invasion and evasion of host immunity (Bacon et al., 2001, Keo et al., 2011, Karlyshev et al., 2008). The polysaccharide structure of the capsule is highly variable between isolates due to the presence of phase-variable genes, similar to *Campylobacter* LOS (Karlyshev et al., 2005a). In addition, the capsule can be modified with ethanolamine glycerol and O-methyl phosphoramidate (MeOPN); the latter being found on about 70% of *C. jejuni* capsules (Maue et al., 2013). The lack of capsular MeOPN residues leads to enhanced *C. jejuni*-induced cytokine response in murine-derived dendritic cells (DCs) (Rose et al., 2012). MeOPN negative mutants lose virulence in a *Galleria mellonella* larvae survival model suggesting an important role in bacterial virulence (Champion et al., 2010).

1.2.4 Flagella

Presence of flagella on either side of the pole provides *C. jejuni* the ability to be motile and colonise the small intestine (Guerry, 2007, Hendrixson and DiRita, 2004). The flagellum is composed of a hook-basal body, embedded in the cytoplasm and inner membrane, and an extracellular filament (Lertsethtakarn et al., 2011). The extracellular filament is composed of two structural flagellin proteins, FlaA and FlaB. FlaA is the major structural component and essential for motility while mutations in FlaB have only little effect on motility (Guerry et al., 1991, Sommerlad and Hendrixson, 2007). The motility system is an essential survival strategy for *Campylobacter* as it allows transit through the GI tract; *flaA* mutated strains fail to colonise the avian gut (Guerry, 2007, Wassenaar et al., 1993). In addition, the flagellar apparatus also functions as a T3SS, transporting the effector protein, Cia invasion antigens and FlaC into the host cell (Guerry, 2007). This process requires the hook-basal body and either FlaA or FlaB filaments (Konkel et al., 2004).

C. jejuni escapes recognition by Toll-like receptor 5 (TLR5) due to amino acid changes in the N-terminal domain of the flagellin molecule, which is centrally positioned within the TLR5 recognition site (Smith et al., 2003). This phenomenon has also been noted in other ε-proteobacteria such as *Helicobacter pylori* and possibly provides strategic advantages to these enteropathogens by evading TLR5-mediated immunity (Andersen-Nissen et al., 2005) .

Approximately 10% of the *Campylobacter* flagellin protein is O-linked glycosylated with sialic acid-like structures, including pseudaminic acid and legionaminic acid, that are essential for successful flagellin assembly, motility and colonisation in chicken (Howard et al., 2009, Ewing et al., 2009). Studies from our laboratory suggest that flagellin-associated pseudaminic acid moieties may interact with the immunomodulatory receptor, Sialic-acid binding Ig-like lectins 10 (Siglec10) on host-cells. This interaction promotes the secretion of Interleukin 10 (IL-10), an anti-inflammatory cytokine which may provide a mechanism for asymptomatic colonisation (Stephenson et al., 2014).

1.3 Colonisation of the gastrointestinal (GI) tract

1.3.1 Transfer through the upper digestive tract

In order to colonise the small intestine, *C. jejuni* needs to survive the transit through the upper part of the GI tract. The acidic condition of the stomach (normally around pH 2.0) acts as major chemical defence against ingested pathogens. *In-vitro C. jejuni* is pH sensitive and survives less than 60min at pH 3.0 (Rotimi et al., 1990). This evident pH-sensitivity stands in contrast to the low inoculation dose required to establish *Campylobacter* infection in humans as this requires bacterial survival in the stomach (Black et al., 1988). It has been proposed that enteric pathogens are protected from killing under acidic conditions when inoculated onto certain food sources. When administered on ground beef, *C. jejuni* survives acidic conditions of pH 2.5 while being sensitive to killing at pH 5 in isolation (Waterman and Small, 1998). Interestingly, *C. jejuni* has also been shown to inhabit protozoa and use them as a vehicle which may also enable its successful stomach transit (Axelsson-Olsson et al., 2010).

1.3.2 Mucus barrier function

Once through the stomach, *C. jejuni* colonises the jejunum and ileum of the lower part of the small intestine (Black et al., 1988). In order to establish adherence to the IECs, the bacterium needs to cross the mucus layer that covers the gut lining. The mucus layer is about 700µm thick with mucin glycoproteins being its main component (Linden et al., 2008). Mucins are heavily O-glycosylated

and consist to 70% of carbohydrates providing important properties such as water holding capacity, protease resistance and a high charge density (Moncada et al., 2003). Together with the other antimicrobial agents that are part of the mucus layer, such as defensins, lysosomes, collectins and nitric oxide, it forms a physical barrier and cell-toxic environment for invading pathogens (Alemka et al., 2012).

The exact mechanisms on how *C. jejuni* manages to circumvent the mucus barrier are not completely understood however, some bacterial factors are known to be essential. Motility and chemotaxis are crucial for bacterial colonisation as shown in animal studies (Kanji et al., 2015, Nachamkin et al., 1993, Yao et al., 1997). *C. jejuni* is highly motile and interestingly, motility increases in mucin-like solutions of high viscosity suggesting its ability to adapt to the mucus environment (Szymanski 1995). The characteristic spiral, corkscrew-like shape of the *C. jejuni* rod confers advantage when passing through viscous media (Shigematsu et al., 1998). Additionally, the relatively short OS-chains of *Campylobacter* LOS may not form optimal interactions with the mucin glycoproteins, promoting movement towards the epithelial apical surface (McSweegan and Walker, 1986).

C. jejuni primarily colonises the mucus-filled crypts of the lower small intestine (Beery et al., 1988). The navigation of *C. jejuni* through the GI tract is mediated by chemotactic signals provided by an extracellular chemical milieu. Carbon sources and electron donors including L-asparagine, formate, D-lactate and mucus function as attraction agents. Energy taxis is thought to be the primary force of promoting the translocation of *C. jejuni* towards its optimal niche for colonisation (Vegge et al., 2009, Lertsethtakarn et al., 2011). However, studies report additional signalling function by other GI components including bile salts and pancreatic amylase (Rivera-Amill et al., 2001, Jowiya et al., 2015). Jowiya et al. found the promotion of biofilm formation when *C. jejuni* was co-cultured with α-amylase. Co-culture induced the secretion of a novel, previously unknown α-dextran which increases bacterial adherence and human IEC invasion *in-vitro* as well as bacterial virulence and colonisation of the avian host (Jowiya et al., 2015).

1.3.3 Intestinal epithelial cell (IEC) adherence and invasion

C. jejuni expresses several potential adherence factors on its surface including the fibronectin binding proteins CadF and FlpA (Flanagan et al., 2009, Konkel et al., 1997). The best studied adhesion factor, CadF, is a 37kDa protein expressing a fibronectin-binding domain (Konkel et al., 2005). CadF expression is essential for maximal bacterial binding to human IECs while mutations in the binding domain significantly impair cellular adherence (Monteville and Konkel, 2002, Krause-Gruszczynska et al., 2007b).

IEC invasion is facilitated by various host and bacterial factors however the molecular details are still lacking. Invasion of *C. jejuni* is promoted by a low-oxygen environment, sialyated LOS and the presence of a polysaccharide capsule (Mills et al., 2012, Louwen et al., 2008, Bacon et al., 2001). Interestingly, Corcionivoschi et al. report that upon interaction with human IECs, *C. jejuni* induces the generation of reactive oxygen species (ROS) *via* epithelial NADPH-oxidases altering capsule formation and bacterial virulence. Exposure to ROS leads to the loss of the polysaccharide capsule and decreased bacterial motility and cellular invasion (Corcionivoschi et al., 2012)

The close proximity of *C. jejuni* with IECs stimulates the secretion of Cia proteins which are implicated in maximal invasion by the modulation of host cell signalling (Rivera-Amill et al., 2001, Neal-McKinney and Konkel, 2012, Samuelson et al., 2013). *C. jejuni* is internalised by a microtubule-dependent mechanism following the activation of a Rho GTPase-associated signalling cascade and rearrangement of the cytoskeleton (Monteville et al., 2003, Krause-Gruszczynska et al., 2007a, Hu and Kopecko, 1999). Following internalisation, *C. jejuni* localises in specific cytoplasmic compartments, referred to as *Campylobacter*-containing vacuoles (CCVs). CVVs form immediately after host cell entry and facilitate intracellular survival by avoiding delivery to the lysosome (Watson and Galan, 2008). CiaI proteins are thought to contribute to bacterial survival by preventing co-localisation of CVVs with the lysosomal marker, cathepsin D (Buelow et al., 2011).

1.3.4 Interaction with the intestinal microbiome

Complex commensal microbial communities reside in a healthy human and animal gut; these communities by their sheer number and diversity are thought to protect the host from potential pathogenic colonisation and invasion (Round and Mazmanian, 2009). *C. jejuni* fails to colonise the murine GI tract under normal conditions but colonisation and disease can be induced in gnotobiotic mice and in mice harbouring a modified microbiome (Bereswill et al., 2011, Haag et al., 2012b). Analysis of the intestinal microbial communities of poultry abattoir workers suggests that the higher abundance of specific bacterial genera in the microbiome, including *Bacteroides* and *Escherichia* species, increases the risk of these individuals to be colonised with *Campylobacter* (Dicksved et al., 2014). A number of recent publications confirm the suggested protective effect of commensal bacteria by showing that the administration of health-associated bacterial strains significantly reduces *Campylobacter* colonisation of chicken intestines (Cean et al., 2015, Ghareeb et al., 2012, Bratz et al., 2015). Co-culture of *C. jejuni* with a combination of probiotic-associated bacterial strains of the *Lactobacillus* and *Bifidobacterium* genus reduces the ability of the

pathogen to adhere and invade human IECs confirming a protective role of the resident bacterial community (Alemka et al., 2010).

C. jejuni infection is associated with long-term effects on the composition of the commensal flora in humans and mice (Lone et al., 2013a, Dicksved et al., 2014). It has been hypothesised, that the relative high incidence of chronic GI conditions (IBS, IBD) following *Campylobacter* infection is linked with post-infectious dysbiosis (Mukhopadhya et al., 2012, Bennet et al., 2015). Despite being two very distinct clinical conditions, the onset of IBS and IBD are both associated with bacterial infection and a number if IBD patients in clinical remission report IBS symptoms (Fukuba et al., 2014, Minderhoud et al., 2004, Kalischuk and Buret, 2010). Apart from its impact on the microbial composition, *C. jejuni* disrupts epithelial tight-junction proteins including occludin and claudin-4 facilitating the translocation of non-invasive *E. coli* strains through the epithelial layer (MacCallum et al., 2005b, Lamb-Rosteski et al., 2008). *C. jejuni* also promotes the transcellular uptake of non-invasive bacteria *via* lipid-raft mediated endocytosis (Kalischuk et al., 2009). In a recent study, Reti *et al.* observed upregulation of virulence factors in non-invasive *E.coli* upon exposure to *C. jejuni* or *C. jejuni*-conditioned media. Further, *C. jejuni* elevated *E.coli* flagellar gene expression and enhanced bacterial adhesion and epithelial pro-inflammatory responses cytokine immunity (Reti et al., 2015). It is interesting to speculate that interactions with other intestinal bacterial species may be linked with long-term inflammatory events observed following *Campylobacter* infection.

1.4 Mediators of host immune response

1.4.1 Toll-like receptors (TLRs)

Toll-like receptors (TLRs) are a family of pathogen recognition receptors (PPRs) that play a key role in host innate immunity. At least eleven members of the mammalian TLR family are known to date (Takeda and Akira, 2005). They recognise a broad range of microbial and cell stress associated signals referred to microbial associated molecular patterns (MAMPs) and danger associated molecular patterns (DAMPs). TLRs are transmembrane proteins and comprise of a ligand-binding C-terminal leucine-rich repeat (LRR) domain, a single membrane spanning domain, and an N-terminal cytoplasmic Toll/IL-1R (TIR) homologous interacting domain that, upon activation, interacts with a variety of adaptor molecules initiating downstream signalling cascades. TLRs 1, 2, 4, 5 and 6 bind to microbial components, while TLRs 3, 7, 8 and 9 recognise RNA and DNA from most organisms, including viral and microbial origin (Takeda and Akira, 2005).

C. jejuni engages in the majority with TLR2 and TLR4/MD-2 while stimulation of TLR9 by bacterial DNA only results in low levels of cytokine response (de Zoete et al., 2010, Dalpke et al., 2006). As previously described, *Campylobacter* produces a modified flagella that is unresponsive to TLR5 recognition. Signalling *via* TLR5 is important for the immune outcome to many enteric pathogens including *Salmonella*; evasion of this axis may be critical for *C. jejuni* survival and infection at mucosal sites (Andersen-Nissen et al., 2005).

TLR4-mediated recognition of LPS/LOS plays a crucial role in the host immunity to many Gram-negative pathogens including *C. jejuni* (Stephenson et al., 2013, Rathinam et al., 2009b, Huizinga et al., 2015). LPS/LOS-TLR4 signalling requires several co-factors including LPS binding protein (LBP), CD14 and MD-2. LBP is a soluble shuttle protein which binds to LPS/LOS and mediates the association with CD14. CD14 exists as soluble protein or anchored to the host cell surface and facilitates the transfer of LPS/LOS to the TRL4/MD-2 receptor complex. Dimerisation of soluble MD-2 with TLR4 provides a platform for recognition of the lipid A moiety of LPS/LOS (Lu et al., 2008).

Hexaacylated *C. jejuni* LOS activates human TLR4/MD-2 (de Zoete et al., 2010). Following ligand binding, TRL4 signalling engages with two downstream pathways depending on the adaptor domains involved. The Myeloid differentiation primary response gene (MyD88)-dependent signalling pathway initiates the generation of pro-inflammatory cytokines *via* NF-κB and the mitogen-activated protein kinases (MAPK) (Chang and Karin, 2001). The MyD88-independent signalling pathway depends on the recruitment of Toll-interleukin-1 receptor domain-containing adaptor inducing interferon-β (TRIF) and mediates the induction of Type I interferons and interferon-inducible genes. The TRIF-pathway signals *via* interferon-regulatory transcription factor 3 (IRF3) but is also plays a role in late-phase activation of NF-κB and AP-1 (Yamamoto et al., 2003). *C. jejuni* induces cooperative signalling through TLR4-MyD88 and TLR4-TRIF axes which play a significant role in the development a T helper 1 (Th1) type immunity in humans (Rathinam et al., 2009a). Interestingly, *C. jejuni* fails to induce the TRIF-pathway mediated secretion of Interferon β (INF-β) in chicken which may prevent LOS-induced systemic inflammation in the avian host (de Zoete et al., 2010). Predicted ligands for TLR2 are *C. jejuni* surface lipoproteins such as JlpA (Jin et al., 2003). While TLR2 is activated by *C. jejuni* it is not essential for the development of an inflammatory response and may play a more protective role by promoting mucosal integrity (Stahl et al., 2014, Rathinam et al., 2009a).

1.4.2 Nucleotide Oligomerisation Domain-like receptors (NLRs)

Another important PRR family are the nucleotide oligomerisation domain (NOD)-like receptors (NLRs) which are located in the cytosol. NLRs are multi-domain proteins comprising a C-terminal LLR domain that senses MAMPs, a centrally located NOD which is critical for activation, and a variable N-terminal effector region consisting of either a caspase recruitment domain (CARD) or a pyrin domain (PYD). (Franchi et al., 2009). NOD1 and NOD2 are two NLRs that recognise the peptidoglycan (PGN) subcomponents: muropeptide (iE-DAP) and muramyl dipeptide (MDP), respectively. PGN is a cell wall component of both Gram-negative and Gram-positive bacteria. Activation of NOD1 and NOD2 triggers the formation of a complex containing receptor-interacting serine/threonine kinase-2 (RIP-2) and cellular inhibitor of apoptosis (clAP) 1 and 2 leading to NF-κB and MAPK cascade activation (Moreira and Zamboni, 2012).

C. jejuni activates NOD1, but not NOD2, in IECs. Engagement of NOD1 triggers the secretion of Interleukin-8 (IL-8) and human β-defensin 2 (hBD2) while the reduced expression of NOD1 results in an increase of intracellular bacteria (Zilbauer et al., 2007). However, *C. jejuni* can activate NOD2 in a human embryonic kidney (HEK) reporter cell-line and NOD2 signalling is critical in controlling *Campylobacter* infection and inflammation in a IL-10 knock-out mouse model (Sun and Jobin, 2014, Al-Sayeqh et al., 2010). Taken together, this data suggests the requirement and activation of NOD receptors may differ in the immune and non-immune compartments in an ongoing infection.

An important function of NLRs is the activation of caspase-1 *via* the formation of a multi-protein platform termed the 'Inflammasome' (Figure 1.4). Activation of some members of the NLR family, including NLRP1, NLRP3 and NLRP4, leads to the recruitment of the adaptor protein, apoptosis associated speck-like protein containing a CARD (ASC) which induces the cleavage of pro caspase-1 into its active form. Active caspase-1 then initiates activation of IL-1β and IL-18, two important pro-inflammatory cytokines, and also triggers cell death *via* pyroptosis. Transcription levels of NLRs and proIL-1β are controlled by TLRs or NOD1/2-mediated NF-κB activation. The Inflammasome therefore requires two signals, one to initiate transcription of essential components and a second signal to induce its activation (Latz et al., 2013).

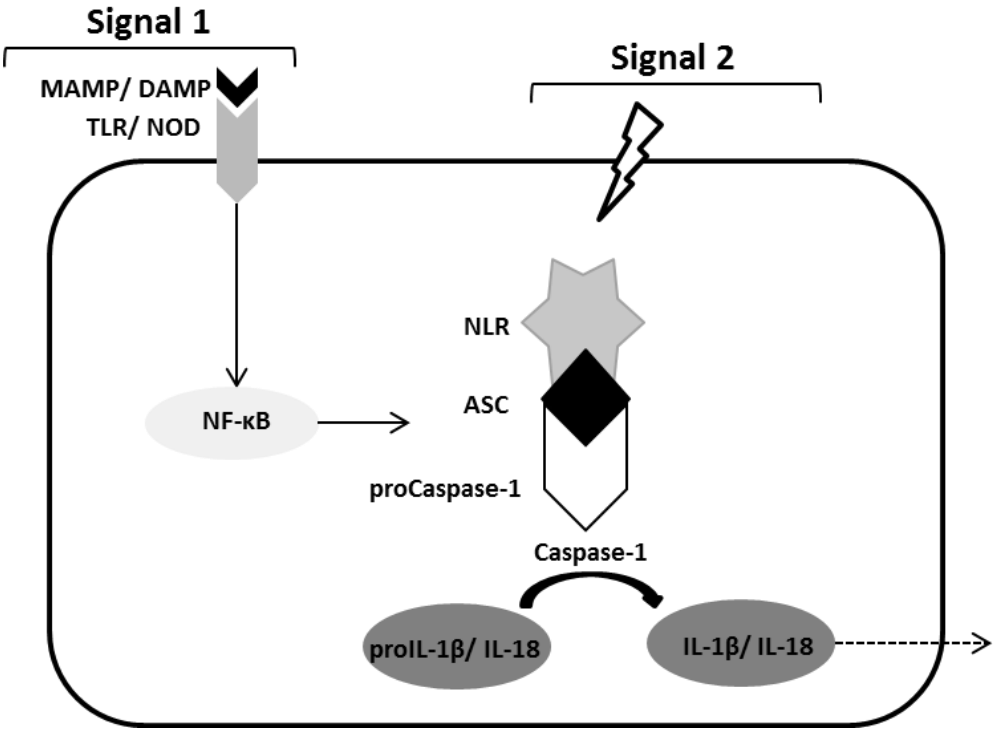


Figure 1.4 Generic model of Inflammasome activation (Adapted from Latz et al., 2013)

Compared to *Salmonella* and enteropathogenic *E. coli* (EPEC), *C. jejuni* is a poor inducer of IL-1β secretion in mouse and human DCs and *ex-vivo* cultures of colonic biopsies (Stephenson et al., 2014, Edwards et al., 2010). Elevated IL-1β levels can be observed in inflamed tissue of *C. jejuni* infected *Il10*-KO mice providing evidence for Inflammasome activation in this species (Sun et al., 2012b, Al-Banna et al., 2012). Bouwman *et al.* report modest activation of the NLRP3 Inflammasome in human macrophages upon co-culture with *C. jejuni* but the implication of this axis in human disease is still unclear (Bouwman et al., 2014).

1.4.3 Sialic-acid binding Ig-like receptors (Siglecs)

Sialic-acid binding Ig-like receptors (Siglecs) are Ig-type lectins primarily expressed on the surface of immune cells. Siglecs play an important role in differentiating self from non-self cells by differential binding to sialic acid residues which are embedded in the diverse glycan structures expressed on the surface of mammalian cells and many microbes (Macauley et al., 2014). Siglecs are transmembrane proteins composed of an N-terminal V-set Ig-like domain that mediates binding to sialic acid, followed by a varying number of C2-set Ig-like domains. Siglecs are categorised into two subsets based on their sequence similarity. The first sub-category includes Sialoadhesin (Siglec-1), Siglec-2, myelin-associated glycoprotein (MAG, Siglec-4) and Siglec-15, all of which contain 25% to 30% sequence identity and have clear orthologues in all mammalian species examined to date. In contrast, the second sub-group of CD33-related Siglecs share 50% to 99% sequence homology to CD33 but there are important differences in the expression repertoire amongst mammalian species. Humans express nine members of this group of Siglecs including CD33 (Siglec-3), Siglec-5, 6, 7, 8, 9, 10, 11, 14 (Crocker et al., 2007).

Siglecs have regulatory function and can influence the host immunity *via* various routes including direct interaction with bacteria, inhibition of PRR-signalling and modulation of cytokine production (Macauley et al., 2014). The sialyation status of many *C. jejuni* isolates has been implicated with disease severity and the onset of post-infectious GBS. LOS of GBS-associated strains bind to sialoadhesin leading to enhanced uptake and increased production of pro-inflammatory interleukin-6 (IL-6) by human macrophages (Heikema et al., 2013, Heikema et al., 2010). Sialyated *C. jejuni* LOS also bind to Siglec-7 on human monocytes and natural killer (NK) cells suggesting greater complexity of microbial sialic acid crosstalk with the host immune system (Avril et al., 2006). Additionally, *C. jejuni* flagella interact with Siglec-10 *via* the sialic acid-like structures, pseudaminic acids. Binding of *C. jejuni* flagella to Siglec-10 induces the secretion of IL-10 in human and mouse DCs potentially promoting the anti-inflammatory immune axis (Stephenson et al., 2014).

1.4.4 Cytokine responses to *C. jejuni*

PRR engagement by microbes stimulates the generation of cytokines and chemokines which are important messengers responsible for the induction and progression of an adequate immune response. Broadly, cytokines can be pro-inflammatory (e.g. tumor necrosis factor-α (TNF-α), IL-1β, IL-6), anti-inflammatory (TGFβ, IL-10), bactericidal (INF-γ) and immunomodulatory (IL-6, IL-12, IL-18, IL-4 and IL-10) (Lacy and Stow, 2011). Chemokines including IL-8, monocytes chemottractant protein-1 (MCP-1) and CCL5/RANTES function as chemoattractants promoting the recruitment of leukocytes to the site of infection (Graves and Jiang, 1995). Cytokines are also crucial mediators of adaptive immunity and T-cell differentiation. Pro-inflammatory cytokines, including INF-γ, IL-2 and TNF-α, are associated with Th1-mediated immunity responsible for the killing of intracellular pathogens. Anti-inflammatory cytokines associated with Th2 type include IL-10 as well as IL-4, IL-5 and IL-13 which are associated with helminth infection, allergy and asthma (Berger, 2000). A third subset of cytokines belongs to the Th17 lineage which includes IL-17A, IL-17F, IL-21 and IL-22, and are implicated in extracellular bacterial infection and many autoimmune diseases (Ouyang et al., 2008).

Interaction of *C. jejuni* with IECs promotes the NF-κB dependent secretion of IL-8 and MCP-1 (Hu and Hickey, 2005). In co-culture with DCs and macrophages *C. jejuni* stimulates the generation of cytokines including TNF-α, IL-6, IL-12 and IL-23 (Edwards et al., 2010, Rathinam et al., 2009a, Heikema et al., 2013).

A polymorphism in the gene encoding IFN-γ is associated with an increased risk of acquiring *Campylobacter* enteritis suggesting Th1 driven immune responses (Nielsen et al., 2012). Co-culture of *C. jejuni* with colonic biopsies shows a marked induction of INF-γ confirming the importance of this cytokine in promoting bacterial elimination. Additionally, T-cells cultured in *C. jejuni* conditioned DCs supernatants significantly induce the secretion of INF-γ, IL-22 and IL-17A expanding Th1 and Th17 immunity (Edwards et al., 2010).

1.4.5 The complement system

The complement system comprises a large number of enzymes and adaptor proteins and is one of the major defence mechanisms by which the body recognises pathogens (Thurman and Holers, 2006). Complement stimulation sets off an array of proteolytic cascades that act against invading pathogens by direct killing or by stimulating microbial uptake by professional phagocytes. Additionally, the complement system has important functions in immune regulation and presents a bridge between innate and adaptive immunity by modulating T-cell immunity and the natural antibody repertoire (Ricklin and Lambris, 2007). To ensure and contain overt activation, it is not surprising that the complement system is tightly regulated. Under homeostatic conditions, many components are present in an inactive zymogen form, and multiple regulatory mechanisms exist at each sequential step of the signalling cascade.

The complement system can be initiated by three distinct pathways: the classical pathway, the lectin pathway and the alternative pathway (Figure 1.5). All pathways converge in the formation of a C3 convertase facilitating activation of the C3 zymogen into C3a, a potent anaphylatoxin, and the effector protein, C3b.

Classical pathway

C1q serves as the first recognition molecule in the classical pathway. C1q is a tulip-like glycoprotein composed of six copies of three polypeptide chains: the A-, B- and C-chain. Each chain has a short N-terminal region, followed by a collagen-like region and a C-terminal globular head region (ghA, ghB or ghC) which is the ligand recognition domain (Kishore et al., 2003). Activation of the classical pathway is primarily initiated by interaction of C1q with immune complexes (mainly IgG or IgM) on the bacterial surfaces (Figure 1.5). Upon binding of the gh region with Igs, conformational changes to the C1q tulip-structure occur leading to autoactivation of the serine-proteases C1r and C1s which, together with C1q, form the C1 complex (Arlaud et al., 2002). The C1 complex induces cleavage of C4 to C4b which cleaves C4b-bound C2 to form the C3 convertase, C4b2a. C1q ghB is the principal binding sites for IgG-binding molecules while the ghC module is specific for IgM (Kishore et al., 2004).

Despite the preferential binding to immune complexes, C1q can interact with a number of other molecules including the serum components C-reactive proteins (CRP) and serum amyloid P component (SAP) and it can also directly bind to LPS on the bacterial surface (Veerhuis et al., 2003, McGrath et al., 2006, Roumenina et al., 2008). Thus, while the classical pathway is typically antibody dependent it can also be activated by antibody independent routes.

Graph removed due to copyright restrictions.

Figure 1.5 Mechanisms of complement activation. Adapted from (Gorsuch et al., 2012)

Lectin pathway

The lectin pathway is activated in the absence of immune complexes and C1q, instead host mannose binding lectins (MBL), fi*coli*ns (Fcn) or collectin-11 serve as microbial sensor (Figure 1.5). These recognition molecules bind to microbial surfaces *via* specific carbohydrate structures including Mannan, GalNAc and GlcNAc moieties (Schwaeble et al., 2011, Matsushita et al., 1996). Upon microbial engagement, these sensors can form complexes with three serine proteases MBL-associated serine proteases (MASP-1, MASP-2 and MASP-3) of which only MASP-2 is essential to form lectin-pathway activation (Vorup-Jensen et al., 2000). Just like C1q, activated MASP-2 cleaves C4 and subsequently C2 to form the C3 convertase, C4b2a. Neither MASP-1 nor MASP-3 can cleave C4 and are therefore not capable to form the C3 convertase (Schwaeble et al., 2011). MASP-1 appears to contribute to activation of the lectin pathway by activation of MASP-2 or enzymatic cleavage of C4b-bound C2 (Kocsis et al., 2010, Takahashi et al., 2008). The function of MASP-3 was long unknown but recent discoveries suggest an important role in the alternative pathway (Iwaki et al., 2011, Sekine et al., 2013)

Alternative pathway

In contrast to the classical and the lectin pathway, the alternative pathway is independent of a specific host microbial sensor. Activation is initiated by a process also referred to as 'tick-over' activation of C3 (Figure 1.5). Spontaneous hydrolysis of C3 leads to C3(H_2O) which is capable of binding with factor B (fB) to form C3bB. The serum protease Factor D (fD) then cleaves the inactive C3bB into C3bBb which serves as C3 convertase of the alternative pathway. C3bBb convertases cleave C3 molecules to C3b which interact with fB to generate more C3bBb convertase. The alternative pathway can therefore also function as an amplification loop for the classical and the lectin pathway *via* fB binding to accumulating C3b (Thurman and Holers, 2006). This proteolytic activation cascade is further enhanced by the presence of properdin which stabilises protein-protein interactions and serves as positive regulator of the alternative pathway (Hourcade, 2006). MASP-3 is thought to facilitate the activation of fD from its inactive pro-form thus plays an essential role in alternative pathway activation (Wilhelm Schwaeble, University of Leicester; personal communications).

Activation of all pathways leads to the deposition of C3b on the surface of pathogens. Since C3b is a potent activator of the alternative pathway, it is quickly cleaved into inhibitory iC3b, a process mediated by factor H (fH) and factor I (fI) (Cunnion et al., 2004). C3b and iC3b function as opsonins and facilitate microbial recognition and uptake by professional phagocytes *via* the complement receptors 1 (CR1), -3 (CR3) and -4 (CR4). C3b/iC3b have differing receptor affinity, C3b

preferentially binds to CR1 while iC3b is high affinity ligand to CR3 which represents the primary route of uptake for most pathogens (Newman et al., 1985). The degree of C3b/iC3b found on the cell surface can be modulated by various bacterial and host factors and may direct the alternative pathway activation and the route of bacterial uptake (Schneider et al., 2006).

Apart from its role in the alternative pathway and phagocytosis, C3b has an additional effector function as it initiates the terminal phase of the complement system leading to bacterial lysis (Figure 1.6). C3b forms a multimeric complex with C4b2b or C3bBb yielding the C5 convertases, C4b2bC3b or C3bBbC3b. The C5 convertases cleave C5 to C5a, which similar to C3a, functions as an anaphylatoxin, and C5b). The latter associates with C6 to C5b6 which binds with C7 resulting in a hydrophobic complex that targets the lipid bi-layer of the cell membrane (mC5b-7; Figure 1.6). The binding of C8 facilitates membrane insertion followed by the assembly of 12 to 18 molecules of C9. Upon polymerisation, the C5b-9 complex forms a pore-forming ring structure also referred to as membrane attack complex (MAC) (Tegla et al., 2011, Berends et al., 2014). This complement-induced cell lysis plays a crucial role in the clearance of pathogens, most notably Gram-negative bacteria, however the exact molecular details are still not fully elucidated (Kondos et al., 2010).

Gram-positive bacteria are protected from complement mediated killing probably due to the thick PGN layer presented on the surface which may prevent MAC insertion (Joiner et al., 1984). A recent study however observed the deposition of MAC on several Gram-positive bacteria which might suggest an as yet undiscovered defence mechanism against these bacteria (Berends et al., 2013). However, since the MAC targets the lipid bi-layer it has activity towards many other cells, including host cells. The tight regulation of the system is therefore crucial to prevent host damage and may also play a role in the maintenance of cell and tissue homeostasis (Tegla et al., 2011) .

Figure 1.6 Assembly of the membrane attack complex (MAC). Adapted from (Berends et al., 2014)

Complement components are primarily located in the plasma where they account for approximately 3g/L and constitute >15% of the globular fraction (Walport, 2001). Complement may also play a role in the GI tract but its presence in the intestinal lumen is not comprehensively understood. IBD patients show evidence for C3b and MAC proteins on the epithelial surface (Halstensen et al., 1990, Halstensen et al., 1992). Others report increased levels of C3 and C4 proteins in the small intestine of individuals suffering from CD and those with severe enteral infections (Ahrenstedt et al., 1990, Riordan et al., 1997). *Ex-vivo* explants of CD patients suggest IECs as a source of the complement proteins C4, C3 and fB (Laufer et al., 2000, Sugihara et al., 2010, Ostvik et al., 2014). The expression of these complement proteins are commonly found in various IEC cell-lines, including T84, Caco-2 and HT-29, suggesting a potential role of the alternative pathway in the intestine (Bernet-Camard et al., 1996, Andoh et al., 1993). The lectin pathway, on the other hand, might not be involved as MBL cannot be detected in the mucosa of humans or mice (Muller et al., 2010). Similarly, studies fail to detect MAC-associated proteins by gene and protein expression analysis indicating a serum specific location of terminal complement activation (Andoh et al., 1996, Andoh et al., 1993).

Complement-mediated interactions with *Campylobacter* are to date unknown. A number of studies report sensitivity of *C. jejuni* to human serum implying terminal complement activation (Blaser et al., 1985, Keo et al., 2011). The lack of capsule markedly enhances serum sensitivity which suggests a protective role in complement-mediated killing of *C. jejuni* (Keo et al., 2011, van Alphen et al., 2014, Maue et al., 2013). *C. fetus*, in comparison, is less sensitive to human serum possibly due to the presence of cross-reactive surface proteins which prevent binding of C3b (Blaser et al., 1987, Blaser et al., 1988). Interestingly, *C. fetus* infection is associated with septicaemia whereas only a few cases report systemic infection with *C. jejuni* (Wagenaar et al., 2014, Pacanowski et al., 2008). Given its potential implication in GI health, one might speculate that the complement system might play a greater role in the host immune response to the *Campylobacter* genus.

1.5 Cellular innate immune response

1.5.1 Neutrophils

Neutrophils, also referred to as polymorphonuclear cells (PMN), account for 50% to 60% of peripheral blood leukocytes thus are the most abundant circulating immune cell in humans (Sadik et al., 2011). They are typically the first immune cells recruited to the site of infection and play a crucial role in the early host immune defence against invading pathogens (Fournier and Parkos,

2012, Kolaczkowska and Kubes, 2013, Kruger et al., 2015). Neutrophils eliminate pathogens by multiple mechanisms, primarily by phagocytosis, secretion of antimicrobial granules and reactive oxygen species (ROS) band the release of neutrophil extracellular traps (NETs).

Phagocytosis

Neutrophils are professional phagocytes and are capable of rapidly internalising and degrading foreign organisms and necrotic cells (van Kessel et al., 2014). Phagocytosis is initiated upon ligation of phagocytic receptors expressed on neutrophils with both opsonised and non-opsonised particles (Lee et al., 2003). There are two principal classes of phagocytic receptors, the Fc receptors (FcγR and FcαR) and complement receptors (CR). FcγR, including FcγRI (CD64), FcγRIIA (CD32), FcγIIIB (CD16) recognise IgG-coated particles *via* the antibody Fc-binding domain. Engagement with the receptor leads to phosphorylation of the intracellular immunoreceptor tyrosine-based activation motif (ITAM) which induces actin polymerisation and initiates particle engulfment (Aderem and Underhill, 1999).

FcγRIIA and FcγRIIIB are abundantly expressed on neutrophils and both receptors have been implicated in promoting phagocytosis (Indik et al., 1995, Nimmerjahn and Ravetch, 2006). Enhanced phagocytosis is observed upon activation of both receptors in neutrophils suggesting a synergistic effect between the receptors is likely (Chuang et al., 2000). FcR also stimulate neutrophil effector function(s) including the NADPH-oxidase complex, production of ROS and granule secretion (Nordenfelt and Tapper, 2011). FcαRI (CD89) is the best characterised receptor for IgA and is constitutively expressed on human neutrophils (van Egmond et al., 1999a). Neutrophils efficiently bind and internalise IgA-coated particles. Similar to FcγRs, FcαRI promotes Ig-mediated uptake and neutrophil activation including ROS generation and the release of NETs (Bakema and van Egmond, 2011, Aleyd et al., 2014).

The three CR involved in phagocytosis are CR3 (CD11b/CD18) and CR4 (CD11c/CD18) and CR1 (CD35). CR3 and CR4 belong to the family of integrins. They are heterodimers of different α-chains (CD11b or CD11c, respectively), and share the common β-chain CD18. Both receptors bind iC3b with high affinity and mediate particle internalisation (Aderem and Underhill, 1999). CR3 is the best characterised CR and the primary phagocytic receptor on neutrophils whilst CR4 is implicated in macrophage associated microbial clearance (Schwartz et al., 2012).

CR1 is a multifunctional, transmembrane glycoprotein consisting of a large extracellular lectin-like complement binding domain and a short cytosolic domain (Aderem and Underhill, 1999). CR1 is a high affinity receptor for C3b but can also bind C4b, iC3b and C1q (Krych-Goldberg and Atkinson,

2001). Neutrophils must be activated prior to CR1 associated phagocytosis as C3b complexes fail to be internalised in unstimulated cells. CR1 is not directly involved in phagocytosis but seems to function as a co-factor facilitating the uptake of complement opsonised particles *via* CR3 (Brown, 1991, Ishibashi and Arai, 1990). CR1-bound C3b is more accessible to proteolytic cleavage by factor I to iC3b which provides ligands for CR3 and also downregulates excessive complement activation (Furtado et al., 2008).

Internalisation by CR3 requires a second activation step which is protein kinase C dependent and can be induced by phorbol esters, TNF-α or bacterial LPS (Caron et al., 2000). CR3 is a multi-ligand receptor interacting with a range of molecules and elicits phagocytic capacity by direct binding to LOS of *Neisseria meningitidis* (Jones et al., 2008). Interestingly, in contrast to FcR which induces phagocytosis by particle engulfment *via* membrane protrusion, CR-mediated phagocytosis is a more passive process where complement-opsonised particles seem to sink into the cell cytoplasm. Internalisation by FcR is sensitive to cytochalasin B while CR3 mediated uptake is much less effected suggesting the latter to be partly independent of microfilament formation (Kaplan, 1977, Allen and Aderem, 1996). In addition, some studies note a difference in the release of pro-inflammatory mediators upon FcR- or CR-mediated phagocytosis. Internalisation with FcR is tightly coupled with the generation of ROS which is less prominent following CR-mediated phagocytosis in macrophages (Aderem et al., 1985, Wright and Silverstein, 1983). This effect however is cell type specific and in neutrophils CR3 and FcR potently activate the respiratory burst (Lofgren et al., 1999)

FcR and CR interact with each other and can produce cooperative effects. Ligation with FcR may induce the activation of CR which is essential for complement-mediated phagocytosis (Ehlenberger and Nussenzweig, 1977). Interestingly, both receptors are required for the efficient uptake and killing of *Streptococcus pneumoniae*. While bacterial uptake is mediated *via* CR1 and CR3, neutrophils are only capable of eliminating internalised bacteria when co-opsonised with IgA (Janoff et al., 1999). Additionally, CR3 deficient, FcαRI expressing mice have normal phagocytic capacity but show impaired ability to lyse engulfed targets (van Egmond et al., 1999b). Taken together, opsonisation of pathogens influences the mode of phagocytosis and may modulate its fate upon internalisation by neutrophils.

Degranulation and Respiratory burst

Independent of the receptors involved, particles are engulfed into membrane-derived vacuoles upon internalisation. Sealing of this phagosomal vacuole initiates its maturation process which is essential for effective killing of its microbial content. Neutrophils contain a large number of

granules that rapidly fuse with the phagosome and release their cytotoxic contents (Lee et al., 2003, Nordenfelt and Tapper, 2011). Neutrophil granules can be divided into three types: azurophilic, specific and gelatinase granules. Azurophilic (primary) granules are the most abundant type fusing with the phagosome. They contain a vast array of antimicrobial substances including lytic enzymes such as myeloperoxidase (MPO), neutrophil elastase (NE), cathepsin G, as well as α-defensins, a group of antimicrobial peptides (Borregaard and Cowland, 1997).

Another defining feature of neutrophil effector function is the generation of ROS which is regulated by the NADPH-oxidase complex. In macrophages, this process is dependent on plasma membrane associated NADPH-oxidases which engulf the target during internalisation, whilst neutrophils are able to recruit additional NADPH-oxidase primarily *via* specific granules. Hence the degree of ROS produced in neutrophils exceeds in magnitude that observed in macrophages (Karlsson and Dahlgren, 2002). Respiratory (oxidative) burst is generated by assembly of the NADPH-oxidase complex which is composed of cytosolic and membrane-bound subunits, in the case of neutrophils the latter are primarily provided by specific granules. The cytosolic components p47phox, p67phox and RAC2 and associate with the granule/membrane components gp91phox and p22phox which catalyses the reduction of molecular oxygen to superoxide anions (Winterbourn and Kettle, 2013). In cooperation with MPO, superoxide anions are further transformed into an array of ROS including hydrogen peroxide (H_2O_2), hydroxyl radicals (HO·), hypochlorous acid (HOCl) and singlet oxygen ($\cdot O_2^-$) (Klebanoff, 2005). The formation of ROS results in an excess of electrons inside the phagocytic vacuole which is compensated by the influx of positively charged K^+ and H^+ ions. This changes the vacuolar pH and triggers the activation of antimicrobial enzymes, thus bacterial killing (Segal, 2005).

The respiratory burst provides neutrophils with exceptional capacity to eliminate microbial pathogens but bears the problem of minimising damage towards themselves and the surrounding tissue. Despite being generated in the phagosome, most ROS are able to diffuse through membranes and into the extracellular space (Winterbourn and Kettle, 2013). On the other hand, ROS production initiates neutrophil cell death with subsequent removal by other phagocytes which are important mechanisms for the resolution of inflammation (Kennedy and DeLeo, 2009).

Neutrophil extracellular traps (NETs)

Neutrophils can kill extracellular pathogens by the release of NETs. NETs consist of smooth filaments derived from granular components, histones and other cytoplasmic proteins which are released upon the induction of a unique form of cell death, termed NETosis (Brinkmann et al., 2004). The underlying molecular events are not completely understood but NETosis is closely

interlinked with the respiratory burst activity (Fuchs et al., 2007). ROS, MPO and NE are essential for NET formation and patients presenting with deficiencies in these components lack the ability to produce NETs (Bianchi et al., 2009, Papayannopoulos et al., 2010). One important function of NETs is to trap microbes preventing their dissemination from the initial infection site. The arsenal of antimicrobial proteins including granule proteases and peptides, ion chelators and histones, allows for the direct elimination of pathogens (Bartneck et al., 2010, Brinkmann et al., 2004). NETs also bind microbial secretory proteins and inactivate them by cathepsin G and proteinase 3 (Averhoff et al., 2008). While an increasing bulk of evidence reveals a critical role of NETs in the elimination of pathogens, in excess they are associated with tissue damage and persistent inflammation stressing the importance of post-infectious neutrophil clearance (Brinkmann and Zychlinsky, 2012).

The early stage of campylobacteriosis manifests with a significant infiltration of neutrophils into the epithelium and subepithelial lamina propria and their transmigration into the crypts leading to abscess formation and occasional granulomata (van Spreeuwel et al., 1985). Neutrophils are the predominant leukocytes found in *C. jejuni* infected tissue which suggests an important role of this cell type in mediating disease development. Neutrophil migration to the infection site can be initiated by a number of endo- and exogenous factors. Murphy *et al.* report the recruitment and transepithelial migration of neutrophils following co-culture of human IECs with *C. jejuni* and *C. coli.* The production of bacterial derived N-formyl peptides and the epithelial enzyme 12-lipoxygenase (12-LOX) are implicated in the process (Murphy et al., 2011). Many bacterial species secrete N-formyl peptides, including N-formyl-Met-Leu-Phe (fMLP), which are potent chemoattractants and induce neutrophil chemotaxis, degranulation and NET production (Southgate et al., 2008). 12-LOX mediate the production of eicosanoids, which are fatty acid derivatives and they possess chemotactic properties. *C. jejuni* infected IECs also release other chemokines including IL-8, another potent neutrophil attractant (MacCallum et al., 2005a, Zheng et al., 2008). Using a germ-free, *Il10* knock-out mouse model Sun *et al.* identify the influx of neutrophils as main cellular mechanism driving inflammation in response to *C. jejuni*. Histologic markers of inflammation, neutrophil accumulation and colonic mRNA levels of *Il1b*, the chemoattractant *Cxcl2* and *Il17a* were diminished after blockage of phosphatidylinositol 3-kinase-γ (PI3K-γ) (Sun et al., 2013). PI3K-γ is an important signalling protein mainly expressed in immune cells and mediates cell migration towards a chemotactic signal (Li et al., 2000).

Taken together, these results suggest an important role for neutrophils in campylobacteriosis. *C. jejuni* induces the secretion of chemotactic agents with involvement of the intestinal epithelium

and promotes neutrophil migration probably *via* PI3K-γ signalling. The role of bacterial-neutrophil interactions in humans however are currently unknown.

1.5.2 Macrophages

Resident intestinal macrophages represent the largest population of mononuclear cells in the body (Lee et al., 1985). These gut-associated macrophages are primarily located in the lamina propria and play a crucial role in the protection of the mucosa by the regulation of inflammatory responses to invading pathogens. Microbial translocation through the epithelial barrier activates mucosal macrophages triggering TLR and NLR signalling. During inflammation, additional blood monocytes are recruited to the intestinal mucosa inducing their differentiation and accumulation at sites of infection (Smith et al., 2011). Macrophages are antigen presenting cells (APCs) and modulate immune responses *via* the generation of various cytokines. They are also potent phagocytes and play an important role in inflammatory resolution by scavenging apoptotic cells, including neutrophils (Fujiwara and Kobayashi, 2005).

While neutrophils predominate during the early phase, monocytes are the main leukocyte population found in colonic tissue of patients at the late stage (7 to 14 days) of *Campylobacter* infection (van Spreeuwel et al., 1985). Co-culture of *C. jejuni* with a human macrophage-like cell-line induces the NF-κB dependent secretion of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α (Jones et al., 2003). Murine and human macrophages internalise *C. jejuni* (Klancnik et al., 2009, Heikema et al., 2013). Interestingly, *C. jejuni* was shown to survive in macrophages for up to seven days probably due to upregulation of catalase activity which provides protection from phagosome-mediated oxidative stress (Day et al., 2000, Kiehlbauch et al., 1985).

1.5.3 Dendritic cells

Dendritic cells (DCs) are classical APCs that bridge innate and adaptive immune responses. Upon stimulation, tissue resident DCs migrate to the lymph nodes where they prime naïve T cells and promote the polarisation into Th1, Th2 or Th17 effector subclasses (de Jong et al., 2002). Human monocyte derived DCs readily internalise and eliminate *C. jejuni*. DC maturation triggers the production of pro-inflammatory and anti-inflammatory cytokines including IL-6, IL-8, IL-10, IL-12 and INF-γ (Hu et al., 2006). *C. jejuni* induces signalling through both TLR4-MyD88 and TLR4-TRIF axes in DCs mediating maximal Th1 specific immune induction (Rathinam et al., 2009a, Edwards et al., 2010). Additionally, *C. jejuni* LOS is involved in T cell polarisation *via* Siglec receptor engagement. Modifications of LOS sialic acid moieties on *C. jejuni* isolates modulate interaction with sialoadhesin and Siglec-7 and promote the differentiation of Th1 or Th2 class immune

response (Bax et al., 2007). The interaction of DC Siglec-10 with *C. jejuni* flagella on the other hand may stimulate IL-10 production and promote anti-inflammatory events (Stephenson et al., 2014).

1.5.4 Adaptive immunity

Acquired immunity is an important factor to *Campylobacter* infection in the less developed and developed world (Havelaar et al., 2009). Patients with campylobacteriosis develop specific IgG, IgA and IgM antibody to *C. jejuni* which remain elevated for up to 50 days post-infection. The increase of plasma IgA levels are primarily associated with enhanced immunity to infection (Walz et al., 2001, Herbrink et al., 1988). Additionally, the consumption of breast milk containing high antibody titres of dimeric secretory IgA reduces the risk of *Campylobacter* associated diarrhoea in children (Ruizpalacios et al., 1990).

C. jejuni infection is associated with the predominant expansion of Th1 and Th17 memory cells (Edwards et al., 2010, Rathinam et al., 2009a, Stahl et al., 2014). Th1 cells produce INF-γ evoking cell-mediated immunity by the stimulation of professional phagocytes. A strong Th1 response is also implicated in autoimmune disorders including IBD and Th1/Th2 axis is implicated in the development of GBS following *C. jejuni* infection (Nyati et al., 2011). Interestingly, Th17 cells produce IL-17, a cytokine associated with the recruitment of neutrophils to the infection site (Ruddy et al., 2004). Th17 cells further promote antimicrobial activity by the secretion of IL-26, a IL-10 related cytokine, that promotes direct bacterial killing via membrane-pore formation (Meller et al., 2015).

While Th1 and Th17 immune response likely aids the clearance of *C. jejuni* during infection, the over activation of cellular immunity is linked with inflammation and tissue damage. Pre-exposure to *C. jejuni* is likely to exert a protective effect as memory cells may promote a more rapid bacterial clearance preventing excessive inflammation and tissue damage due to an overactive cellular immune response (Tribble et al., 2010).

1.6 Hypothesis and Aims

In the present study we wished to elucidate factors involved in innate immunity to the *Campylobacter* genus. We hypothesised that human neutrophils play an important role in *Campylobacter*-mediated pathogenesis which may be modulated by bacterial and host factors. Since bacterial glycosylation is an important driver of *C. jejuni* virulence we propose that modulation of surface glycans act as important mediators of the human immune response to the *Campylobacter* species.

The specific aims of the project were:

1. To investigate neutrophil-mediated uptake of *C. jejuni*.
2. To characterise the mechanisms of complement activation by *C. jejuni*.
3. To delineate *C. concisus* LOS structure and function

CHAPTER II

MATERIALS & METHODS

2.1 Bacterial culture

2.1.1 *Campylobacter jejuni*

2.1.1.1 Bacterial strains

Two *C. jejuni* reference strains have been used in this study. Strain 81-176 was originally isolated from a milk-borne campylobacteriosis outbreak in Minnesota, USA and is a widely accepted reference strain in *Campylobacter* research (Korlath et al. 1985). Strain 11168H is a hypermotile variant of the NCTC 11168 clinical isolate first described by Martin Skirrow in 1977 (Karlyshev et al. 2002). Isogenic mutant bacteria stem from strain 11168H background and were created by insertion of a kanamycin resistance cassette. Mutant strains utilised in this study are listed in Table 2.1. Strains and mutants were a kind gift from Professor Brendan Wren (London School of Hygiene and Tropical Medicine, UK).

Gene	Phenotype	Reference
kpsM	Capsular polysaccharide (CPS) negative	(Karlyshev et al. 2001)
neuB	Lacks N-acetyl neuraminic acid synthetase presenting with sialic acid negative LOS	(Linton et al. 2000)
waaF	Lacks a heptosyltransferase truncating the OS chain to one heptose after the KDO residue	(Oldfield et al. 2002)

Table 2.1 List of *C. jejuni* isogenic mutants derived from parental wild-type strain 11168H

2.1.1.2 Culture conditions

C. jejuni strains were cultured on 5% blood agar (BA) plates under microaerobic conditions in a 37°C incubator. BA plates were prepared by the addition of BA base (Oxoid) to MilliQ water (0.22µm-filtered) followed by autoclaving. Agar was left to cool (~37°C) before 5% pre-warmed defibrinated horse blood (Oxoid) was added. Plates were inoculated with frozen bacterial stocks (Microbank) and cultures were passaged three times a week for up to four weeks. Plates were placed in a 2.5L gas jar (Oxoid) and oxygen was reduced by addition of microareobic generating sachet (Campygen, Oxoid). Isogenic mutants were cultured on BA plates containing 25µg/mL kanamycin (Sigma). Overnight bacterial cultures were used for co-culture experiment.

2.1.2 *Campylobacter concisus*

2.1.2.1 Bacterial strains

C. concisus strains used in this study were originally isolated from faeces of patients presenting with gastrointestinal (GI) conditions. Bacterial isolates were a kind gift from Dr. Georgina Hold (University of Aberdeen, UK), Dr. David Guiliano (University of East London, UK) and Professor Henrik Nielsen (Aarburg University, Denmark). Detailed descriptions are listed in Table 2.2.

***C. concisus* isolates**	**Clinical phenotype**	**Source**	**Reference**
B38	Paediatric IBD	Georgina Hold (University of Aberdeen, UK)	(Hansen et al., 2013)
B124	Paediatric IBD	Georgina Hold (University of Aberdeen, UK)	(Hansen et al., 2013)
NCTC 12408	Paediatric enteritis	David Guiliano (University of East London, UK)	(Figura et al., 1993)
2010-112825	Adult gastroenteritis	Henrik Nielsen (Aarhus University, Denmark)	(Kirk et al., 2015)
2010-131105	Paediatric gastroenteritis	Henrik Nielsen (Aarhus University, Denmark)	(Kirk et al., 2015)
2010-347972	Adult gastroenteritis	Henrik Nielsen (Aarhus University, Denmark)	(Kirk et al., 2015)

Table 2.2 List of *C. concisus* isolates

2.1.2.2 Culture conditions

C. concisus strains were cultured on 5% BA plates fortified with 0.5% yeast extract (Oxoid). *C. concisus* require microaerobic, hydrogen-enriched culture conditions which were generated in a 2.5L gas jar by the addition of a Campygen sachet and a falcon containing 0.7g sodium borohydride (Sigma) dissolved in 7mL MilliQ water helping to generate a microaerobic (~5% O_2), hydrogen enriched (~7% H_2) atmosphere required for *C. concisus* growth (Duffy et al., 2008). Plates were inoculated with frozen bacterial stocks (Microbank) and passaged every five days for up to one month. For co-culture experiments bacteria were cultured for three days.

2.1.3 Bacterial quantification

C. jejuni and *C. concisus* strains were cultured for one day or three days, respectively, before colonies were harvested and suspended in sterile phosphate buffered saline (PBS, Gibco). Optical

density (OD) was determined by the use of a spectrophotometer (Jencons U-1800) at 600nm wavelength (OD_{600}). Bacterial suspensions were adjusted to an OD 1 and subjected to a 10-fold serial dilution. 10µL were spotted in triplicates on BA plates and incubated for two days or four days, respectively. Colonies were counted and the number of Colony forming units (CFU)/mL were determined. For *C. jejuni* strains and mutants as well as *C. concisus* strains an OD reading of 1 equalled $3x10^9$ CFU/mL.

2.1.4 Bacterial preparation for co-culture experiments

For co-culture experiments bacteria were collected from a BA plate with the help of a bacterial loop and suspended in PBS. Absorbance was determined by spectrophometry and adjusted to an OD 0.5 prior to co-culture experiments.

2.1.5 Inactivation of bacterial cells

Bacteria were suspended in PBS and adjusted to an OD 0.5. For heat-inactivation, 2mM $MgCl_2$ was added to the suspension prior to incubation in a water-bath at 56°C for 45min. Antibiotic killing was achieved by addition of Gentamicin (250µg/mL) for 1h. Bacterial cells were fixed in 0.5% PFA for 20min. After treatment, bacteria were washed three times and resuspended in PBS to an OD 0.5. Suspensions were spotted on a BA-plate to confirm successful killing.

2.2 CHO cell-lines

Chinese Hamster Ovary (CHO) cell-line either mock-transfected or transfected with CR1, CR3 or CR4 were cultured in selective F12 Ham Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% FBS and 250µg/mL geneticin (G418, Sigma). Accutase® (Thermo Fisher) was used to gently detach cells from the culture dish. Cells were grown to 80% confluency and passaged twice a week. Transfected cells were a kind gift from Dr. Suzan Rooijakkers (University Medical Center Utrecht, Netherlands; CR1) and Dr. Hannah Jones (UCL Institute of Child Health (ICH), UK; mock, CR3, CR4).

2.3 Venous blood preparation

2.3.1 Isolation of neutrophils and peripheral blood mononuclear cells (PBMCs)

Venous blood (~20mL) was regularly obtained from consented, healthy volunteers at ICH. 15mL blood was collected in heparin (1.000 U/mL) containing falcon tubes. Leukocytes were isolated as previously described (Nauseef, 2007). Heparinised blood was diluted 1:1 with sterile filtered 3% Dextran-500/PBS solution (Sigma), mixed by gentle inversion and left for 45-60min to allow for

erythrocyte sedimentation. The straw-coloured buffy coat was collected and layered onto Hypaque-Ficoll (GE Lifescience) at a ratio of 2:1. Cells were separated by density centrifugation at 600g for 20min. PBMCs were carefully collected from the interface of the Hypaque-Ficoll and the upper liquid layer using a sterile Pasteur pipette. Pelleted neutrophils were harvested after removal of the liquid layer and hypotonic lysis of residing erythrocytes. Collected cells were washed twice with Hank's balanced salt solution (HBSS) prior to suspension in RPMI+10% FBS. The levels of neutrophil and PBMC purity were regularly checked by flow cytometry using neutrophil and monocyte specific surface marker CD66b (FITC; BD Biosciences) and CD14 (PerCP; eBioscience), respectively (Figure 2.1). Monocyte contamination in the neutrophil fraction was typically less than 1%.

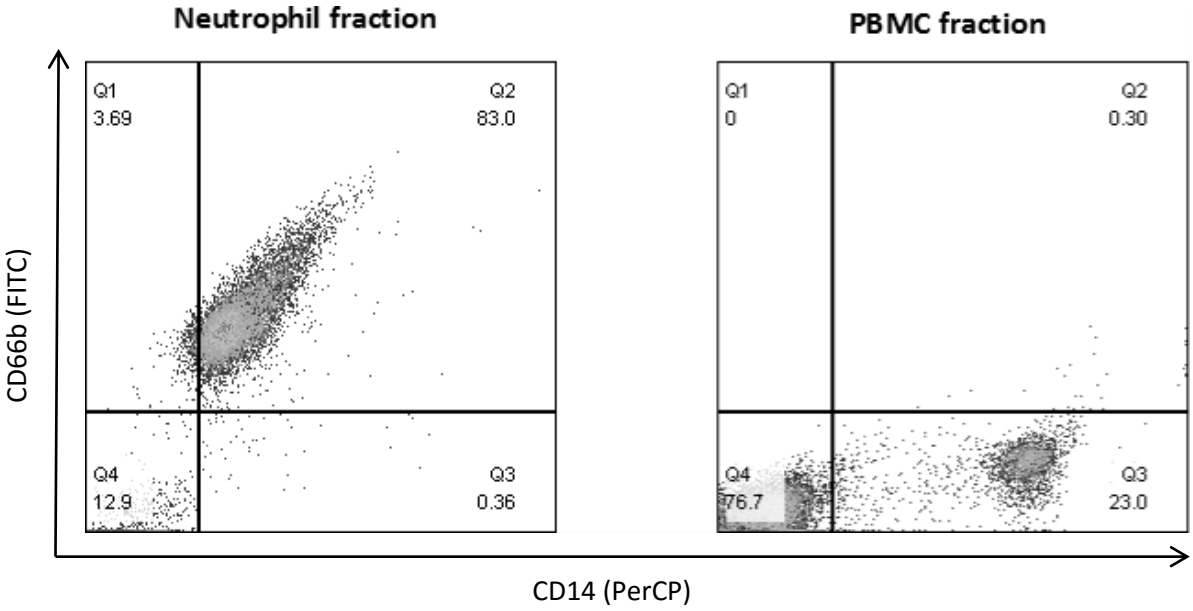


Figure 2.1 Neutrophil and peripheral blood mononuclear cell (PBMC) purity after Dextran-Ficoll extraction.

Cells were stained with the neutrophil marker, CD66b (FITC) and the monocyte maker CD14 (PerCP) and cell purity was assessed by flow cytometry.

2.3.2 $CD14^+$ monocytes bead separation

PBMCs were washed once with pre-chilled MACS buffer (PBS containing 2mM EDTA, 1% FBS) prior to resuspension in 80µL MACS buffer and 20µL magnetically labelled anti-CD14 beads (Miltenyi Biotec). PBMCs were incubated at 4°C for 30min followed by washing with MACS buffer. Cells were resuspended in 500µL MACS buffer and passed through an LS MACS column placed in a MACS magnet (Miltenyi Biotec). The column was washed three times with MACS buffer and the $CD14^-$ negative cell population was collected. The column was then removed from the magnetic field and the $CD14^+$ cells were collected by passing 5mL MACS buffer through the column with the help of a sterile plunger provided.

2.3.3 Serum collection

5mL collected blood was used to prepare autologous normal human serum (NHS). Blood was transferred into a heparin-free falcon tube and left to coagulate for 45min. Tubes were subjected to centrifugation at 4000rpm for 15min to separate the fluid phase from the cellular content. Serum was collected and aliquots were stored at -80°C until use.

2.3.4 Heat-inactivation of serum

After collection, one serum aliquot was used to prepare heat-inactivated serum (HIS). Serum was placed into a water bath at 56°C for 20min to inactivate the complement system.

2.3.5 Detection of antibody titres by enzyme-linked immunosorbent assay (ELISA)

C. jejuni 81-176 was suspended in PBS and adjusted to an OD 1. Bacteria were fixed by addition of 0.5% paraformaldehyde (PFA) for 30min. Bacterial cells were washed once in PBS and adjusted to an OD 0.05. Microtitre plates were coated with 100µL of the suspension and incubated overnight at 4°C. Plates were washed and non-specific binding was blocked with 1% bovine serum albumin (BSA) at room temperature (RT) for 1h. Serial diluted serum was added and plates were incubated for 1h at RT. Adherent immune complexes were detected with horse radish peroxidase (HRP)-conjugated mouse anti-human IgG or IgA (Southern Biotech) or alkaline phosphatase (AP)-conjugated mouse anti-human IgM (BD Biosciences) antibody for 1h at room temperature. Plates were washed and 50µL Tetramethylbenzidine (TMS, eBiosciences) substrate was added until colour developed followed by 25µL stop solution (2N H_2SO_4) to detect HRP-conjugated antibodies, or p-nitrophenylphosphate (pNPP, Sigma*)* substrate was added to detect AP-conjugated antibodies, respectively. Plates were subjected to an ELISA plate reader to measure absorbance at 450nm (TMS) or 405nm (pNPP). As an assay control, serum-incubation and antibody detection was prepared in parallel in an untreated control plate (not bacterial coated). Little non-specific binding to the microtitre plate was observer (Figure 2.2). Antibody titres were calculated by

adjusting the values to an OD 0.6 and multiplied by the dilution factor. A reference serum was included to each plate in order to normalise the expression.

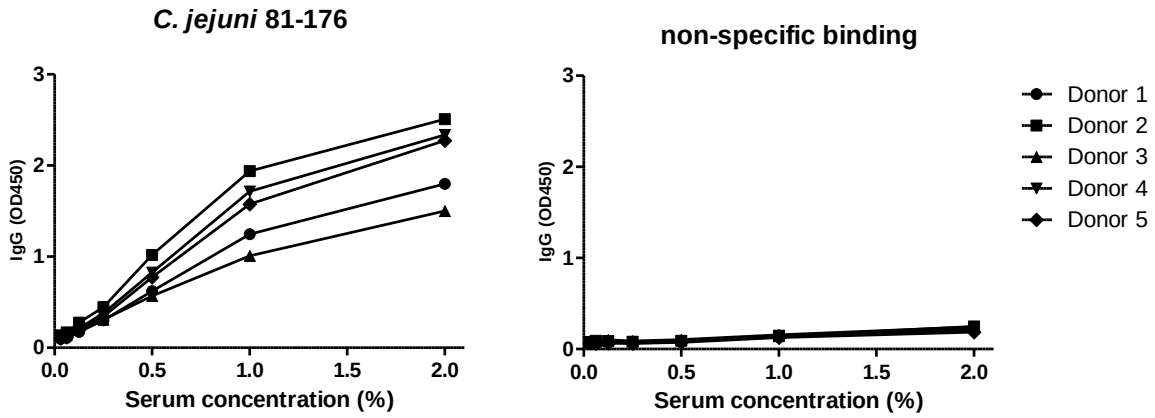


Figure 2.2 ELISA IgG- results of a *C. jejuni* coated (left) and an uncoated microtitre plate (right, non-specific binding)

2.3.6 Depletion of immune complexes

Serum IgA, IgG and IgM were depleted according to a protocol kindly provided by Professor Sanjay Ram (University of Massachusetts, USA) (Agarwal et al., 2014). After the collection of serum (4mL) all steps were performed at 4°C. Serum was treated with EDTA (10mM) to prevent complement activation and NaCl solution (1M) to reduce C1q loss during the depletion procedure. Wash columns were prepared containing Protein G sepharose (Sigma) for IgG depletion, anti-human IgA agarose (Sigma) or anti-human IgM agarose (Sigma). Prior to use, columns were equilibrated three times with wash buffer (PBS, 10mM EDTA, 1M NaCl). Diluted serum was applied to the columns and incubated with gentle rocking at 4°C for 1h to allow for antibody adhesion. After incubation, depleted sera were collected into falcon tubes by centrifugation at 1000g for 1min. Sera were diluted with PBS 0.1mM EDTA (1:3 ratio) and concentrated to 2mL using a spin concentration tube (Amicon, 10.000MW cut-off) at 5000g at 4°C. Ice-cold PBS was added and sera were reconstituted to 1mL. Successful depletion was confirmed by ELISA (Figure 2.3). Prior to use 2mM Ca^{2+} and 2mM Mg^{2+} were added to depleted sera to counter the inhibitory effect of residual EDTA on complement activation.

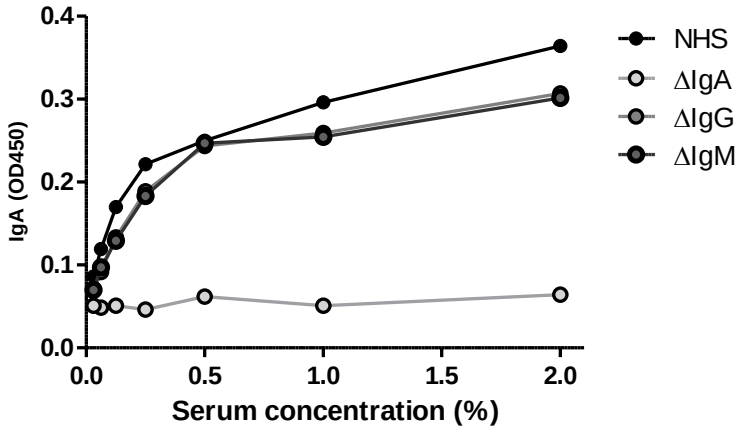


Figure 2.3 Representative ELISA result of serum IgA after antibody depletion

2.4 Bacterial Phagocytosis

2.4.1 FITC labelling of bacteria

Fluorescein isothiocyanate (FITC, Sigma) was diluted in PBS (saturated solution) and passed through a 0.22µm filter to sterilise the solution and remove residual particles. Bacteria were suspended in PBS and adjusted to an OD 0.5. 500µL of bacterial suspension were mixed with the equal volume of FITC solution and incubation in the dark for 1h with gentle rocking. Bacteria were pelleted at 10.000g for 2min and washed three times with PBS. Where indicated, labelled bacteria were then subjected to serum opsonisation. Labelling was assessed by flow cytometry.

2.4.2 Serum opsonisation

For opsonisation with serum, bacteria were adjusted to an OD 0.5 in PBS and incubated with 20% normal human serum (NHS) or heat-inactivated serum (HIS) at gentle agitation for 30min. Bacterial cells were washed twice in PBS and re-adjusted to an OD 0.5. Serum treatment had no effect on bacterial viability as determined by CFU counts (data not shown).

2.4.3 Detection of surface opsonins by Western blot analysis

10% SDS-polyacrylamide gels were prepared using a Gel cast system (1.5mm, Bio Rad) as follows:

Resolving Gel:

2.5mL Resolving Buffer (1.5M Tris, 0.4% SDS, pH 8.8)

3.5mL 30% Acrylamide/ Bisacrylamide (Sigma)

4mL MilliQ water

100µL 10% Ammonium Persulfate (APS, Sigma)

10µL Tetramethylethylenediamine (TEMED, Sigma)

Stacking Gel:

1.2mL Stacking Buffer (0.5M Tris, 0.4% SDS, pH 6.8)

1.2mL 30% Acrylamide/ Bisacrylamide (Sigma)

1.2mL MilliQ water

120µL 10% APS

10µL TEMED

Opsonised bacterial samples were prepared as described above. 100µL of bacterial suspension were transferred into an eppendorf tube and bacterial cells were pelleted. The supernatant was removed and cell lysis and protein denaturation was achieved by addition of 20µL PBS and 4µL 6x Laemmli buffer (12% Sodium dodecyl sulphate (SDS, Sigma), 10% β-mercaptoethanol (Sigma), 47% glycerol (Sigma), 0.6% bromophenol blue (Fisher scientific), 0.06M Tris HCl, pH 6.8). Samples were incubated at 95°C for 10min prior to transfer to the SDS-gel. SeeBlue protein ladder (Life Technologies) served as control. Proteins were separated using a mini-protean cell unit (Bio Rad) at constant 120V for approximately 90min in Running buffer (25mM Tris, 190mM glycine, 0.1% SDS). Proteins were transferred onto a nitrocellulose membrane at constant 200mA for 70min in ice-cold Transfer Buffer (25mM Tris, 190mM glycin, 20% methanol). Membranes were blocked with 5% milk (Marvel) for 1h at RT prior to detection with specific antibodies as indicated. Primary antibodies were applied in PBS or Tris buffered saline (TBS) containing 0.1% Tween-20 (PBS/TBS-T 0.1%), 5% milk at 4°C overnight. The membrane was washed three times in PBS/TBS-T 0.1% prior to incubation with HRP-conjugated secondary antibody at 4°C for 4h. To detect the proteins of interest, membranes were subjected to enhanced chemiluminescence (ECL, GE Healthcare) followed by exposure to x-ray film (ECL hyperfilm, GE Healthcare) and development.

2.4.4 Bacterial phagocytosis by flow cytometry

Isolated neutrophils were suspended to a concentration of $2x10^6$ cells/mL and transferred into sterile tubes. FITC-labelled bacteria and, were applicable, opsonised bacteria were added at multiplicity of infection (MOI) 10. At the indicated time points, 50µL aliquots of the co-culture were transferred into FACS tubes and cells were fixed by addition of 500µL 4% PFA for 10min. Cells were washed once in FACS buffer (PBS containing 0.2% BSA and 0.02% sodium azide) and 40µL 0.04% Trypan blue was added to quench adherent bacteria. After 10min, cells were washed

three times in FACS buffer and resuspended in 50µL Cell-fix (Roche). Cells were stored at 4°C until analysis by FACSCalibur. Results were analysed using the FlowJo software (Tree Star).

2.4.5 Fluorescent microscopy

Isolated neutrophils were suspended to a concentration of $2x10^6$ cells/mL. 0.5mL of the cell-suspension were transferred onto a culture slide (Falcon) and cells were allowed to adhere for 30min. Opsonised, FITC-labelled bacteria were added at MOI 100 and the co-culture was allowed to proceed at 37°C. After the indicated incubation time, cells were carefully washed with PBS and fixed with 4% PFA for 10min. Cell-adherent bacteria was detected by the addition of polyclonal goat anti-*Campylobacter* antibody (BacTrace®, KPL) followed by donkey anti-goat AlexaFluor®568 (Life Technologies) secondary antibody for 30min, each with intermittent washing steps. Glass slides were allowed to dry before addition of one drop of Vectashield (Vecta) to stain the cell nuclei. A cover glass was placed on the slide and sealed around the edges. Images were obtained by fluorescent microscopy (Letica Upright).

2.4.6 Phagocytosis inhibition

Inhibition of phagocytosis was achieved by addition of 10µg/mL Cytochalasin D (cytD, Sigma) to the cells for 30min, prior to bacterial co-culture. DMSO served as vehicle control.

2.4.7 Receptor blocking

Phagocytic receptors were blocked by addition of 20µg/mL blocking antibodies for 15min, prior to bacterial co-culture. The following blocking antibodies were utilised: anti-human CD16 (BioLegend), CD89 (AbD SeroTec), CD35 (GeneTex), CD11b (mAblCRF44, N. Hogg Cancer Research, UK), CD18 (Endogen, Perbio Science) and CD11c (N. Hogg Cancer Research, UK). Mouse anti-human IgG1 antibody (BioLegend) served as isotype control.

2.5 CHO cell-line co-culture

500µL cell suspensions ($5x10^5$ cells/mL) of CR1, CR3 and CR4 transfected CHO cells and mock control were transferred into 24-well plates and allowed to adhere at 37°C for 2h. FITC-labelled, opsonised *C. jejuni* were added at MOI 100. After 4h, cells were fixed with 4% PFA and gently removed from the plates by the help of Accutase®. Cells were stained for the expression of CR1/CD35 (PE, eBioscience), CR3/CD11b (PerCP, eBioscience) or CR4/CD11c (Pe, BD Biosciences) and analysed by flow cytometry. Receptor-bacterial cell interaction was determined by quantifying double positive cells expressing the surface marker and FITC-bacterial signal.

2.6 Neutrophil effector function

2.6.1 Expression of CR1 and CR3 on the neutrophil surface

Neutrophils were monitored for CR1 and CR3 protein expression in response to bacterial co-culture. $2x10^6$ cells/mL neutrophil suspensions were co-cultured with *C. jejuni* at MOI 10. After 1h, 100µL were transferred into FACS tubes, washed once with FACS buffer, and stained with CD35 (PE, eBioscience) and CD11b (PerCP, eBioscience) antibodies. Unstimulated cells served as control. Samples were analysed by flow cytometry.

2.6.2 Neutrophil Elastase secretion

Neutrophils were suspended at a concentration of $1x10^7$ cells/mL and 50µL aliquots transferred into 96-well plates. Opsonised or non-opsonised bacteria were added at MOI 10 and the co-culture was incubated at 37°C for 1h. Total amount of elastase was determined by addition of 0.1% TritonX (Promega) to one well. After 1h, 50µL Elastase Substrate 1 (Calbiochem) was added and incubated for 1h at 37°C. Colorimetric changes were assessed at 405nm. Readings were normalised to a cell-free control and expressed as % of released elastase/total amount of elastase.

2.6.3 Neutrophil oxidative burst

Neutrophils were suspended at a concentration of $2x10^6$ cells/mL and 1µM DHR 123 (Life Technologies) was added. 100µL of the cell suspension were transferred into 96-well plates and opsonised or non-opsonised bacteria were added at MOI 10. After 1h cells were washed once in PBS and analysed by flow cytometry.

2.6.4 Neutrophil bactericidal activity

Neutrophils ($2x10^6$ cells/mL) were co-cultured with opsonised bacteria at MOI 0.1 at 37°C for 4h. Aliquots were taken at time 0 and over time and subjected to serial dilution. Dilutions were spotted onto BA plates and incubated for 48h. CFUs were counted to determine bacterial survival over time. HIS served as control.

2.7 C1q experiments

2.7.1 Bacterial opsonisation with C1q

Recombinant C1q (recC1q) protein and maltose-binding protein (MBP)-linked globular head regions (gh) of C1q (ghA, ghB and ghC) and antibodies were a kind gift from Dr. Uday Kishore (Brunel University, UK) (Kishore, 2003). Bacteria were suspended to an OD of 0.5 in PBS containing

4mM Ca^{2+}. 250µL bacterial suspensions were incubated with 20µg/mL recC1q, ghA, ghB or ghC at 37°C for 1h. Cells were washed once and resuspended in PBS.

2.7.2 C1q-binding by flow cytometry

50µL opsonised bacterial suspension were washed in FACS buffer and incubated with rabbit anti-human C1q antibody or mouse anti-MBP antibody followed by anti-rabbit or anti-mouse AlexaFluor®488 secondary antibody (Life Technologies). Protein-binding was analysed by flow cytometry.

2.7.3 C1q-binding by ELISA

Microtiter plates were coated with 100µL PFA-fixed *C. jejuni* at an OD 0.5. Non-specific binding was blocked by 3% BSA at RT for 1h. C1q and gh proteins were subjected to serial dilution in PBS/4mMCa^{2+} and 100µL aliquots were transferred to the plates followed by incubation at 37°C for 1h. Adherent proteins were detected by rabbit anti-human C1q antibody or mouse anti-MBP antibody following HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibody, respectively. TMS substrate was added until colour developed followed by stop solution. Absorbance was determined at 450nm.

2.7.4 C1q-binding by Western blot analysis

100µL opsonised bacterial suspensions were pelleted and boiled in Laemmli buffer for 10min. Samples were subjected to mini-protean SDS-gel electrophoresis (10% acrylamide gel) followed by protein transfer onto a nitrocellulose membrane. Membranes were blocked with 5% milk at RT for 1h. Proteins were detected by rabbit anti-human C1q antibody or mouse anti-MBP antibody following HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibody.

2.8 Bacterial-mediated Complement activation

2.8.1 Total C3b deposition by ELISA

Protocols used to assay for complement activation and mouse sera were kindly provided by Professor Wilhelm Schwaeble (University of Leicester, UK) (Ali et al., 2012). Bacteria were adjusted to an OD 0.5 and fixed in 0.5% PFA for 30min. Bacteria were pelleted and suspended in coating buffer (eBioscience). Microtiter plates were coated with 100µL bacterial suspension and incubated at 4°C overnight. 10µg/mL mannan (Promega) was used as control. Wells were blocked with 0.1% BSA in TBS at RT for 1h then washed with wash buffer (TBS-T 0.05%). Serum samples were serial diluted in BBS buffer (4mM barbital (Sigma) 145mM NaCl (Sigma), 2mM $CaCl_2$, 1mM $MgCl_2$, pH 7.4). Serum dilutions were added to the plates and incubated at 37°C for 1h. Plates

were washed and bound C3b was detected using rabbit anti-human C3c antibody (Dako) for 1h followed by AP-conjugated goat anti-rabbit IgG for 1h. pNPP substrate was added until colour developed and absorbance was determined at 405nm.

2.8.2 Alternative pathway C3b deposition by ELISA

Assays were performed in absence of Ca^{2+} allowing only for alternative pathway activation. Microtiter plates were coated and blocked as described above. Sera were diluted in Ca^{2+}-free BBS buffer (4mM barbital, 145mM NaCl, 8mM EGTA, 5mM $MgCl_2$, pH 7.4). Serum dilutions were added to the plates and incubated at 37°C for 1h. 10µg/mL zymosan(Promega) was used as control. The first wash was performed with Ca^{2+}-free BBS buffer to prevent undesired complement activation. Bound C3b was detected as described above.

2.8.3 C3b deposition by Western blot analysis

C. jejuni was suspended in PBS and normalised to an OD 0.5. 100µL bacterial suspension were transferred into Eppendorf tubes, washed once in PBS and resuspended in 50µL PBS containing 20% NHS, 0.15mM $CaCl_2$, 0.5mM $MgCl_2$. Samples were incubated at 37°C for the indicated time period. After incubation, reactions were stopped by addition of 1mL ice-cold PBS containing 10mM EDTA followed by extensive washing in PBS. Samples were boiled in Laemmli buffer and subjected to a mini-protean SDS-gel electrophoresis (10% acrylamide gel) followed by protein transfer onto a nitrocellulose membrane. Membranes were blocked for 1h and incubated with rabbit anti-human C3c antibody (Dako) followed by HRP-conjugated goat anti-rabbit (Dako) secondary antibody.

2.8.4 Serum survival assay

Bacteria were suspended to an OD 0.5 in PBS containing 20% NHS and incubated at 37°C to allow for complement activation. Samples were taken at time 0 and after 1h, 2h and 4h and subjected to serial dilution. Four dilutions were spotted onto BA plates and incubated for 48h. CFUs were determined and expressed as $\log_{10}$ values.

2.9 Cytokine secretion

2.9.1 Co-culture with human blood leukocytes

1mL heparinised blood or isolated leukocytes at $2x10^6$cells/mL were transferred into sterile falcon tubes and stimulated with ultrapure LPS (*E.coli*, Sigma) or co-cultured with bacteria at various MOIs. Samples were subjected to centrifugation at 3000rpm (blood samples) or 1500rpm (leukocytes) for 5min and supernatants were collected for cytokine analysis.

2.9.2 Inhibitory studies

Where indicated, cells were pre-treated for 30min with 50µM caspase-1 inhibitor IV (Ac-YVAD-AOM, Calbiochem), 10µg/mL Elafin (Anaspec) or 10µM cathepsin G inhibitor 1 (Calbiochem) or DMSO vehicle control prior to LPS stimulation or bacterial co-culture.

2.9.3 Cytokine secretion by ELISA

Cytokine secretion was quantified by Sandwich ELISA according to the manufacturer's protocol. ELISA kits were obtained from eBioscience for IL-β and TNF-α (Ready-Set-Go!®) or Peprotech for IL-8. Microtiter plates were coated with capture antibody overnight to allow for antibody binding. Non-specific binding was blocked with 1% BSA at RT for 1h. Samples and serial diluted standards were transferred to the plates in duplicates and incubated at RT for 2h. Biotin-conjugated detection antibody was added and incubated at RT for 1h followed by incubation with HRP-conjugated avidin at RT for 30min. TMS substrate solution was added until colour developed followed by stop solution. Absorbance was measured at 450nm. Cytokine concentrations were determined by utilising a standard curve.

2.9.4 Intracellular cytokine expression by FACS analysis

50µL aliquots from a whole blood sample were transferred into FACS tubes and stained with specific cell surface markers for lymphocytes (CD3, FITC; BD Biosciences), monocytes (CD14, PerCP; eBioscience) and neutrophils (CD66b, APC; eBioscience) in the dark at 4°C for 30min. Cells were washed once with FACS buffer prior to erythrocyte lysis using 1mL red blood cell lysis buffer (BD Biosciences) at RT for 15min. Leukocytes were washed twice with FACS buffer before addition of 1% PFA for 10min. Cells were permeabilised by addition of FACS buffer containing 0.05% saponin (Sigma) for 10min. Cells were washed with FACS buffer (1800rpm after permeabilisation) and stained for intracellular, bioactive IL-1β or isotype control (both BD Biosciences) at 4°C for 30min. Cells were stored in Cell-fix (Roche) at 4°C until analysis by flow cytometry.

2.9.5 Quantitative polymerase chain reaction (qPCR)

Neutrophils were stimulated with LPS or *C. jejuni* 81-176 for 5h. After stimulation cells were washed once with PBS before addition of 1mL TRIzol solution (Life Technologies). 200µL chloroform (Sigma) was added and mixed prior to centrifugation at 12.000rpm at 4°C for 20min. The RNA containing, aqueous layer was transferred into a fresh tube and an equal volume of ice-cold isopropanol (Sigma) was added. Samples were mixed and left over night at -20°C to allow for RNA precipitation. RNA was pelleted at 8000rpm at 4°C for 10min. RNA pellets were washed twice with 70% ethanol and left to dry. Samples were suspended in RNAse/DNAse fee water (Gibco) and RNA was quantified by Nanodrop (Thermo Scientific).

cDNA was generated using 1-5µg RNA with 1µL oligo dT (Bioline) and 1µL random hexamer (Bioline) and incubated (Thermocycler, Techne TC-512) at 70°C for 10min. Samples were immediately quenched on ice for 5 min to ensure optimal primer annealing. 4µL dNTPs, 0.5 µL Bioscript enzyme and Reaction buffer (5x) (all Bioline) were added and cDNA was synthesised at 25°C for 10min followed by 42°C for 1h. A final cycle at 75°C for 10min ensured enzyme deactivation.

SYBR® Green qPCR was performed in duplicates using the Rotor-gene 6000 (Qiagen). 2 µL cDNA (diluted 1:2) was added to 18µL master mix containing 10µL SYBR® Green (Sigma) and 5pmol forward and reverse primer (listed in Table 2.3). RNAse/DNAse free water was used to adjust the volume and samples were subjected to thermo cycling utilising the following conditions:

1x cycle	Initial denaturation	95°C	10min
40x cycles	Denaturation	95°C	15sec
	Annealing	Table 2.3	30sec
	Extension	72°C	30sec
1x cycle	Final extension	72°C	10min

Results were analysed using the Rotor Gene 6000 Software. Relative levels of transcription to housekeeping genes, RPLP0 and GAPDH, were determined by the following equation: $\Delta ct=2^{(housekeeping\text{-}gene\ of\ interest)}$

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing temperature (°C)
nlrp3	TCTCATGGATTGGTGAACAGC	GGTCCCCCAGAGAATTGTCA	58
Il1b	CCAAAAGATGAAGGGCTGCT	AGAAGGTGCTCATGTCCTCA	58
Il18	*Commercially purchased* (Qiagen)		60
GAPDH	CCTGGAGAAACCTGCCAAGTATG	AGAGTGGGAGTTGCTGTTGAA	58
RPLP0	GCAATGTTGCCAGTGTCTG	GCCTTGACCTTTTCAGCAA	58

Table 2.3 List of qPCR primers

2.10 Lipooligosaccharide (LOS) structural analysis

LOS purification and analysis was performed at the University of California San Francisco (UCSF) under the close guidance of Dr. Constance John and Dr. Nancy Philips. Protocols and reagents used were a kind gift from Professor Gary Jarvis (UCSF, USA).

2.10.1 Lipooligosaccharide (LOS) purification

Bacteria were harvested from BA plates, pelleted and stored at -80°C until use. Bacterial cultures of approximately 15 BA plates per strain were used for LOS purification. Bacterial pellets were suspended in 25mL of 50mM sodium phosphate buffer (pH 7.0) containing 5mM EDTA. Hen egg lysozyme (4mg/mL, Sigma) was added and incubated overnight at 4°C under gentle stirring. Suspensions were incubated at 37°C for 20min prior to addition of 50mM sodium phosphate (pH 7.0) containing 20mM $MgCl_2$ to make up a volume of 100mL. DNAse (1µg/mL, Sigma) and RNAse (2µg/mL, Sigma) were added and incubation allowed to precede at 37°C for 1h followed by 60°C for 1h. Proteinase K (20µg/mL, Sigma) was added and incubated at 50°C for 1h. Suspension was heated to 70°C and 100mL pre-equilibrated 90% phenol (Sigma) was added and mixed thoroughly prior to cooling on an ice-bath for 15min. The LOS containing aqueous phase was separated by centrifugation at 18.000g for 15min and transferred into a fresh falcon tube. The solution was dialysed against water using a 1000 MWCO-membrane at 4°C for 12-16h to remove any remaining phenol. After dialysis, solution was frozen at -20°C before being lyophilised for 12-16h. The pellet was resuspended in 10mL MilliQ water and subjected to ultracentrifugation at 100.000g at 4°C for 4h. The LOS pellet was resuspended in 1mL MilliQ water, allowed to freeze at -20°C before being lyophilised. Purified LOS was stored at -20°C until use.

2.10.2 LOS profile by silver stain

SDS-gel electrophoresis

Purified LOS was suspended in loading buffer (Bio Rad). Mini-Protean gels were run with 1µg LOS per well using pre-cast 7-15% TRIS-glycine gradient gels (Bio Rad) at constant 120V.

12.9% polyacrylamide long gels were prepared using a gel-casting system (Protean II xi cell, Bio Rad) as follows:

Resolving gel:

7mL Resolving Buffer (1.88M Tris, pH 8.8)

15mL 30% Acrylamide/ Bisacrylamide (Sigma)

12.2mL MilliQ water

300µL APS (50mg/mL)

10µL TEMED (Sigma)

Stacking gel:

1mL Stacking Buffer (1.25M Tris, pH 6.8)

1mL 30% Acrylamide/ Bisacrylamide (Sigma)

7.8mL MilliQ water

100µL SDS

100µL 10% APS

5µL TEMED

Long gels were run with 10µg per well at 4°C. The apparatus was set at constant 25mA while samples were run through the spacer, and 55mA for the remaining run.

Silver staining

Gels were fixed for 1h in fixing solution (40mL methanol, 5mL acetic acid, 55mL MilliQ water). Gels were washed twice in water for 5min with shaking prior to incubation in 1.4% periodic acid for 10min. Gels were washed eight times for 15min. Silver stain solution (2.8mL NaOH (1N), 2.5mL concentrated NH_4OH, 5mL silver nitrate (20%), 140mL water) was added for 10min followed by washing three times for 15min. Gels were developed in developing solution (10mg citric acid, 100µL formaldehyde, made to 200mL in water). At desired colour reaction was stopped by addition of 5% acetic acid for 30min.

2.10.3 O-deacylation of LOS

LOS of some strains was O-deacylated prior to MS analysis. Approximately 300µg of lyophilised LOS was transferred into an Eppendorf tube and 200µL anhydrous hydrazine (Sigma) was added. The samples were incubated at 37°C for 2h with intermittent vortexing. The reaction was stopped by addition of 1mL pre-cooled acetone (-20°C). O-deacylated LOS samples were pelleted at 12.000g for 20min, washed once in acetone and resuspended in 500µL MilliQ water. Samples were frozen at -20°C before being lyophilised.

2.10.4 Hydrogen fluoride (HF) treatment of LOS

Phosphoesters were partially removed by hydrogen fluoride (HF) treatment. Approximately 300µg of lyophilised LOS were transferred into Eppendorf tubes and cold 48% aqueous HF was

added to make a 10mg/mL solution. Samples were incubated at 4°C for 16-20h. Excess HF was removed using a SpeedVac (Thermo Savant) with an in-line trap.

2.10.5. Matrix preparation

The MS matrix was prepared by dissolving nitrocellulose transblot membrane (Bio Rad) in acetone:isopropanol (1:1) to a concentration of 15mg/mL. 2,4,6-trihydroxyacetophenone (THAP, Sigma) was dissolved in methanol to 200mg/mL and added to the nitrocellulose solution at a 4:1 ratio. Small drops (~0.5µL) of matrix were spotted onto the MS plate and were allowed to dry before the samples were added.

2.10.6 LOS sample preparation

Lyophilised LOS was suspended to 10mg/mL in a mixture of methanol:water (1:3) containing 5mM EDTA. An aliquot was mixed with and equal volume di-basic ammonium citrate (20mM); the mixture was desalted by the use of Dowex 50WX8-200 cation-exchange beads (Sigma). Approximately 1µL sample was transferred onto the MS matrix using a glass capillary pipette and allowed to dry.

2.10.7 Mass spectrometry

Matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF) MS analysis was performed on a Synapt G2 high definition MS (HDMS) system (Waters) in 'sensitivity mode'. Spectra were obtained in negative- or positive-ion mode operating a neodymium doped yttrium aluminium laser at 355nm and 200Hz. For composition analyses, tandem mass spectrometry (MS/MS) was performed. Fragment ions were separated from molecular ions by ion-mobility separation MS (IMS-MS). The instrument was calibrated using the masses of the monoisotopic ions for porcine renin substrate, bovine insulin and insulin B-chain. Spectra were smoothed and base-line corrected using MassLynx software. Lipid A structures were generated by using the ChemBioDraw Ultra software.

2.11 *Galleria mellonella* model of infection

Injection of *G. mellonella* larvae were performed by Dr. Dagmar Alber (ICH/UCL, UK). *Campylobacter* strains were suspended to an OD 1. The laevae were inoculated with 10µL of bacterial suspension (~$3x10^7$ CFUs) or PBS as control. *G. mellonella* were kept at 37°C and survival was monitored over seven days. Ten *G. mellonella* larvae were infected for each bacterial strain.

2.12 Statistics

Unpaired or paired t-test was performed for comparison of two normally distributed data sets. For comparison of more than two groups in the same experiment one-way ANOVA was performed using the Tukey post-test to account for multiple comparison analysis. When more than one variable was analysed the data sets were compared by two-way ANOVA with Bonferroni post-test. Statistical analysis was performed using the GraphPad 5 software.

CHAPTER III

NEUTROPHIL-MEDIATED PHAGOCYTOSIS OF

CAMPYLOBACTER JEJUNI

3.1 Background

Invasion of the gut mucosa by *C. jejuni* is accompanied by a vast influx of neutrophils to the site of infection (Wassenaar and Blaser, 1999). Neutrophils serve as an important first line defence system. Internalisation and clearance of pathogens by these professional phagocytes is critical in controlling the acute phase of infection. Mode of phagocytosis and the subsequent signalling cascades are largely determined by both host and bacterial factors (Witko-Sarsat et al., 2000).

The two main mechanisms of bacterial phagocytosis are non-opsonic and opsonic (Underhill and Ozinsky, 2002). Non-opsonic phagocytosis is initiated when neutrophils directly interact with the bacterial surface. The LOS of *Neisseria meningitidis* has been shown to bind to complement receptor 3 (CR3) providing an important serum-independent mechanism of bacterial uptake (Jones et al. 2008). Another example for this can be found in some strains of *Helicobacter pylori* which induce neutrophil activation and internalisation by binding to cell surface gangliosides *via* the virulence factor, sialic acid binding adhesion (SabA) (Unemo et al., 2005).

For opsonin-dependent phagocytosis two principal classes of receptors have been described (Underhill and Ozinsky, 2002). These include Fc receptor (FcR) and the complement receptor (CR) family interacting with bacterial-bound immunoglobulin (Ig) or components of the complement complex, respectively. Igs are thermo-stable opsonins as they retain functionality after heat-treatment which distinguishes them from the heat-labile complement system. IgA and IgG are primarily involved in Fc-mediated phagocytosis and interact with FcαR or FcγR, respectively (Soltis et al., 1979b). Many studies provide evidence for neutrophil-mediated phagocytosis of Ig-coated particles including beads, bacteria and fungi (Gresham et al., 1990, Serrander et al., 2000, Wenisch et al., 1999). While IgG is regarded as a classical immune component involved in phagocytosis, a recent publication highlights the importance of IgA-mediated uptake. In this study IgA-coating of *Staphylococcus aureus* and beads enhanced phagocytosis and induced neutrophil extracellular trap (NET) formation suggesting and important contribution of IgA in the elimination of this pathogen (Aleyd et al., 2014).

The complement system is an important contributor in the efficient clearance of many pathogens. Activation of the enzyme cascade results in the deposition of complement components (C3b, iC3b, C1q) on the bacterial surface enabling recognition by CR1, CR3 and CR4. The most detailed mechanism known to date is the interaction between iC3b and CR3 (Fossati-Jimack et al., 2013, Hyams et al., 2010, Suhonen et al., 2000). CR3 binds iC3b with high affinity while its precursor C3b is the preferred ligand for CR1. C3b cleavage to iC3b is facilitated by factor H (fH) co-factor together with factor I (fI). CR3 expression as well as optimal opsonisation with iC3b has shown to

be crucial in the clearance of many pathogens including *Bordetella pertussis*, *Candida Albicans* and *Streptococcus pneumonia* (Gordon et al., 1986, Gazendam et al., 2014b, Mobberley-Schuman and Weiss, 2005). Interestingly, *N. meningitides* fH-binding protein facilitates cleavage of surface-bound C3b to iC3b resulting in significant enhancement of CR3 binding (Agarwal et al., 2010, Schneider et al., 2006).

CR1 is a high-affinity receptor for C3b but can also associate with C4b, MBL and C1q (Ghiran et al., 2000, Klickstein et al., 1997, Tas et al., 1999). Historically, CR1 was considered to be a phagocytic co-factor facilitating FcγR- or CR3-mediated uptake rather than being directly involved in particle internalisation (Underhill and Ozinsky 2002). Porteu *et al.* incubated neutrophils with C3b-coated particles and demonstrated a significant increase in CR1 expression and ligand-receptor binding. Internalisation, however, was only achieved when beads were additionally coated with IgG, implicating FcγR in these events (Porteu et al., 1987). CR1 also has a role in the cleavage of C3b to iC3b hence promoting particle uptake by CR3 (Seya et al., 1990). Both, CR1 and CR3 in concert are involved in the phagocytosis of *Franciscella tularensis* (Schwartz et al., 2012).

While neutrophils employ a range of receptors to recognise intrinsic and extrinsic (serum-acquired) bacterial components, many pathogens have evolved to evade neutrophil-mediated killing. One strategy of action is to escape phagocytosis by the help of virulence factors, like the expression of polysaccharide capsules by *N. meningitides* and *S. pneumonia*, or the secretion of *S. aureus* extra fibrinogen binding protein (Efb). These molecules either hinder deposition of C3b on the bacterial surface or actively cleave enzymatic components of the complement cascade preventing optimal opsonisation and uptake (Hyams et al., 2010, Kugelberg et al., 2008, Ko et al., 2013)

Other pathogens have developed mechanisms that improve their survival after internalisation. Once internalised, bacteria are engulfed and directed to the phagosome (Kaplan, 1977) . Intracellular granules then fuse with the phagosome, forming the phagolysosome which initiates the release of their antimicrobial contents. Apart from an assortment of antimicrobial proteins and metalloproteases, granules discharge peroxidases important for the generation of ROS. Together with the NADPH-oxidase complex, which assembles on the phagosomal membrane, this leads to the induction of a respiratory burst with pivotal function in bacterial killing (Urban et al., 2006).

Upon contact with neutrophils, *C. albicans* was found to upregulate expression of oxidative stress genes, including superoxide dismutase, catalase and glutathione peroxidase, enabling it to

neutralise ROS more efficiently and resulting in resistance to oxidative killing (Rubin-Bejerano et al., 2003). Others like *S. pyrogenes* and *H. pylori* have evolved ways to escape killing by preventing the formation of neutrophil phagolysosome or redirecting the assembly of the NADPH-oxidase complex away from the phagosome to the plasma membrane (Allen et al., 2005, Medina et al., 2003).

Neutrophils employ various mechanisms to eliminate pathogens including phagocytosis. The adherence of pathogens to phagocytic receptors is accompanied by the activation of a range of signalling pathways involved in their engulfment, recruitment of the phagosome machinery and subsequent bacterial elimination (Underhill and Ozinsky, 2002). While multiple studies have been conducted to investigate phagocytosis and mechanisms of immune evasion for many important pathogens, such as *N. meningitides*, *S. aureus*, *H. pylori* and *Streptococcus* species, surprisingly little is known for *C. jejuni*. A few studies report neutrophil-mediated bacterial uptake in humans (Keo et al., 2011, Walan et al., 1992, Bernatowska et al., 1989). The purpose of this study was to assess if and how *C. jejuni* is phagocytosed by neutrophils and to elucidate the bacterial and host factors involved in this process.

3.2 Phagocytosis of *Campylobacter jejuni* is serum-dependent

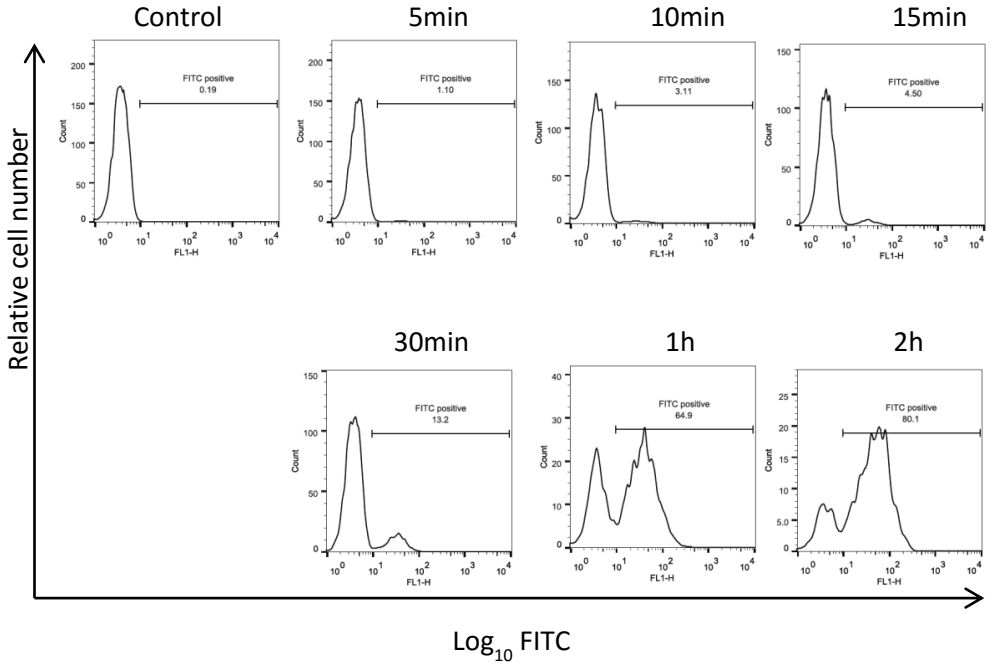
Whole blood was co-cultured with FITC-labelled *C. jejuni* strain 81-176 to assess neutrophil-mediated bacterial phagocytosis. The FITC from the surface adherent bacteria was quenched with trypan blue, so that primarily internalised FITC-positive cells were assessed by flow cytometry. Trypan blue treated cells without the addition of bacteria were used as control. Minimal uptake was observed in the first 15min of co-incubation, however a measurable increase was observed at 30min which steadily increased up to 2h. Bacterial uptake from one representative donor is shown in Figure 3.1 A. Cumulative data from five donors is shown in Figure 3.1B. These observations suggest that neutrophils phagocytose *C. jejuni* by 30min of co-culture; the increase between 30min and 2h was found not be to statistically significant (Figure 3.1).

In order to determine host factors involved in bacterial uptake, neutrophils were isolated and co-cultured with *C. jejuni* that were previously non-opsonised or opsonised with heat-inactivated serum (HIS) or active normal human serum (NHS). Interestingly, only NHS-opsonised bacteria showed significant phagocytosis whereas uptake of HIS and non-opsonised bacteria were minimal (Figure 3.2 A). These findings were independent of the time of co-culture (Figure 3.2 B). It was important to show that the mode of opsonisation had no impact on the degree of bacterial fluorescent labelling (Figure 3.2 C).

To assess if the uptake was dependent on bacterial viability, neutrophils were co-cultured with NHS-opsonised, live and inactivated bacterial cells (by heat-treatment, gentamicin and PFA). The rate of uptake was found to be independent of bacterial viability (Figure 3.3).

To confirm bacterial internalisation, assays were performed in the presence of cytochalasin D (cytD). CytD significantly reduced (42.1% and 24.3% FITC-positive cells in non-treated and cytD treated cells, respectively) the number of FITC-positive cells in trypan blue treated (quenched) cells whereas little effect was noted on non-quenched cells (Figure 3.4 A). CytD inhibits actin-dependent phagocytosis but does not hinder cell adherence confirming that trypan blue quenching serves as a good method to assess internalised bacteria however it should be noted that the uptake was only partly inhibited by cytD treatment suggesting either incomplete quenching of adherent bacteria or a actin-independent mode of uptake. Trypan blue quenching was utilised in all future experiments. Bacterial engulfment was further confirmed using a differential staining method to distinguish surface associated *versus* internalised *C. jejuni* and then analysed by fluorescent microscopy (Figure 3.4 B).

A



B

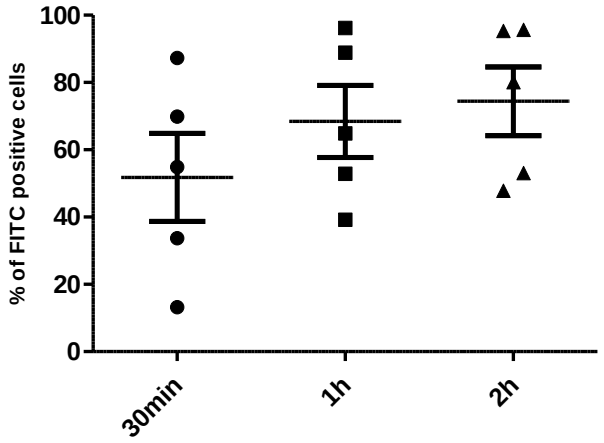


Figure 3.1 Neutrophils phagocytose *C. jejuni* in a time-dependent manner.

Heparinised human blood (1mL) from healthy donors was incubated with FITC-labelled *C. jejuni* 81-176 ($5x10^7$CFU). Samples were taken after the indicated incubation times and erythrocytes were lysed with red blood cell lysis buffer. Leukocytes were fixed with 4% PFA and adherent bacteria were quenched by addition of trypan blue. Cells were washed and the percentage of FITC-positive neutrophils was determined by FACSCalibur. Non-infected, trypan blue treated cells served as control. **(A)** Time-dependent uptake from one representative individual; **(B)** Percentage uptake mean±SEM of five donors. One-way ANOVA with Tukey post-test was performed for statistical analysis. No stars: not significant

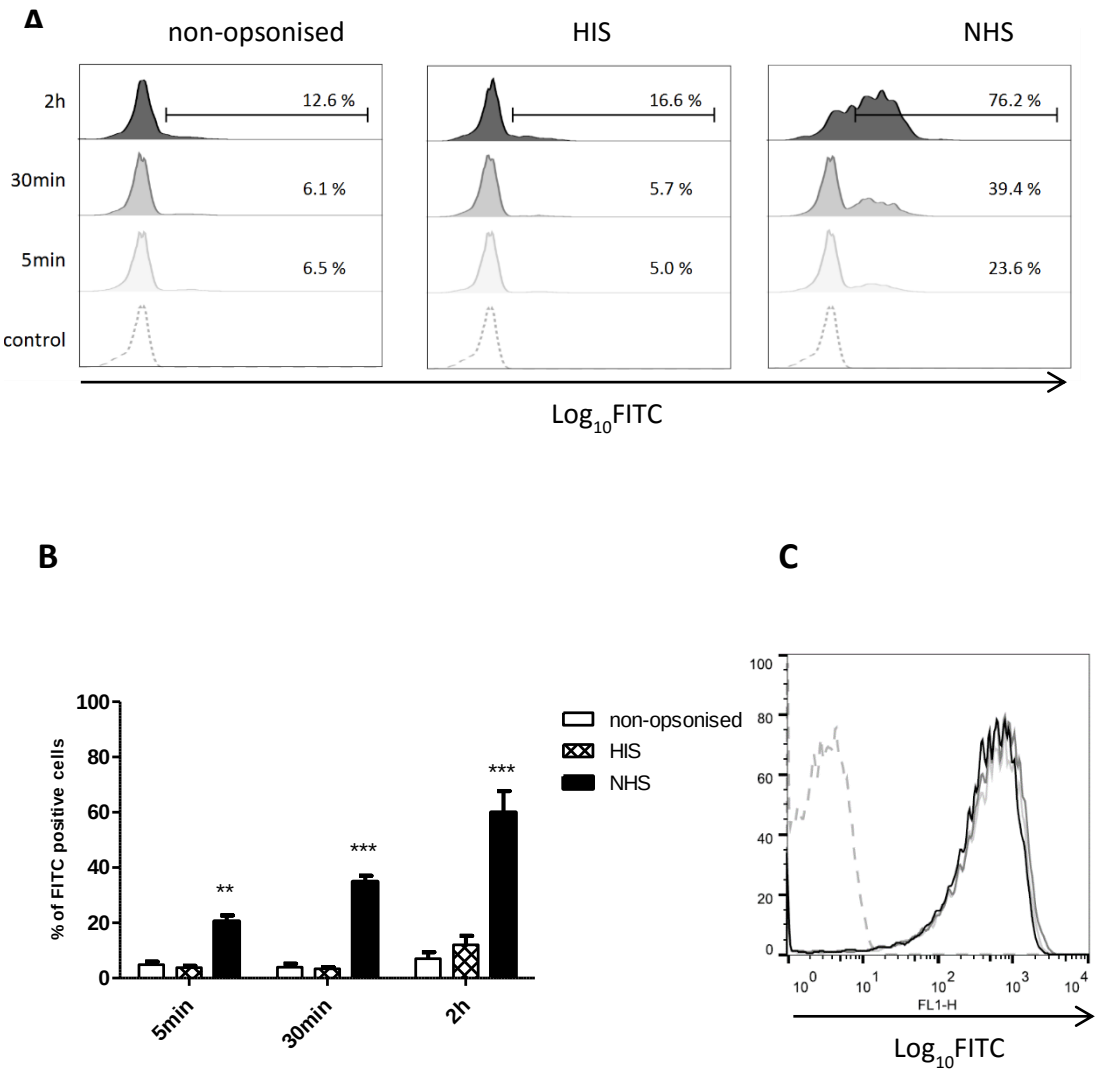


Figure 3.2 Serum-dependent phagocytosis of *C. jejuni.*

Human neutrophils were co-cultured *with C. jejuni* 81-176 (MOI 10). Prior to co-culture, FITC-labelled bacteria were either non-opsonised or opsonised with 20% autologous heat-inactivated serum (HIS) or normal human serum (NHS). **(A)** Time-dependent bacterial uptake from one representative donor. **(B)** Time-dependent uptake (mean±SEM) from three individual donors. **(C)** FACS analysis of FITC-labelled bacteria: non-opsonised (light grey), HIS (dark grey), NHS (black); non-labelled control (dash). Two-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant; * $p < 0.05$; ** $p<0.01$; *** $p<0.001$

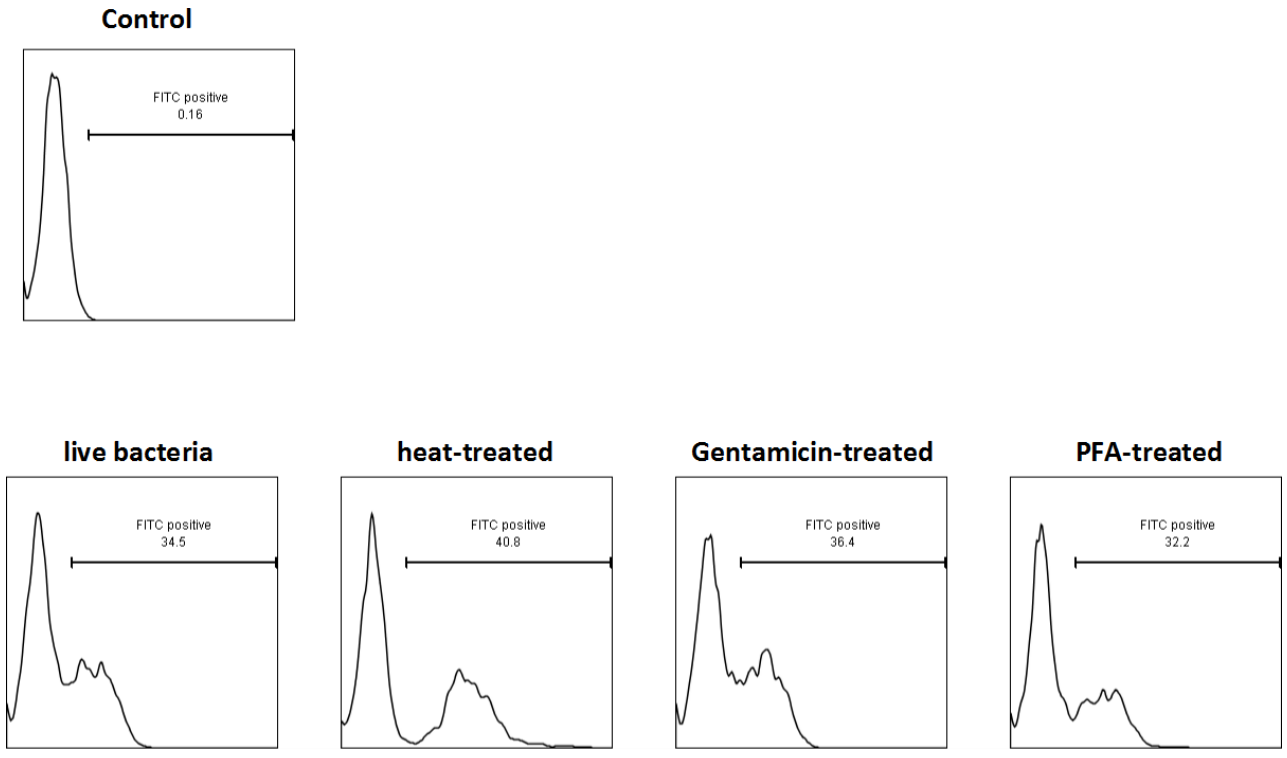


Figure 3.3 Neutrophil phagocytosis of live and killed *C. jejuni*

Isolated human neutrophils were co-cultured *with* FITC-labelled, NHS-opsonised *C. jejuni* 81-176 at an MOI of 10. Prior to co-culture, bacteria were live or inactivated by heat-treatment (56°C, 30min), gentamicin (250µg/mL, 1h) or PFA (0.5%, 30min). Rate of phagocytosis was assessed by flow cytometer.

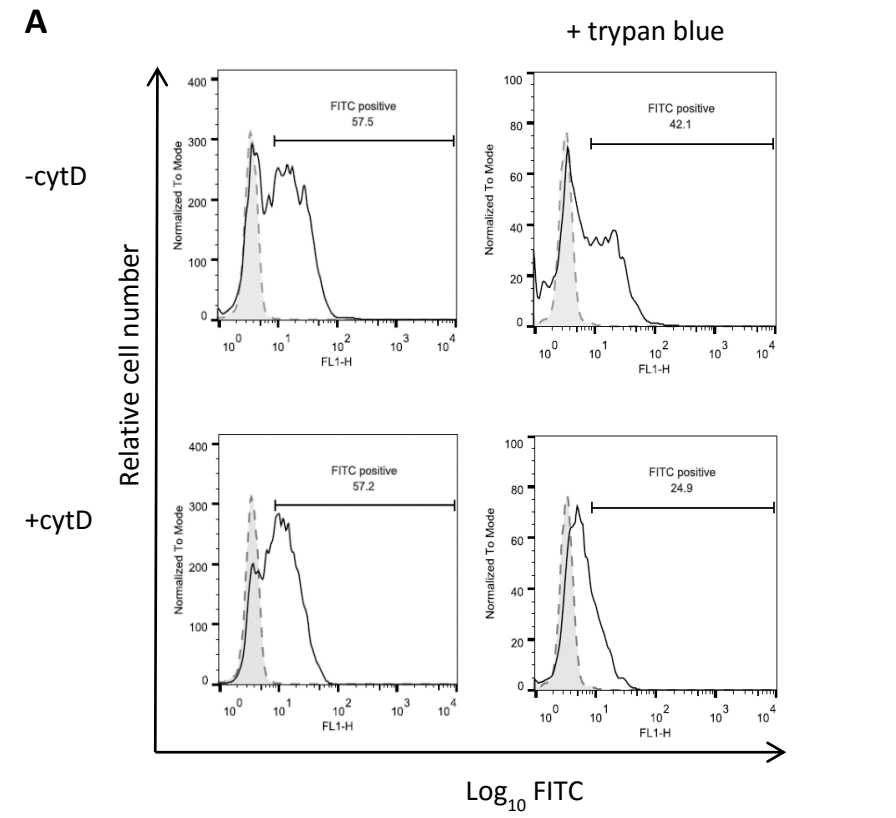


Figure 3.4 Neutrophil-mediated internalisation of serum-opsonised *C. jejuni.*

(A) Human neutrophils were co-cultured with FITC-labelled, NHS-opsonised *C. jejuni* 81-176 (MOI 10). Where indicated, cytochalasin D (cytD) was added to the cells 30min prior to co-culture. After 1h, cells were fixed and trypan blue was added in order to quench external, adherent bacteria. Samples were analysed by FACSCalibur. Data shows one representative of three individual experiments. **(B)** Neutrophils were allowed to adhere onto glass slides prior to bacterial co-culture (MOI 100). At the indicated time-points, cells were fixed with 4% PFA and adherent bacteria were detected with goat anti-*Campylobacter* antibody followed by AlexaFluor®568 secondary antibody. Cell nuclei were stained with DAPI and appear in blue. External bacteria appear in red whereas internalised bacteria remain green.

Serum antibodies are generally thermostable at 56°C (Soltis et al., 1979a). As we found minimal effect of HIS on bacterial uptake, it was important to record if serum employed in the study did indeed contain antibodies to *Campylobacter.* Next, serum IgA (Figure 3.5 A), IgG (Figure 3.5 B) and IgM (Figure 3.5 C) titres to *C. jejuni* were investigated in ten healthy donors. Despite positivity for all three antibody types, minimal uptake of HIS-opsonised bacteria was observed suggesting that antibody-mediated internalisation is not a major pathway for neutrophil phagocytosis of *C. jejuni* and that FcR-independent mechanisms are at play. To test this, binding to FcγIIIR (CD16) and FcαRI (CD89) were inhibited by specific blocking antibodies prior to exposure to bacteria. Blocking of FcγIIIR and FcαRI had minimal effect on bacterial phagocytosis supporting the notion that *C. jejuni* is phagocytosed by human neutrophils *via* antibody-FcR independent pathways (Figure 3.6).

3.3 Phagocytosis is primarily mediated by Complement receptor CR1

Our study provided evidence that FcR receptors are unlikely to play a major role in *C. jejuni* uptake. Bacterial uptake was observed to be NHS-opsonin dependent. Since complement receptors (CR1,CR3 and CR4) have previously shown to function as phagocytic receptors of NHS-opsonised particles, we hypothesised the involvement of CRs in uptake of *C. jejuni* (Brown, 1991). In a series of experiments, we employed blocking antibodies to this family of receptors in bacterial uptake assays. Blocking of CR1 significantly impaired phagocytosis by about 70% whereas blocking of CR3 with CD11b had a less prominent, but significant impact on bacterial uptake. No effect was observed with blocking of CR4 (Figure 3.7). Collectively, this suggests that CR1 plays a major role in *C. jejuni* phagocytosis while the involvement of CR3 was less clear. CR3 is composed of two protein subunits (CD11b and CD18). Interestingly, the modest reduction of uptake by CR3 was only observed when CD11b or CD11b and CD18 were blocked simultaneously whereas no change was observed with blocking CD18 alone (Figure 3.8). These results suggest that CR1 serves as the primary route of uptake of *C. jejuni* 81-176.

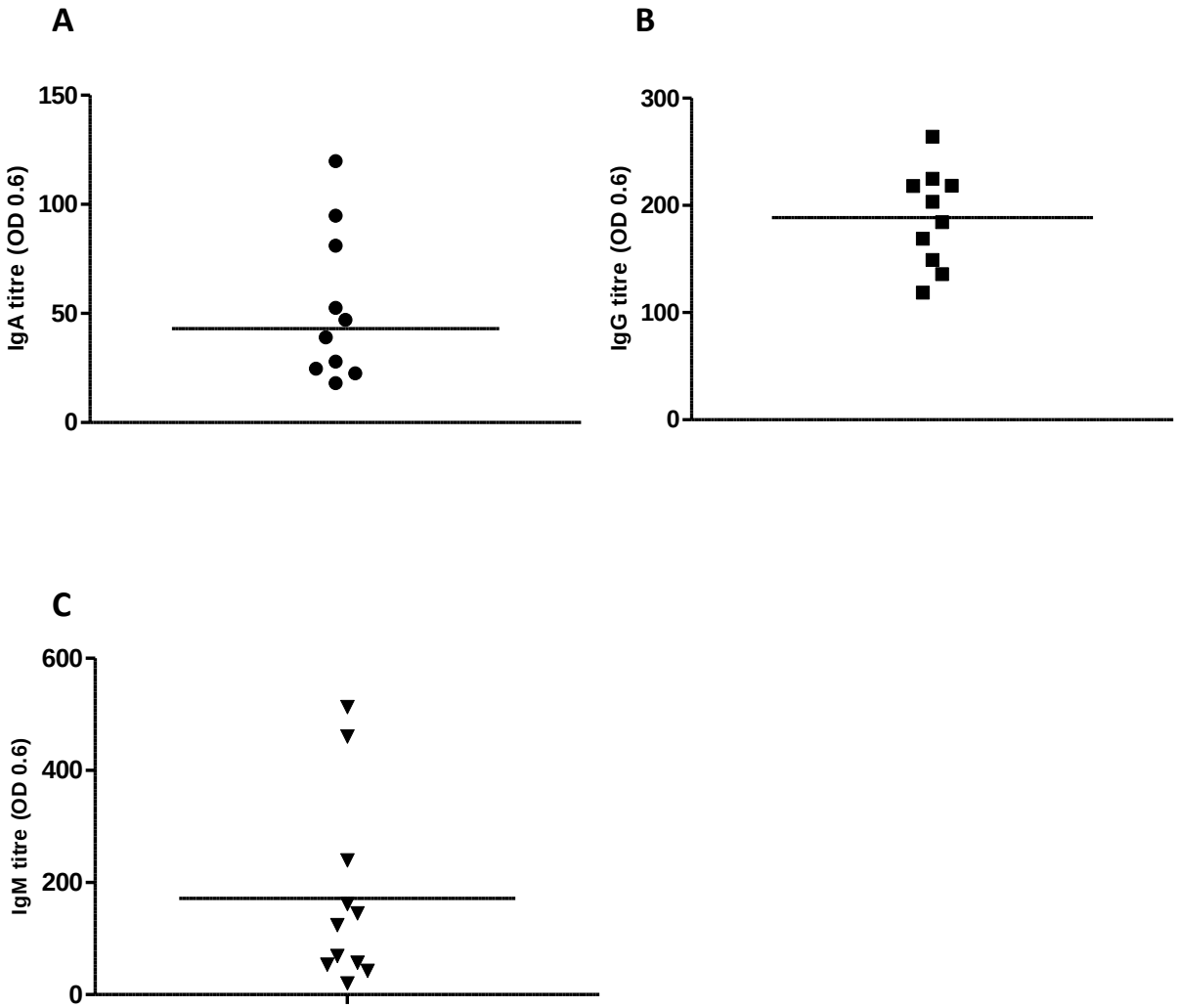


Figure 3.5 *C. jejuni* specific immune complexes in serum of healthy individuals.

Microtiter plates were coated overnight with PFA-fixed *C. jejuni* 81-176. Plates were washed and non-specific binding was blocked with 3% BSA for 1h at room temperature. Serial diluted serum from ten individuals was added and plates were incubated 1h at room temperature. **(A)** IgA and **(B)** IgG were detected by HRP-conjugated anti-human IgA or IgG; **(C)** IgM was detected by AP-conjugated anti-human IgM. Antibody levels were quantified at 450nm or 405nm, respectively. Serum titres were normalised to an OD of 0.6. Bars represent the mean of individual values.

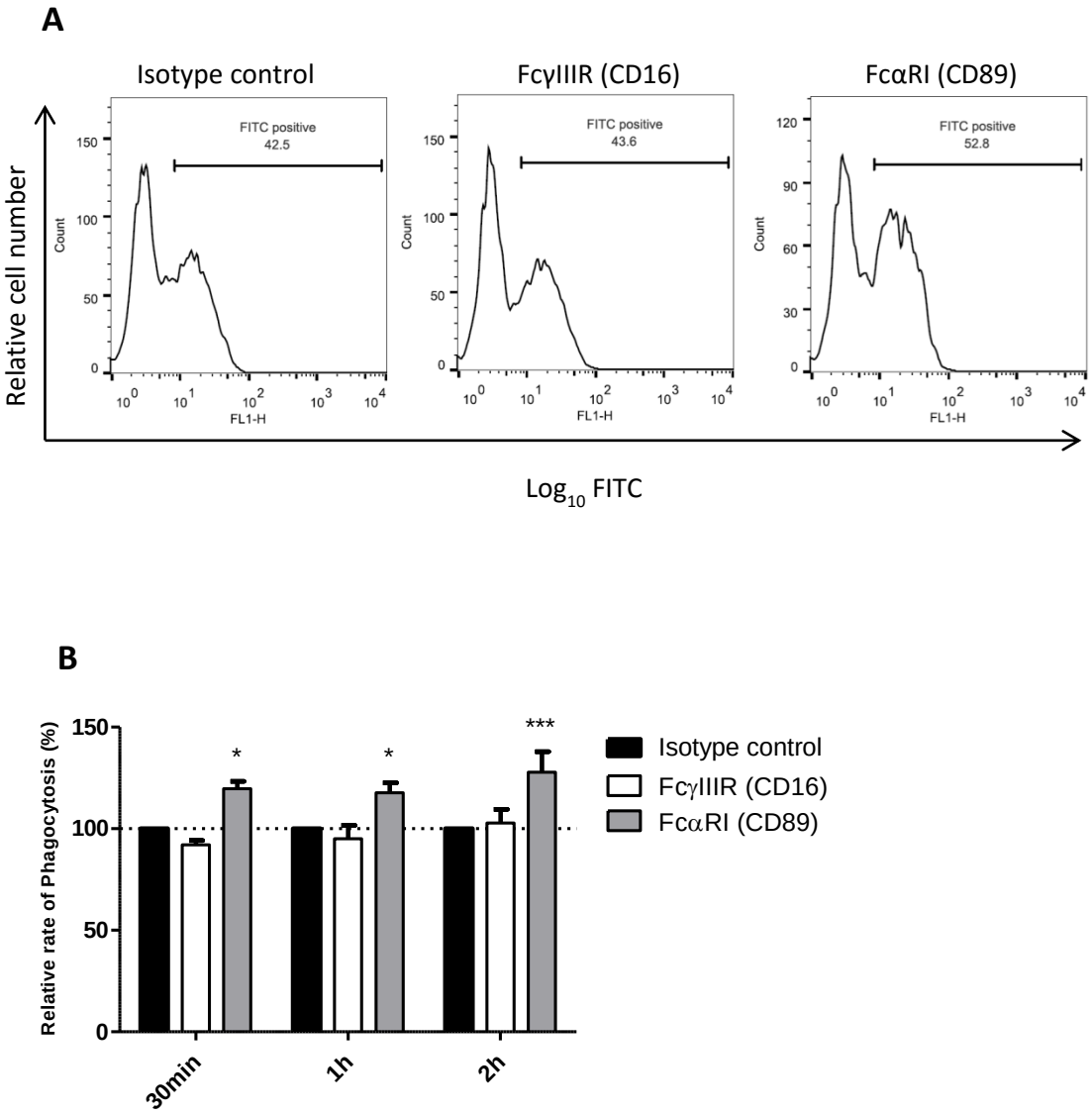


Figure 3.6 Role of Fc-receptors FcγIIIR and FcαRI in *C. jejuni* phagocytosis.

Human neutrophils were incubated with anti-human CD16 (FcγIIIR), CD89 (FcαRI) or isotype control (20µg/mL) for 30min prior to co-culture with FITC-labelled, NHS-opsonised bacteria (MOI 10). Data shows **(A)** bacterial uptake after 1h of co-culture from one representative experiment and **(B)** rate of phagocytosis compared to the isotype control as mean±SEM of six individual experiments. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant; * $p < 0.05$; *** $p < 0.001$

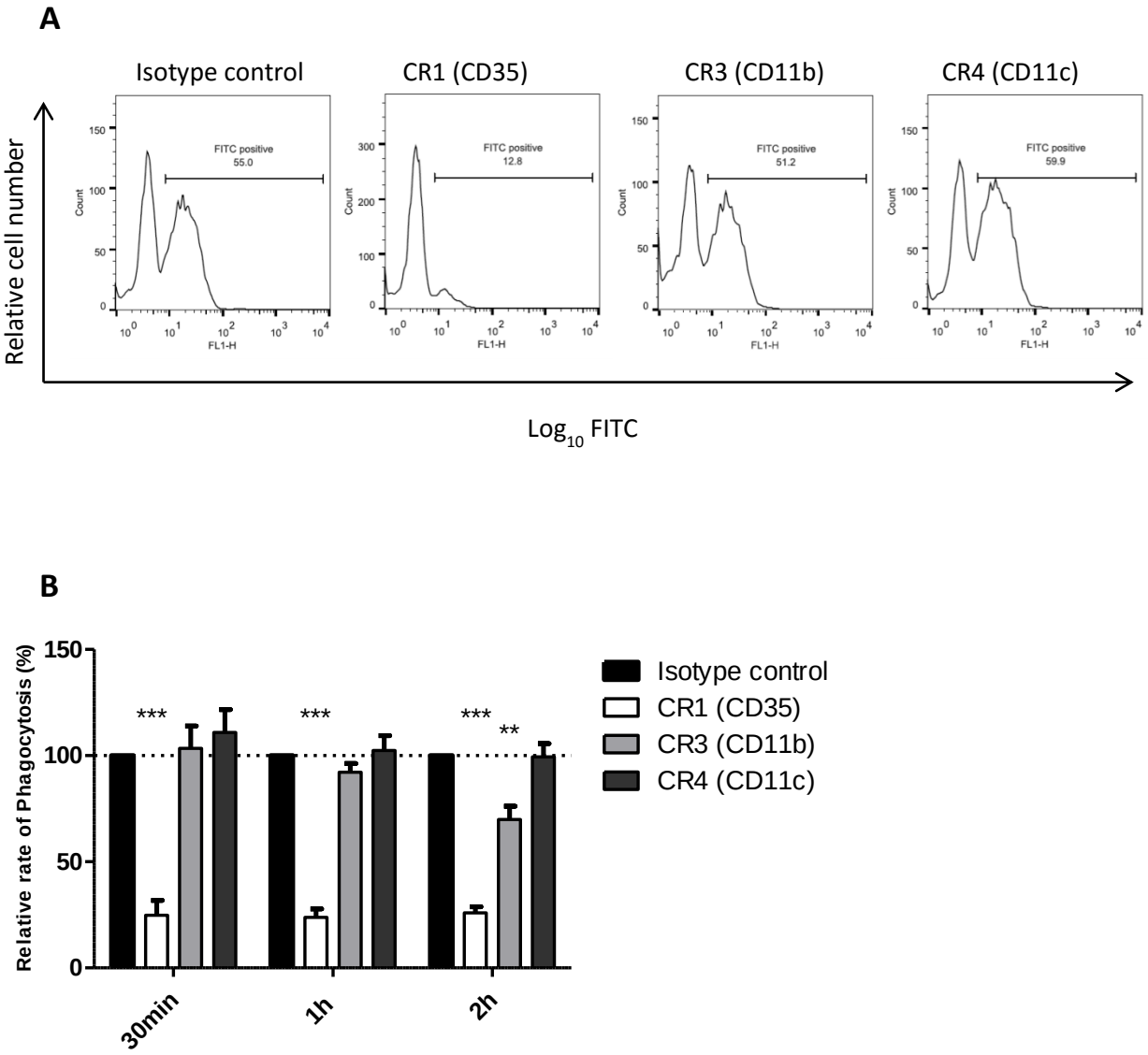


Figure 3.7 Role of complement receptor (CR) in *C. jejuni* phagocytosis.

Human neutrophils were incubated with anti-human CD35 (CR1), CD11b (CR3), CD11c (CR4) or isotype control (20µg/mL) for 30min prior to co-culture with FITC-labelled, NHS-opsonised *C. jejuni* 81-176 (MOI 10). **(A)** Bacterial uptake of one representative experiment after 1h of co-culture. **(B)** Rate of phagocytosis in the presence of antibodies compared to the isotype control over time and is presented as mean±SEM of four donors. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant; ** p< 0.01; *** p<0.001

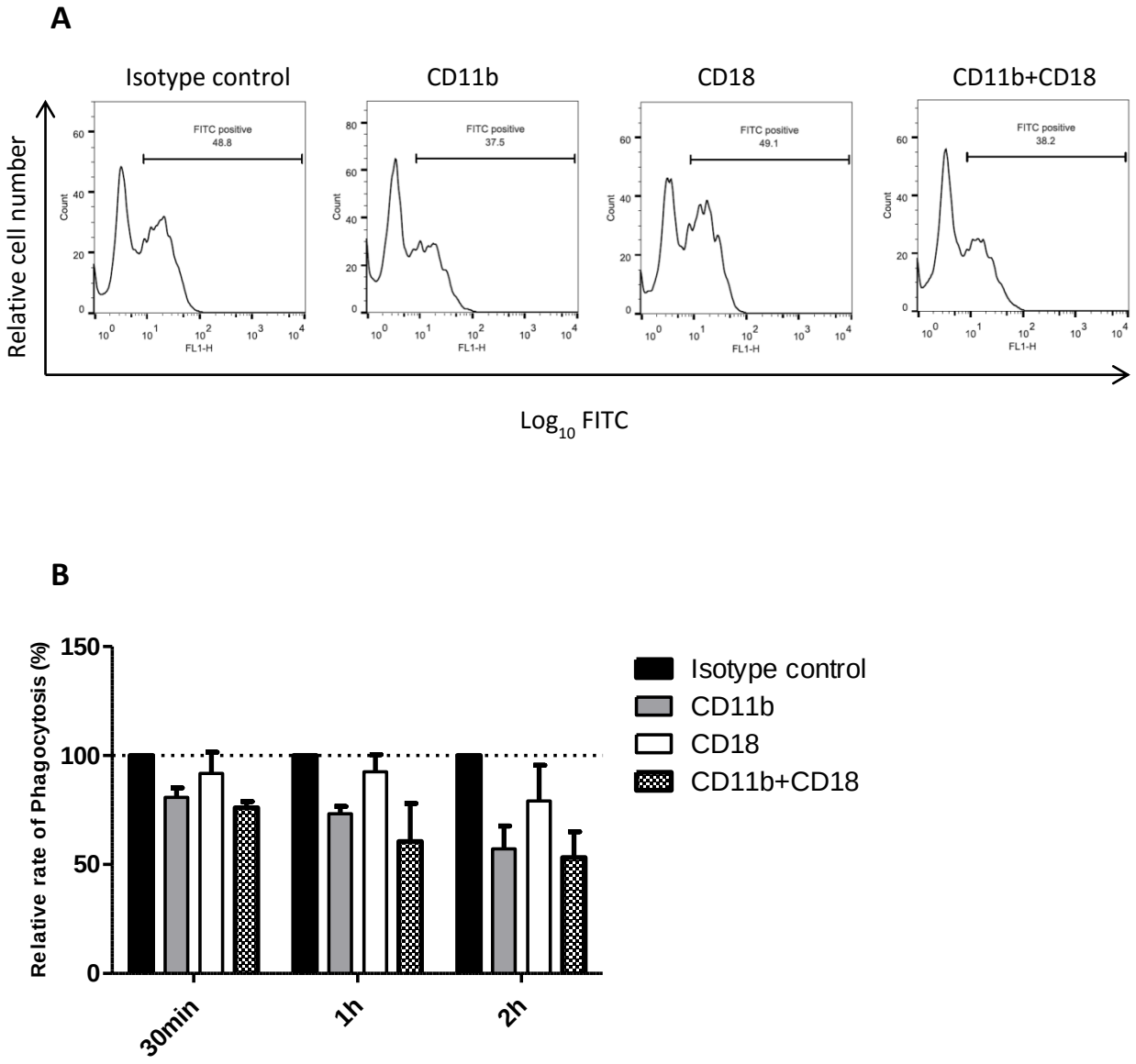


Figure 3.8 Rate of bacterial uptake after blocking of CD11b and CD18.

Human neutrophils were incubated with blocking antibodies to CD11b, CD18 or both prior to co-culture with FITC-labelled, NHS-opsonised *C. jejuni* 81-176 (MOI 10). The rate of phagocytosis was detected over time. Data is presented as relative values to the isotype control. **(A)** representative of one individual 1h post co-culture. **(B)** Mean±SEM of two individual donors.

To further confirm our findings, CR1, CR3 and CR4 transfected CHO cells as well as mock transfected controls were co-cultured with *C. jejuni* 81-176. Significant bacterial binding was only observed in the presence of CR1-expressing cells and only when bacteria were previously NHS-opsonised (Figure 3.9 A, B). In contrast to *C. jejuni*, significant binding to CR3-CHO cells was observed with *N. meningitidis* suggesting a species specific mechanism (Figure 3.9 C).

To exclude the possibility that these observations were phenotypic to *C. jejuni* 81-176 we included *C. jejuni* 11168H as an additional reference strain. NHS-dependence and rate of phagocytosis of 11168H strain was found to be comparable to the 81-176 strain, suggesting a conserved mechanism for *C. jejuni* (Figure 3.10).

Next, we wished to identify bacterial moieties that may contribute to this process. For this purpose, a panel of *C. jejuni* 11168H isogenic mutants lacking surface structures was employed. An increase of uptake was consistently observed with *C. jejuni* mutant *kpsM* (which lacks the capsular polysaccharide) but this increase failed to reach significance (Figure 3.11 A). *C. jejuni* LOS is terminally sialyated, interestingly co-culture with asialyated *neuB* and OS-truncated *waaF* mutants showed modest reduction in the rate of phagocytosis (3.11 A). Despite the increased phagocytic rate for *kpsM,* co-culture with CR-expressing CHO cells did not lead to enhanced binding to CR1 nor additional interaction with CR3 or CR4 (Figure 3.11 B).

We assessed the presence of CR1 and CR3 on the surface of human neutrophils and both receptors were found constituently expressed and expressions were upregulated after co-culture with *C. jejuni*; these observations were independent of the mode of opsonisation (Figure 3.12).

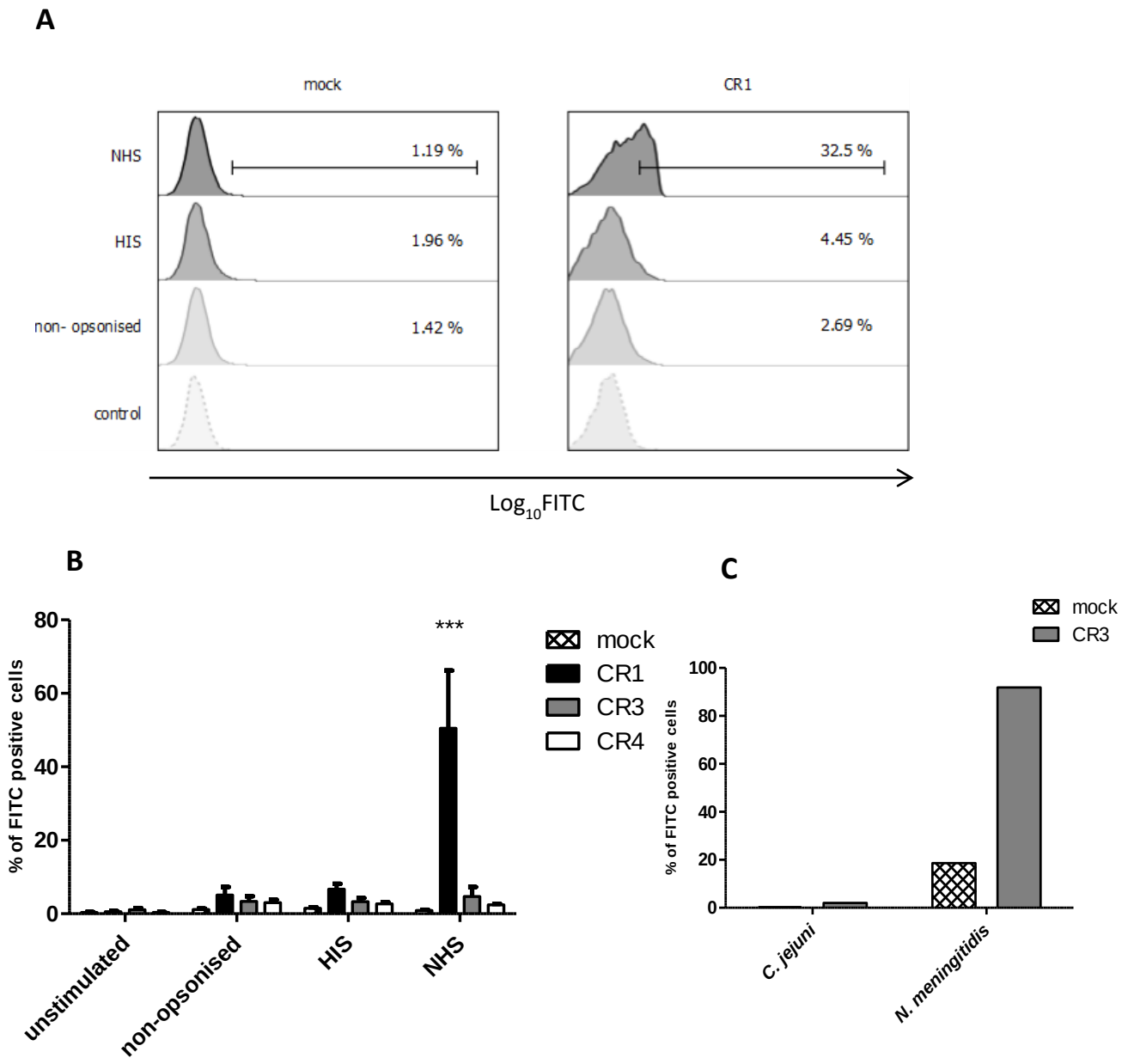


Figure 3.9 *C. jejuni* binding to CR transfected Chinese hamster ovary (CHO) cells.

Chinese hamster ovary (CHO) cells stably transfected with CR1, CR3, CR4 or mock-transfected controls were transferred into 24-well plates at a concentration of $5x10^5$ cells/mL and were allowed to adhere. After 2h cells were co-cultured with FITC-labelled *C. jejuni* 81-176 (MOI 100) that were non-opsonised, HIS or NHS-opsonised. 4h post-culture, cells were collected and the rate of FITC-positive cells was assessed by FACSCalibur. **(A)** One representative experiment showing the degree of adherence to mock-transfected and CR1 expressing cells. **(B)** Mean±SEM bacterial adherence from three independent experiments. **(C)** Adherence of FITC-labelled, NHS-opsonised *C. jejuni* and *N. meningitidis* to CR3-transfected CHO cells. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant; *** $p<0.001$ compared with the mock-transfected controls.

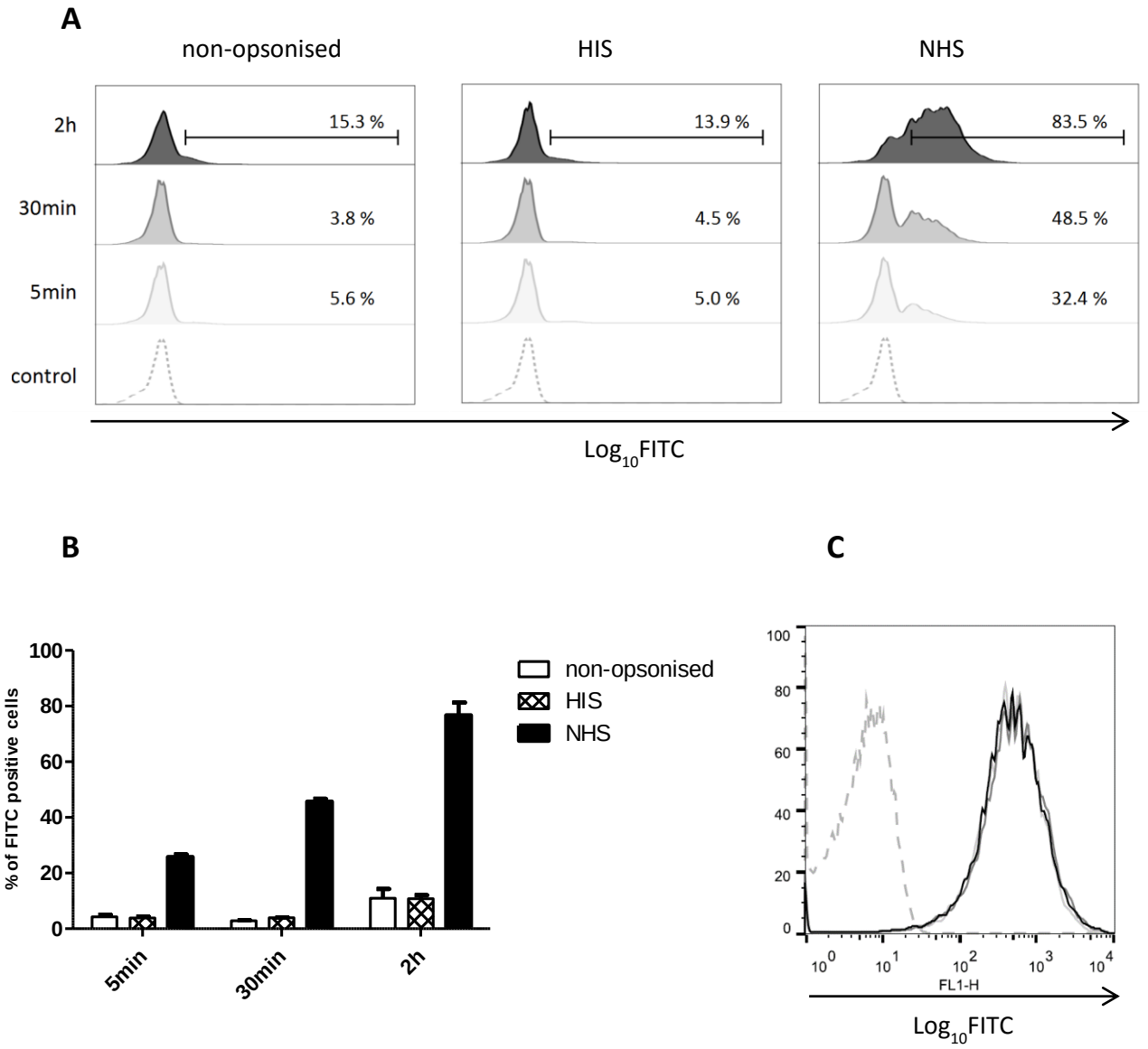


Figure 3.10 Rate and mode of phagocytosis of *C. jejuni* 11168H.

Human neutrophils were co-cultured *with C. jejuni* 11168H (MOI 10). Prior to co-culture, FITC-labelled bacteria were non-opsonised or opsonised with HIS or NHS. **(A)** Time-dependent phagocytosis from one representative donor. **(B)** Mean±SEM of bacterial uptake from two individual donors. **(C)** Fluorescent labelling of FITC-labelled bacteria: non-opsonised (light grey), HIS (dark grey), NHS (black); non-labelled control (dash).

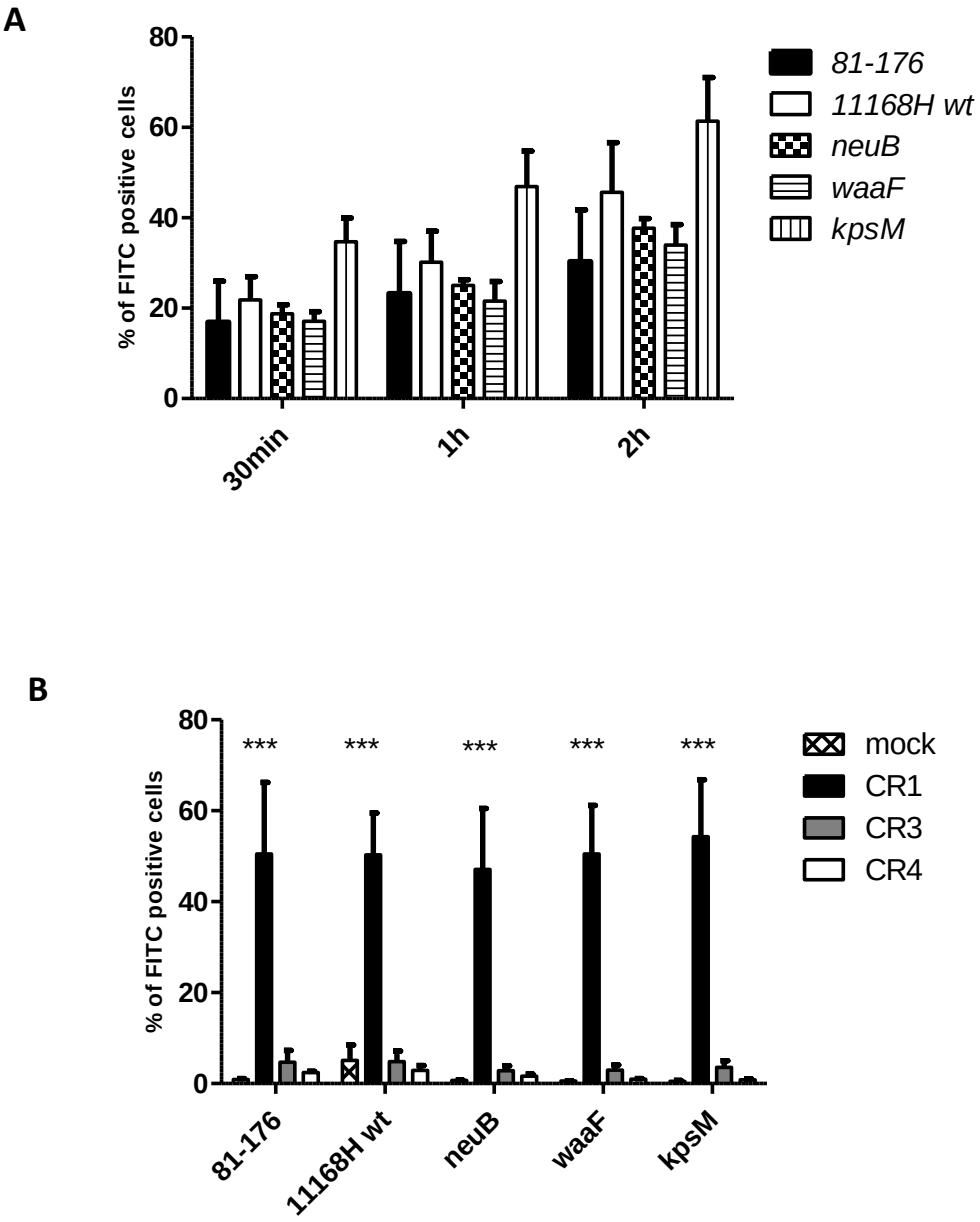


Figure 3.11 Role of capsule and LOS in bacterial uptake and binding to complement receptor CR1. **(A)** Human neutrophils were co-cultured with FITC-labelled, NHS-opsonised *C. jejuni* stains 81-176, 11168H wild-type (wt) and its isogenic mutants (MOI 10). FITC-positive cells were assessed by FACSCalibur. **(B)** CR1, CR3 and CR4 transfected CHO cells and mock transfected controls were co-cultured with FITC-labelled bacteria (MOI 100). FITC-positive cells were assessed after 4h. Mean±SEM of three independent experiments. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant; *** $p<0.001$

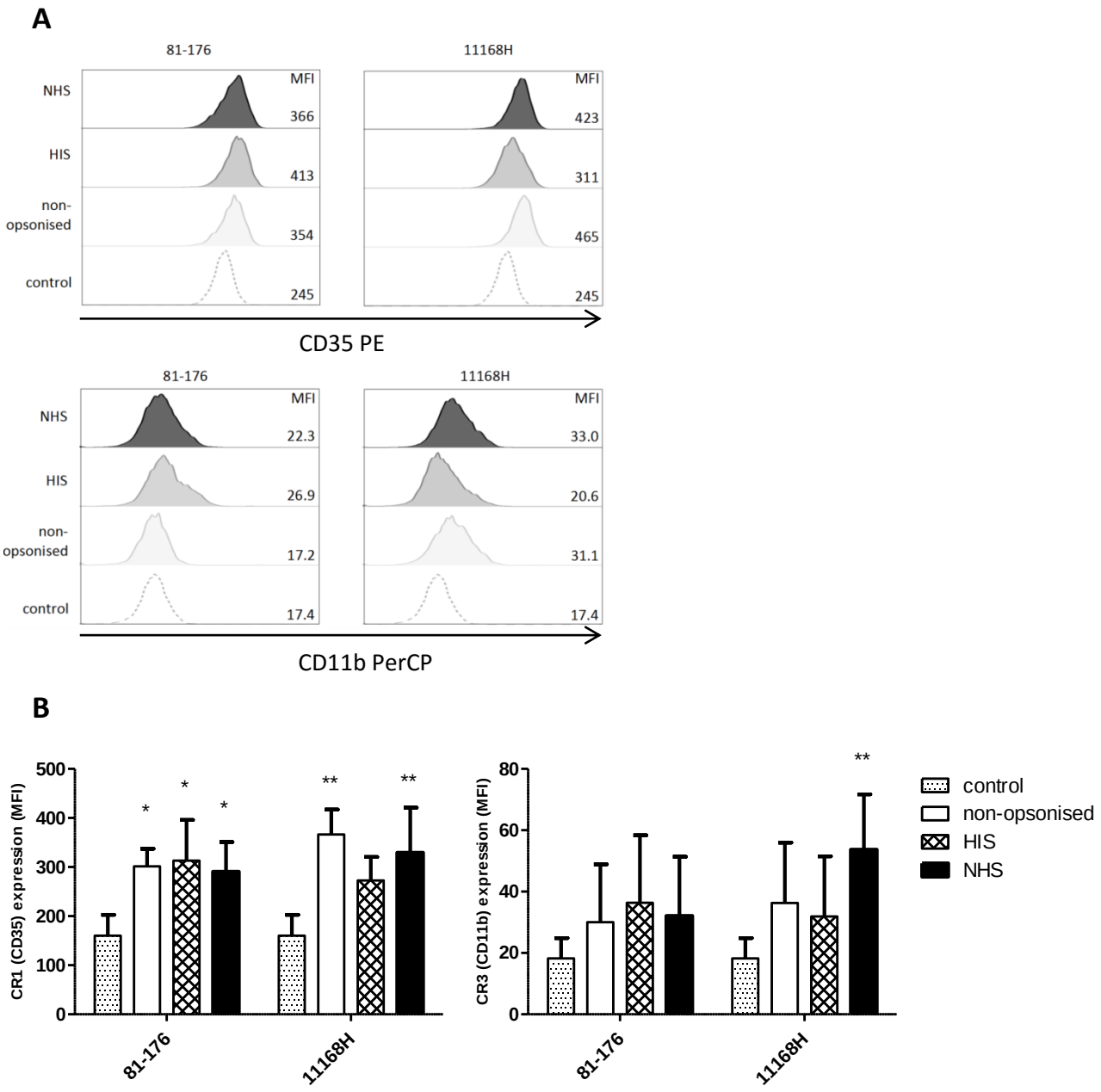


Figure 3.12 Neutrophil CR1 and CR3 expressions in response to *C. jejuni.*

Human neutrophils were co-cultured with non-opsonised or opsonised *C. jejuni* strains 81-176 and 11168H (MOI 10). After 1h, cells were stained for CR1 (CD35) and CR3 (CD11b) expression and analysed by FACSCalibur. **(A)** Representative CD35 and CD11b Mean fluorescent index (MFI) in response to 81-176 and 11168H strains. **(B)** CD35 and CD11b MFI mean±SEM of three individual donors. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant; * $p< 0.05$; ** $p< 0.01$

3.4 C3b serves as potential ligand to CR1-mediated phagocytosis

Pathogen mediated activation of the complement cascade leads to the opsonisation with C3b which is further cleaved into inhibitory iC3b. C3b and its breakdown product iC3b are ligand for CR1 and CR3 receptor, respectively. (Gordon et al., 1987, Liu and Niu, 2009). As *C. jejuni* showed significant binding to CR1, we hypothesised that C3b complexes with the bacterium promoting its binding to the receptor. Complement activation was mediated by bacterial incubation in the presence of NHS followed by Western blot analysis in order to assess the deposition of C3b related components to the bacterial surface. Positive aggregation of C3b to *C. jejuni* 81-176 was observed and a time-dependent increase in iC3b was recorded. Using *N. memingitidis* in contrast, we observed earlier deposition of iC3b (at 5min) and while C3b was not detected (Figure 3.13 A).

C3b/iC3b deposition was detected on both *C. jejuni* reference strains as well as the isogenic mutants (Figure 3.13 B). It should be noted that despite the enhanced uptake by neutrophils, *kpsM* mutant appears to present with increased C3b turnover resulting in lower C3b levels expressed on its surface. In summary, we were able to detect C3b on the *C. jejuni* surface which could potentially be a CR1 binding site.

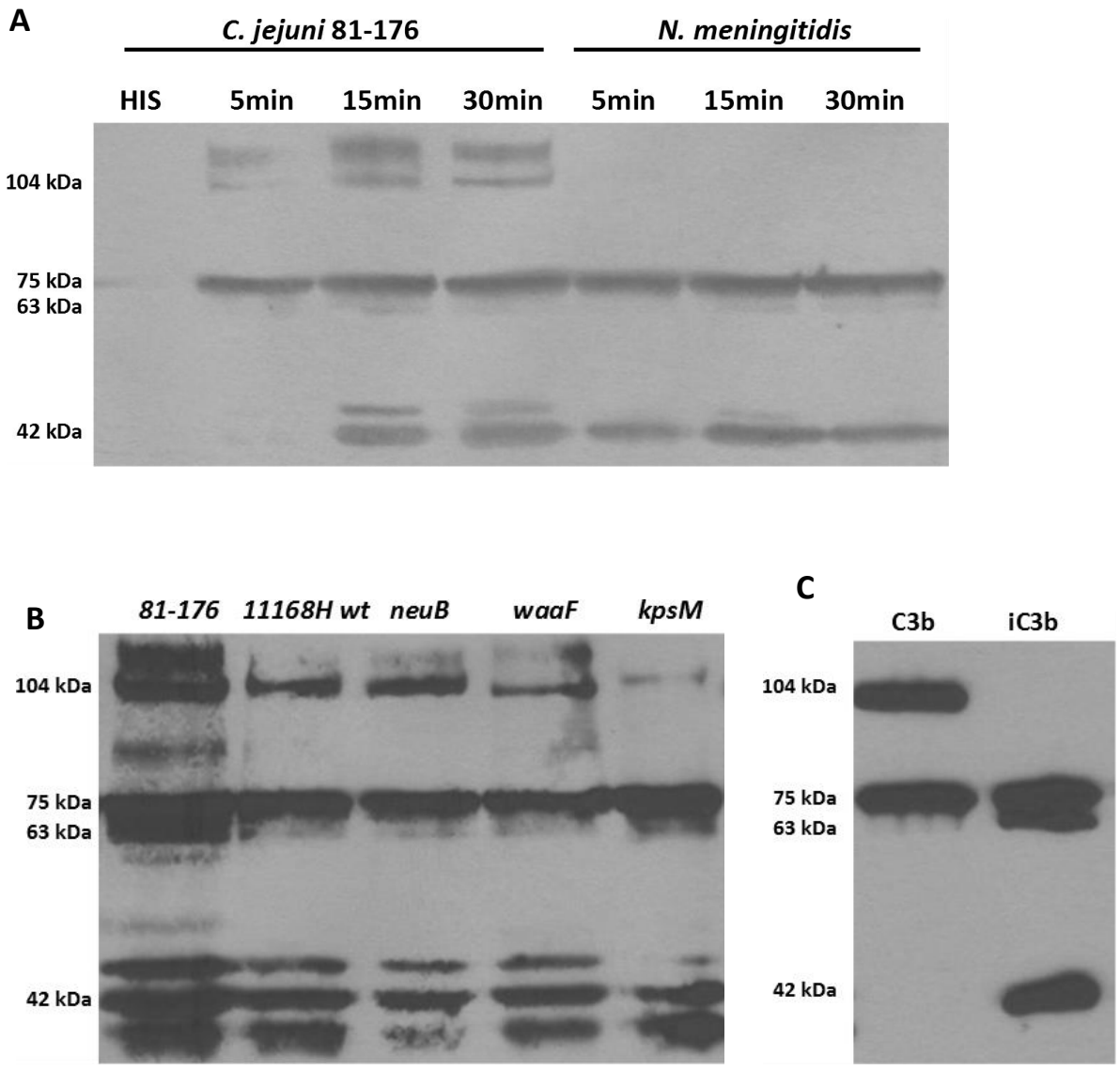


Figure 3.13 Detection of C3b and iC3b deposition on the *C. jejuni* surface

(A) Bacterial suspension of *C. jejuni* 81-176 and *N. meningitidis* were normalised to an OD 0.5 and were incubated in the presence of 20% NHS. At the indicated incubation times the reaction was stopped by PBS containing 10mM EDTA, bacterial cells were pelleted and extensively washed. Laemmili buffer was added and subjected to Western blot analysis. Visualised protein bands indicate for the deposition of C3b (104kDa and 75kDa) and iC3b (75kDa, 63kDa and 42kDa). HIS served as negative control. **(B)** *C. jejuni* strains 81-176 and 11168H wt with respective isogenic mutants were incubated in the presence of NHS for 30min to assess for C3b/ iC3b deposition. **(C)** Recombinant C3b and iC3b protein (10ng) served as controls. Blots are representative of three independent experiments.

3.5 Direct binding of C1q to *C. jejuni* does not modulate phagocytosis

CR1 is a multifactorial receptor and was found to bind C4b, MBL and C1q in addition to its favoured ligand C3b (Ghiran et al., 2000, Klickstein et al., 1997, Tas et al., 1999). C1q binds to *S. typhimurium* LPS *via* its ghB subunit (Roumenina et al. 2008). In order to determine if C1q is directly involved in *C. jejuni* uptake we first sought to assess adherence of recombinant C1q (recC1q) protein to the bacterium. Binding of recC1q was not detected by FACS analysis (Figure 3.14 A) but results by ELISA and Western blot suggest positive adherence to the bacterial surface (Figure 3.14 B, C). ELISA data indicated ghB as the primary bacterial binding domain which is in line with previous findings published on *S. typhimurium* LPS (Figure 3.14 B). Western blot analysis also revealed the presence of ghB on the bacterium, as identified by protein size, suggesting this to be the binding site to the bacterial surface (Figure 3.14 C). Both reference strains and the isogenic mutants yield similar levels of adherence for C1q and ghB proteins (Figure 3.15). Collectively, we show for the first time that C1q can directly bind to *C. jejuni* in the absence of serum antibody.

To determine if C1q opsonisation promotes CR1-dependent internalisation, CR1 expressing CHO cells were co-cultured with *C. jejuni* previously incubated with recC1q. Neither adherence to CR1 nor any of the other CR receptors was observed in the presence of recC1q (Figure 3.16 A). Further, C1q-opsonisation failed to promote bacterial uptake by neutrophils in the presence and absence of HIS (Figure 3.16 B).

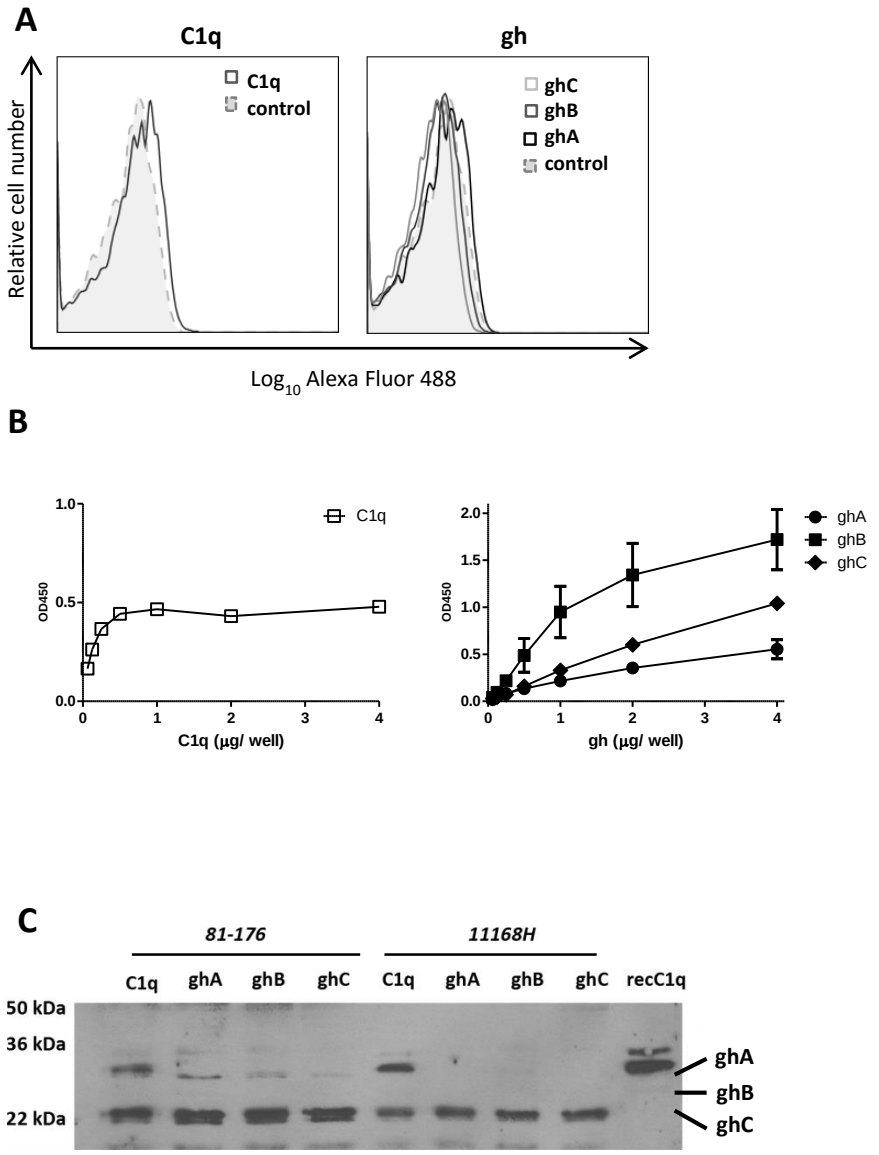


Figure 3.14 Globular head (gh) domain mediated binding of C1q to *C. jejuni.*

(A) *C. jejuni* 81-176 was suspended in PBS containing 4mM Ca^{2+}. Recombinant C1q (recC1q) protein and globular head modules ghA, ghB and ghC were added to a final concentration of 30µg/mL. After 1h, cells were washed and incubated with anti-C1q or anti-MBP followed by Alexa Fluor®488 secondary antibodies. Fluorescence was assessed by FACSCalibur. **(B)** ELISA plates were coated with PFA-fixed *C. jejuni* 81-176 overnight. Wells were washed and non-specific binding was blocked with 3% BSA. Recombinant proteins were added in serial dilution and incubated for 1h. Binding was detected with anti-C1q or anti-MBP followed by respective HRP-conjugated secondary antibodies. TBM substrate was added and absorbance was determined at 450nm. Mean±SEM of two independent experiments. **(C)** Binding of recC1q and gh proteins to *C. jejuni* 81-176 and 11168H strains were assessed by western blot analysis. Cell-free recC1q served as control.

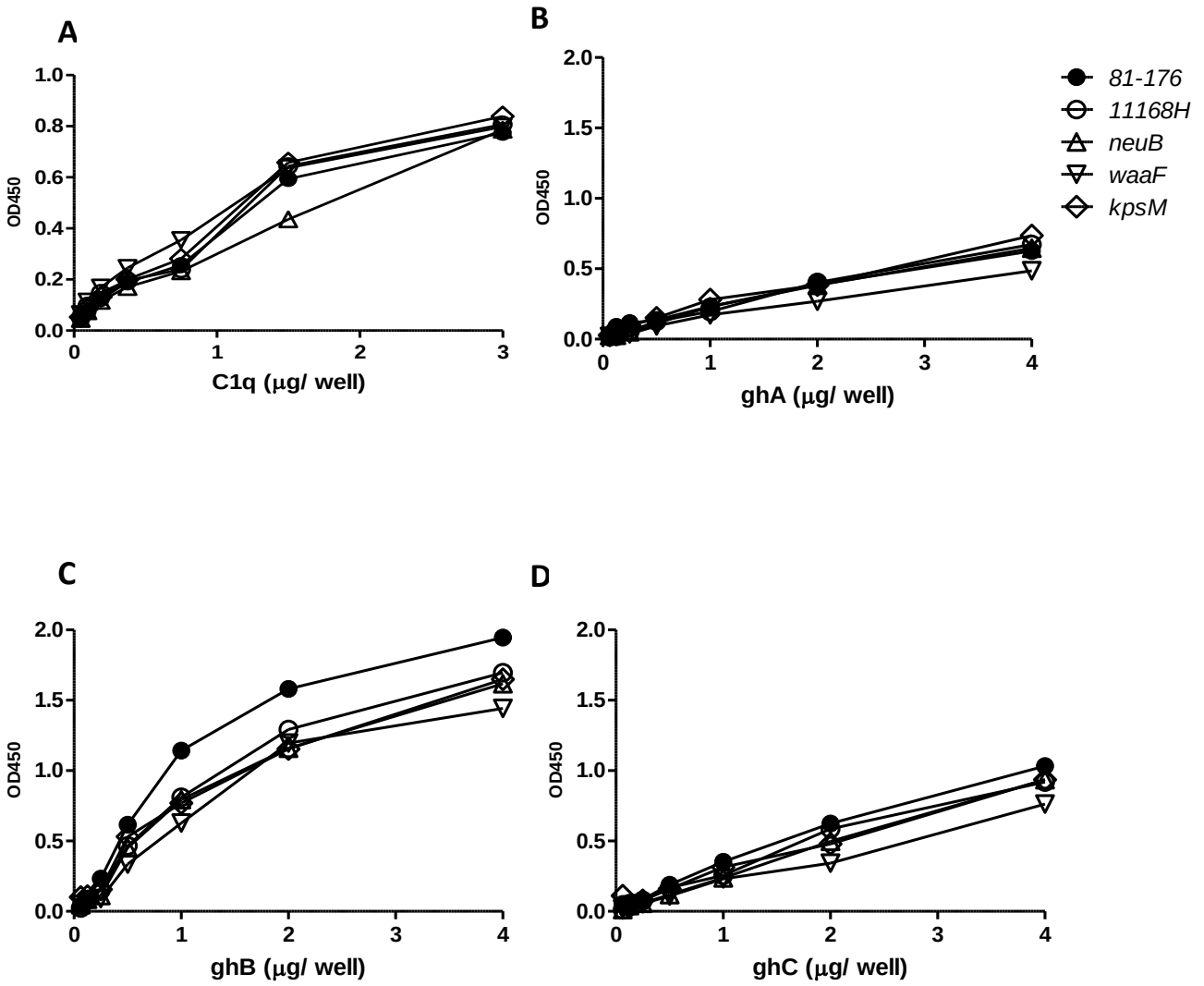


Figure 3.15 Role of capsule and LOS in bacterial interaction with C1q.

Microtiter plates were coated with *C. jejuni* strains 81-176, 11168H wt and the isogenic mutants *neuB, waaF* and *kpsM*. Wells were washed and non-specific binding was blocked with 3% BSA. Recombinant proteins were added in serial dilution and incubated for 1h. Binding of **(A)** C1q or **(B)** ghA, **(C)** ghB and **(D)** ghC was detected with anti-C1q or anti-MBP antibodies. Absorbance was detected at 450nm.

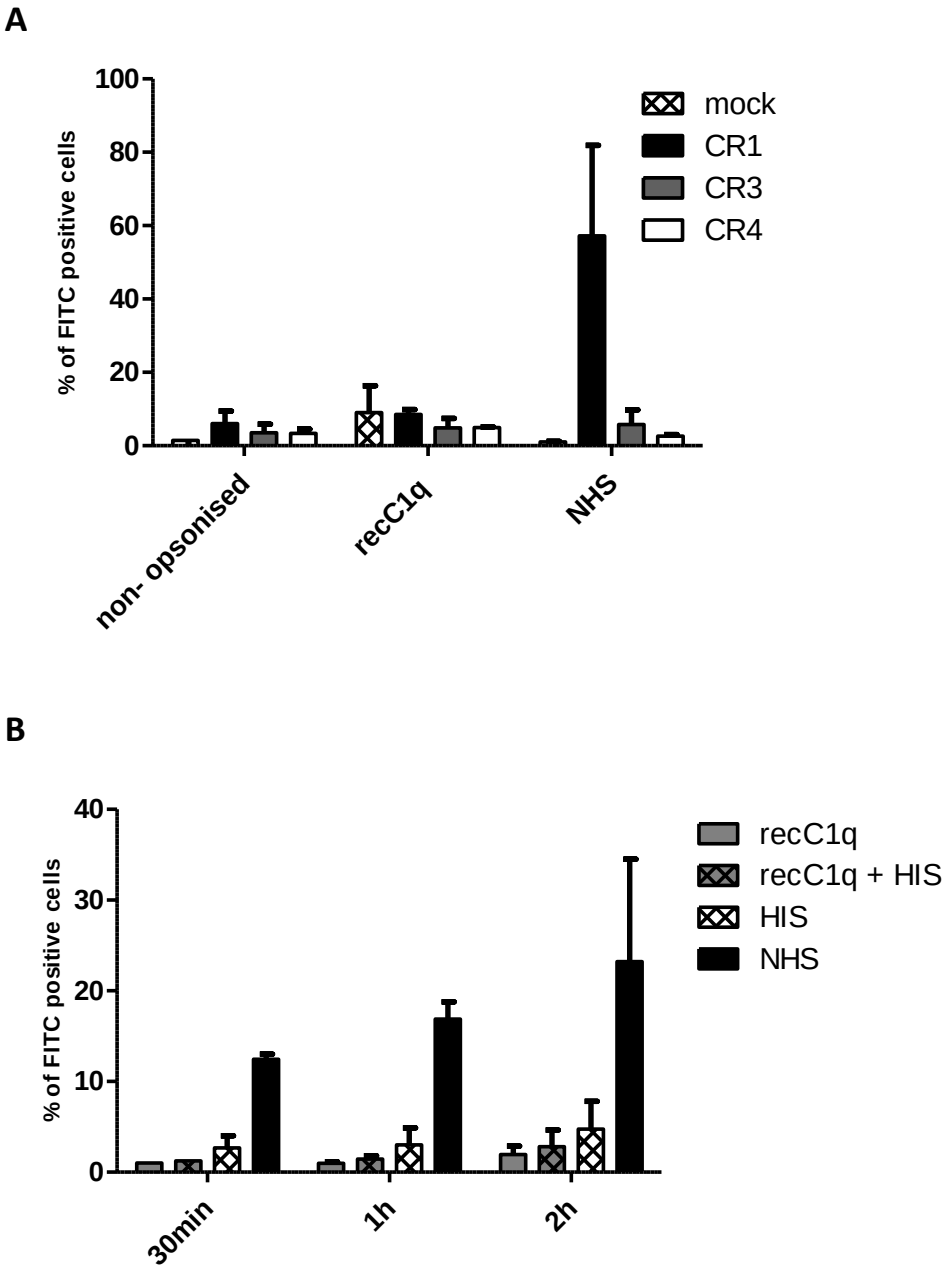


Figure 3.16 Role of C1q in CR1-binding and phagocytosis of *C. jejuni.*

FITC-labelled *C. jejuni* 81-176 was opsonised with recombinant C1q (recC1q) or serum. **(A)** CR1, CR3 and CR4-expressing CHO cells were co-cultured with non-opsonised, recC1q or normal human serum (NHS) opsonised bacteria (MOI 100) for 4h. Mock transfected cells served as control. **(B)** Human neutrophils were co-cultured with recC1q or recC1q+HIS opsonised *C. jejuni* 81-176 (MOI 10). HIS and NHS served as controls. Rate of FITC-positive cells was assessed by FACSCalibur. Mean±SEM of two individual experiments.

3.6 *C. jejuni*-mediated neutrophil ROS production and bacterial clearance

Finally, we wanted to determine if *C. jejuni* activates neutrophil signalling and induces bacterial clearance. An increase in ROS generation was observed upon co-culture with *C. jejuni* and was highest after NHS-opsonisation although HIS-opsonised bacteria also exerted a significant effect (Figure 3.17 A). No increase in the release of neutrophil elastase was observed upon bacterial infection (Figure 3.17 B). Most strikingly, we did not observe bacterial clearance within 4 hours of neutrophil co-culture with *C. jejuni*, a finding that requires further investigation (Figure 3.17 C).

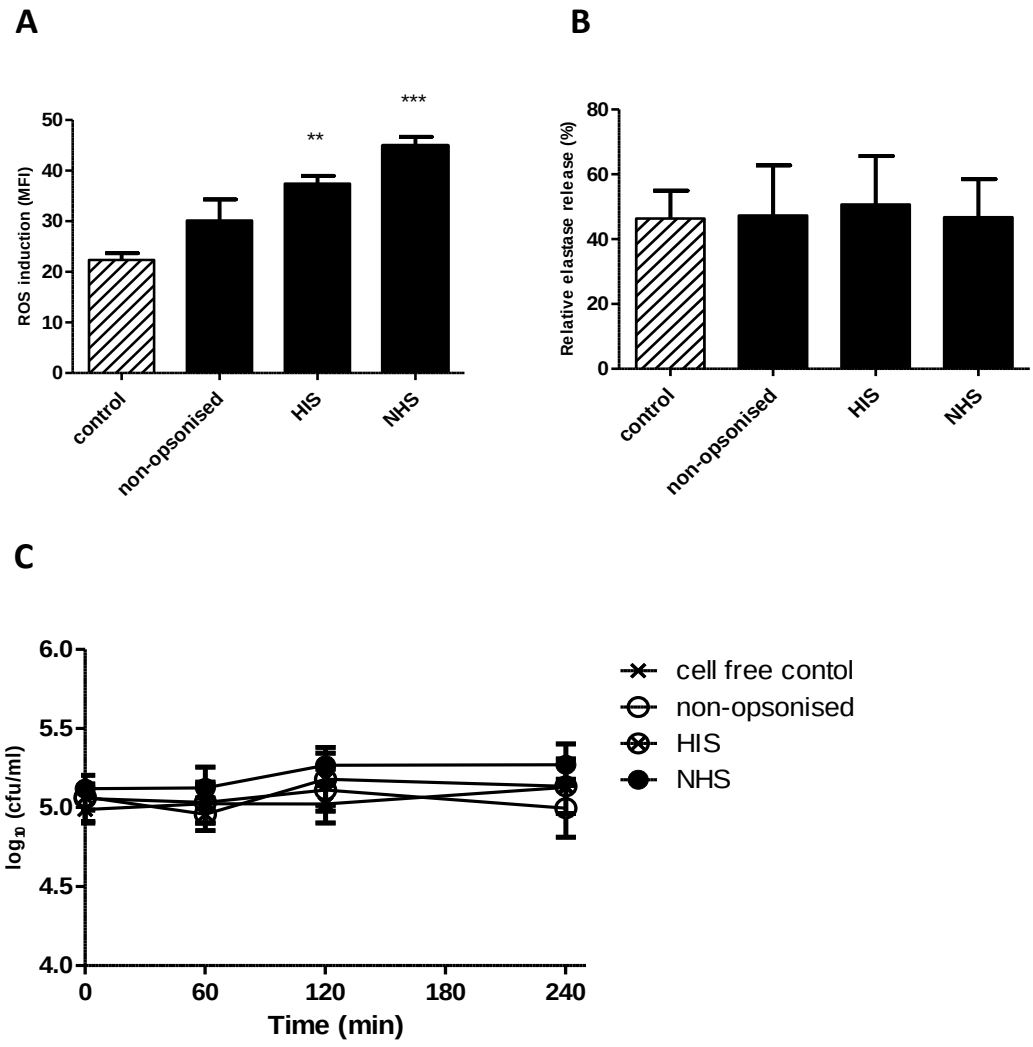


Figure 3.17 Neutrophil activation and bacterial clearance upon *C. jejuni* co-culture.

(A) DHR 123 was added to human neutrophils to detect the generation of neutrophil ROS. Neutrophils ($2x10^9$/mL) were co-cultured with non-opsonised, HIS or NHS opsonised *C. jejuni* 81-176 (MOI 10). After 1h the formation of fluorescent rhodamine was measured by FACSCalibur. **(B)** 100µL neutrophil suspension ($1x10^7$ cells/mL) were transferred into a 96-well plate. *C. jejuni* was added (MOI 10) and the co-cuture was incubated for 1h. Elastase substrate was added and further incubated for 1h. Colorimetric changes were assessed at 450nm. Total neutrophil elastase content was determined by addition of 0.1% TritonX. **(C)** Neutrophils were co-cultured with *C. jejuni* (MOI 0.1) and bacterial counts were assessed over time. Media containing NHS-opsonised *C. jejuni* served as cell free control. Mean±SEM of three independent experiments. One-way ANOVA with Tukey post-test was performed for statistical analysis. No stars: not significant; ** $p<0.01$; *** $p<0.001$

3.7 Discussion

Phagocytosis is a major first step in neutrophil-mediated clearance of pathogens. Infection with *C. jejuni* promotes a vast influx of neutrophils to the GI tract where they are known to contribute to tissue pathology and disease. At present, how neutrophils contribute to bacterial uptake and clearance remain ill-defined. Here, we report for the first time that *C. jejuni* is taken up by neutrophils in a whole blood environment and that internalisation happens almost entirely in a serum-dependent manner. Despite the presence of immune complexes in serum, bacterial uptake was found to be independent of FcR-activity. Instead, CR1 was identified as the main CR in *C. jejuni* recognition. Our observations also indicate a potential role for the CR3 receptor in host-pathogen interactions.

Several studies report FcR-mediated phagocytosis of pathogens including *E. coli*, *S. aureus* and *C. albicans* (Aleyd et al., 2014, Gazendam et al., 2014a, Grommes et al., 2012). Segal *et al.* showed the efficient uptake of IgG-opsonised latex beads by neutrophils. Phagocytosis and closure of the phagosome occurred in less than 20s, followed by fusion with specific and azurophilic granules (Segal et al., 1980). Similar findings were observed with IgG-opsonised zymosan particles where phagosome sealing was accompanied by a decrease in granule markers CD63 and CD66b on the cell surface suggesting their recruitment to the lysosomal vacuole (Tapper and Grinstein, 1997). In our study, we found positive deposition of IgG, IgA and IgM on the surface of *C. jejuni* however FcR-mediated phagocytosis was not observed. Instead to our surprise, inhibition of FcαRI (with anti-CD89) led to statically significant increase in phagocytosis suggesting a potential role for the Fc family of receptors in C. *jejuni* engagement. A recent study using latex beads of various sizes with increasing concentrations of IgG complex noted that the uptake of small particles (0.5μm) by macrophages was significantly affected by the density of surface bound Fc-ligands, further the impact of ligand density diminished with larger particles (>2 μm). (Pacheco et al., 2013). It is interesting to speculate that immune complexes bound to *C. jejuni* surface may be insufficient to promote recognition by FcR. *C. jejuni* is a spiral-shaped rod of ~4 μm in length and 0.5 μm in width hence this mechanism might not be relevant for its uptake. However, in an unfavourable environment *C. jejuni* can change morphology from its characteristic spiral to a coccoid shape with approximately <1μm diameter (Ng et al., 1985). It would be interesting to further investigate the Ig-density accumulating on the bacterial surface and assess the efficiency of uptake of the two forms of *C. jejuni*.

The vital role of complement in enhancing neutrophil mediated phagocytosis is well described (Yuste et al., 2010, Underhill and Ozinsky, 2002, Gasque, 2004). Activation of the complement

system leads to the deposition of C3b and iC3b on the bacterial surface serving as ligands to CR1 and CR3, respectively. The best described phagocytic receptor is CR3 which binds iC3b with high affinity and plays an important role in the clearance of pathogens (Mollnes et al., 2002, Mobberley-Schuman and Weiss, 2005). In our study, the presence of complement was key in mediating uptake of *C. jejuni*. In addition, blocking of CR1 significantly impaired bacterial uptake, whereas a potential effect of CR3 was observed but not until 2h post co-culture. This mode of phagocytosis was similar in both *C. jejuni* strains tested. There was a distinct trend of increased phagocytosis in the presence of the capsule mutant and a trend for decrease in the presence of the LOS mutant was noted when compared to the wt strain. Further studies are needed to clarify the potential role of these two bacterial moieties in uptake.

CR1 is a multi-ligand receptor and binds C3b, but has also binding sites for C1q, C4b and MBL. Western blot analysis confirmed the presence of C3b on the bacterial surface suggesting it may be involved in uptake by CR1. C1q has been reported to bind to Gram-negative bacteria including *S. typhimurium* LPS via the ghB domain (Roumenina et al., 2008). Utilising recombinant proteins we were able to show C1q-binding to *C. jejuni*, and binding was primarily mediated *via* ghB. It would be interesting to explore if the binding of ghB to *C. jejuni* may occur *via* the bacterial LOS, as seen in the case of S. *tymphimurium.* Our findings currently do not support a direct role for C1q deposition in bacterial uptake as C1q-opsonised bacteria failed to adhere to CR1 nor did they promote neutrophil-mediated phagocytosis. While it seems unlikely that bacterial uptake is induced by direct interaction between C1q and CR1, C1q has an important role in the activation of the classical arm of the complement cascade which leads to the deposition of C3b/iC3b to the bacterial surface. The contribution of C1q and the classical pathway in complement activation by *C. jejuni* is discussed in more detail in Chapter IV.

Predominant binding of opsonised bacteria to CR1 was further confirmed by CR-expressing CHO cell experiments. A recent publication showed significantly reduced uptake of *F. tularensis* by utilising anti-CR1, and to a lesser extent anti-CR3 antibodies (Schwartz et al., 2012). Fallman *et al.* utilised complement-opsonised yeast particles to assess the roles of CR1 and CR3 in neutrophil-mediated uptake. They observed ~50% reduction in particle ingestion when CR1 was blocked, inhibition of CR3 impaired uptake by 30% while simultaneous inhibition of both receptors completely blocked particle engulfment (Fallman et al., 1993). Another study determined the adherence of C3b-opsonised sheep erythrocytes to CR1, CR3 and CR1/CR3 co-expressing CHO cells. Erythrocytes readily bound to CR1 but binding was transient in the presence of factor I (fI), presumably due to C3b ligand cleavage to iC3b. While C3b opsonisation failed to induce

recognition by CR3 alone, stable adherence to cells was observed in primary monocytes and CR1/CR3 co-transfected CHO cells even in the presence of fI. The authors suggest that the adhesion to cells expressing CR1 and CR3 is a dynamic process where the function of CR1 is to generate ligands for CR3, raising the hypothesis that CR3 engagement is secondary to CR1 (Sutterwala et al., 1996). We observed an effect of CR3 blockade on bacterial uptake 2h post co-culture suggesting dynamic changes in the contribution of CR1 and CR3 are most likely in *C. jejuni* uptake and phagocytosis. While we could not identify an interaction of *C. jejuni* and CR3 transfected CHO cells it would be interesting to repeat the experiment with CR1/CR3 co-transfected cells. A potential involvement of CD11b downstream of receptor binding is similarly intriguing and requires further investigation.

Effective clearance of pathogens by neutrophils requires the fusion of the phagolysosome, ROS production and the release of cytotoxic antimicrobial granule contents after internalisation. Our findings suggest the generation of neutrophil ROS in response to *C. jejuni*, however neutrophils failed to promote bacterial killing. Research suggests profound differences between the type of receptors involved in microbial phagocytosis and the signalling cascade triggering pathogen clearance. IgG induced phagocytosis is characterised by membrane extension and the rearrangement of the actin cytoskeleton, a pathway inhibited by cytD. Engulfment of C3b-coated particles is only inhibited at higher concentrations of cytD and appears to include a membrane activation independent process. Moreover, FcR engagement readily induces ROS production while superoxide anions are only observed after internalisation of C3b-coated particles (Hed and Stendahl, 1982, Underhill and Ozinsky, 2002). A study on human monocyte-derived macrophages further showed differences between the CRs as they showed that serum- opsonised *S. typhi* is primarily recognised by CR1 in contrast to its family member *S. typhimurium* where uptake is mediated *via* CR3. Engagement of CR1 herein, was closely correlated with prolonged survival of the strain suggesting the involvement of CR in bacterial intracellular fate whilst the mechanisms of action remain unknown (Ishibashi and Arai, 1996). Since we observed ROS generation in HIS- and NHS-opsonised bacteria it is possible that multiple receptors are involved in the process.

We did not observe neutrophil-mediated bacterial killing. It is interesting that other publications indicate intracellular survival of *C. jejuni* in epithelial cells and macrophages (Day et al., 2000, Watson and Galan, 2008). Further studies are required to verify these findings and identify mechanisms that promote bacterial survival.

CHAPTER IV

CAMPYLOBACTER JEJUNI-MEDIATED COMPLEMENT ACTIVATION

4.1 Background

The complement system is an evolutionary conserved component of innate immunity; the presence of a primitive form of the complement system in invertebrate deuterostomes such as the sea urchins confirms its ancient ancestry (Al-Sharif et al., 1998). All higher vertebrates share an almost identical set of complement genes providing protection against invading microorganisms through antibody dependent and independent mechanisms; primarily by opsonophagocytosis, generation of chemoattractants and lysis of Gram-negative bacteria by the membrane attack complex (MAC) (Nonaka and Kimura, 2006). Three different pathways have been recognised to trigger complement activation which are known as classical (largely antibody-dependent), lectin and alternative pathway (both antibody-independent; for details, see Chapter I, 1.4.5).

While complement driven immunity has a crucial function for clearance of infectious agents, pathogens have evolved various strategies to escape complement-mediated killing. Some bacteria release proteases that degrade complement components into smaller, non-functional fragments. For example, *Pseudomonas spp.* express *Pseudomonas* elastase (PaE) and alkaline protease (PaAP) enzymes which facilitate degradation of IgG and C1q thus reducing classical pathway activation (Rooijakkers and van Strijp, 2007). Similarly, *S. aureus* produces a Staphylokinase (SAK) which targets IgG and C3b/iC3b deposition on the bacterial surface cleaving them to inactive subunits (Rooijakkers et al., 2005). Other strategies interfere with complement effector function by the expression of proteins that bind to complement regulators or lead to the recruitment of complement inhibitors. *N. meningitides* expresses a fH-binding protein which recruits fH to the bacterial surface and accelerates the breakdown of C3b to iC3b leading to down-regulation of the alternative pathway and increased bacterial survival in serum (Schneider et al., 2006).

The capsule *N. meningitidis*, is an important virulence factor, which resists complement-mediated killing by inhibiting IgM-mediated C4b deposition resulting in disruption of the classical pathway (Agarwal et al., 2014). In addition, capsule and LPS/LOS of some Gram-negative bacteria contain sialic acids, like N-acetyl neuraminic acid (NeuAc), which are well described negative regulators of the alternative pathway (Meri and Pangburn, 1990). Sialic acid is a common terminal sugar of mammalian cell membranes and is thought to protect the host cell from spontaneous complement activation. Some bacteria, including *Neisseria sp.* utilise molecular cell mimicry to evade immune recognition and counter complement-mediated killing (Jarvis and Vedros, 1987).

While mechanisms of complement activation and evasion have been recognised as important factors in understanding pathogenesis to *S. pneumonia*, *N. meningitidis* and *S. aureus*, to date very

little is known about its role in *C. jejuni* infection and disease pathogenesis. Although the presence of complement (see, Chapter III) was found to be crucial for neutrophil-mediated phagocytosis of *C. jejuni* the mode of complement activation and potential evasion mechanisms employed by *C. jejuni* remain unknown. The aim of this study was to identify the mechanisms involved in complement activation by *C. jejuni* and to assess the impact of *Campylobacter* glycosylation and polysaccharide capsule on complement activation.

4.2 The role of *C. jejuni* polysaccharide in bacterial-mediated complement activation

Wild-type (wt) *C. jejuni* 81-176 and 11168 strains were found to activate the complement system in human serum. This activation was assessed in three ways. Firstly, plates were coated with bacteria and incubated with dilution series of normal human serum (NHS). C3b deposition was quantified by ELISA. A dose dependent increase in C3b deposition was detected in response to the two *C. jejuni* wt strains (Fig 4.1 A). No significant difference in C3b deposition was noted between the two strains. Secondly, rates of phagocytosis of the two NHS-opsonised *C. jejuni* strains increased and were comparable over time, although the 11168H strain showed a trend for greater phagocytosis (Figure 4.1 B).

We show C3b deposition occurs in both strains, therefore as the third assessment, we went on to look at the downstream events of C3b-deposition. We specifically investigated the activation of the membrane attack complex (MAC) which has potent bactericidal activity against Gram-negative bacteria (Berends et al., 2014). Bacteria were co-cultured with heat-inactivated serum (HIS) and NHS and the numbers of viable bacteria were quantified over time. *C. jejuni* CFU counts decreased by approximately 1-2 log over a 4h time-period only in the presence of NHS confirming the crucial role of the complement system in these cellular events (Figure 4.1 C). Similarly to phagocytosis, a trend for greater killing was observed in response to strain 11168H.

To investigate where on the bacterial surface C3b binding occurred we used *C. jejuni* 11168H wt strain and its isogenic mutants and performed similar experiments as described in Figure 4.1. The mutant lacking sialic acid residues (*neuB*) showed similar levels of C3b deposition in comparison to the parental strain while slightly enhanced values were observed with the capsule-negative (*kpsM*) strain (Figure 4.2 A). Interestingly, the capsule negative mutant, where increased C3b deposition was observed, also showed enhanced uptake, however this was not significant. (Figure 4.2 B). The uptake of *neuB* and the OS-truncated *waaF* mutant were minimally reduced (Figure 4.2 B). The lack of capsule significantly promoted serum bactericidal activity (2 log reduction in the wt compared to 5 log reduction in the *kpsM* mutant). Little effect was observed for *neuB* and *waaF* mutants (Figure 4.2 C). This data suggests that removal of the capsule results in increased C3b binding site availability.

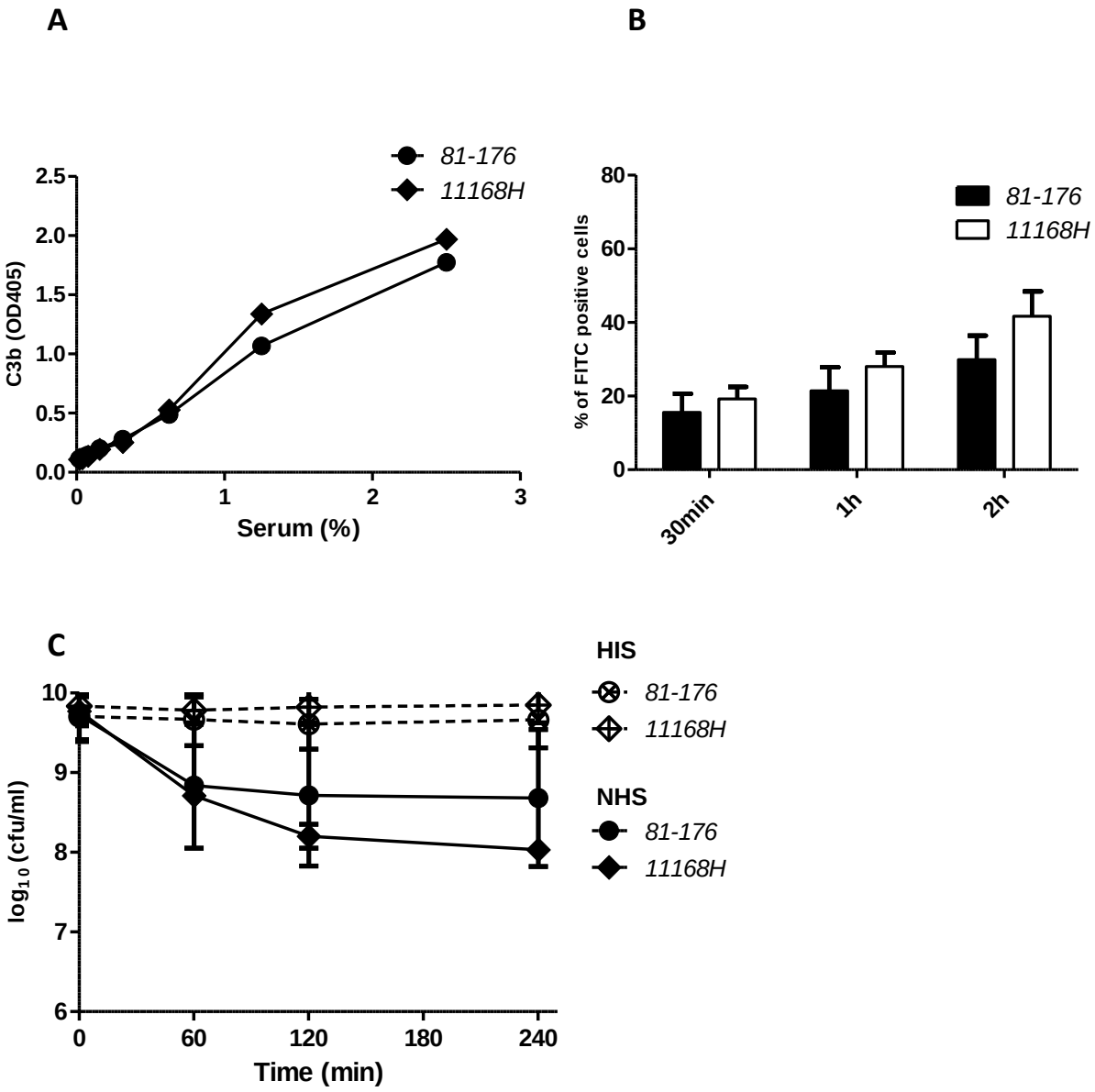


Figure 4.1 *C. jejuni* induces complement activation in human serum.

(A) Microtiter plates were coated with *C. jejuni* wt 81-176 and 11168H strains and degree of C3b deposition in the presence of serial-diluted normal human serum (NHS) was determined by ELISA. Absorbance was determined at 405nm and expressed as median values of one experiment performed in duplicates. **(B)** Human neutrophils were co-cultured with FITC-labelled, NHS-opsonised bacteria (MOI 10) and the rate of uptake was assessed by FACSCalibur. Data represent mean±SEM of five independent experiments. **(C)** Serum bactericidal activity was assessed by co-incubation with 20% NHS and bacterial survival was sampled over time. Heat inactivated serum (HIS) served as control. Data is presented as mean±SEM of three independent experiments. 2-way ANOVA with Bonferroni post-test was performed to determine statistical significance between strains. No stars: not significant

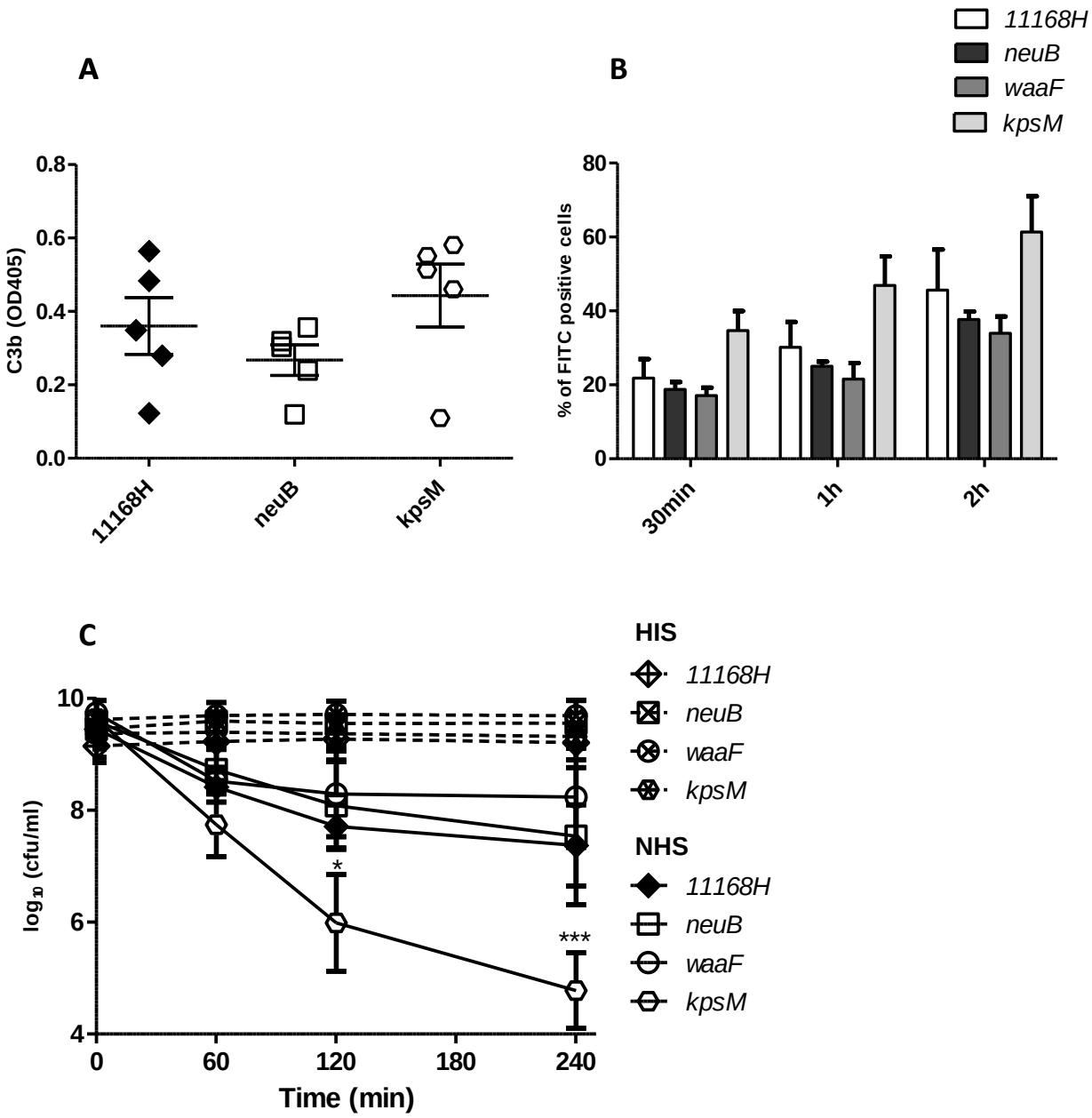


Figure 4.2 Impact of *C. jejuni* capsule and LOS on neutrophil phagocytosis and serum bacterial lysis activity.

(A) Microtiter plates were coated with *C. jejuni* 11168H wt and isogenic asialiated *neuB*, OS-truncated *waaF* and a-capsulated *kpsM* mutants and C3b deposition in the presence of 1% NHS was determined by ELISA. Mean±SEM of duplicates of five independent experiments. One-way ANOVA with Tukey post-test **(B)** Human neutrophils were co-cultured with FITC-labelled, NHS-opsonised *C. jejuni* wt strains and isogenic mutants (MOI 10) and rates of uptake were assessed by FACSCalibur. Mean±SEM of three independent experiments. **(C)** Serum bactericidal activity was assessed by co-incubation with 20% NHS and bacterial survival (CFUs) was assessed over time. HIS served as control. Mean±SEM of three independent experiments. 2-way ANOVA with Bonferroni post-test. No stars: not significant; * $p < 0.05$; *** $p<0.001$

4.3 Role of Ca^{2+} in *C. jejuni*-mediated Complement activation

We observed serum complement activation and C3b-deposition in response to *C. jejuni* as determined by ELISA and neutrophil-mediated uptake. Next, we wished to identify the complement pathway(s) involved in this deposition. This was done by using the requirement or not of Ca^{2+} or Mg^{2+} ions in the pathway. Activation of the classical and the lectin pathway requires Ca^{2+} and Mg^{2+} whereas the alternative pathway is solely Mg^{2+} dependent (Des Prez et al., 1975). Prior to neutrophil co-culture, bacteria were opsonised with 20% NHS in the presence of either EDTA (chelating both Mg^{2+} and Ca^{2+} ions) or EGTA+Mg^{2+} (chelating Ca^{2+} only) to block total complement and classical/lectin pathway activation, respectively. The rate of phagocytosis was reduced by both, EDTA and EGTA+Mg^{2+} in isolated neutrophils, suggesting all three pathways may play a role in neutrophil-mediated *C. jejuni* phagocytosis (Figure 4.3 A). Serum bactericidal activity was also found to be partially inhibited by both chelating agents (Figure 4.3 B).

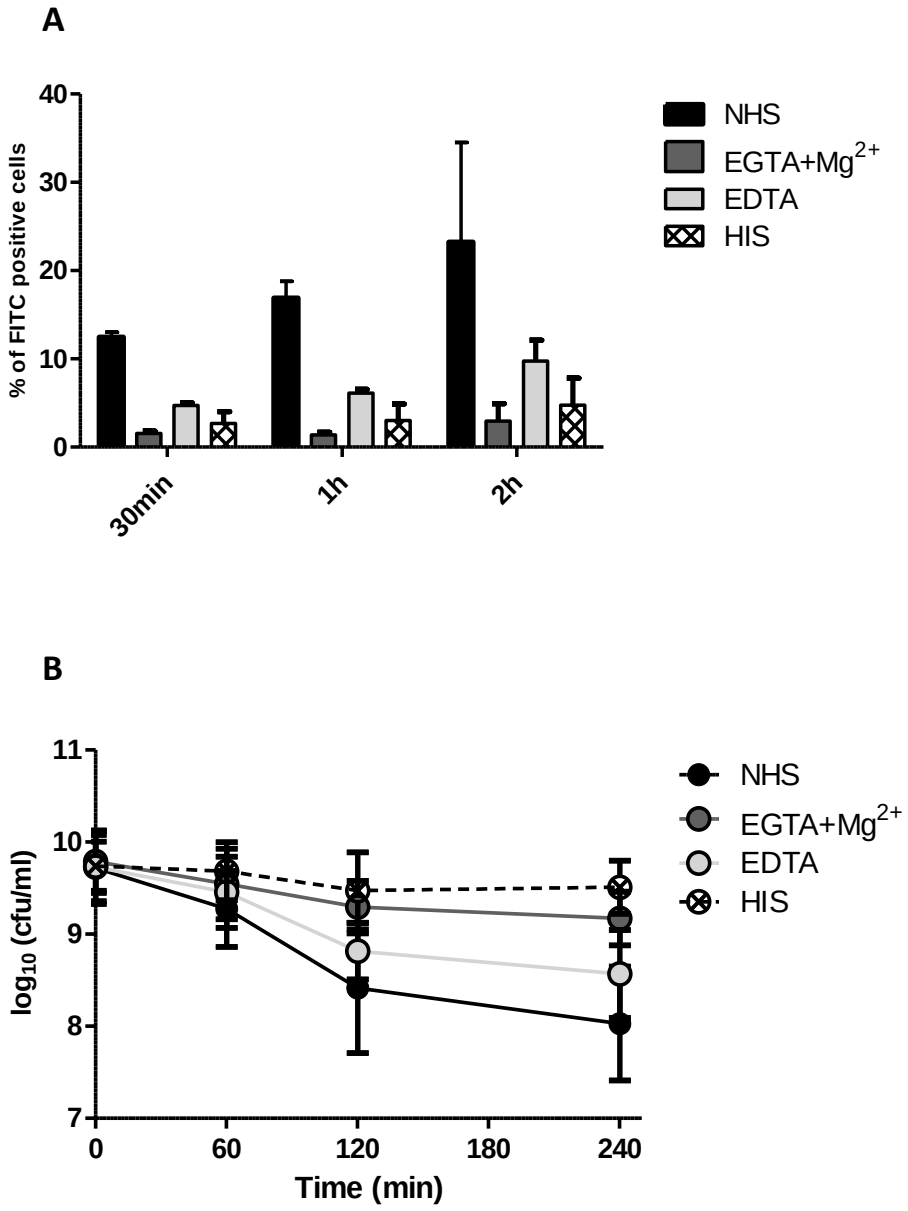


Figure 4.3 Ca^{2+} and Mg^{2+}-mediated neutrophil phagocytosis and serum bactericidal activity.

(A) *C. jejuni* 81-176 was opsonised with NHS in the presence of EGTA+Mg^{2+} or EDTA. HIS served as control. Human neutrophils were co-cultured with FITC-labelled, opsonised bacteria (MOI 10) and the rate of phagocytosis was assessed by FACSCalibur. **(B)** *C. jejuni* was incubated in the presence of 20% serum. Samples were taken over time and subjected to serial dilution. CFUs were counted after 48h culture. Data is presented as mean±SEM of **(A)** two **(B)** three independent experiments. 2-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant

4.4 Involvement of the classical pathway

For optimal neutrophil-mediated phagocytosis and serum bactericidal activity, our observations indicated a requirement for Ca^{2+} and Mg^{2+} ions, implicating all three complement pathways. We therefore went on to investigate each pathway individually. The classical complement pathway is dependent on C1q while C4 is required for classical and lectin pathway activation. We utilised C1q and C4 deficient mouse sera and observed a significant reduction in C3b deposition in both deficiency sera when compared to control (wt) (Figure 4.4).

The observed dependence on C1q protein suggested involvement of the classical pathway. The classical pathway is predominantly activated upon binding of specific antibody to the bacterial surface. In order to assess the role of immune complexes in bacterial complement activation, *C. jejuni* 81-176 was either co-incubated in the presence or absence of HIS (source of antibody as determined in Chapter III, Figure 3.4) and baby rabbit complement (RC) as an exogenous source of complement. Opsonisation with RC alone induced phagocytosis by neutrophils in a concentration and time-dependent manner (Figure 4.5 A). In the presence of HIS, bacterial uptake was significantly increased by two-fold (Figure 4.5 B). Bacterial co-culture with RC resulted in modest bactericidal activity to *C. jejuni* 81-176 and was not further enhanced by HIS (Figure 4.5 C). Similar effects on the involvement of antibody were observed using *C. jejuni* strain 11168H (Figure 4.6 A, B). Bactericidal activity was slightly enhanced by the addition of HIS to this bacterial strain (Figure 4.6 C).

Collectively, the results obtained indicated antibody independent and dependent mechanisms of complement activation by *C. jejuni* as activation was significantly enhanced in the presence of HIS but not completely dependent on HIS. In order to further understand the role of antibody in this system, we went on to investigate the involvement of IgA, IgG and IgM, human serum was depleted of each one. Surprisingly, antibody depletion had minimal effect on phagocytosis and bacterial lysis (Figure 4.7).

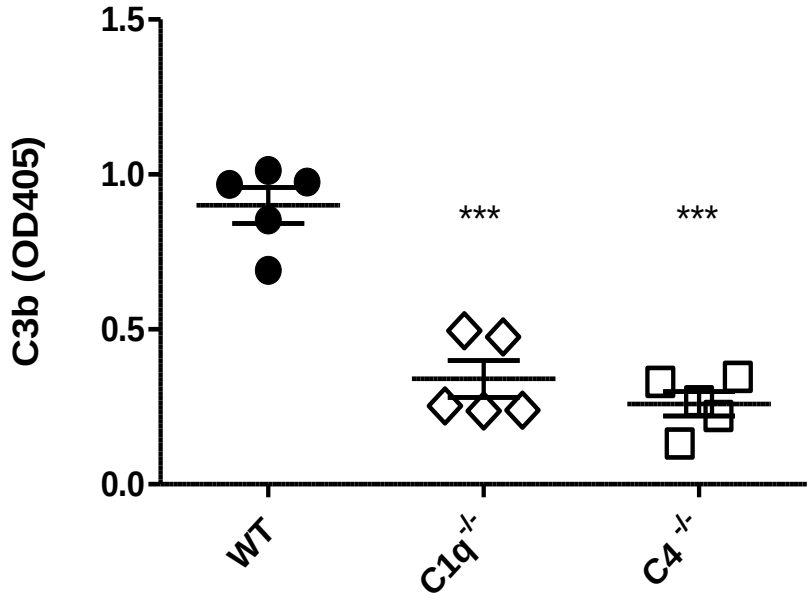


Figure 4.4 Murine C1q and C4-mediated C3b deposition on *C. jejuni* 81-176.

Microtiter plates were coated with *C. jejuni* 81-176 over night and incubated with wild type (wt) murine serum, C1q or C4 deficient murine serum and C3b deposition was determined by ELISA. Bars represent mean±SEM of the five murine sera tested per condition. One-way ANOVA with Tukey post-test was performed for statistical analysis. No stars: not significant; *** $p<0.001$

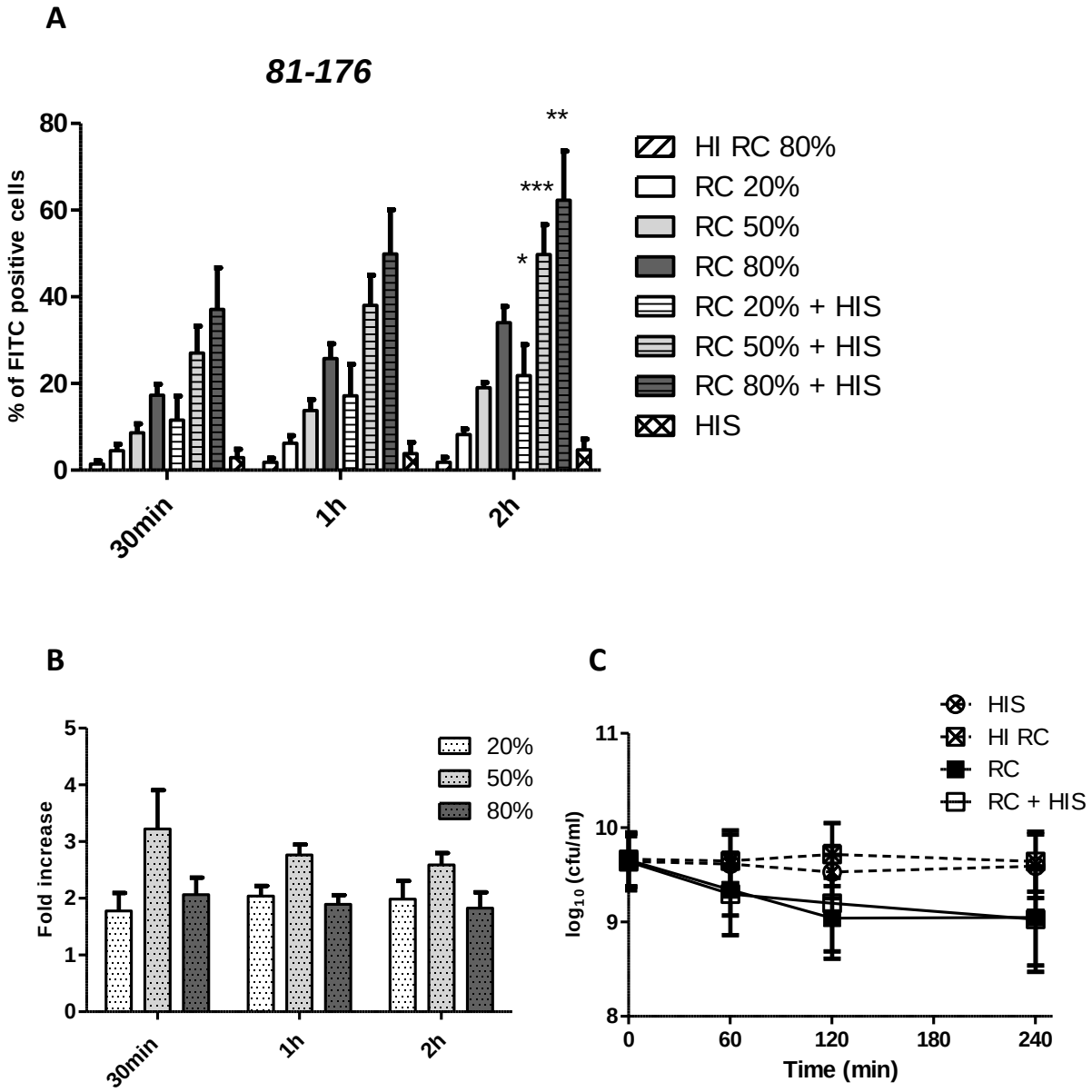


Figure 4.5 Impact of heat-inactivated serum (HIS) on complement-mediated phagocytosis of *C. jejuni* 81-176.

(A) *C. jejuni* 81-176 was incubated with baby rabbit complement (RC) at various concentrations. Where indicated 20% heat inactivated serum (HIS) was added. Heat inactivated RC (HI RC) served as control. Human neutrophils were co-cultured with FITC-labelled, opsonised bacteria (MOI 10) and the rate of phagocytosis was determined by FACS analysis. **(B)** Fold increase in phagocytosis upon bacterial opsonisation with RC+HIS compared to RC alone. **(C)** *C. jejuni* was incubated in the presence of 50% RC with or without HIS. CFUs were assessed over time. Mean±SEM of three independent experiments. 2-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant; * p< 0.05; ** p<0.01; *** p<0.001

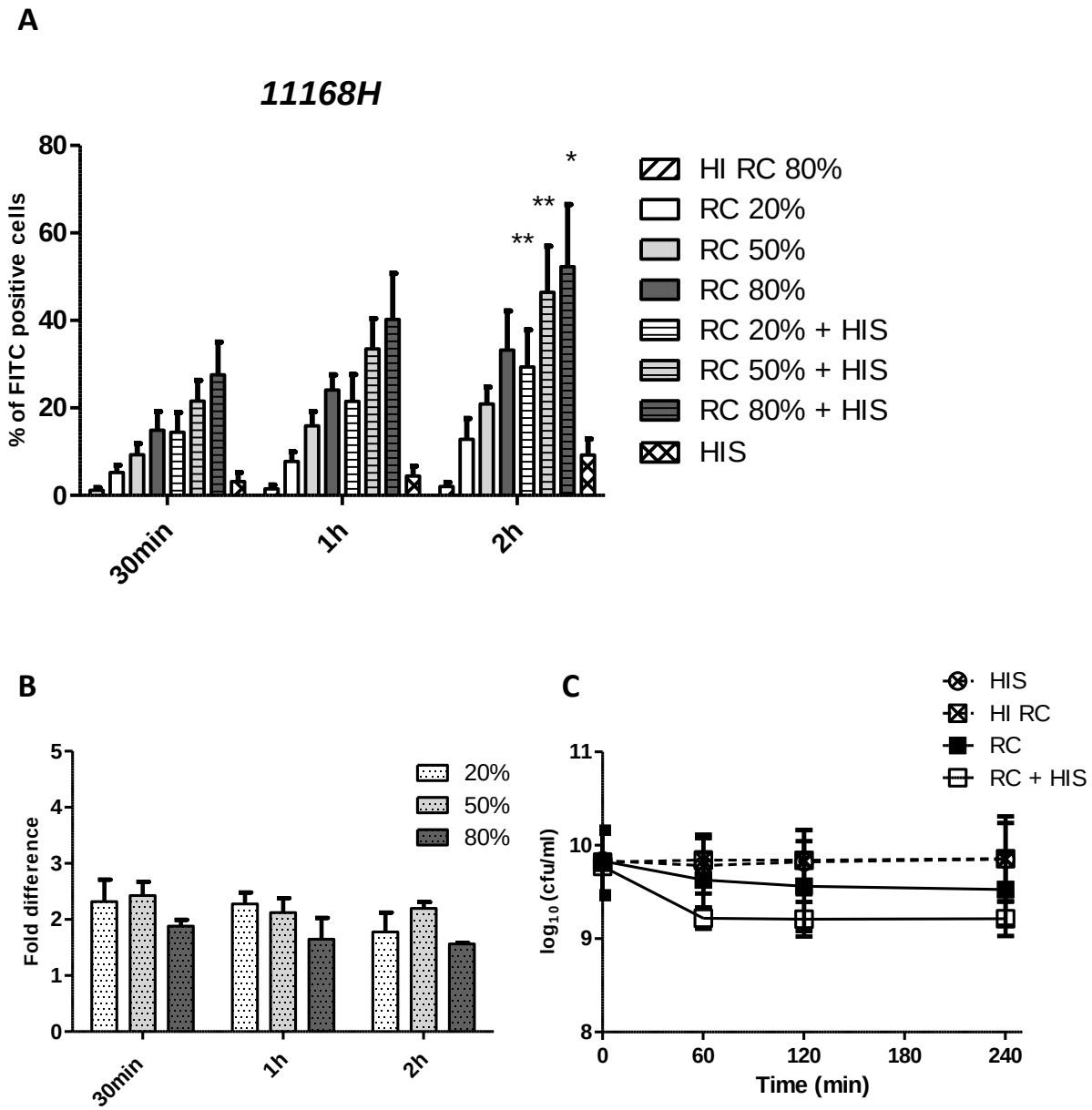


Figure 4.6 Impact of heat-inactivated serum (HIS) on complement-mediated phagocytosis of *C. jejuni* 11168H.

(A) *C. jejuni* 11168H was incubated with baby rabbit complement (RC) at various concentrations. Where indicated 20% heat inactivated serum (HIS) was added. Heat inactivated RC (HI RC) served as control. Human neutrophils were co-cultured with FITC-labelled, opsonised bacteria (MOI 10) and the rate of phagocytosis was determined by FACS analysis. **(B)** Fold increase in phagocytosis upon bacterial opsonisation with RC+HIS compared to RC alone. **(C)** *C. jejuni* was incubated in the presence of 50% RC with or without HIS. CFUs were assessed over time. Mean±SEM of three independent experiments. 2-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant; * $p < 0.05$; ** $p<0.01$

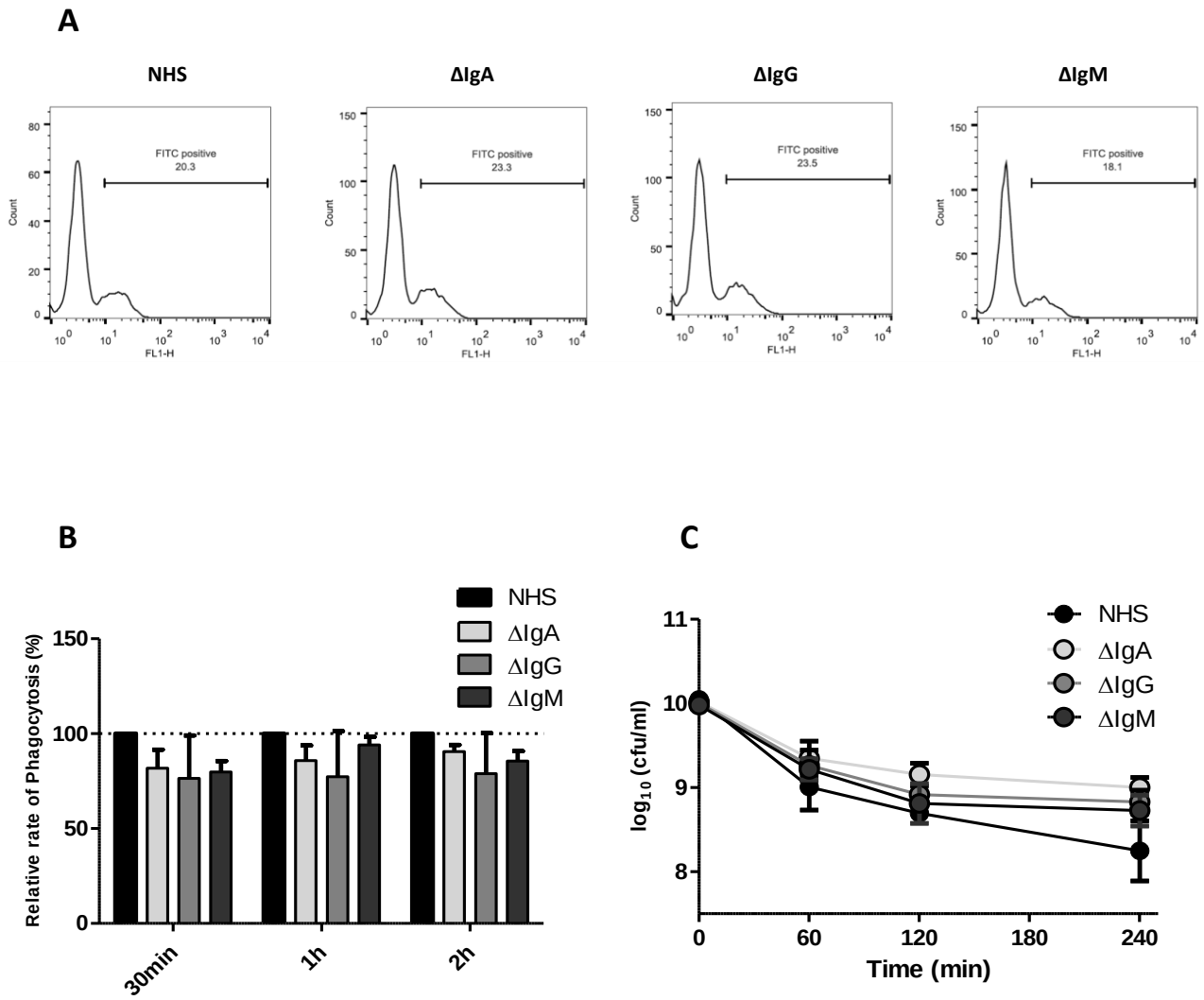


Figure 4.7 Effect of IgA, IgG or IgM depletion on neutrophil phagocytosis and bactericidal activity.
C. jejuni 81-176 was incubated with NHS or serum where immune complex IgA, IgG or IgM were removed by passage over anti-human IgA agarose, protein G Sepharose or anti-human IgM agarose, respectively. **(A), (B)** Human neutrophils were co-cultured with opsonised bacteria (MOI 10) and phagocytosis was assessed over time. Data is presented as **(A)** one representative at 1h co-culture or **(B)** mean±SEM of three independent experiments. **(C)** *C. jejuni* 81-176 were incubated in the presence of 20% serum and CFUs were assessed over time. Mean±SEM of three independent experiments. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant

Although experiments performed in the presence of RC and HIS clearly showed a significant increase in C. *jejuni* 81-176 and 11168H phagocytosis indicating a potential contribution of antibody-mediated pathways, when experiments were performed with antibody depleted serum, no major effect on phagocytosis and serum bactericidal activity towards *C. jejuni* was recorded. The precise roles of antibody-mediated effects on *C. jejuni* phagocytosis require further investigations.

4.5 Role of the lectin pathway in *C. jejuni* complement activation

We observed antibody-independent complement activation following opsonisation of *C. jejuni* with RC which may be related to lectin and/or alternative pathways activation. To determine the involvement of the lectin pathway we utilised murine sera presenting with mutations at various stages of the activation cascade. Sera deficient in MASP-2, and the recognition molecules FcnA and MBL showed significantly lower C3b deposition when compared to the wt controls (Figure 4.8).

The lack of C3b deposition observed in the presence of lectin pathway deficient sera, suggest a pivotal role of the lectin pathway in complement activation to *C. jejuni*, including the recognition molecule MBL. Inherited MBL-deficiency is relatively common in humans (Dahl et al., 2004). In order to confirm our findings obtained from murine sera, we employed serum and plasma samples of MBL-deficient donors. Surprisingly, co-incubation with human MBL-deficient serum/plasma resulted in similar or even enhanced rates of C3b-deposition compared to normal controls. These results were obtained for both *C. jejuni* strains and the isogenic mutants, *neuB* and *kpsM* (Figure 4.9). These results suggest limited involvement of the lectin pathway by *C. jejuni* in humans.

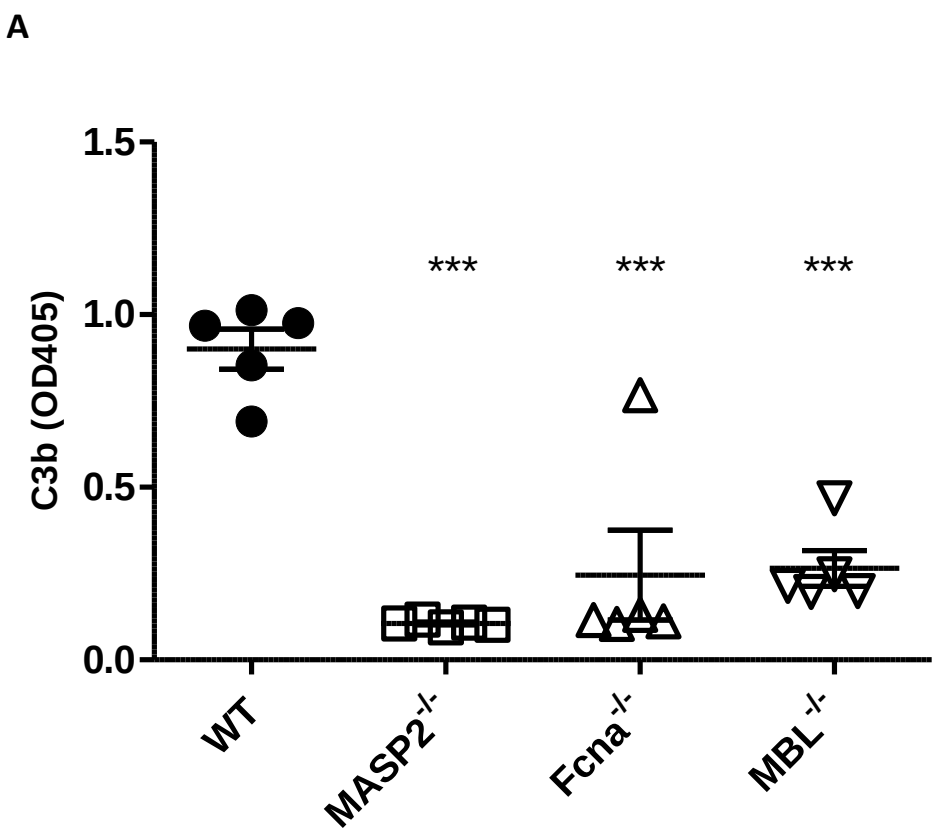


Figure 4.8 Murine lectin pathway-mediated C3b deposition in response to *C. jejuni*.
Microtiter plates were coated with *C. jejuni* 81-176 overnight and incubated with 1% murine serum of wild type (wt) animals and murine MASP-2, MBL and Fcna KO serum. C3b deposition was determined by ELISA. Bars represent mean±SEM of the five sera used per condition. One-way ANOVA with Tukey post-test. No stars: not significant; *** $p<0.001$

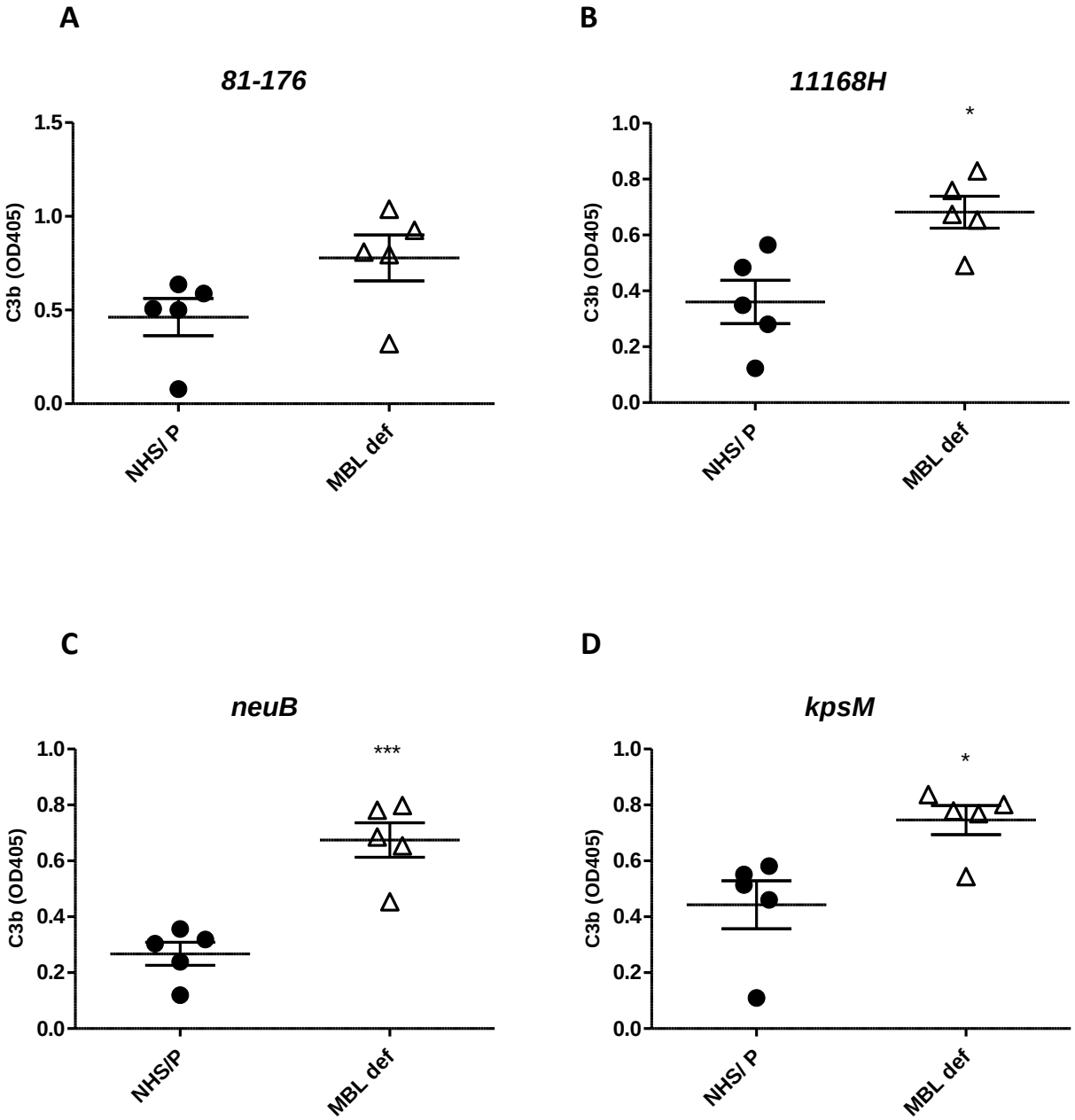


Figure 4.9 C3b deposition by MBL-deficient human serum.

Microtiter plates were coated with *C. jejuni* strain **(A)** 81-176, **(B)** 11168H and the isogenic mutants **(C)** *neuB* and **(D)** *kpsM*. Plates were incubated with 1% NHS or normal human plasma (NHP) and serum or plasma of individuals with MBL-deficiency syndrome (MBL def). C3b deposition was determined by ELISA. T-test was performed for statistical analysis. No stars: not significant; * $p<0.05$**;** *** $p<0.001$

4.6 MASP-3 dependent alternative complement activation

After assessing the implication of the classical and lectin pathway, we next investigated the involvement of the alternative pathway activation in response to *C. jejuni*. Alternative pathway dependent C3b-deposition was assessed using experimental conditions in the presence of Mg^{2+} but absence of Ca^{2+} which leads to inhibition if the classical and lectin pathway. Utilising this experimental approach we observed alternative pathway activation following incubation of mouse serum with *C. jejuni* strains and isogenic mutants followed by analysis of C3b deposition for each strain. The capsule deficient strain showed reduced C3 deposition compared to wt bacteria (Figure 4.10 A).

The alternative pathway lacks a recognition molecule but activation is dependent on the presence of MASP-3 and can be accelerated by properdin (Iwaki et al., 2011, Ali et al., 2014). We failed to observe a difference in C3b deposition on the surface of bacteria between MASP-3 deficient or wt murine sera. The addition of recombinant murine MASP-3 or murine properdin did not promote a significant increase in C3b deposition on the bacterial cell (Figure 4.10 B, C). This again highlights the differences often observed between murine and human data.

Alternative pathway activation to *C. jejuni* strains and mutants was also observed using NHS (Figure 4.11 A). Since we have previously observed profound differences between mouse and human serum in lectin pathway activation we repeated the experiments utilising serum of a MASP-3 deficient donor. In contrast to the observations obtained with murine sera, C3b deposition was reduced in the presence of human MASP-3 deficient sera. Interestingly, C3b deposition was partly restored by the addition of exogenous MASP-3 (Figure 4.11 B, C). These findings confirmed a potential contribution of the alternative pathway activation in response to *C. jejuni* in humans.

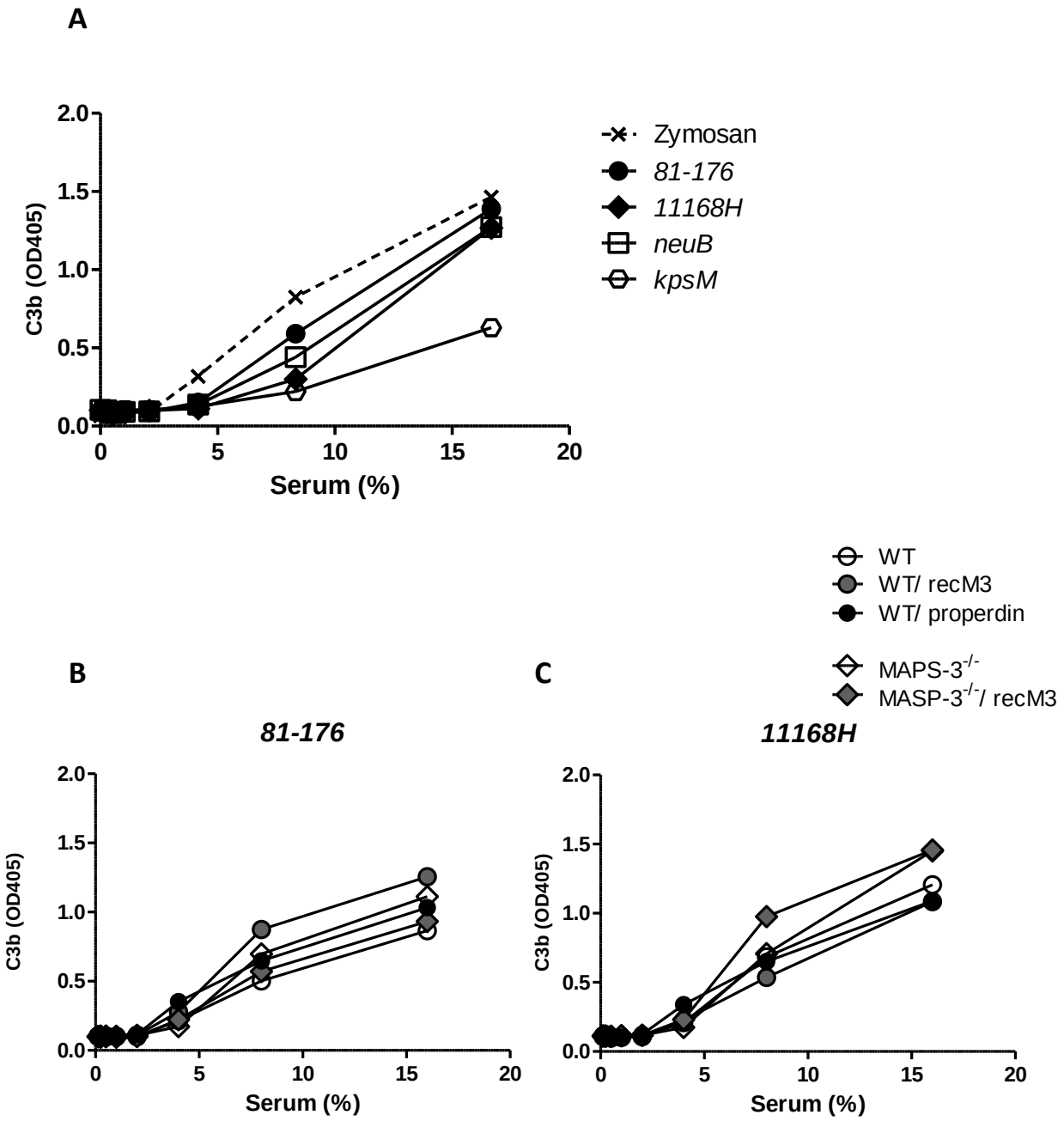


Figure 4.10 Murine MASP-3 does not contribute to alternative pathway activation.

Microtiter plates were coated with *C. jejuni* and serial diluted serum was added in the absence of Ca^{2+} to allow for alternative pathway activation. C3b deposition was determined by ELISA. **(A)** Plates were incubated with strain 81-176, 11168H and respective mutants prior to incubation with wild type mouse serum (wt). Zymosan (10µg/well) served as positive control. **(B), (C)** Plates were coated with *C. jejuni* strain **(B)** 81-176 or **(C)** 11168H and incubated with wt or MASP-3 deficient serum. Where indicated, recombinant MASP-3 (recM3) protein (10µg/mL) or properdin (5µg/mL) were added. Results are presented as means of duplicates.

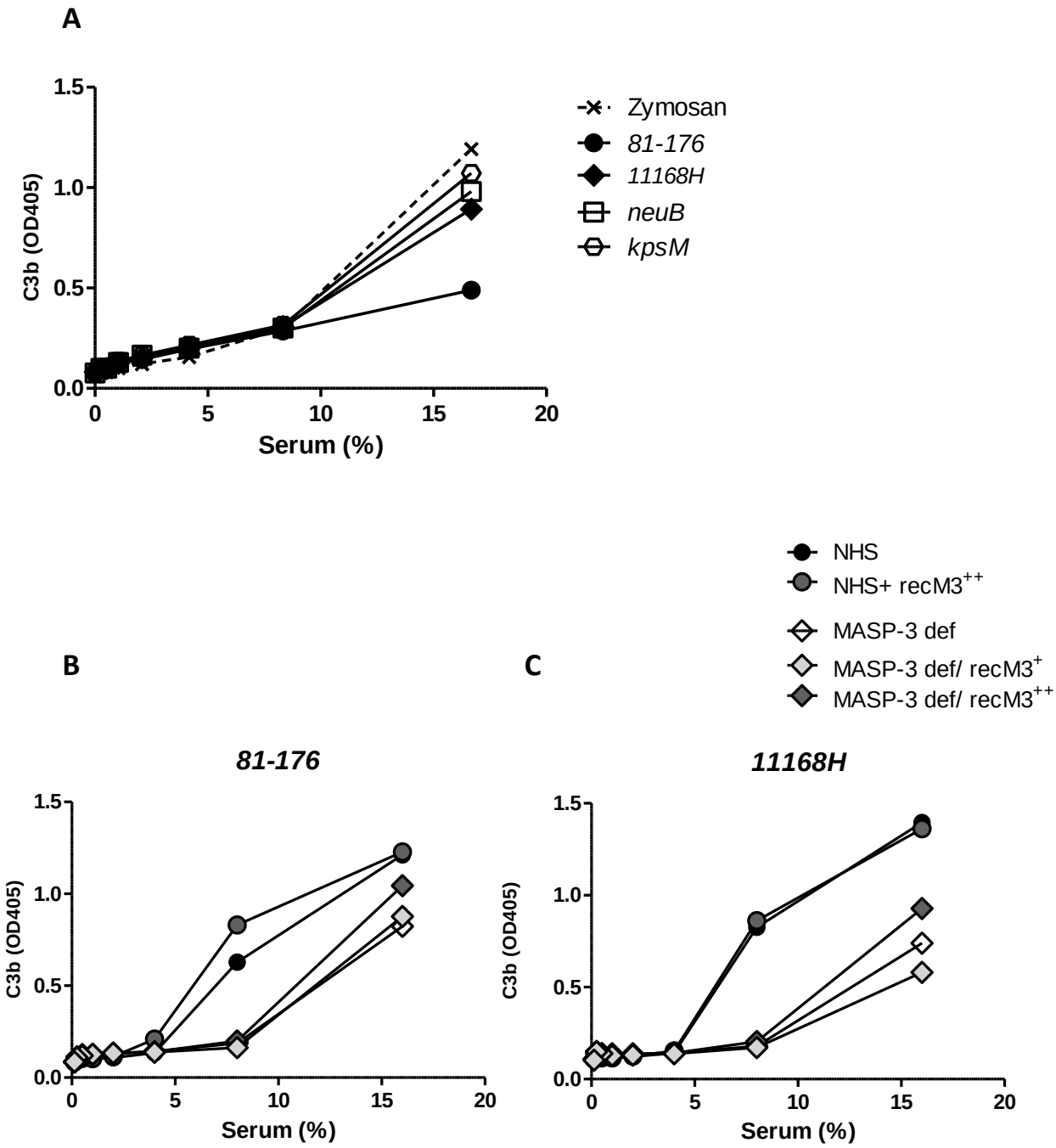


Figure 4.11 Human MASP-3 contributes to alternative pathway activation.

Microtiter plates were coated with *C. jejuni* and serial diluted serum was added in the absence of Ca^{2+} to allow for alternative pathway activation. C3b deposition was determined by ELISA. **(A)** Plates were incubated with strain 81-176, 11168H and respective mutants prior to incubation with NHS. Zymosan (10µg/well) served as positive control. **(B), (C)** Plates were coated with *C. jejuni* strain **(B)** 81-176 or **(C)** 11168H and incubated with NHS or MASP-3 deficient serum. Where indicated, recombinant MASP-3 (recM3) protein was added at increasing concentrations ($^{+}$ 1µg/mL; $^{++}$ 10µg/mL). Results are presented as means of duplicates.

4.7 Discussion

To date very little is known about the interaction of *C. jejuni* with the complement system. Herein, we show that the complement system is activated by *C. jejuni* and that activation results in neutrophil-mediated uptake as well as serum bactericidal activity. Blaser *et al.* have previously reported serum susceptibility of clinical *Campylobacter* isolates after 60min incubation with 10% human serum. The authors observed bacterial clearance (ranged between 0.5-4 log) for *C. jejuni* (Blaser et al., 1985). The differential serum susceptibility observed in *C. jejuni* isolates suggests the involvement of bacterial factors in mediating and/or evading complement activation. We observed moderate levels of bacterial killing for both *C. jejuni* reference strains over a timeframe of 4h. Of the bacterial factors investigated, lack of the polysaccharide capsule markedly enhanced opsonophagocytosis and serum bactericidal activity. The protective function of capsule against serum-mediated killing of *C. jejuni* has been previously reported (Keo et al., 2011, Maue et al., 2013, van Alphen et al., 2014). *C. jejuni* capsule is structurally complex and underlies frequent phase variations, which makes investigations on its functionality very difficult. Van Alphen *et al.* suggest a link between serum survival and the expression of O-methyl phosphoramidate (MeOPN), a molecule frequently contained in the capsule of *Campylobacter* members. Lack of MeOPN significantly reduces serum survival of *C. jejuni* 81-176 to the same level as the capsule negative mutant strain (van Alphen et al., 2014).

To assess the impact of surface glycosylation on complement activation by *C. jejuni* strains we studied isogenic mutants that lack the expression of sialic acid (*neuB)* and with truncation of the LOS outer core region (*waaF*). Both mutations resulted in a modest reduction in complement activation but the observed trends did not reach significance. Sialic acid has been identified as an important virulence component of *C. jejuni* LOS as it is associated with enhanced inflammation, epithelial invasion and increases risk of developing GBS (Stephenson et al., 2013, Louwen et al., 2008, Heikema et al., 2013). While the presence of sialic acid has been reported to inhibit the alternative pathway in *N. meningitides* we could not confirm such association with *C. jejuni neuB* mutant (Jarvis and Vedros, 1987). Our observations are in agreement with Naito *et al.* who also failed to observe increased serum susceptibility of a *C. jejuni* 81-176 mutant lacking the terminal sialic acid of the LOS (*cstII*). However, contrary to our findings, this group showed significant reduction in serum survival for *waaF* mutants (Naito et al., 2010). In contrast, Keo *et al.* observed capsule dependent and *waaF* independent bacterial killing which is in accordance to our findings (Keo et al., 2011). It is possible that the contrasting reports about the role of *waaF* mutation on complement activation derive from different *C. jejuni* parental strains used in the experiments.

The role of such structural changes could vary depending on the complement pathway primarily involved in activation.

Our results suggest that the presence of specific antibody is not essential for complement activation by *C. jejuni* as demonstrated by experiments with RC and Ig depleted serum. However, the addition of antibody (HIS) did significantly enhance complement-mediated uptake proposing an enhancing effect of immune complexes and thus the classical pathway.

Human MBL deficiency had no effect on C3b deposition but our investigations were not comprehensive in order to refute involvement of the lectin pathway on the whole. Our observations suggest that the alternative pathway regulated in a MASP-3 dependent manner is also likely to be important in complement activation. In the literature expanded studies on a serum sensitive *C. jejuni* strain have provided evidence for classical and alternative pathway dependent complement activation. Bactericidal activity was abrogated in the absence of immune complex but the addition of antibody in the form of heat-inactivated serum was found to induce alternative, rather than classical pathway activation. Further, the researchers suggest a IgG independent, possibly IgM dependent mechanism (Blaser et al., 1985). Similar results were obtained by Bernatowaska *et al.* who showed impaired neutrophil-mediated uptake when *C. jejuni* isolates were opsonised in the absence of C2, implicating the classical pathway. In this case however, the researchers report IgG dependent bacterial uptake (Bernatowska et al., 1989).

Taking all these findings together, one possible scenario could be that the classical pathway initiates complement activation. Accumulating C3b (tip-over mechanisms) then triggers the amplification loop of the alternative pathway. Interestingly, this fits with our previous observations where CR1 was identified as the main phagocytic receptor (Chapter III). Both systems are dependent on C3b which is the favoured ligand for CR1 and upon accumulation may also serve as substrate for the alternative pathway. It would be interesting to investigate if the cleavage of C3b to iC3b is impaired in response to *C. jejuni;* a mechanism mediated by fH and fI. This could lead to enhanced activity of the alternative pathway which in turn may contribute to severe damage to the surrounding tissue in an ongoing infection.

We observed compelling differences in complement activation mechanisms between murine and human serum. The most striking difference was the role of the lectin pathway which was shown to be essential in murine sera whilst the lack of MBL was found to be insignificant in humans.

The complement system is a complex machinery of enzymatic reactions and a challenging subject to study. Here, we have shown that it is activated by *C. jejuni* in a multi-faceted way. Based on our

findings we propose the involvement of both the classical and the alternative pathway as summarised in Figure 4.12. Further investigations are needed to unfold the exact mechanisms involved which may provide essential insights into a better understanding of *C. jejuni*-mediated inflammation.

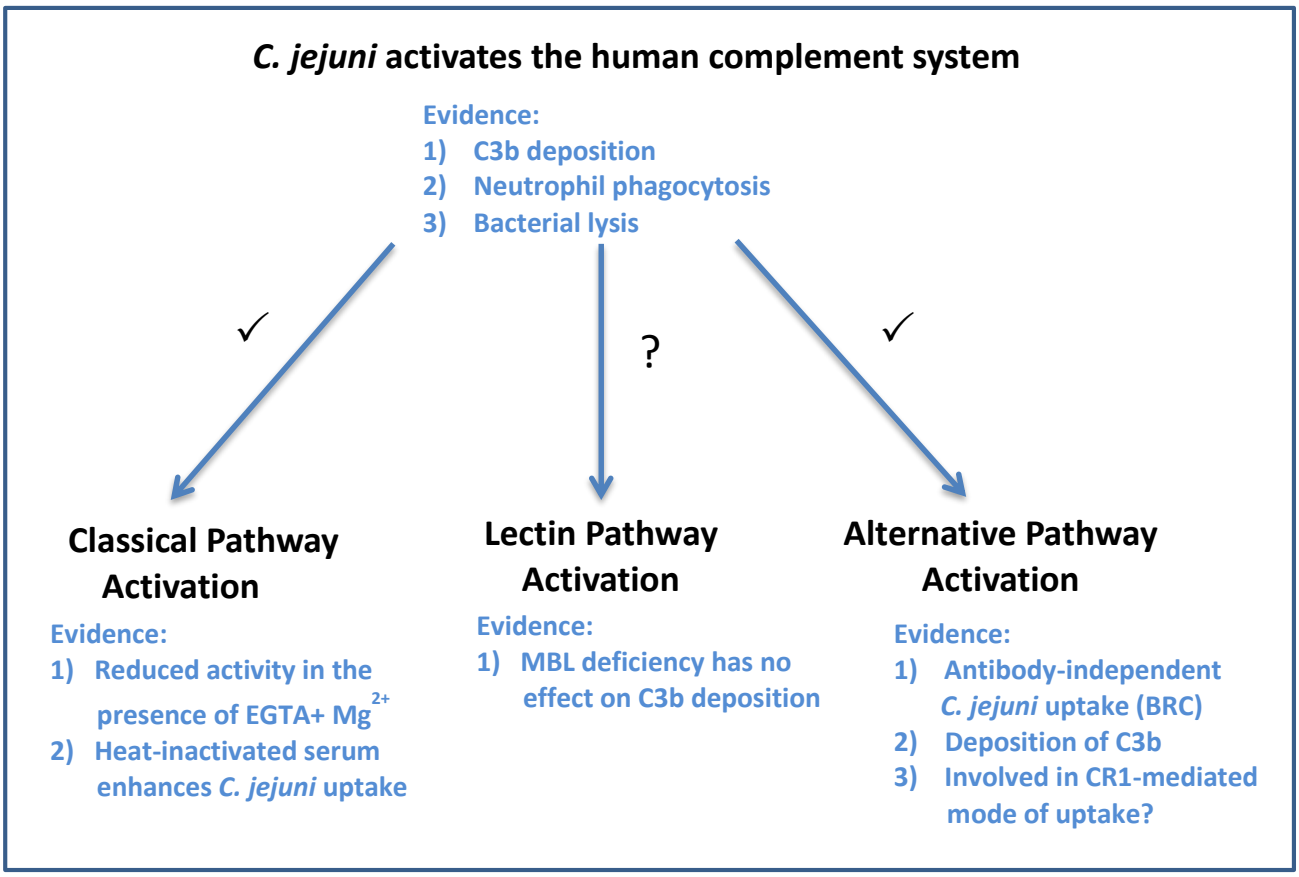


Figure 4.12 Possible mechanism of *C. jejuni* mediated complement activation

CHAPTER V

NEUTROPHIL-DERIVED IL-1B SECRETION IN RESPONSE TO *CAMPYLOBACTER JEJUNI*

5.1 Background

Infection with *C. jejuni* results in a pro-inflammatory cytokine milieu in the intestinal mucosa, including elevated levels of Interleukin-1β (IL-1β). IL-1β is an important pyrogenic cytokine secretion of which is now known to be regulated by the Inflammasome-complex (Sun et al., 2012a). TLR or NOD-mediated NF-κB activation initiates the synthesis of proIL-1β and proIL-18; this pathway is generally referred to as cell-priming. A second danger signal (exogenous or endogenous) is required, which facilitates cleavage of the pro-peptide to its bioactive form. This cleavage is controlled by the 'Inflammasome' a multi-protein platform. Inflammasome activation is mediated by intracellular sensors of the NOD-like receptor (NLR) family, the best studied so far is the NLRP3, which in response to a range of danger signals recruits the adaptor protein apoptosis associated speck-like protein containing a CARD (ASC). The assembly of a large ASC-protein multimer facilitates recruitment and subsequent autolytic cleavage of pro caspase-1 into its active form. Active caspase-1 then initiates the cleavage of proIL-1β and proIL-18 (Latz et al., 2013). Recently, Kayagaki *et al*. proposed an additional Inflammasome activation pathway triggered by intracellular Gram-negative bacteria. This non-canonical pathway requires activation of caspase-11 or caspase 4/5 in mice or humans, respectively, which in contrast to caspase-1, are not constitutively expressed. Intracellular LPS can induce the non-canonical pathway *via* the recruitment of pro caspase-11 (or human caspase 4/5) and subsequent cleavage of proIL-1β/IL-18 occurs *via* a NLRP3, ASC and caspase-1 dependent manner (Kayagaki et al., 2011).

Recently, Bouwman *et al.* investigated *C. jejuni*-induced generation of IL-1β in murine macrophages. Cytokine secretion was found to be mediated by the NLRP3-inflammasome which was independent of cell-priming suggesting canonical pathway activation (Bouwman et al., 2014). In contrast to Bouwman and colleagues, studies from our laboratory suggest minimal Inflammasome activation by *C. jejuni* in human and mouse DCs. When compared to other enteropathogens including Enteropathogenic *E. coli (EPEC)*, *S. typhimurium* and *Clostridium difficile*, *C. jejuni* induced modest IL-1β secretion (Stephenson et al., 2014). Similar findings have been observed in PMA-differentiated, macrophage-like THP-1 cells where *C. jejuni* induced minimal levels of IL-1β in comparison to LPS *plus* ATP, a potent activator of the NLRP3-Inflammasome (own observation; data not shown).

To date, Inflammasome activation has been extensively investigated in monocytes/macrophages and DCs, classic myeloid-cell populations. More recently publications have begun to draw the attention to neutrophil-derived cytokines as a contributor to immune regulation, including IL-1β (Tecchio et al., 2014, Gabelloni et al., 2013). It is important to note that investigations focusing on

cytokine secretion by neutrophils have proven difficult due to various factors and published data remains contradictory. The main concerns lie in the degree of purity of the neutrophils achieved by the various isolation techniques and prominent functional differences between mouse and human neutrophils (Tecchio et al., 2014, Mestas and Hughes, 2004).

Gabelloni *et al.* report the generation of IL-1β in LPS+ATP stimulated human neutrophils. While IL-1β processing was found to be partly caspase-1 dependent, its cleavage was also mediated *via* the neutrophil serine proteases elastase and proteinase-3 as demonstrated by blocking experiments (Gabelloni et al., 2013). Similarly, Karmakar *et al.* suggest an Inflammasome-independent mechanism of IL-1β generation in mouse neutrophils. Using a cornea infection model in response to *Pseudomonas aeruginosa* they found IL-1β secretion to be NLRC4 and caspase-1 independent, instead cytokine release was inhibited in the presence of neutrophil serine protease inhibitors. Neutrophils, not macrophages or DCs were the primary source of IL-1β secretion which was essential for bacterial clearance. Neutrophil elastase negative mice were unable to cleave the cytokine and showed impairement of bacterial clearance (Karmakar et al., 2012).

While human and murine studies show elevated IL-1β levels in inflamed tissue upon *C. jejuni* infection, reports on the activation of the Inflammasome by the bacterium remain contradictory *in-vitro*. In this study we aimed to assess if human neutrophils show markers of Inflammasome-activation and if they contribute to elevated IL-1β levels found in response to *C. jejuni* infection.

5.2 CD14$^+$ monocytes are the main source of IL-1β in response to LPS

Prior to assessing *C. jejuni* induced IL-1β secretion in human neutrophils, we first sought to characterise the potential differential cytokine response amongst blood leukocytes. LPS stimulation served as a trigger for cell-priming and ATP as a well-known trigger for NLRP3-Inflammasome activation (Netea et al., 2009). Stimulation of whole blood with LPS alone mediated significant increase in IL-1β secretion which was further enhanced by the addition of ATP. LPS also triggered the generation of pro-inflammatory TNF-α and IL-8; interestingly unlike IL-1β levels, TNF-α and IL-8 protein levels decreased in the presence of ATP (Figure 5.1).

Next, the amount of cytokine secretion from isolated PBMCs and neutrophils was compared. Low levels of IL-1β were detected in media of stimulated neutrophils, in comparison PBMCs produced significantly higher amount of cytokine (Figure 5.2A). PBMCs were further fractionated into CD14$^+$ (monocytic) and CD14$^-$ (lymphocytic) fractions by positive selection and subjected to LPS+ATP stimulation. CD14$^+$ cells were the major cell type responsible for IL-1β secretion, in contrast minimal secretion was seen in the CD14$^-$ fraction (Figure 5.2 B). These findings were confirmed by FACS analysis where neutrophils and monocytes stained positive for intracellular, cleaved IL-1β in contrast to CD3$^+$ lymphocytes (Figure 5.3). The percentage of IL-1β positive neutrophils was lower when compared to the highly active monocytes. The addition of ATP led to a minor reduction in IL-1β positive cells, most likely indicative of cytokine release (Figure 5.4). mRNA analysis of stimulated neutrophils showed upregulation in *nlrp3* and *Il1b* but failed to reach significance, while *Il18* was found to be constitutively expressed (Figure 5.5).

To elucidate the mechanisms involved in the processing of active IL-1β neutrophils and PBMCs were pre-treated with caspase-1 inhibitor prior to LPS+ATP treatment. Blocking of caspase-1 led to a reduction in active IL-1β in both cell populations. The effect was found to be more prominent in monocytes when compared to neutrophils (Figure 5.6 A, C).

Since neutrophil serine proteases have been proposed to be involved in IL-1β cleavage we assessed the effect of specific inhibitors of cathepsin G (CatG) and of neutrophil elastase and proteinase-3 (Elafin) on the generation of active IL-1β. Blocking of CatG led to a reduction of IL-1β in neutrophils and monocytes whereas Elafin had only a minor effect on both cell types suggesting a possible role of serine proteases in the cleavage of proIL1β (Figure 5.6 B, D).

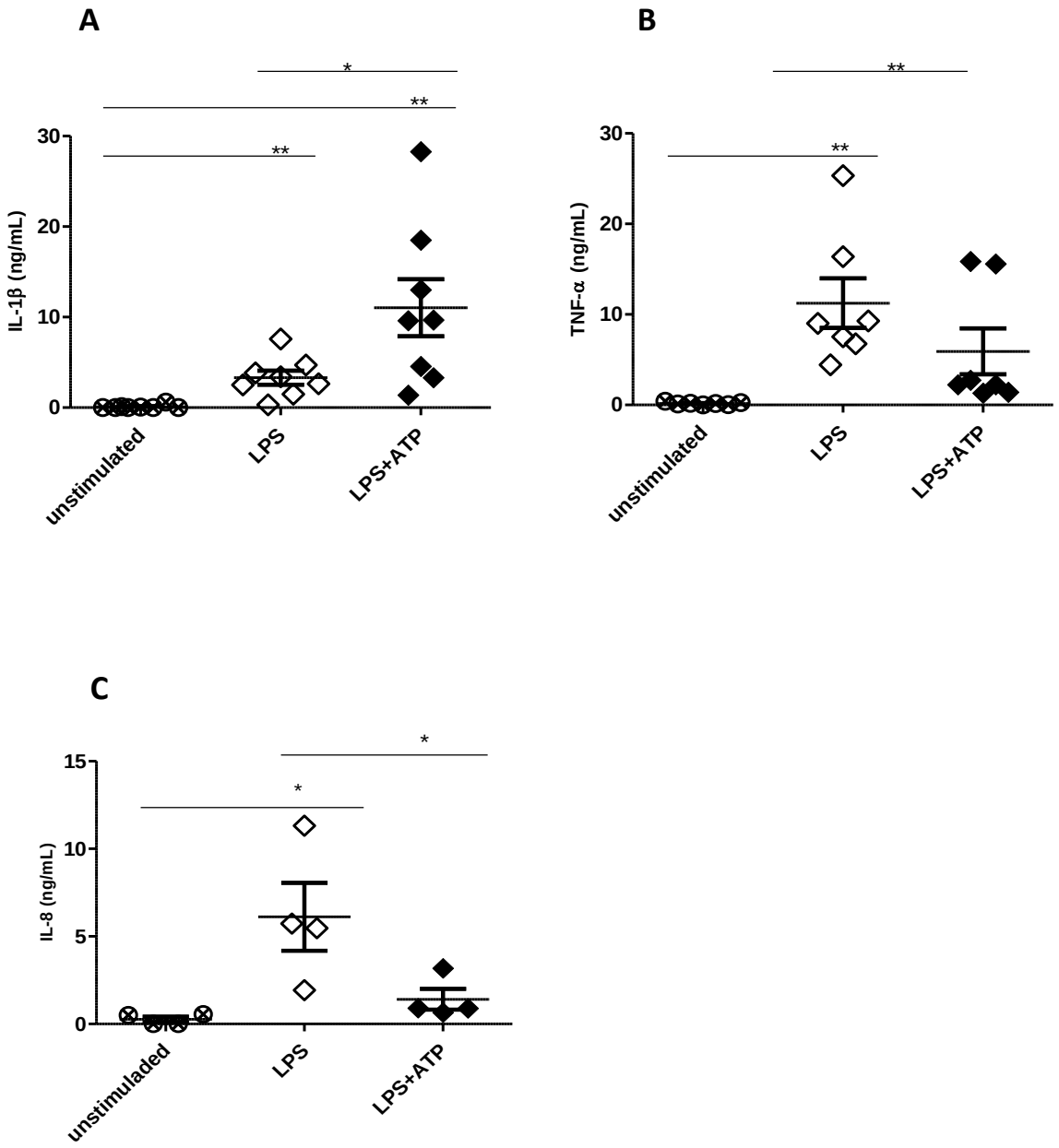


Figure 5.1 Effect of LPS and LPS+ATP on whole blood cytokine secretion.

Heparinised whole blood was stimulated with LPS (200ng/mL) for 5h. Where indicated, ATP (2.5mM) was added for the last 3h of incubation. Secretion of **(A)** IL-β and **(B)** TNF-α and **(C)** IL-8 was determined by ELISA. Data points show single measurement of at least four independent experiments. Bars indicate mean±SEM. One-way ANOVA with Tukey post-test was performed for statistical analysis. No stars: not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$

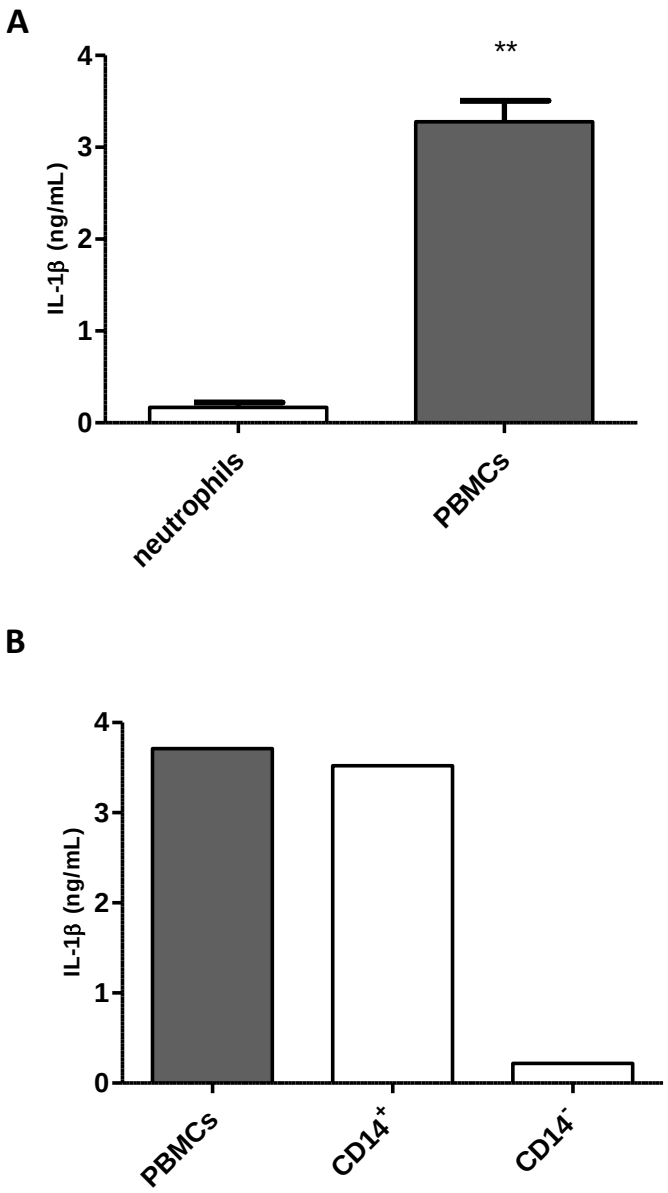


Figure 5.2 Effect of LPS and LPS+ATP on immune-cell specific IL-1β secretion

(A) Human neutrophils and PBMCs ($2x10^6$ cells/mL) were stimulated with LPS (200ng/mL) for 2h followed by ATP (2.5mM) for 3h. IL-1β secretion was determined by ELISA. Mean±SEM of three independent experiments. T-test was performed for statistical analysis. ** $p<0.01$. **(B)** Prior to stimulation, PBMCs of one donor were separated using CD14-magnetic beads to receive $CD14^+$ monocytes and $CD14^-$ lymphocytes.

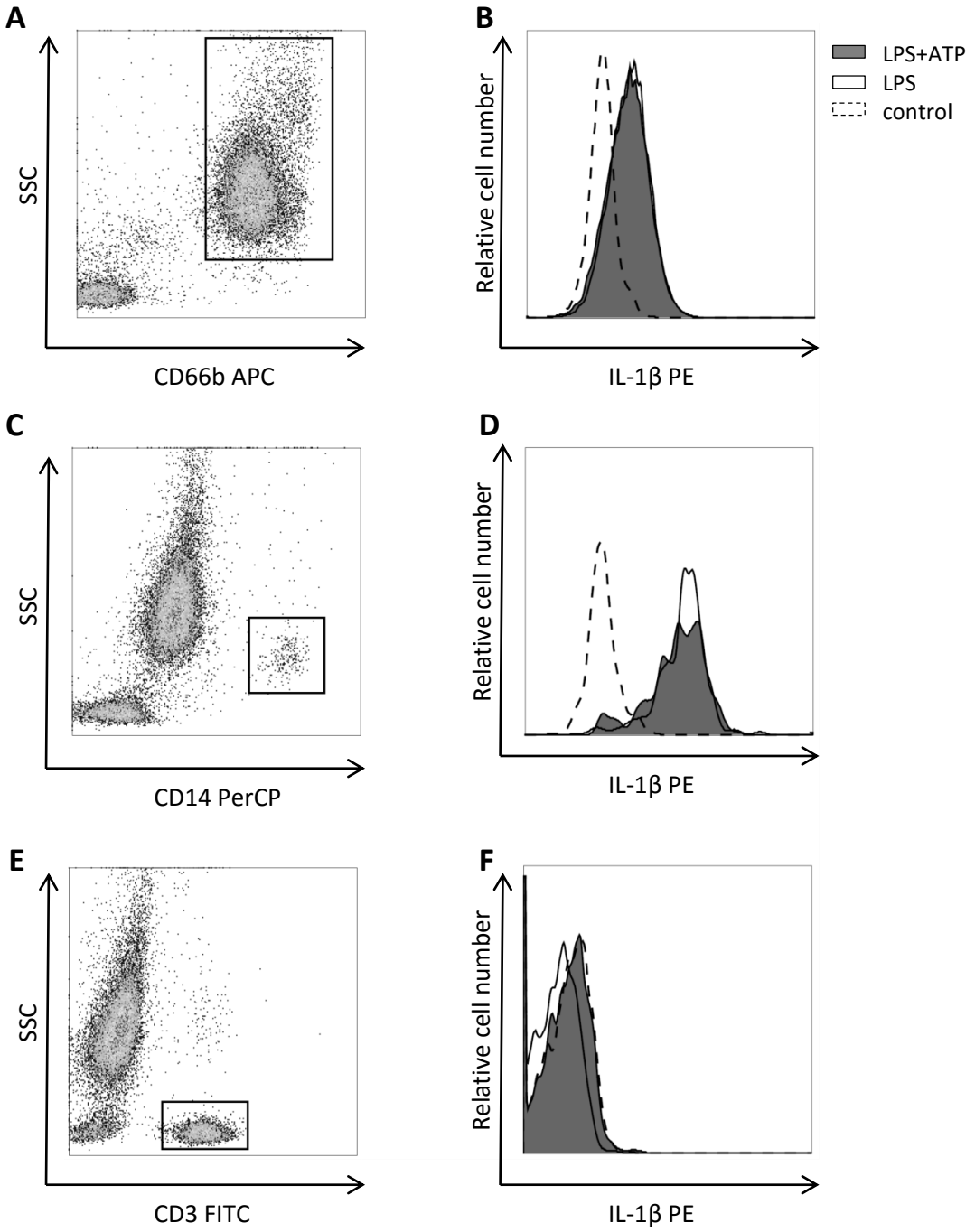


Figure 5.3 Generation of IL-1β in monocyte and neutrophil cells populations.

Heparinised whole blood was stimulated with LPS (200ng/mL) for 5h. Where indicated, ATP (2.5mM) was added after 2h LPS-priming. Leukocytes were stained for specific surface markers and intracellular, bioactive IL-1β prior to analysis by FACSCalibur. Gates were set for **(A), (B)** $CD66b^+$ neutrophils, **(C), (D)** $CD14^+$ monocytes or **(E), (F)** $CD3^+$ leukocyte fractions to determine cell-specific IL-1β secretion. Graphs shown are one representative of two independent experiments.

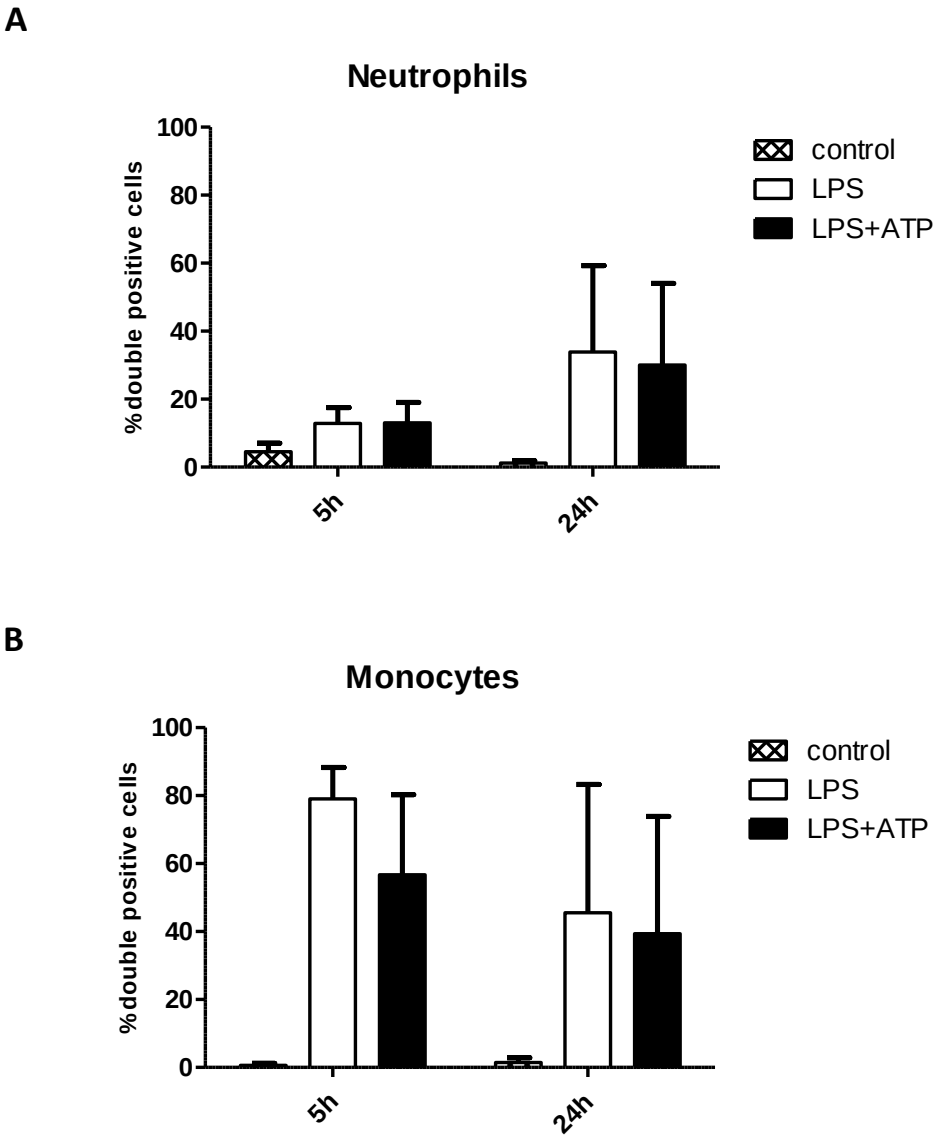


Figure 5.4 LPS-mediated generation of bioactive IL-1β in neutrophils and monocytes.
Heparinised whole blood was stimulated with LPS (200ng/mL) for 5h or 24h. Where indicated, ATP (2.5mM) was added after 2h LPS-priming. Leukocytes were stained for specific surface markers and intracellular, bioactive IL-1β prior to analysis by FACSCalibur. Percentage of double-positive cells for active IL-1β and the surface marker **(A)** CD66b (neutrophils) and **(B)** CD14 (monocytes). Mean±SEM of two independent experiments.

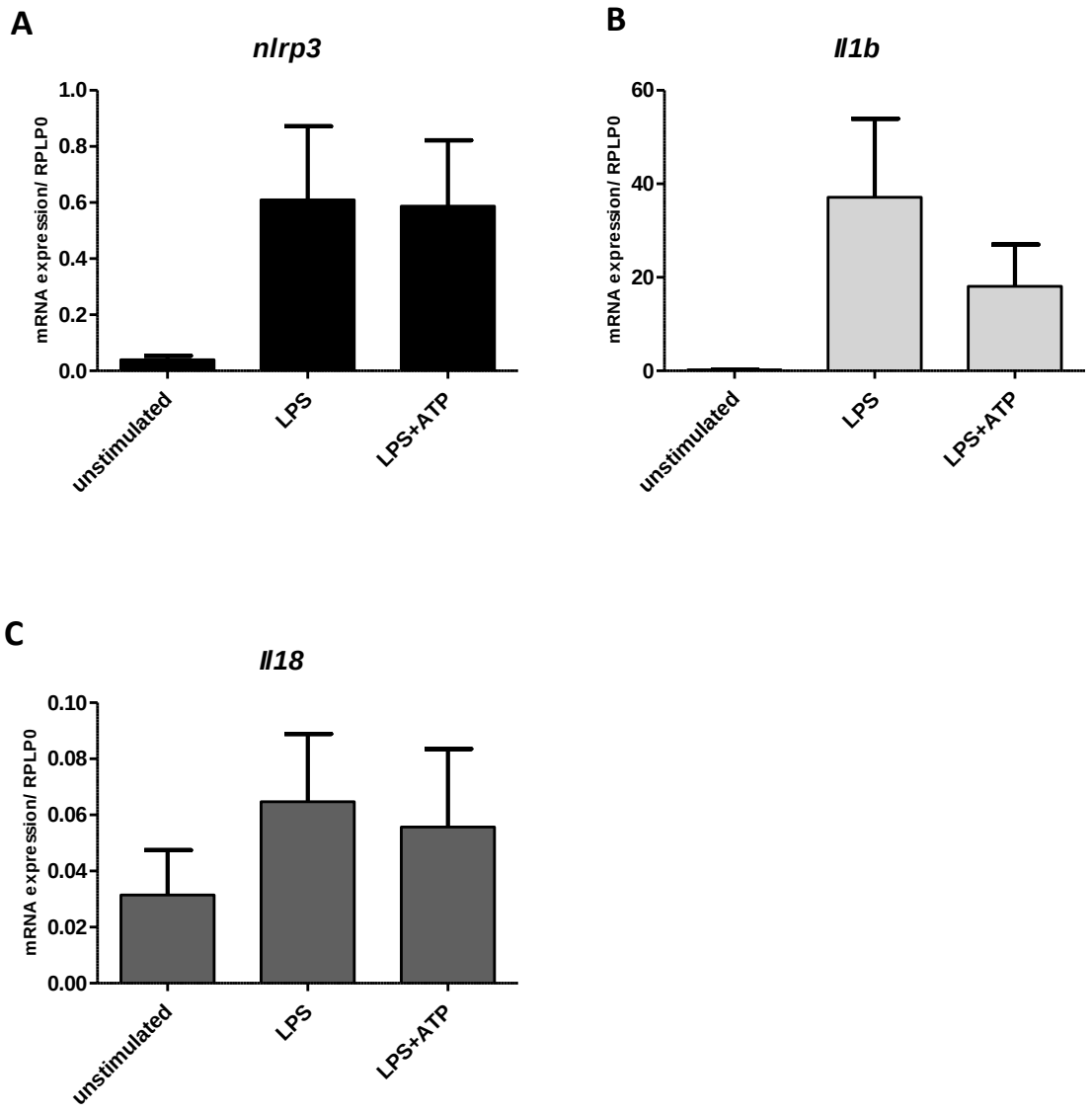


Figure 5.5 LPS-mediated *nlpr3* and *Il1b* mRNA expression in human neutrophils.

Human neutrophils were stimulated with LPS (200ng/mL) for 5h. Where indicated, ATP (2.5mM) was added after 2h. RNA was isolated and expression of **(A)** *nlrp3*, **(B)** *Il1b* and **(C)** *Il18* were determined by qPCR and normalised to the expression of the house keeping gene RPLP0. Mean±SEM of three independent experiments. One-way ANOVA with Turkey post-test was performed for statistical analysis. No stars: not significant

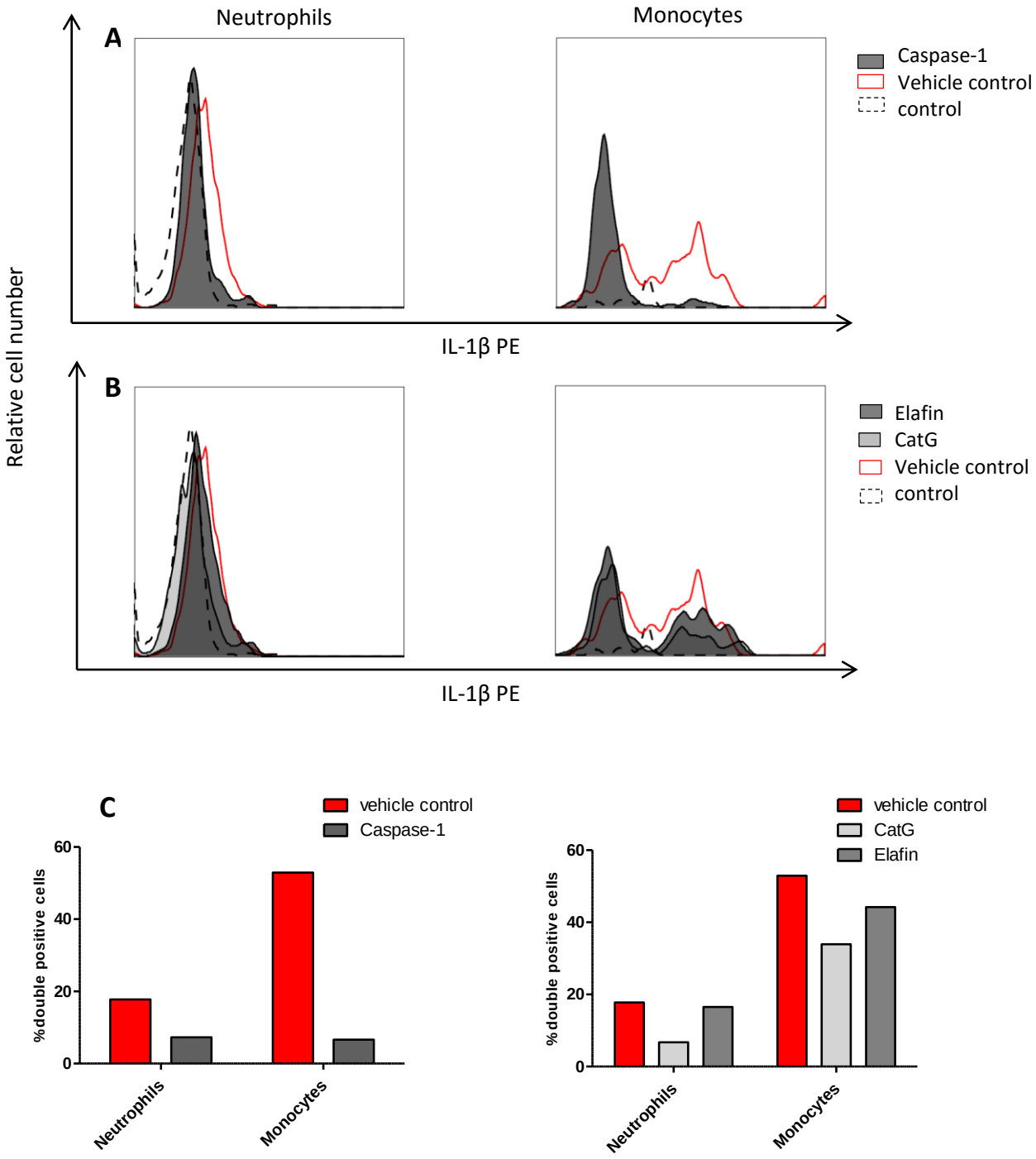


Figure 5.6 LPS-mediated generation of bioactive IL-β is caspase-1 dependent.

Heparinised blood was pre-treated with specific inhibitors for **(A)** caspase-1 (50µM) or **(B)** cathepsin G (CatG, 10µM) and elastase/protease 3 (Elafin, 10µg/mL) for 30min. Cells were stimulated with LPS (200ng/mL) for 2h followed by ATP (2.5mM) for 3h. Leukocytes were stained for specific surface markers and intracellular bioactive IL-1β prior to analysis by FACSCalibur. **(C), (D)** Percentage of double-positive cells for active IL-1β and the surface marker CD66b (neutrophils) and CD14 (monocytes). Data was obtained from one experiment.

5.3 *C. jejuni*-mediated neutrophil IL-1β production

In order to assess *C. jejuni*-induced cytokine profile, whole blood was co-cultured with the bacterium. Significant IL-1β and TNF-α was measured in response to $5x10^6$/mL bacterial inoculum 5h post co-culture. A dose-dependent increase was seen on further increase of the inoculum to $5x10^8$/mL (Figure 5.7 A, B). IL-1β secretion was time-dependent and significantly increased between 3h to 24h (Figure 5.7 C). Interestingly, in contrast maximal levels of TNF-α, were observed as early as 3h post co-culture (Figure 5.7 D).

Next isolated neutrophils and PBMCs from healthy donors were exposed to C. *jejuni* (MOI 10) or to LPS+ATP (positive control). IL-1β secretion from infected neutrophils showed a mean of 150pg/mL, which was comparable to that obtained in response to LPS+ATP stimulation (Figure 5.8 A). As expected, the response from PBMCs was significantly greater (mean 2800pg/mL) which was again comparable to LPS+ATP (Figure 5.8 B). In summary, cytokine analysis suggests that C. *jejuni* has the ability to induce IL-1β secretion in neutrophils and in PBMCs which is comparable to LPS+ATP.

The above observations were confirmed by FACS analysis where the percentage of double-positive cells increased in monocytes; in contrast, a minimal change was observed in neutrophils. This was independent of the initial inoculation dose (Figure 5.9). Interestingly, after 24h a notable increase in IL-1β positive cells was observed in the neutrophil, and not in monocyte population suggesting a delayed response in neutrophils compared to monocytes (Figure 5.10). We also recorded upregulation of *nlrp3* and *Il1b* mRNA levels in neutrophils upon bacterial co-culture which was similar to LPS stimulation, suggesting the involvement of the NLRP3-Inflammasome (Figure 5.11).

Pre-treatment of neutrophils with caspase-1 inhibitor led to a reduction in *C. jejuni*-induced IL-1β secretion. Inhibition of caspase-1 significantly reduced IL-1β secretion in monocytes (Figure 5.12). Taken together, these results suggest *C. jejuni*-mediated Inflammasome activation in neutrophils and monocytes which is in concordance with the observed IL-1β secretion by these cell types.

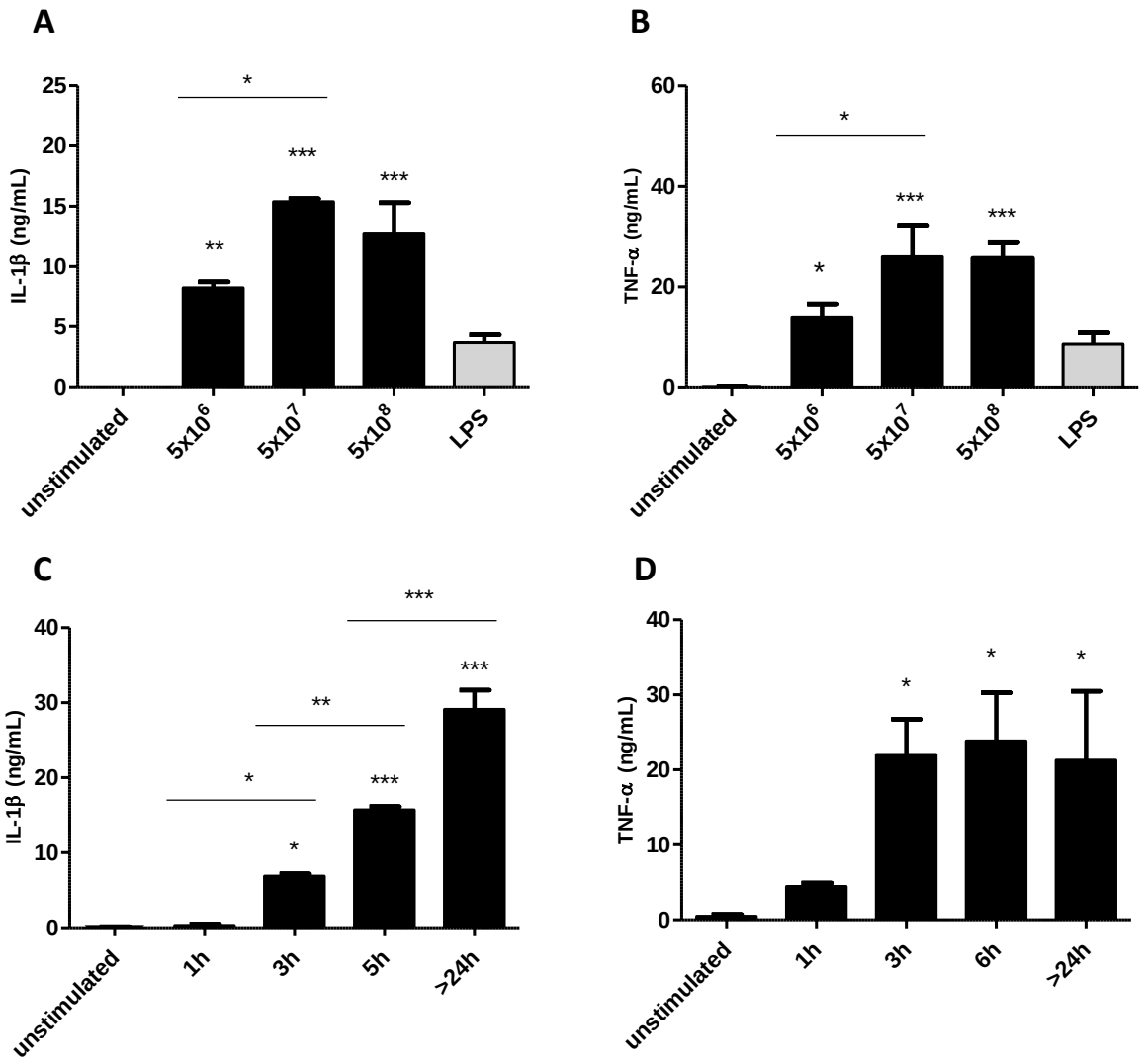


Figure 5.7 *C. jejuni*-mediated cytokine secretion in whole blood.

(A), (B) Heparinised whole blood was co-cultured with *C. jejuni* 81-176 at increasing inoculum (CFU/mL). Treatment with LPS (200ng/mL) served as control. Supernatants were collected 5h post co-culture and subjected to ELISA cytokine analysis for **(A)** IL-1β and **(B)** TNF-α. **(C), (D)** Whole blood was co-cultured with *C. jejuni* 81-176 ($5x10^7$ CFU/mL). Supernatants were collected at the indicated time-points and analysed for **(C)** IL-1β and **(D)** TNF-α secretion. Mean±SEM of three independent experiments. One-way ANOVA with Tukey post-test was performed for statistical analysis. No stars: not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$

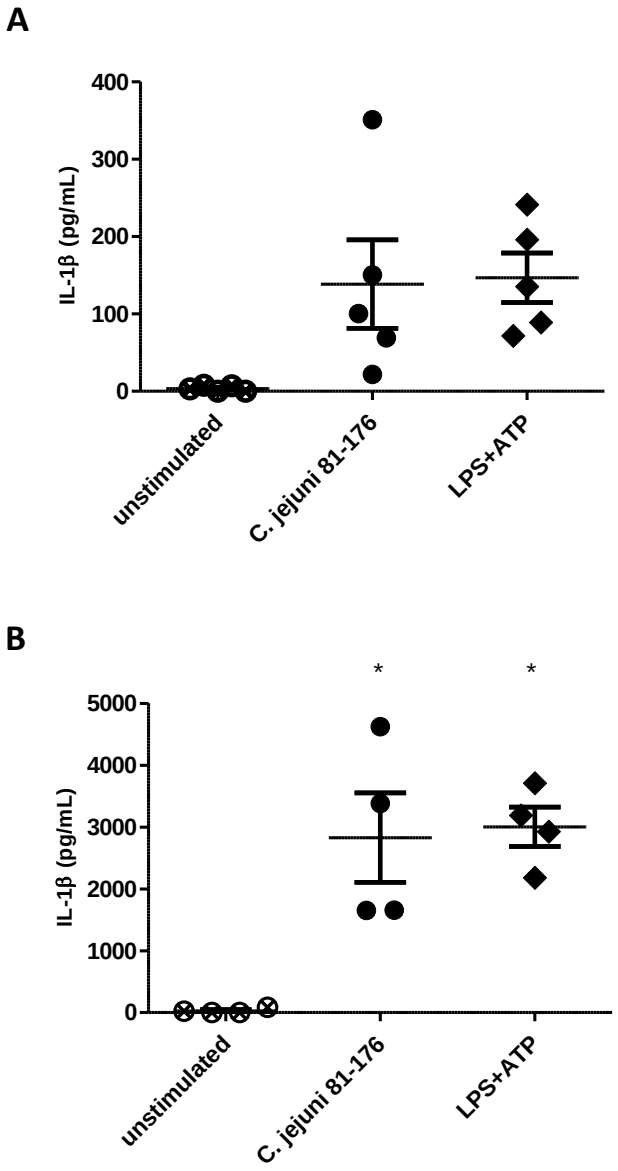


Figure 5.8 *C. jejuni*-mediated IL-1β secretion in by human neutrophils and PBMCs.

Human neutrophils and PBMCs ($2x10^6$ cells/mL) were co-cultured with *C. jejuni* 81-176 at MOI 10 for 5h. Stimulation by LPS (200ng/mL) for 2h followed by addition of ATP (2.5mM) for 3h served as positive control. IL-1β secretion of **(A)** neutrophils and **(B)** PBMCs was determined by ELISA. Mean±SEM of five independent experiments. One-way ANOVA with Tukey post-test was performed for statistical analysis. No stars: not significant; * $p<0.05$.

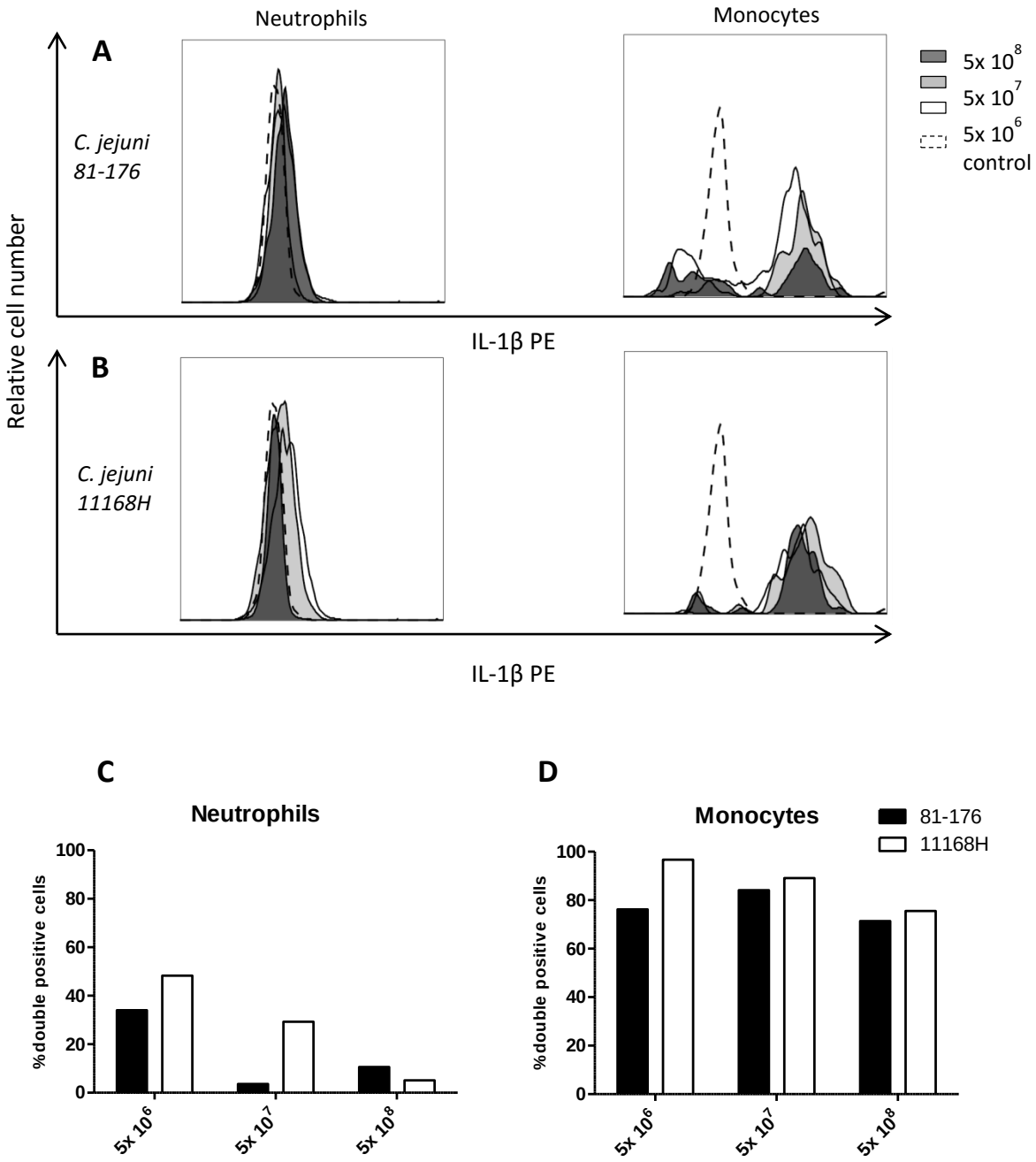


Figure 5.9 Dose-dependent *C. jejuni*-mediated generation of IL-1β in neutrophils and monocytes.

Heparinised whole blood was co-cultured with **(A)** *C. jejuni* 81-176 or **(B)** *C. jejuni* 11168H at increasing bacterial inoculum (CFU/mL) for 5h. Cells were strained for specific cell surface markers and intracellular IL-1β prior to analysis by FACSCalibur. Percentage of double-positive cells for active IL-1β and the surface marker **(C)** CD66b (neutrophils) and **(D)** CD14 (monocytes). Data was obtained from one experiment.

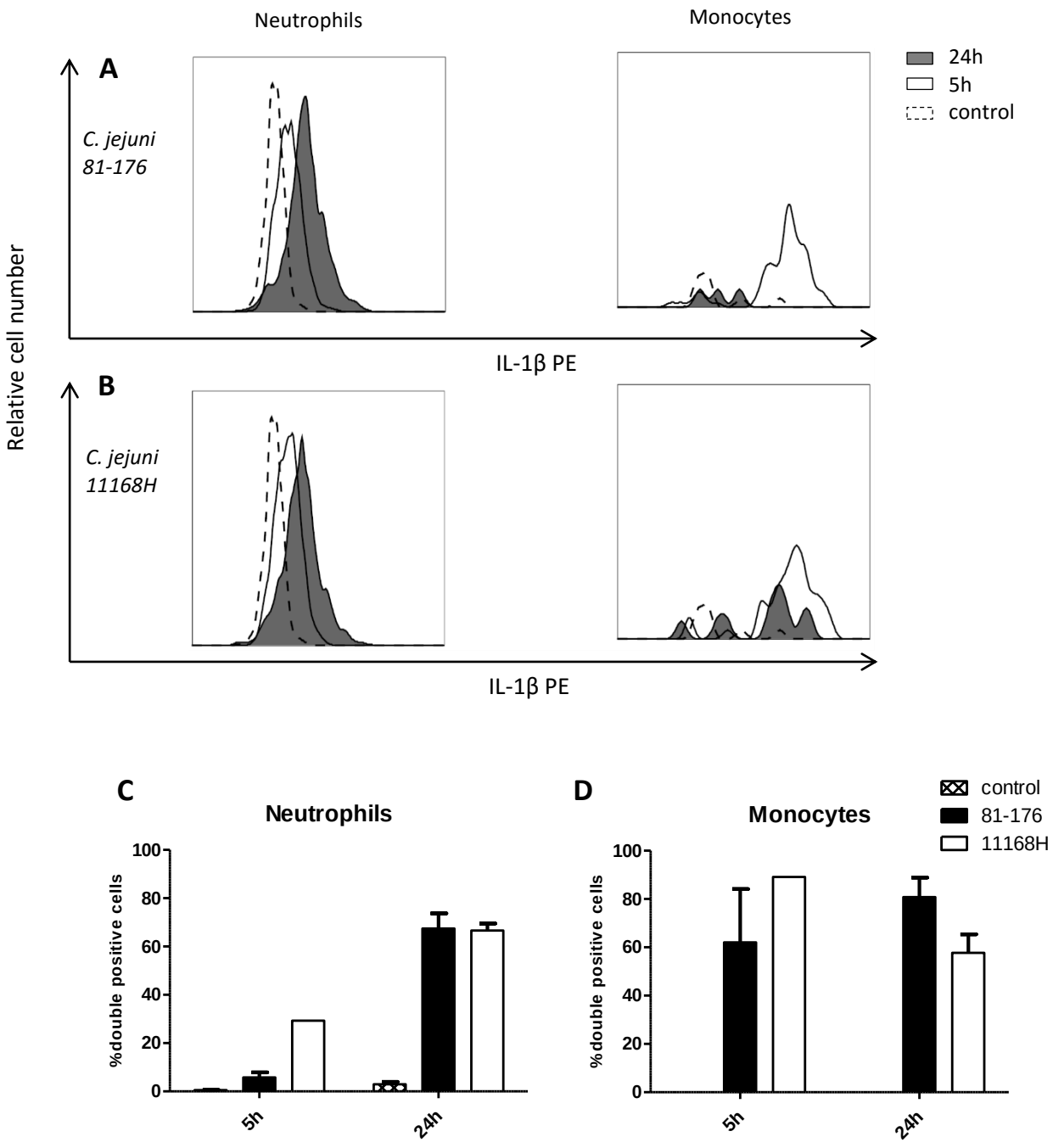


Figure 5.10 Time-dependent *C. jejuni*-mediated generation of IL-1β in neutrophils and monocytes. Heparinised whole blood was co-cultured with **(A)** *C. jejuni* 81-176 or **(B)** *C. jejuni* 11168H ($5x10^7$ CFU/mL) for 5h and 24h. Cells were strained for specific cell surface markers and intracellular IL-1β prior to analysis by FACSCalibur. Percentage of double-positive cells for active IL-1β and the surface marker **(C)** CD66b (neutrophils) and **(D)** CD14 (monocytes). Mean±SEM of two independent experiments.

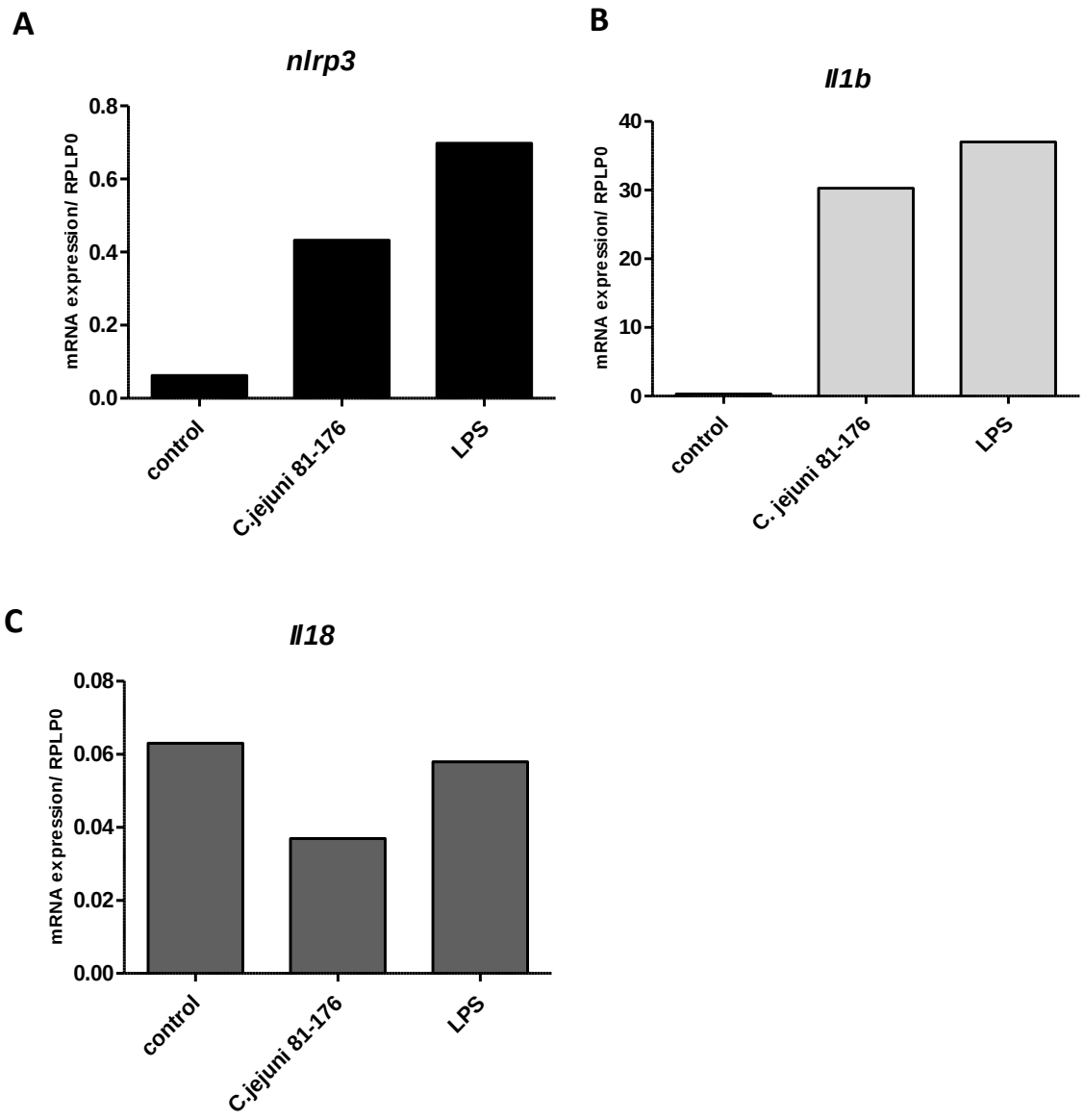


Figure 5.11 *C. jejuni*-mediated *nlrp3* and *Il1b* mRNA expression in human neutrophils.

Human neutrophils were co-cultured with *C. jejuni* 81-176 (MOI 10). Treatment with LPS (200ng/mL) served as positive control. mRNA was isolated and expression of **(A)** *nlrp3*, **(B)** *Il1b* and **(C)** *Il18* were determined by qPCR and normalised to expression of the house keeping gene *RPLP0*. Results were obtained from one experiment.

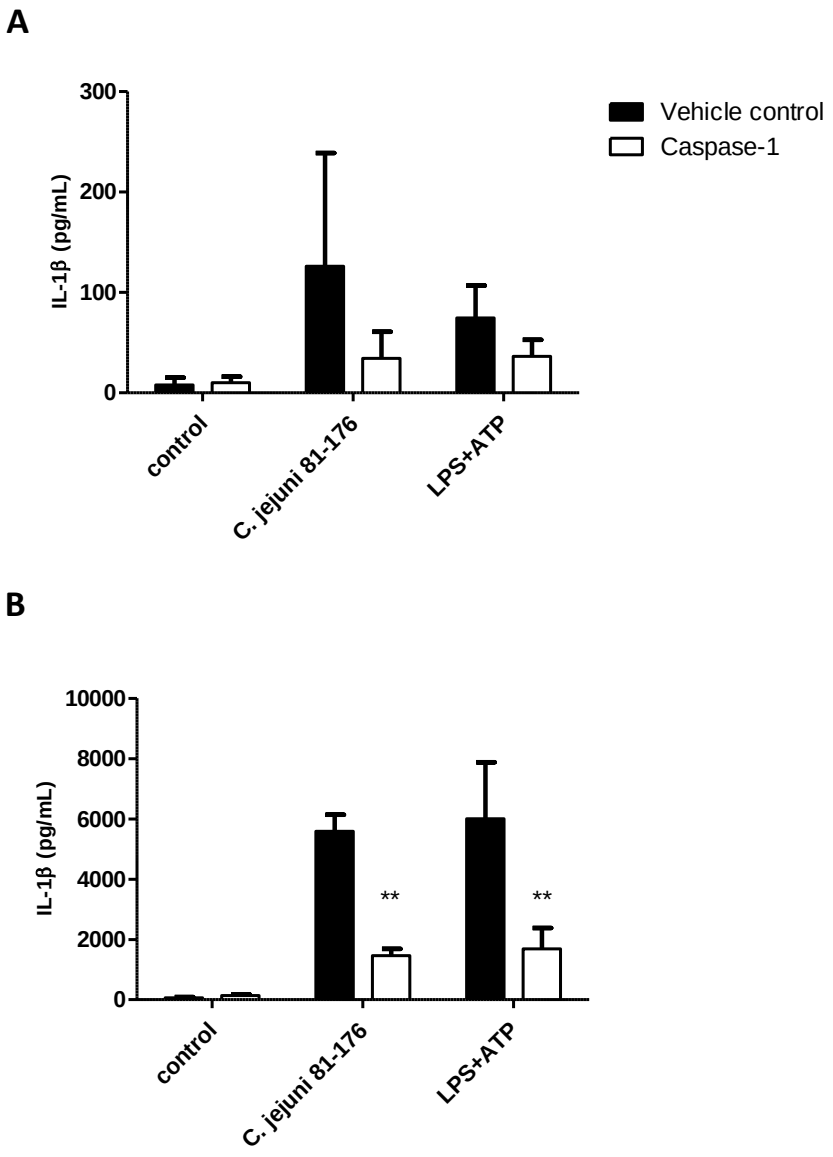


Figure 5.12 *C. jejuni*-induced IL-1β secretion is caspase-1 dependent.

Human neutrophils and PBMCs ($2x10^6$ cells/mL) were pre-treated with caspase-1 inhibitor (50µM) for 30min prior to co-culture with *C. jejuni* 81-176 (MOI 10). Stimulation with LPS (200ng/mL) for 2h followed by addition of ATP (2.5mM) for 3h served as control. IL-1β secretion by **(A)** neutrophils and **(B)** PBMCs was determined by ELISA. Mean±SEM of three independent experiments. One-way ANOVA with Tukey post-test was performed for statistical analysis. No stars: not significant; ** $p<0.01$

5.4 Discussion

Marked infiltration of neutrophils and elevated IL-1β levels are characteristic features found in *C. jejuni* infected tissue (Wassenaar and Blaser, 1999). No studies to date have investigated the role of neutrophils and Inflammasome activation in response to C. *jejuni*. In order to dissect a potential role of neutrophil-derived IL-1β secretion, we first assessed neutrophil cytokine response to the well-defined NLRP3-inflammasome priming and activator combination, LPS *plus* ATP. Upon stimulation of whole blood we observed high levels of IL-1β secretion. Further cell-specific analyses revealed monocytes as the primary source of the cytokine and secretion was found to occur in a caspase-dependent manner.

Neutrophils were the second cell type that contributed to whole blood IL-1β protein (T cells produced minimal amounts). Interestingly, the amount secreted by neutrophils was significantly less when compared to monocyte-derived production. LPS priming led to upregulation of *nlrp3* and *Il1b* mRNA expression. Co-culture with C. *jejuni* resulted in a similar pattern of Inflammasome activation as noted for LPS+ATP, indicating that during human infection, monocytes and neutrophils can be a source of IL-1β cytokine secretion.

Reports on cytokine expression by peripheral blood neutrophils have been and continue to be a topic of debate due to potential effects of contaminating monocytes. Monocytes are potent cytokine secretors so that even with high grade neutrophil purification protocols, the presence of monocyte-derived cytokines remains a possibility. Moreover neutrophils, in comparison to other leukocytes, possess 10 to 20 times less RNA per cell suggesting that a monocyte cell contamination of only 1% can actually result in up to 20% to 30% RNA contamination thus have a significant impact on gene expression analysis (Tamassia et al., 2014). Reported IL-10 secretion by murine and human neutrophils is a good example for the importance of these analytic difficulties (De Santo et al., 2010, Kasten et al., 2010). While the generation of IL-10 has been confirmed in murine neutrophils, highly purified human neutrophils have been shown to lack IL-10 mRNA and protein expression disproving previous publications and stressing the importance of the experimental setting when evaluating neutrophil-derived cytokine production (Davey et al., 2011).

We utilised intracellular FACS analysis to assess the presence of cleaved IL-1β in $CD66b^{+}$ neutrophils and observed a modest increase after LPS-stimulation while, in accordance with the ELISA results, the effect in monocytes was much more prominent. We further observed no or little cytokine production in a WAS patient also presenting with reduced IL-1β secretion, giving validity of our experimental approach. LPS stimulation in macrophages and DCs promotes cell-priming by TLR4 engagement, but this signal is insufficient and a second, distinct danger signal is required for

the Inflammasome activation and cytokine release. Netea *et al.* were the first to demonstrate that human blood monocytes, in contrast to differentiated macrophages or DCs, exert notable IL-1β secretion upon stimulation with TLR2 and TLR4 ligands in absence of a second exogenous stimulus. This is due to the presence of constitutively active caspase-1 and the release of endogenous ATP in monocytes (Netea et al., 2009). Little is known about mechanisms involved in neutrophil Inflammasome activation and further studies are required to assess the activity status of caspase-1. Similar to our findings, Gabelloni *et al.* observed release of IL-1β in LPS-stimulated neutrophils which was further increased by ATP. In both cases, secretion was partly caspase-dependent but a significant, Inflammasome-independent reduction was observed by the addition of the neutrophil elastase inhibitor Elafin while blocking of CatG had no effect (Gabelloni et al., 2013). Direct cleavage of IL-1β by neutrophil serine proteases was also observed in a mouse model upon infection with *P. aeruginosa.* We observed a reduction of IL-1β upon pre-exposure to caspase inhibitor in both monocytes and neutrophils. In contrast to Gabelloni and colleagues, we observed minimal cytokine reduction upon inhibition of CatG while Elafin had little effect on IL-1β generation. Future studies must clarify these findings.

We went on to assess Inflammasome activation in response to *C. jejuni*. Upon bacterial co-culture potent secretion of IL-1β in whole blood and monocytes was detected whereas low levels were found in infected neutrophils. Interestingly, while secretion was found independent to the inoculation dose, an elongated co-culture 24h led to a substantial increase in the cytokine-producing neutrophil population. Caspase inhibitor significantly decreased IL-1β secretion in the monocyte compartment and to a lesser extent in the neutrophil fraction.

Gene expression analysis indicated upregulation of the NLRP3-inflammasome by *C. jejuni*, similarly to LPS stimulation. A recent study by Bouwman *et al.* reported *C. jejuni*-induced NLPR3-mediated Inflammasome activation in murine macrophages and in the monocytic THP-1 cell-line. *Campylobacter* LOS is a potent TLR4-agonist while its flagellum evades recognition by TLR5 indicating a NLRP3 dependent and NLRP4 independent mechanism is likely at play (de Zoete et al., 2010). It was interesting to note that the IL-1β protein levels reported in the publication were approximately ten-fold higher than what has been previously described in differentiated THP-1 cells and were comparable to *S. typhimurium* infection, contradicting observations made by us and others in human and murine DCs (Jones et al., 2003, Stephenson et al., 2014, Hu et al., 2006). Unlike macrophages and DCs, however in this study we did observe potent generation of monocytic IL-1β in response to *C. jejuni*, which may be explained by the presence of constitutively active caspase-1 (Netea et al., 2009).

Taken together, we report for the first time the generation of IL-1β in *C. jejuni*-infected human neutrophils. As this cell-type outnumbers any other immune cell by multi-folds during inflammation, it is possible that IL-1β secretion by neutrophils plays a role in mounting an inflammatory response to *C. jejuni*, especially in the acute phase of infection. Further analyses, in particular by using a highly purified neutrophil population are required to confirm this hypothesis.

CHAPTER VI

CAMPYLOBACTER CONCISUS – ELUCIDATING THE LIPOOLIGOSACCHARIDE (LOS) STRUCTURE AND ITS PRO-INFLAMMATORY POTENTIAL

6.1. Background

C. concisus is a member of the *Campylobacter* genus with yet unclear pathogenic potential. While initial research focused on periodontal disease more recent data suggests a role for these non-*jejuni*/*coli* strains in acute and chronic human GI conditions (Kaakoush and Mitchell, 2012, Nielsen et al., 2013). When compared to control tissue, increased prevalence of *C. concisus* DNA has been recorded in colonic biopsies from adults with ulcerative *coli*tis (Mukhopadhya et al., 2011). Similar results were also observed in faecal samples of paediatric patients with newly diagnosed Crohn's disease (Man et al., 2010b). Other studies however have not seen such an association, and a high prevalence in patients and healthy individuals suggests a more commensal-like nature for these emerging species (Van Etterijck et al., 1996).

C. concisus can attach and invade IECs and induce the secretion of pro-inflammatory cytokines, potentially in a TLR4 dependent mechanism (Man et al., 2010a, Ismail et al., 2013). Bacterial LPS/LOS activates TLR4 and serves as important virulence factors for Gram-negative bacteria. Apart from TLR4 engagement, *C. jejuni* LOS has shown to play a role in bacterial invasion, colonisation and stress survival (Muller et al., 2007, Naito et al., 2010, Louwen et al., 2008). LOS-TLR4 interaction triggers a downstream signalling cascade activating NF-κB transcription factor and subsequent secretion of pro-inflammatory cytokines such as TNF-α and IL-8. The hydrophobic lipid A backbone of *C. jejuni* is hexaacylated and a potent activator of TLR4 (Rathinam et al., 2009a). *H. pylori* on the contrary, another member of the *Campylobacter* genus, is tetraacylated exhibiting low reactivity to TLR4 (Lepper et al., 2005).

C. jejuni is prone to frequent phase variations in the LOS gene loci resulting in great structural heterogeneity amongst strains (Parker et al., 2005). Major differences are observed in the outer core of the oligosaccharide (OS) moiety but variations in lipid A phosphorylation and number of amide linkages are also present (Dorrell et al., 2001). The lipid A disaccharide of *C. jejuni* comprises of either 2-amino-2-deoxy-D-glucose (glucosamine, GlcN) or 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N) thus the number of amide linkages to the acyl chains can vary between two (GlcN-GlcN) to four (GlcN3N-GlcN3N). The lipid A component is hexaacylated with either myristic (C14:O) or palmitic (C16:O) fatty acids; four of which have are hydroxy-fatty acids and are primarily linked to the disaccharide residue (for details, see Chapter I, Figure 1.3). There is great heterogeneity in the positioning and number of phosphate (P) and phosphoethanolamine (PEA) residues bound to the disaccharide backbone (Moran et al., 1991). Amide linkages and phosphorylation has been shown to impact TLR4 activation as reported by us and others (Stephenson et al., 2013, Cullen et al., 2013). While the inner OS core of *C. jejuni* is primarily conserved, large variations in the outer core

region have been found (Dorrell et al., 2001). Of these, especially the number of N-acetylneuarminic acid (NeuAc, sialic acid) residues are known to modulate TLR4 activation (Kuijf et al., 2010, Stephenson et al., 2013, Heikema et al., 2013). Recently, there has been some progress in the understanding of *C. jejuni* LOS and its role in human disease pathogenesis. The structure and heterogeneity of *C. concisus* LOS to date remains unstudied whilst its pathogenic and/or commensal nature in humans is unclear. The aim of this study was to delineate the LOS structure of *C. concisus* isolates and to determine if there is a structural similarity to its pathogenic family member *C. jejuni* which could give insight in its pro-inflammatory potential.

6.2 Heterogeneity between *C. concisus* lipooligosaccharide (LOS) structures

Mass spectrometry is the method of choice for the study of LPS structures through the characterisation of the lipid A and the polysaccharide component. The methodology is well described in a recent review from Banoub and colleagues (Banoub et al., 2010). To assess *C. concisus* LOS, we utilised a total of six *C. concisus* strains of mostly paediatric, GI origin (See, Chapter II, Table 2.2). Sample preparation and MALDI-TOF analysis were conducted under the direct guidance of Dr. Constance John and Dr. Nancy Phillips (University of California San Francisco, USA). LOS was successfully extracted from all isolates however the LOS yield of *C. concisus* B124 was low and analysis was only partly possible. The LOS profile determined by silver staining revealed multiple LOS bands for each sample and great heterogeneity between *C. concisus* isolates. The top bands of the separation pattern suggest the presence of higher mass LOS in most *C. consisus* isolates when compared *to C. jejuni* or *N. meningitides* LOS which were included for molecule size comparison (Figure 6.1). *C. concisus* isolate B124 failed to be visualised in the long gel most likely due to insufficient LOS load (Figure 6.1 B).

High resolution negative-ion MALDI-TOF spectra of intact LOS showed multiple molecular ion LOS residues in each sample with great diversity between strains thus confirming the observations made by silver staining (Figure 6.2). In accordance to the LOS gel profile, the molecular ions of *C. concisus* isolates were much larger than what has been previously reported by negative-ion MALDI-TOF analysis of *C. jejuni* and *N. meningitidis* LOS. We observed molecular ion above *m/z* 5000 in all samples apart from *C. concisus* 2010-112825 (Figure 6.2 D) confirming the absence of higher mass LOS bands by silver staining in the latter (Figure 6.1 B). In comparison, molecular ions for *C. jejuni* 11168H or *N. meningitidis* 89I LOS were observed in the range of *m/z* 4292 and *m/z* 4008, respectively (data not shown).

Differences in the drift time of the fragment ions allowed for the application of ion mobility separation (IMS) MALDI-TOF which was used to separate intact LOS molecular ions, OS and lipid A fragments (Figure 6.3). Most *m/z* of molecular ions and OS fragments observed were unique but similarities were observed in lipid A fragment ions between samples. A similar molecular ion peak was observed at *m/z* 4192.7 for *C. concisus* B124 and 2010-112825 (Table 6.1).

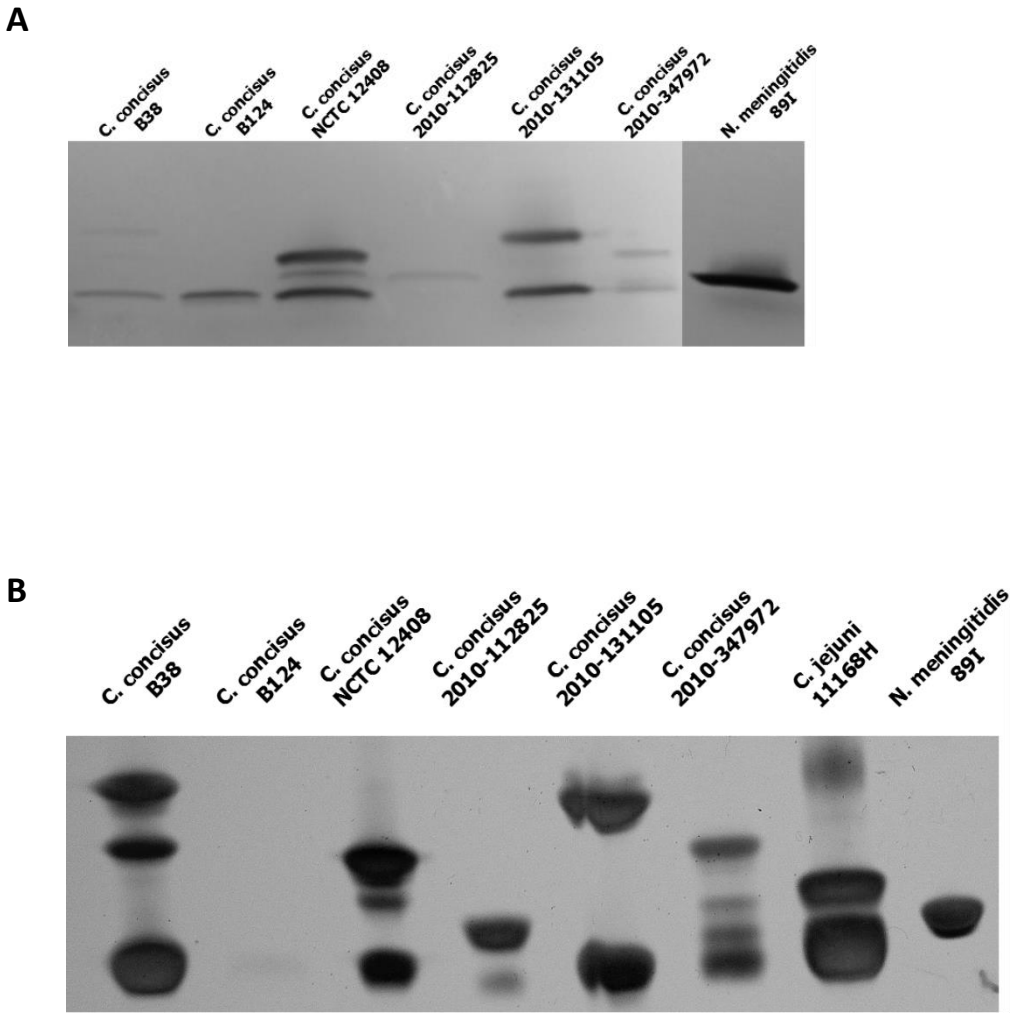


Figure 6.1 Lipooligosaccharide (LOS) profiles of *C. concisus* isolates.

Purified LOS of *C. concisus* isolates were separated by SDS-PAGE and subjected to silver staining using silver nitrate. **(A)** Samples (1µg LOS) were separated by mini-protean SDS-PAGE (12% acrylamide). *N. meningitidis* 98I LOS served as control. **(B)** Samples (10µg LOS) separated by a long gel (12.9% acrylamide) prior to silver staining. *C. jejuni* 11168H and *N. meningitidis* 89I LOS served as controls.

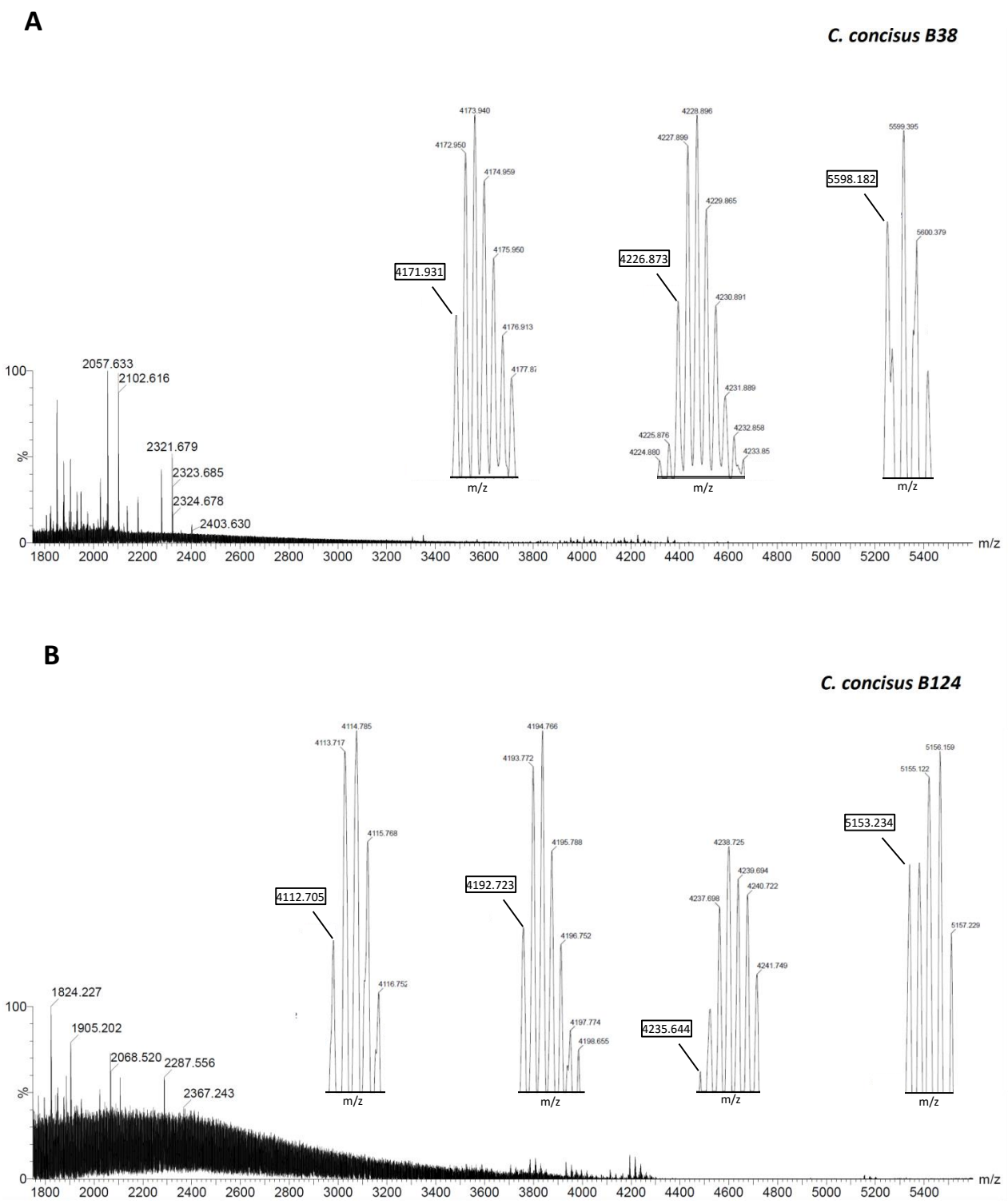


Figure 6.2-A, B Negative-ion MALDI-TOF spectra for intact *C. concisus* LOS.

Negative-ion MALDI-TOF spectra for the LOS of *C. concisus* isolates **(A)** B38 and **(B)** B124. In the insets of the high resolution spectra the labelled masses represent the monoisotopic ions of intact LOS.

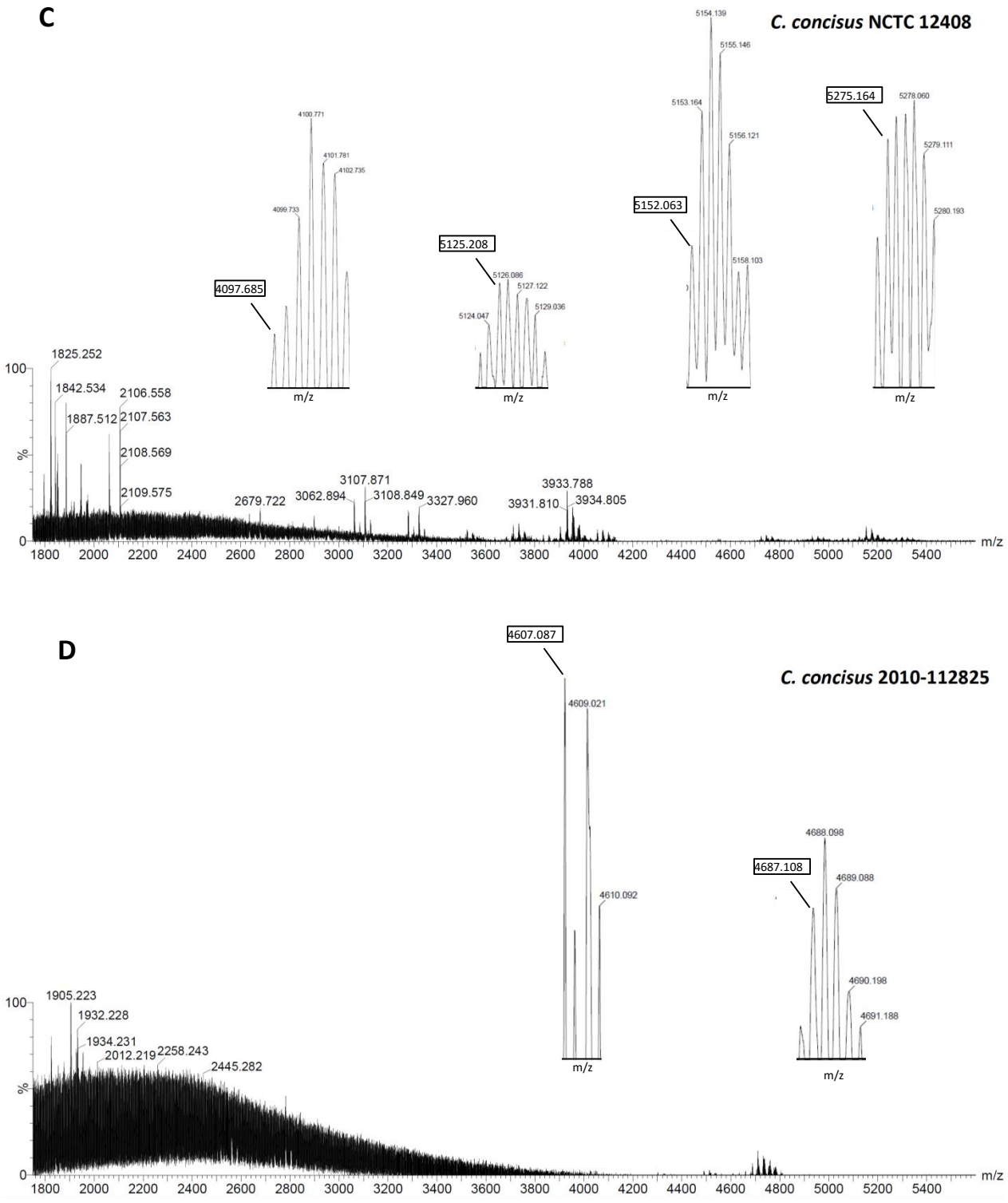


Figure 6.2-C, D Negative-ion MALDI-TOF spectra for intact *C. concisus* LOS.

Negative-ion MALDI-TOF spectra for the LOS of *C. concisus* isolates **(C)** NCTC12408 and **(D)** 2010-112825. In the insets of the high resolution spectra the labelled masses represent the monoisotopic ions of intact LOS.

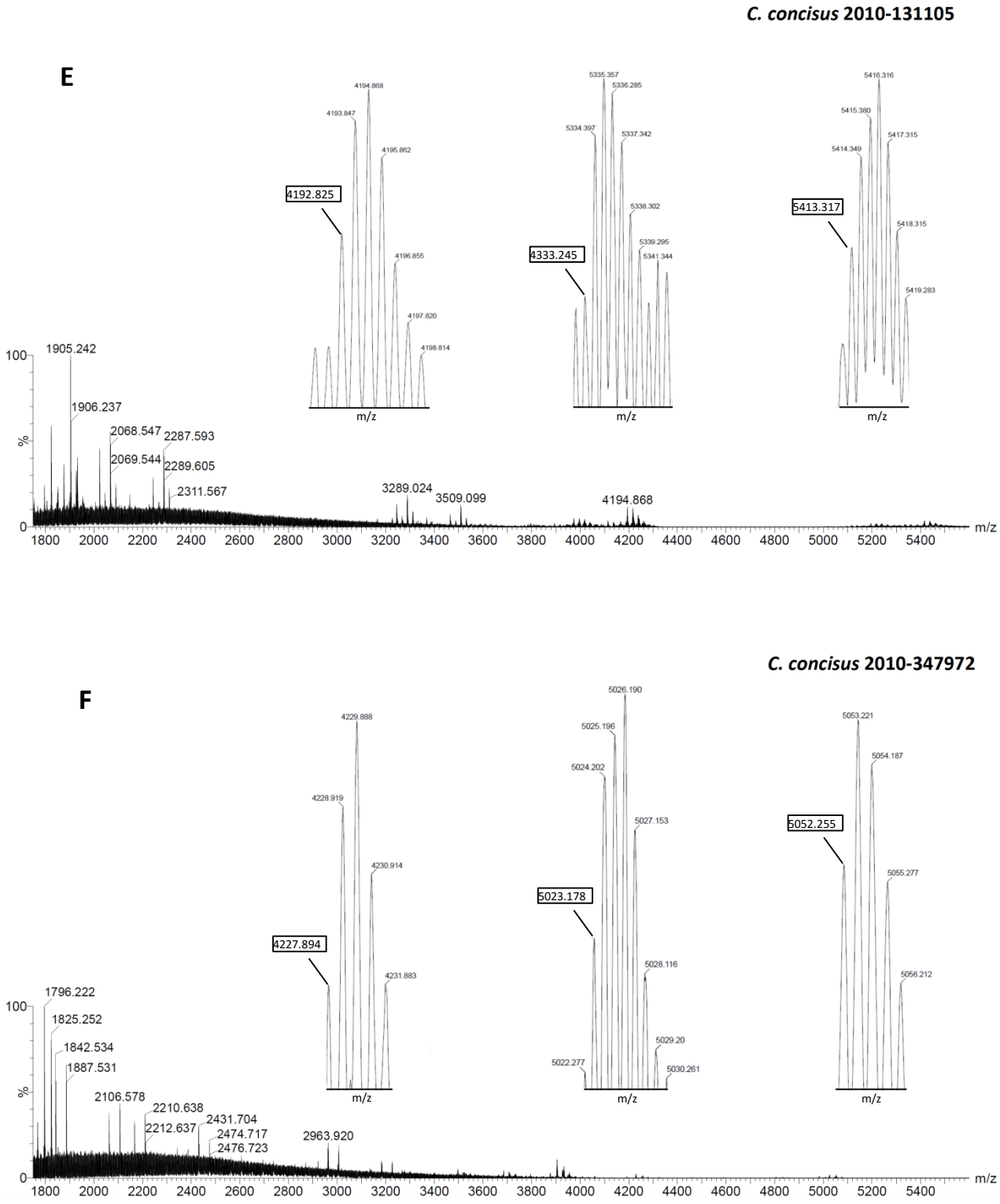


Figure 6.2-E, F Negative-ion MALDI-TOF spectra for intact *C. concisus* LOS.

Negative-ion MALDI-TOF spectra for the LOS of *C. concisus* isolates **(E)** 2010-1311105 and **(F)** 2010-347972. In the insets of the high resolution spectra the labelled masses represent the monoisotopic ions of intact LOS.

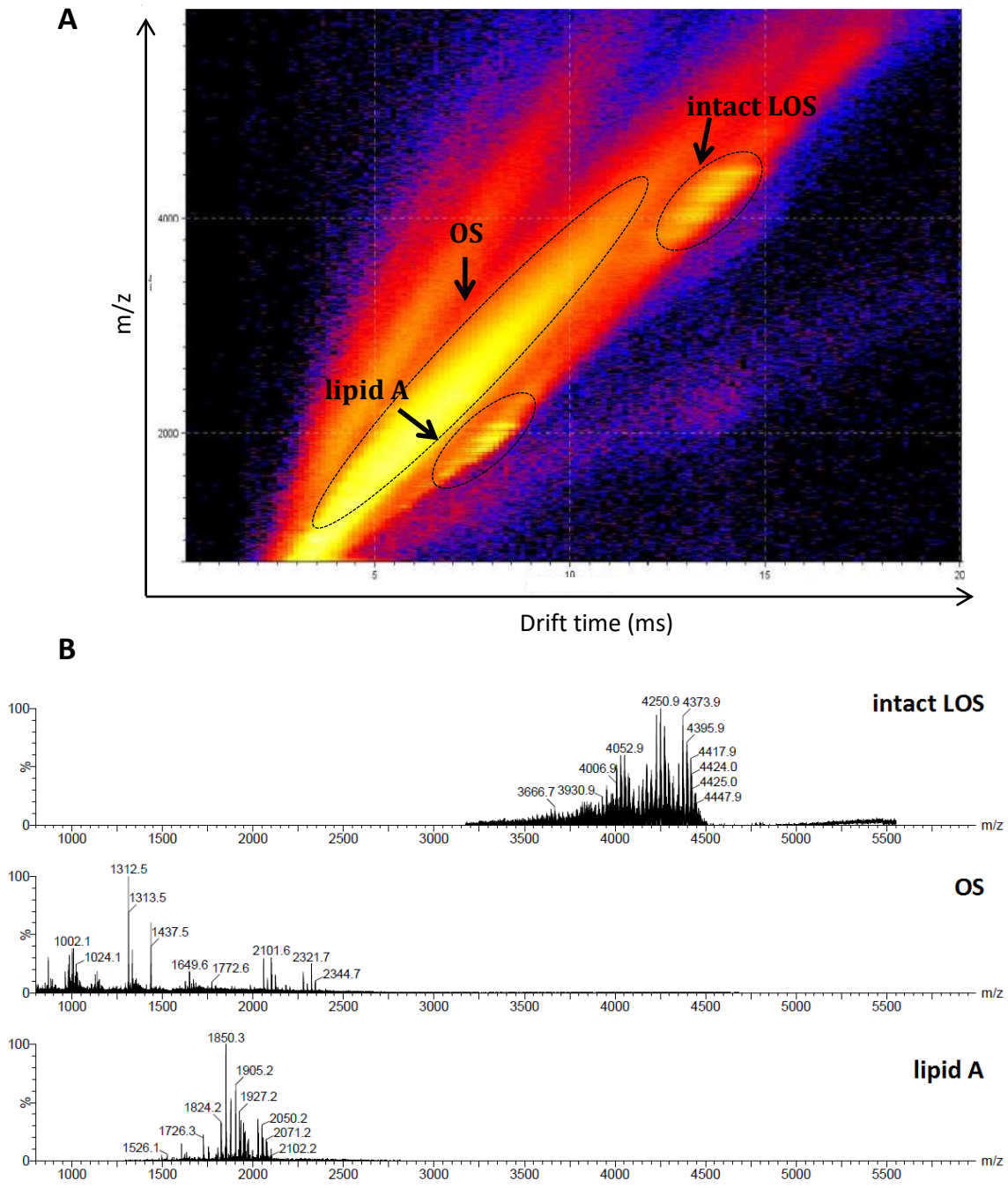


Figure 6.3 LOS fragment separation by Ion mobility separation (IMS) MALDI-TOF.

(A) Spectrum of negative-ion MALDI-TOF analysis with the use of tri-wave ion mobility separation (IMS) of intact LOS of *C. concisus* B38. **(B)** Differences in drift time allow for separation of the intact LOS, oligosaccharide (OS) and lipid A fragments from low molecular weight components of non-LOS origin. Spectra were acquired with the Synapt G2 HDMS mass spectrometer using ion mobility separation (IMS) and analysed with DriftScope and MassLynx software.

C. concisus isolates	Lipid A m/z	OS m/z	Intact LOS $(M-H)^-$
B38	1849.271	2321.679	4171.931
	1904.203	2321.679	4226.873
			5598.182
B124	1824.227	2287.556	4112.705
	1904.208	2287.556	4192.723*
	1947.219	2287.556	4235.644
			5152.573
NCTC 12408			4097.685
	1797.206	3326.924	5125.208
	1824.252	3326.924	5152.063
	1947.237	3326.924	5275.164
2010-112825	1824.207	2781.879	4607.087
	1904.209	2781.879	4687.108
2010-131105	1904.228	2287.593	4192.825*
	1824.263	3508.087	5333.245
	1904.228	3508.087	5413.317
2010-347972	1796.222	2430.710	4227.894
	1796.222	3225.978	5023.178
	1824.260	3225.978	5052.255

Table 6.1 Observed peaks for molecular ions of intact LOS and fragment ions of lipid A and oligosaccharide (OS).

High-resolution negative-ion MALDI-TOF and MALDI-TOF IMS mass spectra revealed prominent peaks for molecular ions of intact LOS and respective oligosaccharide (OS) and lipid A fragments of *C. consisus* isolates.

6.3 Structural analysis of the Lipid A moiety

C. concisus LOS showed similarities in the lipid A ion peaks (Table 6.1). We performed negative-ion IMS-MS/MS analysis to reveal the structural composition of the lipid A ion fragment. Differences in the drift time of the lipid A ion fragment allows for the elimination of fragments of non-lipid A origin resulting in a better quality MS/MS spectrum for interpretation here shown for the lipid A fragment at *m/z* 1824.3 (Figure 6.4).

Next, hydrazine-treatment of LOS was performed as it promotes removal of the O-linked acyl chains from the lipid A moiety whilst the more robust N-linked bonds remain intact. IMS-MS/MS analysis of O-deacylated lipid A fragment at m/z 979.5 was in accordance with the presence of a disaccharide containing two HexN residues with the hydroxy-fatty acids myristic acid (C14:0(3-OH)) and palmitic acid (C:16(3-OH)) on the reducing and non-reducing sugar residue, respectively. We further observed evidence for two phosphate residues. Mass peaks corresponding to B and Y-fragments as well as ring fragmentations (^{0,2}A and ^{0,4}A) gave indication for two lipid A structures, one with phosphate on either end (di-phosphorylated) or two phosphate residues on the non-reducing end (bis-phosphorylated) (Figure 6.5).

We went on to analyse the intact lipid A ion fragments and identified a peak at *m/z* 1824.3 which corresponded to the O-deacylated fragment at *m/z* 979.5. Negative-ion IMS-MS/MS analysis was in accordance with mass losses corresponding to C14:0(3-OH), lauric acid (C12:0) and myristic acid (C14:0) residues (Figure 6.6). Evidence for B- and Y-ions and ^{0,4}A ring fragments indicating the existence of di-and bis-phosphorylated hexaacylated lipid A (Figure 6.7). GC-MS analysis confirmed the presence of C14:0, C14:0(3-OH) and C16:0(3-OH) but unfortunately no evidence for C12:0 was found possibly due to loss during sample preparation (Figure 6.8).

Taken together, we identified a total of five different lipid A masses in the spectra of *C. concisus* LOS (Table 6.2). All lipid A fragments were in accordance with a disaccharide of two HexN residues thus contained two amide linkages. Higher mass fragments above *m/z* 1824.2 corresponded with the fatty acid profile identified for this fragment, there was evidence for the exchange of a C14:0(3-OH) for the C16:0(3-OH) residue for the lower mass fragment *m/z* 1796.2. Differences in the number of phosphate or PEA corresponded to the mass increase of higher mass lipid A fragments observed in *C. concisus* isolates (Table 6.2).

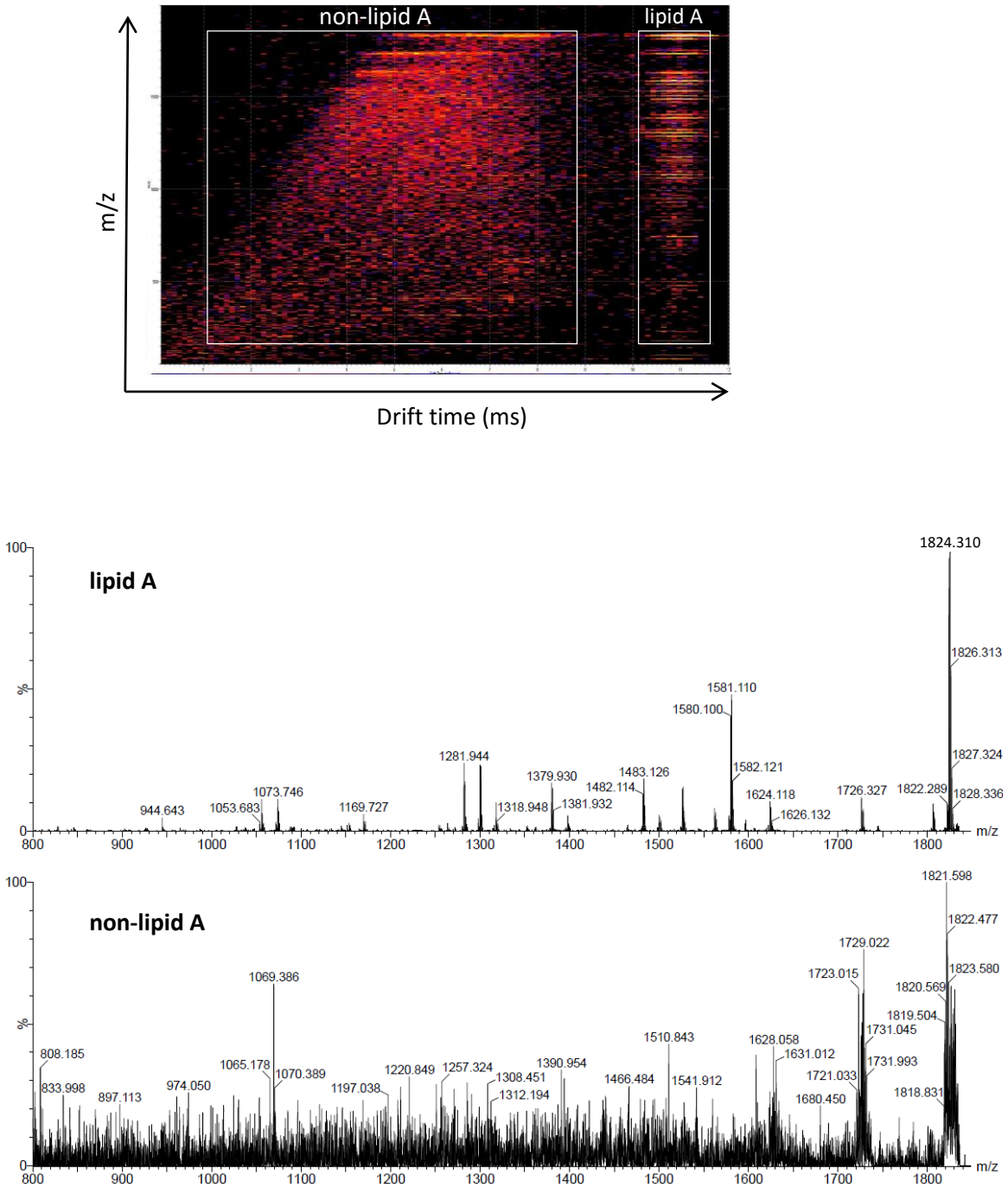
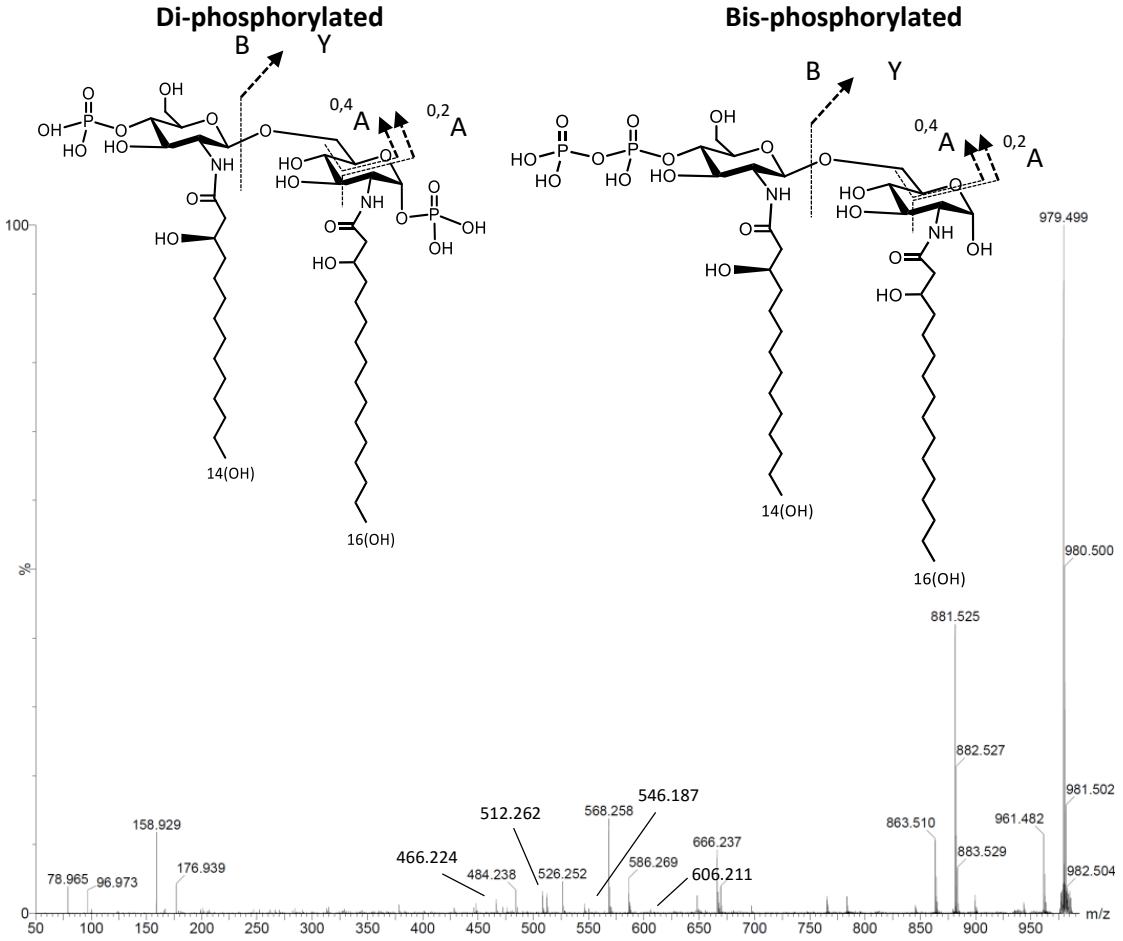


Figure 6.4 IMS-MS/MS separation of Lipid A fragment.

Negative-ion IMS-MS/MS spectra of lipid A fragment (*m/z* 1824.310) of *C. concisus* 2010-131105. Differences in drift time was used to separate fragments originating from the ions of interest (lipid A) from carbohydrate ions and ions of non-lipid A origin (non-lipid A). Spectra were acquired by Synapt G2 HDMS and analysed using DriftScope and MassLynx software.



	Di-phosphorylated		Bis-phosphorylated	
	Calculated m/z	**Observed m/z (Δppm)**	**Calculated m/z**	**Observed m/z (Δppm)**
Ion	979.491	979.499 (8.17)	979.491	979.499 (8.17)
B- ion	466.220	466.224 (8.58)	546.187	546.188 (1.83)
Y- ion	512.262	512.265 (5.28)	432.296	N/A
^{0,4}**A**	526.263	526.252 (-20.90)	606.229	606.211 (-29.69)
^{0,2}**A**	586.241	586.269(47.76)	666.208	666.237 (43.53)

Figure 6.5 Proposed structure and negative-ion MS/MS spectrum of O-deacylated lipid A fragment (*m/z* 979.499) of *C. concisus* 2010-131105.

Mass peaks observed in the MS/MS spectrum of the O-deacylated lipid A fragment were consistent with B, Y, ^{0,4}A and ^{0,2}A ion fragments indicated in the proposed lipid A structures. Fragment ions are listed in the table and are in accordance with the presence of di- and bis-phosphorylated lipid A moieties.

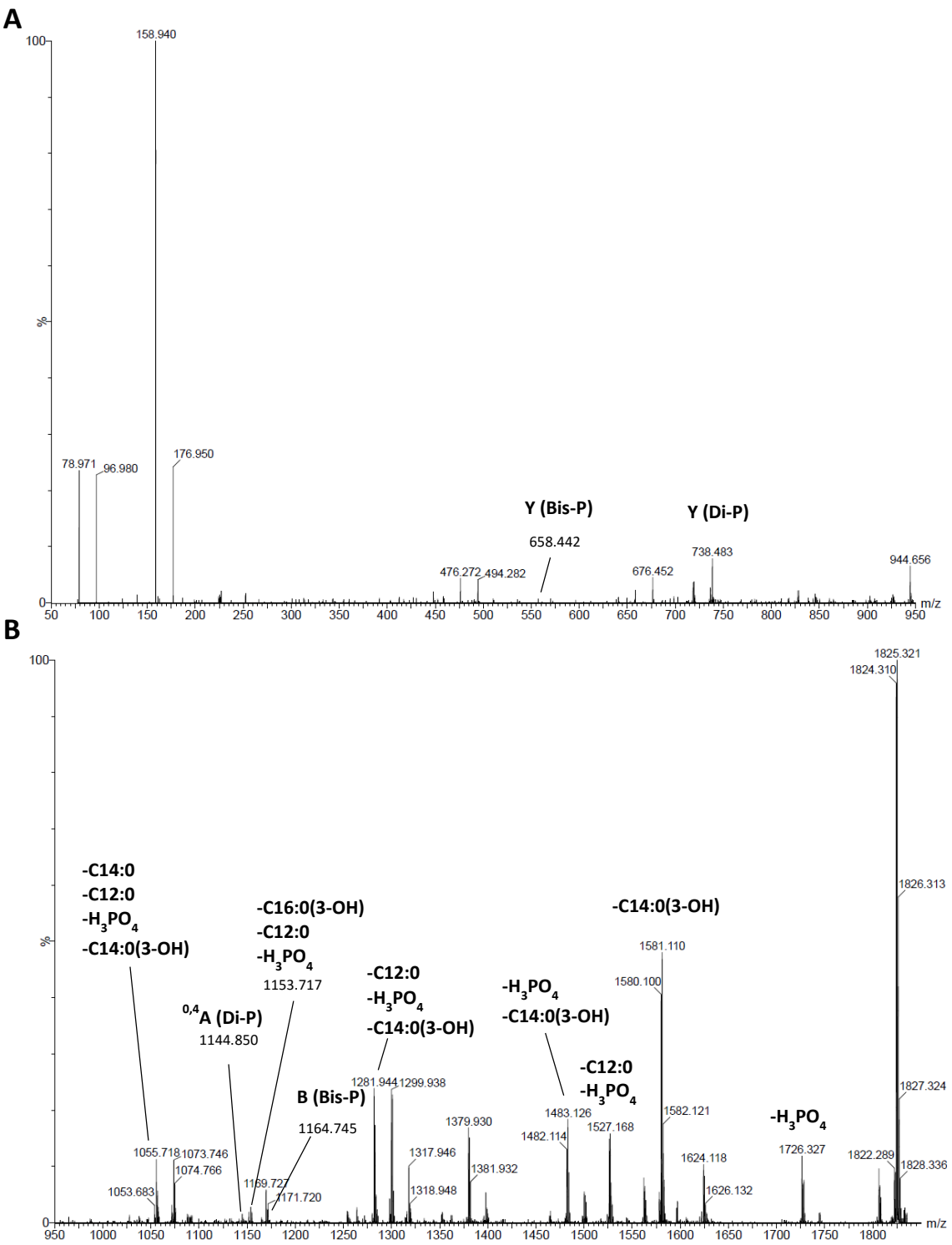
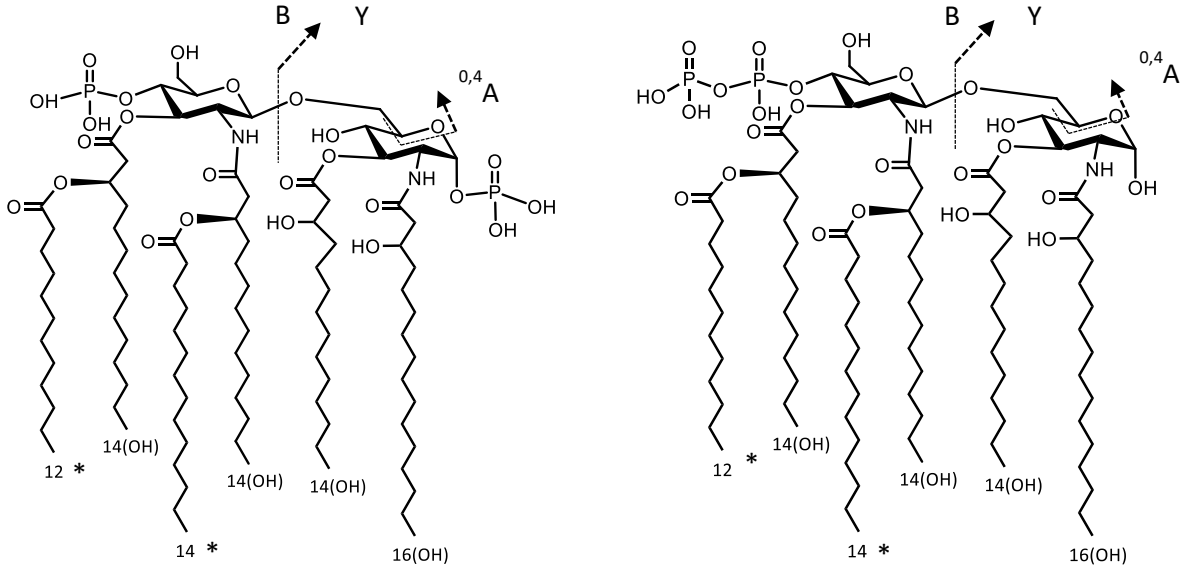


Figure 6.6 Negative-ion IMS-MS/MS spectra of lipid A fragment M-H$^-$ (*m/z* 1824.310) of *C. concisus* 2010-131105.

(A) Low mass and **(B)** high mass negative-ion IMS-MS/MS spectra of *C. concisus* LOS. Indicated mass losses were in accordance with the fatty acids hydroxy-myristic acid (C14:0(3-OH), hydroxy-palmitic acid (C16:0(3-OH)), myrisitic acid (C14:0), lauric acid (C12:0), and phosphate (P). Mass peaks are consistent with Y-type fragment ions for bis-phosphorylated (Y Bis-P) and di-phosphorylated (Y Di-P) were also observed.

Di-phosphorylated **Bis-phosphorylated**



	Di-phosphorylated		Bis-phosphorylated	
	Calculated m/z	**Observed m/z (Δppm)**	**Calculated m/z**	**Observed m/z (Δppm)**
Ion	1824.243	1824.310 (36.73)	1824.243	1824.310 (36.73)
B- ion	1084.779	N/A	1164.745	1164.861 (99.59)
Y- ion	738.456	738.483 (36.56)	658.489	658.452 (-56.19)
^{0,4}A	1144.822	1144.850 (24.46)	1224.788	N/A

Figure 6.7 Structure and fragment ions observed in negative-ion IMS-MS/MS spectrum of lipid A fragment *m/z* 1824.310 of *C. concisus* 2010-131105.

Mass peaks observed in the MS/MS spectrum of the lipid A fragment (*m/z* 1824.310) were consistent with B, Y, ^{0,4}A and ^{0,2}A ion fragments indicated in the proposed lipid A structures. Fragment ions are listed in the table and are in accordance with the presence of di- and bis-phosphorylated lipid A moieties. Asterisk (*) indicates potential interchange in the positions of two fatty acids.

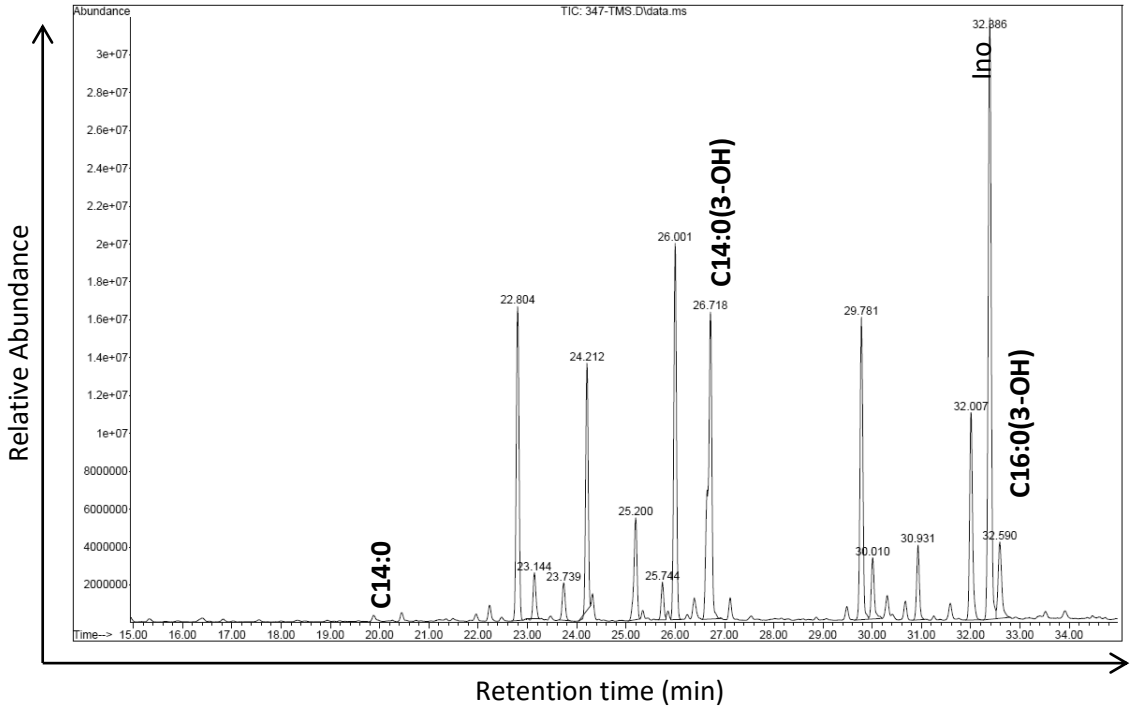


Figure 6.8 GC-MS spectrum of *C. concisus* LOS after trimethylsilyl (TMS) derivatisation.

Trimethylsilyl (TMS) derivatives of hydrolysed LOS of *C. concisus* 2010-347972 was subjected to GC-MS analysis. Elution peaks observed were in accordance with hydroxy-myristic acid (C14:0(3-OH), hydroxy-palmitic acid (C16:0(3-OH)) and myrisitic acid (C14:0). Sample preparation and GC-MS analysis were performed by Dr. Biswa Choudhury (Glycotechnology Core Resource, University of California San Diego).

Calculated m/z	**1796.211**	**1824.243**	**1849.274**	**1904.209**	**1947.251**
Proposed structure	HexN-HexN 4 C14:0(3-OH) C14:0 C12:0 2P	HexN-HexN 3 C14:0(3-OH) C16:0(3-OH) C14:0 C12:0 2P	HexN-HexN 3 C14:0(3-OH) C16:0(3-OH) C14:0 C12:0 2P PEA ($-H_3PO_4$)	HexN-HexN 3 C14:0(3-OH) C16:0(3-OH) C14:0 C12:0 3P	HexN-HexN 3 C14:0(3-OH) C16:0(3-OH) C14:0 C12:0 2P PEA
Observed m/z (Δppm)					
B38			1849.271 (-1.81)	1904.216 (3.72)	
B124		1824.227 (-8.57)		1904.208 (-0.48)	1947.219 (-16.52)
NCTC 12408	1796.203 (-4.63)	1824.241 (-0.90)			1947.237 (-7.28)
2010-112825		1824.275 (17.74)		1904.218 (4.77)	
2010-131105		1824.263 (11.16)		1904.228 (10.03)	
2010-347972	1796.222 (5.95)	1824.260 (9.52)			

Table 6.2 Proposed lipid A structures of *C. concisus* isolates.

Proposed structures of prominent lipid A peaks observed in negative-ion mass spectra of intact LOS of *C. concisus* isolates**.** Exact mass units were used for calculation of the following molecular masses (in Da) based on proposed compositions, as follows: Hexosamine (HexN), 161.0688; Hydroxy-myristic acid (C14:0(3-OH), 226.3592, Hydroxy-palmitic acid (C16:0(3-OH)), 254.2246; Myrisitic acid (C14:0), 210.3598; Lauric acid (C12:0), 182.1671; Phosphate (P), 79.9663; Phosphoethanolamine (PEA), 123.0085. Differences between observed and calculated exact masses for the negative ion peaks observed were expressed in parts per million (ppm).

6.4 Composition analysis of the oligosaccharide (OS) moiety

While the lipid A moiety was found to be relatively conserved between the strains we identified unique mass peaks (*m/z*) corresponding to OS fragment ions in the LOS spectra of each isolate. Positive-ion MS/MS analysis revealed an inner core fragment (*m/z* 1888.6) to be at least partly present in each strain (Table 6.3). The observed mass losses were in accordance with 2-keto-3-deoxyoctulonosonic acid (KDO) and heptose (Hep) residues which are typically present in the inner core OS region found to be assembled on lipid A. We further identified a common fragment (*m/z* 1221.4) which was present in the LOS spectra of NCTC 12408 and 2010-131105 (Table 6.3). We utilised HF-treatment of the LOS of *C. consisus* NCTC12408 in order to remove phosphoester residues from the sample. The comparison of positive-ion MALDI-TOF spectra of untreated and HF-treated LOS samples suggested the existence of one phosphate residue as part of the inner core fragment (*m/z* 1888.6) (Figure 6.9). This was confirmed by MS/MS analysis of the untreated and HF-treated sample and mass losses were in accordance to typical OS residues such as KDO, Hep, hexose (Hex) and hexosamine (HexN).

We frequently observed a mass loss of *m/z* 175 possibly corresponding to hexaminuronic acid (HexNA) (Figure 6.10). MS/MS analysis of all samples allowed the proposal of parts or the full composition of the OS moiety. Results suggest similarities in the inner core fragment adjacent to lipid A but great differences in the composition and molecular mass of the total OS unit. Interestingly, there was no evidence for the existence of sialic acid in any of the isolates (Table 6.4).

Components	Inner core m/z	Common structure m/z
Proposed composition	KDO 3Hep 3Hex HexN 2HexNA P Me	2Hex HexN 2HexNA HexNAc dHex Me Na
Calculated m/z	1888.562	1221.421

C. concisus isolate	Inner core m/z (Difference from 1888.5)	Common structure m/z
B38	1314.564 -KDO -Hep -Hex	
B124	1314.589 (As determined by 2010-131105)	
NCTC 12408	2108.568 +KDO	1221.393
2010-112825	1314.589 -KDO -Hep -Hex	
2010-131105	1314.564 -KDO -Hep -Hex	1221.482
2010-347972	2108.606 +KDO	

Table 6.3 Oligosaccharide (OS) fragment ions observed in positive-ion MALDI-TOF MS spectra.
Positive-ion MALDI TOF analysis determined oligosaccharide (OS) masses (*m/z*) of *C. concisus* isolates. OS of all isolates contained the same or parts of a core structure (*m/z*) corresponding to *m/z* 1888.5. Observed mass differences consistent with additional or missing monosaccharide components from the inner core fragment were indicated as (+) or (-), respectively. A common structure (*m/z* 1221.421) was also observed in NCTC 12408 and 2010-131105. LOS yield of B124 was insufficient to permit detailed MS analysis. The observed common OS fragment is very likely contained in the OS fragment of 2010-131105. 2-keto-3-deoxyoctulonosinic acid (KDO); Heptose (Hep); Hexose (Hex); Hexosamine (HexN); Hexosaminuronic acid (HexNA); N-acetylhexosamine (HexNAc); Methyl (Me); Phosphate (P); Sodium (Na)

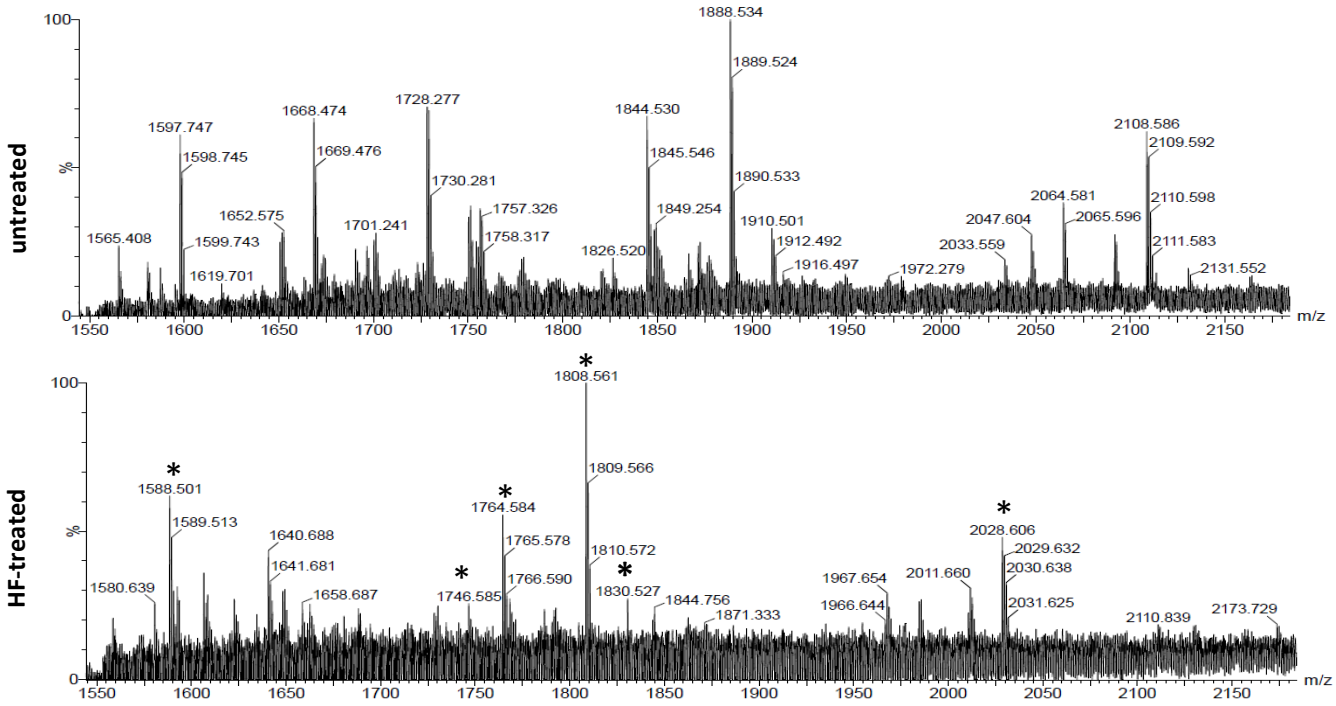


Figure 6.9 Positive-ion MALDI-TOF spectra of untreated and HF-treated LOS of *C. concisus* NCTC12408. LOS of NCTC12408 was treated with hydrogen fluoride (HF) to remove phosphoester moieties from the sample. Positive-ion MALDI-TOF spectra of untreated (top) and HF-treated (bottom) LOS. Asterisk (*) in the spectrum of the HF-treated LOS was used to indicate for mass peaks which were in accordance with the loss of phosphate (*m/z* 79.966) after treatment.

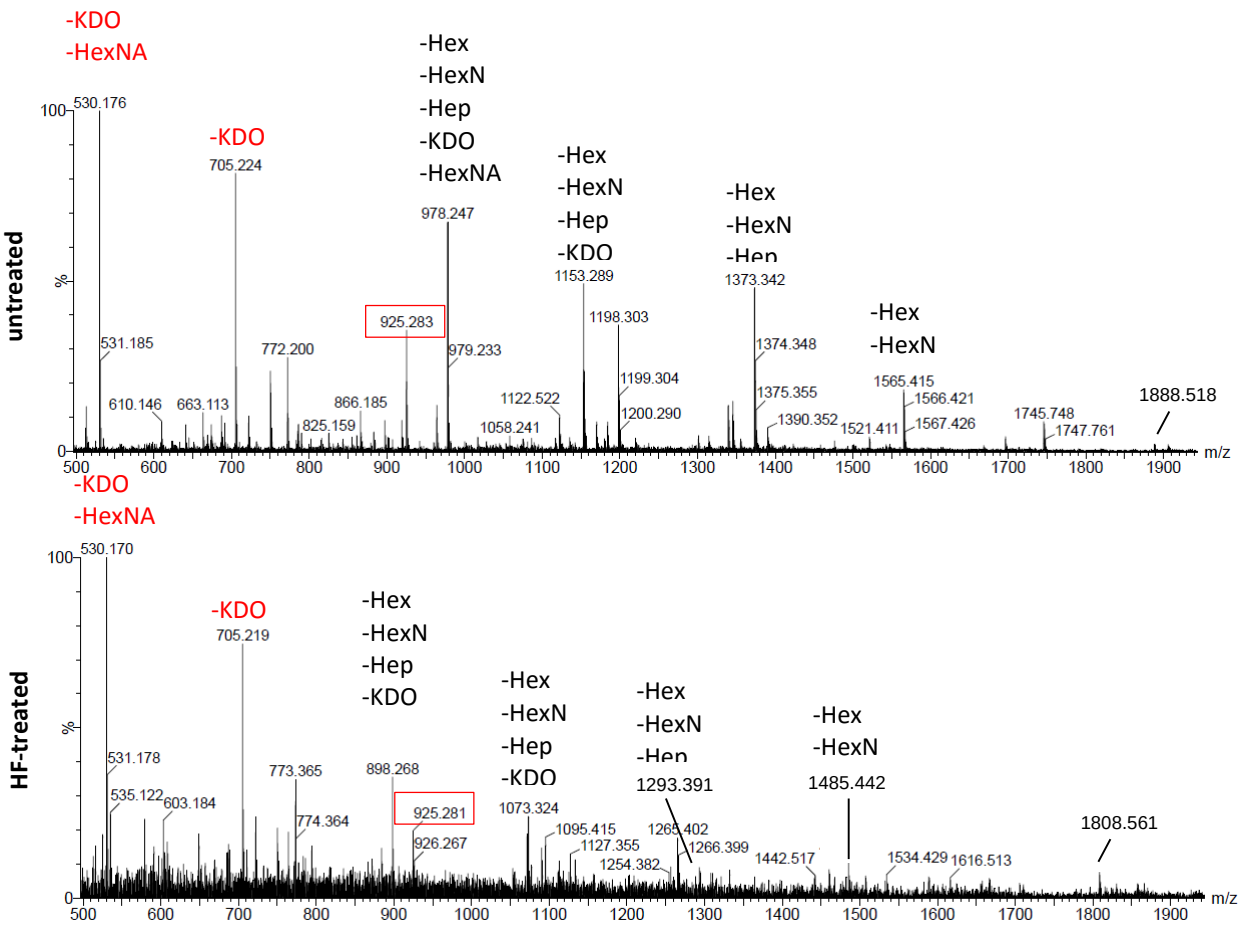


Figure 6.10 Structural analysis of inner core fragment ion *m/z* 1888.5.

LOS of NCTC12408 was treated with hydrogen fluoride (HF) to remove phosphoester moieties from the sample. Positive-ion MS/MS spectra of untreated OS fragment (*m/z* 1888.518; top) and HF-treated fragment (*m/z* 1808.5561; bottom) samples are shown. Masses for the fragment ions observed are indicative of the presence of known carbohydrate moieties. Red indicates for a fragment without phosphate which is observed in both spectra.

Hexose (Hex); Heptose (Hep); Hexosamine (HexN); Hexosaminuronic acid (HexNA); 2-keto-3-deoxyoctulonosinic acid (KDO)

C. concisus isolate	Proposed composition	Calculated m/z	Observed m/z (ppm)
B38	2KDO 4Hep 4Hex 2HexNA P Me Na	2323.657	2323.717 (25.73)
B124	Not determined		
NCTC 12408	2KDO 3Hep 5Hex 2HexN 3HexNA P 2Me HexNAc dHex Na	3329.033	3328.950 (-25.05)
2010-112825	KDO 2Hep 2Hex HexN 2HexNA P Me HexA*	N/A	2782.886
2010-131105	2KDO 3Hep 4Hex HexN 2HexNA P Me 3HexNAc dHex 2Pen	3290.054	3290.061 (2.26)
	2KDO 2Hep 2Hex HexN 2HexNA P Me HexNAc*	N/A	2288.556
2010-347972	2KDO 3Hep 4Hex HexN 2HexNA P Me 2HexNAc Pen 2Na*	N/A	3227.992
	2KDO 3Hep 5Hex HexN 2HexNA P Me	2432.726	2432.699 (-11.07)

Table 6.4 Proposed oligosaccharide composition of *C. concisus* isolates.

Proposed compositions of oligosaccharide (OS) peaks observed in positive-ion mass spectra of *C. concisus* LOS. B124 LOS was insufficient for detailed analysis and the composition was not determined. Where indicated with an asterisk (*) only parts of the structure were determined and mass weights were not calculated (N/A not applicable). Differences to observed positive-ion masses are expressed as parts per million (ppm) where the full structure could be proposed. Exact mass units were used for calculation of the following molecular masses (in Da) based on proposed compositions, as follows: Deoxyhexose (dHex), 146.0579; 2-keto-3-deoxyoctulonosinic acid (KDO), 220.0583; Heptose (Hep), 192.0634; Hexose (Hex), 162.0528; Hexosamine (HexN), 161.0688; Hexosaminuronic acid (HexNA), 175.0481; N-acetylhexosamine (HexNAc), 203. 0794; Methyl (Me), 14.0157; Pentose (Pen), 132.0423; Phosphate (P), 79.9663; Sodium (Na), 21.9898

To confirm our observations and identify the stereoisomeric nature of monosaccharides, *C. concisus* 2010-347972 LOS was further analysed by Dr. Biswa Choudhury (Glycotechnology Core Resource, University California San Diego). GC-MS elution peaks confirmed the existence of KDO and Hep and were consistent with the hexoses, mannose (Man), galactose (Gal) and glucose (Glc). Hexaminuronic acid (HexNAc) observed in the variable part of the OS were in accordance with acetylgalactosamine (GalNAc) and acetylglucosamine (GlcNAc) (Figure 6.11). High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed to assess for the existence of HexNA corresponding to the observed mass loss of *m/z* 175.

HexNA is not commonly found in LOS/LPS but galactosaminuronic acid (GalNA) has been described in the core region of *B. pertussis* LPS (Geurtsen et al., 2009). Lacking a suitable standard, LPS of *B. pertussis* Bp536Δwlb with truncated polysaccharide moiety (only present in the GalNA-containing inner core) was used as control for HPAEC-PAD analysis to assess for HexNA in our sample. We observed a region with similar elusion peaks in the *C. concisus* and *B. pertussis* LOS sample but peaks were not identical. If the region was in accordance with HexNA residues the observed discrepancies may be due to different stereoisomers present in *C. concisus* and *B. pertussis* LOS/LPS (Figure 6.12). Further analysis is needed to confirm the presence of HexNA in *C. consisus* LOS.

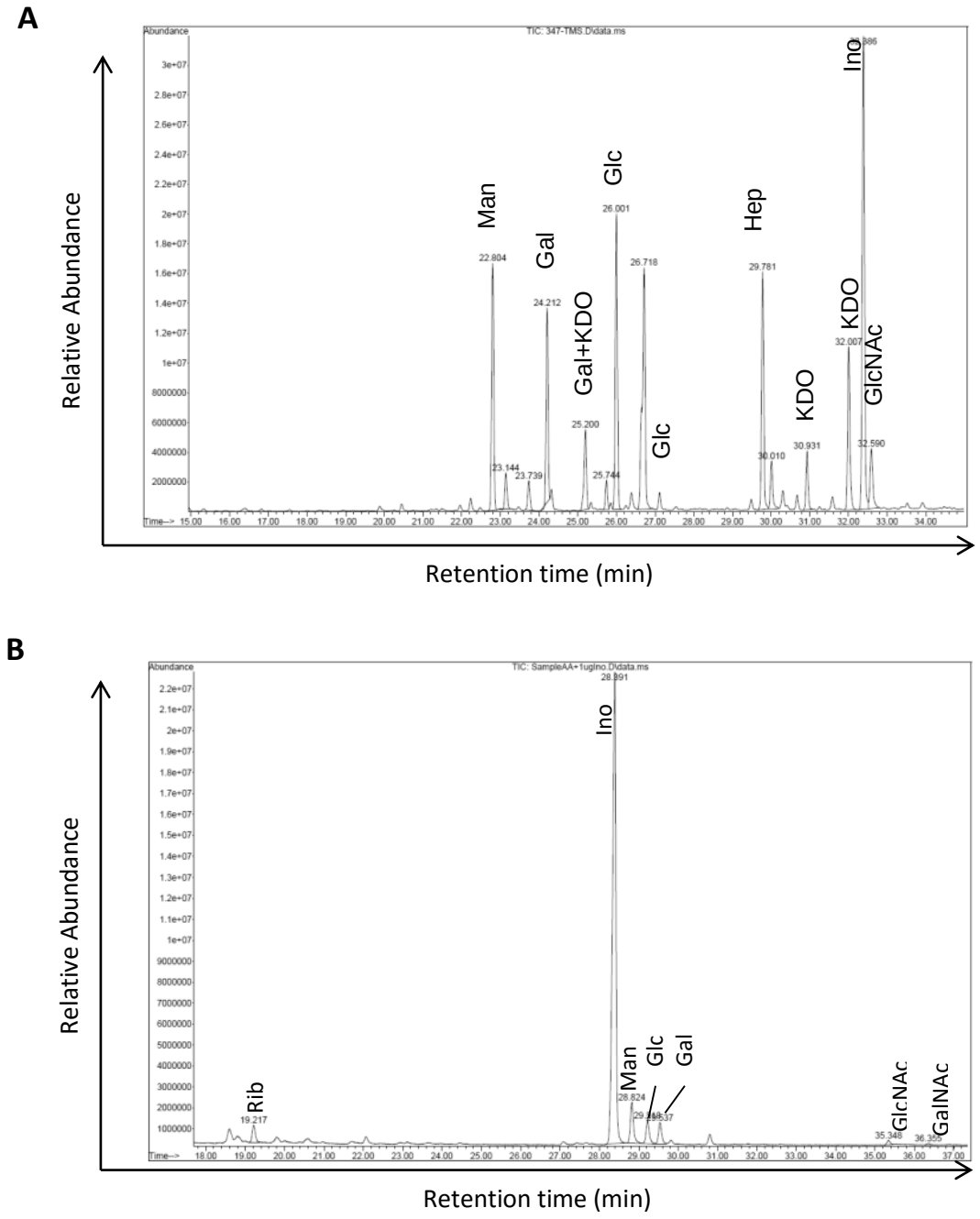


Figure 6.11 GC-MS analysis of the LOS of *C. concisus* 2010-347972.

GC-MS analyses of LOS of *C. concisus 2010-347972* were performed to identify monosaccharide components. GC-MS of **(A)** Trimethylsilyl (TMS) derivatives and **(B)** aldiol acetate derivatives reveals the presence of the three hexoses mannose (Man), galactose (Gal), and glucose (Glc). N-acetylhexosamine residues were identified as acetylgalactosamine (GalNAc) and acetylglucosamine (GlcNAc) and ribose (Rib) was identified as pentose. Further peaks eluded in accordance with 2-keto-3-deoxyoctulonosinic acid (KDO) and D-D-glycero-heptose (Hep). Sample preparation and analyses were performed by Dr. Biswa Choudhury (Glycotechnology Core Resource, University California San Diego).

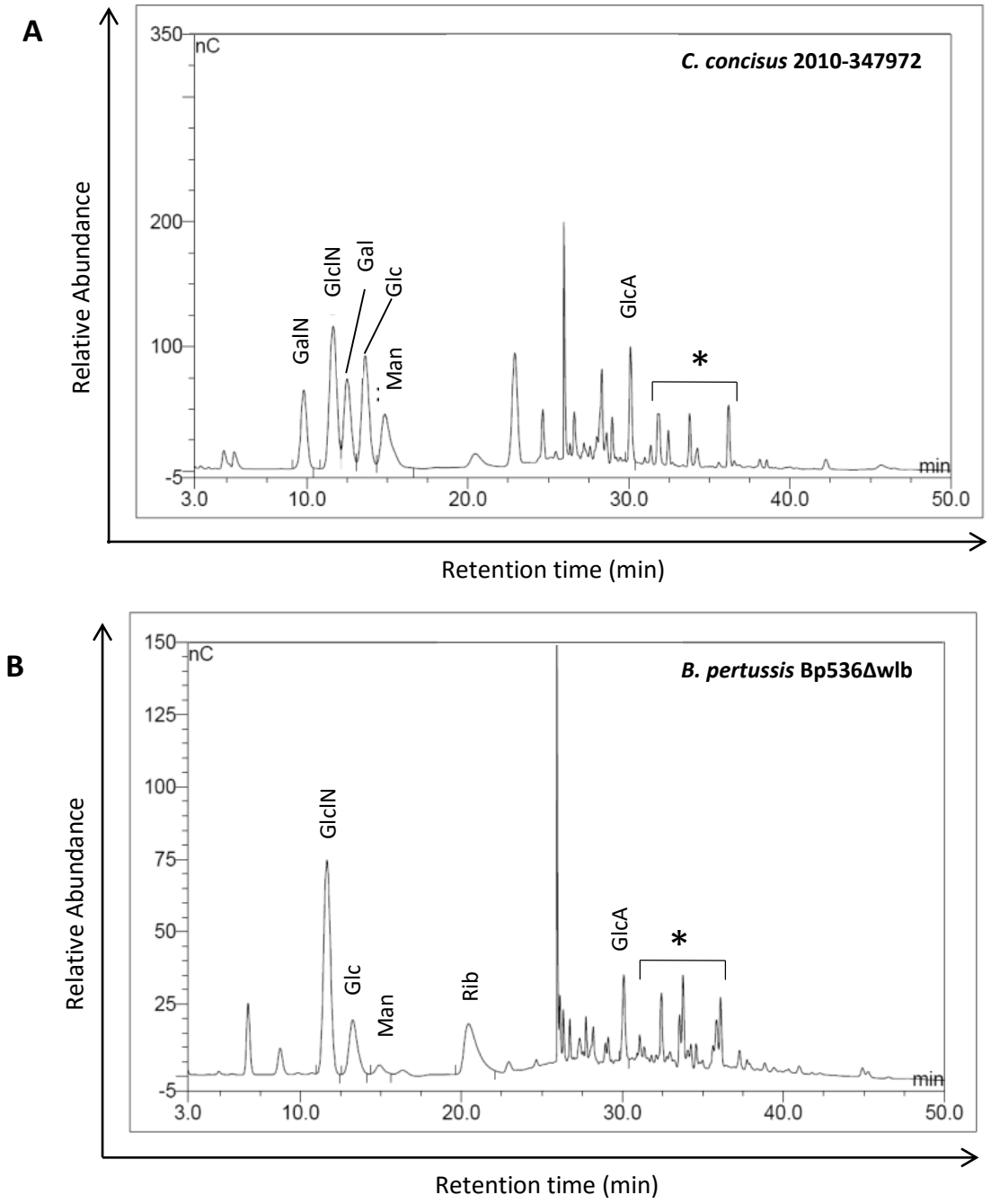


Figure 6.12 HPAEC-PAD analysis of the LOS of *C. concisus* 2010-347972.

(A) HPAEC-PAD analysis of LOS of *C. concisus 2010-347972* was performed to identify monosaccharide components. Peaks eluded in accordance with the hexoses mannose (Man), galactose (Gal), and glucose (Glc), the hexosamines galactosamine (GalN) and glucosamine (GlcN) and Glucuronic acid (GlcA). **(B)** *B. pertussis* Bp546Δwlb mutant was chosen for comparison as it presents with a truncated OS composed of hexaminuronic acid (HexNA). MS/MS analysis of *C. concisus* OS showed mass losses consistent with HexNA (*m/z* 175). Asterisk (*) indicate a region in the chromatogram with peaks of similar but non-identical retention time between 30-40min. The lack of a suitable standard prevented further identification. Sample preparation and analyses were performed by Dr. Biswa Choudhury (Glycotechnology Core Resource, University of California San Diego).

6.5 Pro-inflammatory potential of *C. concisus* isolates

LOS-mediated TLR4 signalling cascade has been identified as important factor of the pro-inflammatory immune response to *C. jejuni* (Rathinam et al., 2009a). Monocytes are potent cytokine secretors in response to C. *jejuni* (see, Chapter V). Co-culture of *C. concisus* isolates with PBMCs from several donors resulted in cytokine secretion. No significant difference was noted in IL-8 production between the various isolates and *C. jejuni*. There was a trend for less TNF-α secretion amongst isolates compared to *C. jejuni*. The variation in cytokine secretion was more pronounced for IL-1β, as there was a statistically significant increase in response to *C. jejuni* compared to all the isolates tested (Figure 6.13 A). Overall, these findings suggest a lower pro-inflammatory potential of *C. concisus* when compared to its family member *C. jejuni.*

To further assess bacterial virulence, we infected *Galleria mellonella* with *C. concisus* isolates and *C. jejuni* 81-176 and monitored their survival over time. Again, *C. concisus* isolates were less virulent when compared to *C. jejuni* with 60-100% larvae survival in response to the isolates *versus* 10% survival with *C. jejuni* after seven days of infection (6.13 B).

Finally, we assessed activation of the complement system by *C. concisus* isolates which might play a role in mediating immune response to *C. jejuni* infection (Chapter III, IV). While most isolates exhibited similar or slightly reduced rates of C3b deposition we observed a significant increase by *C. concisus* 2010-112825 (Figure 6.14). This needs to be confirmed by further analyses.

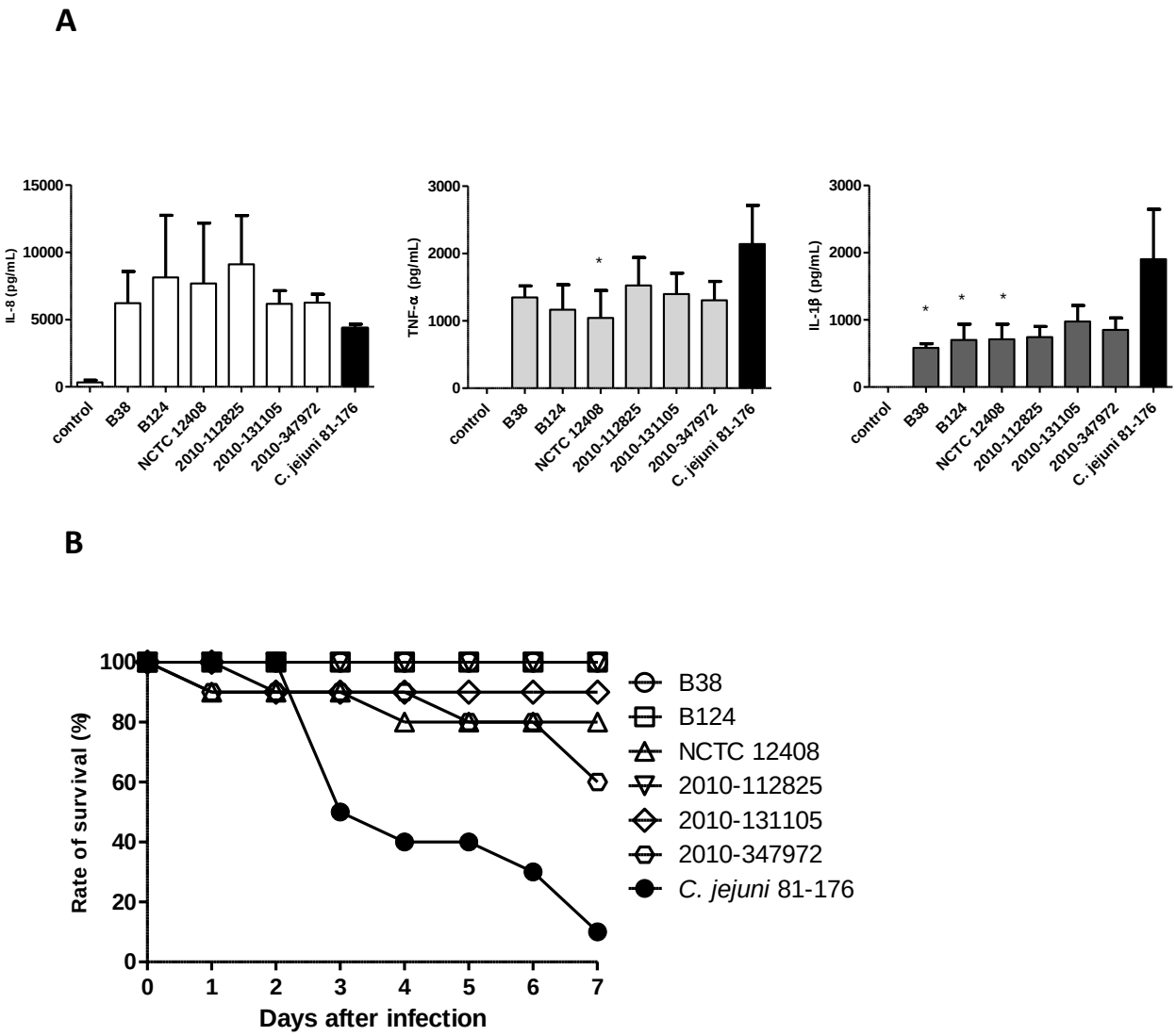


Figure 6.13 Inflammatory potential and virulence of *C. concisus* isolates.

(A) PBMCs were co-cultured with bacteria at MOI 10. Supernatants were collected after 5h and cytokine release for IL-8, TNF-α, and IL-1β was determined by ELISA. Mean±SEM of three independent experiments. Statistical differences to *C. jejuni* 81-176 were determined by one-way ANOVA with Tukey post-test. **(B)** *Galleria mellonella* (n=10) were injected with 10µl bacterial suspension (~10^7 CFUs) and the rate of survival was monitored over seven days. Injection of caterpillar was performed by Dr. Dagmar Alber (UCL, London). In both experiments *C. jejuni* 81-176 served as control. No stars: not significant; * p< 0.05; ** p<0.01; *** p<0.001

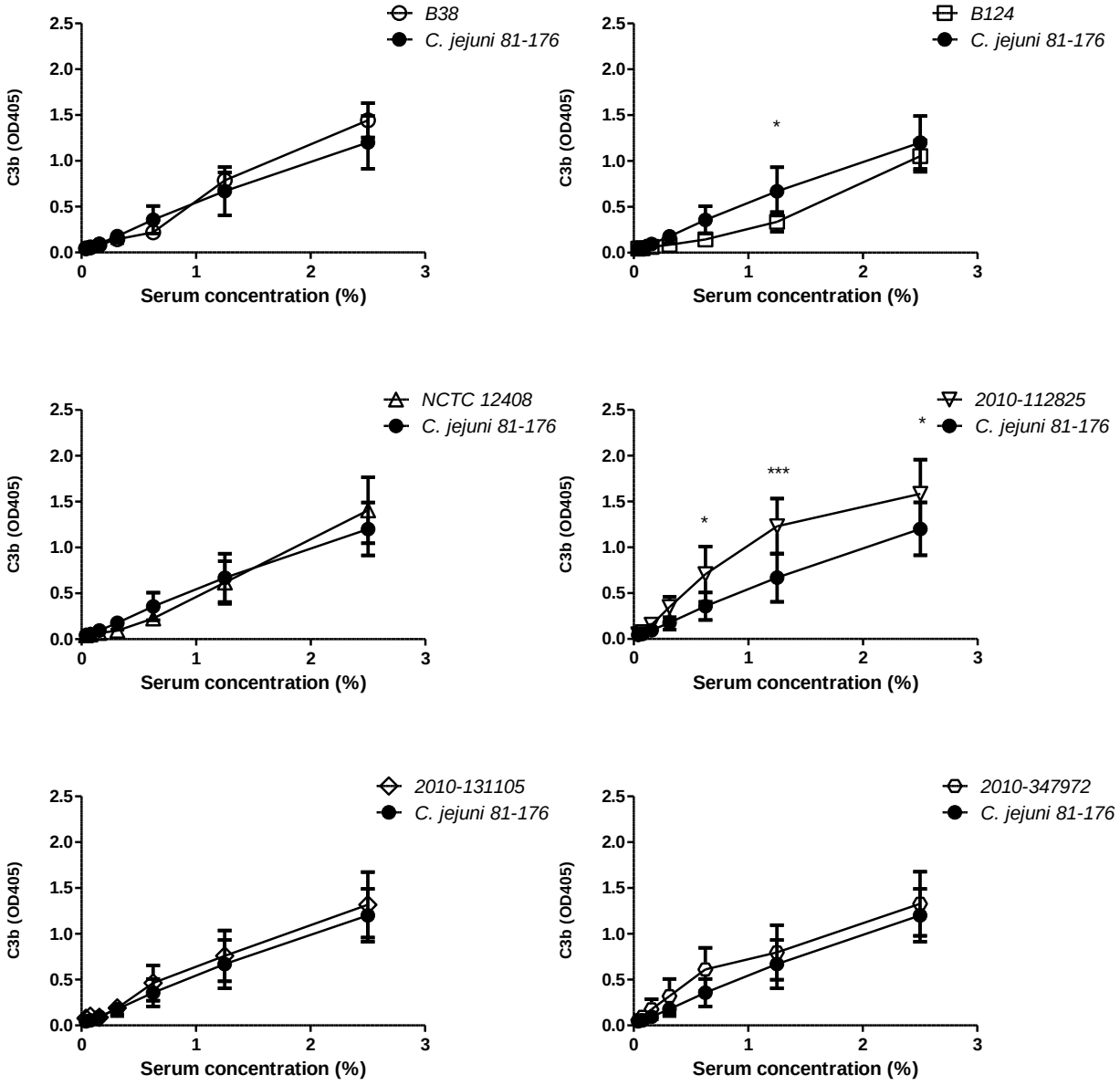


Figure 6.14 Complement activation by *C. concisus* isolates.

Microtiter plates were coated with *C. concisus* isolates and *C. jejuni* 81-176 and C3b deposition to serial diluted normal human serum (NHS) was determined in by ELISA Mean±SEM of three independent experiments. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis. No stars: not significant; * p< 0.05; *** p<0.001

6.6 Discussion

In this study we aimed to investigate for the first time the structural composition of *C. concisus* LOS. We observed that the LOS moieties of six isolates facilitated in this study were of larger molecular weight when compared to *C. jejuni* or *N. meningitides* LOS. In addition, we observed great heterogeneity between the isolates. The heterogeneous nature of *C. jejuni* LOS has been previously described and was ascribed to frequent phase variations in the LOS gene loci (Parker et al., 2005). Modifications were reported to primarily affect the outer region of the OS while inner core OS and the general structure of the lipid A was found to be primarily conserved (Dorrell et al., 2001). In line with this, we observed structural similarities in the lipid A and a common inner core OS region to be present in all six *C. concisus* isolates while great variations in the outer OS regions were observed.

C. concisus was found to have a hexaacylated lipid A similarly to *C. jejuni* LOS (Figure 6.15). Both contain four hydroxy-fatty acids, but in most *C. concisus* lipid A moieties, one C14:0(3-OH) was exchanged with 16:0(3-OH). The remaining two secondary fatty acids were also assigned to the reducing hexosamine-unit but were identified as C14:0 and C12:0 in contrast to two C16:0 acyl chains reported in *C. jejuni* lipid A (Moran et al., 1991, Szymanski et al., 2003).

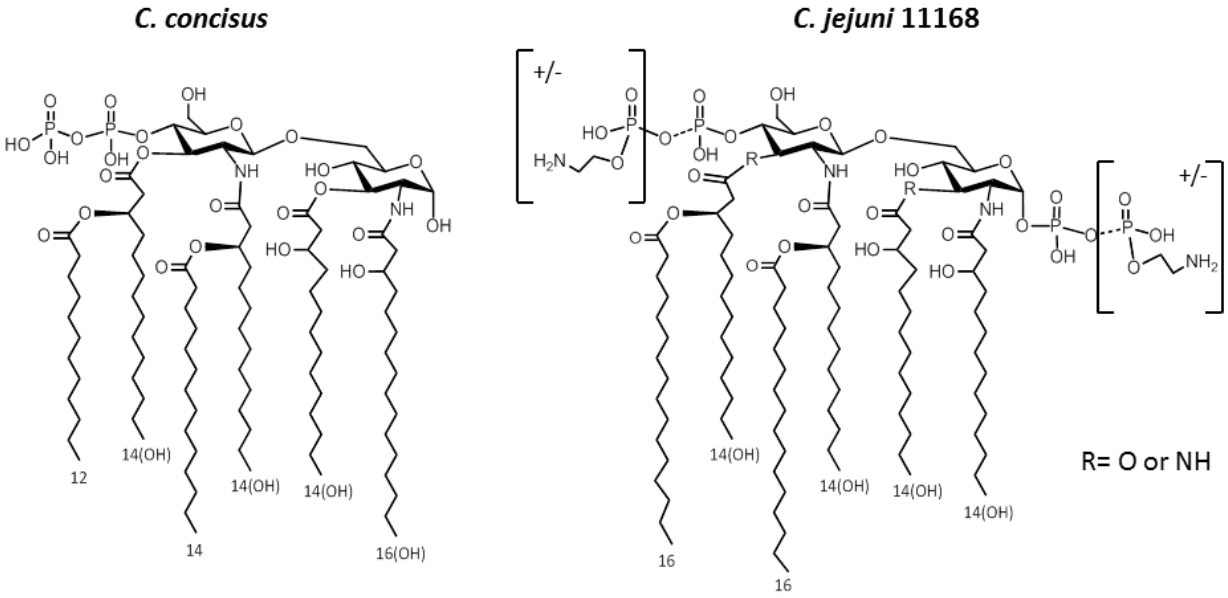


Figure 6.15 Schematic structures of *C. concisus* and *C. jejuni* lipid A moieties.

Schematic examples for a bis-phosphorylated *C. concisus* lipid A (left, this study) and *C. jejuni* 11168 lipid A (right, adapted from Szymanski et al., 2003).

Hexaacylation is a pre-requisite for optimal engagement with TLR4 and its downstream signalling cascade (Park et al., 2009). Despite that, after co-culture with PBMCs we observed a lower pro-inflammatory cytokine profile in PBMCs to *C. concisus* isolates when compared to *C. jejuni.*

Apart from the number of acyl-chains, other modifications of LOS/LPS have been recognised to influence engagement with TLR4 (Maeshima and Fernandez, 2013). Analysing a panel of livestock and non-livestock associated *C. jejuni* isolates, Stephenson *et al.* reported that an increased number of amide linkages, phosphorylation, and sialic acid status correlated with TLR4 activation and TNF-α synthesis by THP-1 cells and monocytes (Stephenson et al., 2013). While *C. jejuni* strains exhibit up to four amide-linkages in the lipid A moiety depending on the disaccharide backbone, we only observed evidence for the presence of HexN and therefore two amide-linkages in all six *C. concisus* isolates. *C. jejuni* LOS has been reported to be heavily phosphorylated generally containing one or two P and up to three PEA residues on the lipid A and one PEA residue attached to the OS moiety (Stephenson et al., 2013).

The presence of *C. jejuni* surface PEA has been associated with bacterial virulence by various mechanisms. A recent publication identified the EptC gene encoding a novel PEA transferase modifying PEA expression on flagella rod protein, N-linked glycans and lipid A (Scott et al., 2012). EptC also catalyses the addition of PEA to lipid A and the Hep on the inner core of OS resulting in enhanced recognition of TLR4, resistance to antimicrobial peptides and facilitating the ability of *C. jejuni* to colonise and survive in the avian and murine host (Cullen et al., 2013).

Variations in PEA and pyrophosphorylation (PP, Bis-P) of lipid A play an important role in TLR4 signalling of the *N. meningitidis* and *N. gonorrhoeae* while PEA and PP were found absent in the lipid A moiety of most commensal *Neisseria* strains which also exhibit a lower pro-inflammatory potential *in-vitro* (Liu et al., 2010, John et al., 2012). In *C. concisus* LOS, there was no evidence for PEA in the OS moiety but instead we found one P as part of the inner core of the OS moiety. Of the multiple lipid A peaks observed, only three *C. concisus* isolates showed evidence for a lipid A moiety containing PEA while most major lipid A peaks were consistent with non-PEA containing lipid A. The analytical methods used did not allow for the interpretation of the relative abundance of PEA lipid A nor were we able to quantify differences between the isolates or *C. jejuni*. However, given that we observed maximal one PEA in *C. concisus* LOS compared to maximal four PEA residues reported in *C. jejuni,* it is interesting to speculate that *C. concisus* LOS may exhibit comparably less pro-inflammatory phosphorylation. Phosphorylation of *C. concisus* LOS and a possible link to reduced virulence should be subject of further investigations.

LOS sialyation is an important virulence factor of *C. jejuni* and is associated with enhanced inflammation, colonisation and the onset of (GBS) (Heikema et al., 2013, Stephenson et al., 2013, Louwen et al., 2008). Previous work on *C. jejuni* isolates reported the presence of up to two sialic acid residues on the terminal end of OS and increased sialyation significantly enhanced TLR4-mediated TNF-α secretion when compared to strains without sialic acid (Stephenson et al., 2013). We have not observed any evidence for sialic acid in any of the six *C. concisus* strains analysed. Further, there was no evidence for the annotation of sialic acid related gene loci neuB, cst-II or cst-III in the genome of *C. concisus* isolates 2010-131105 and 2010-347972 (Dr. Georgina Hold, University of Aberdeen; personal communication). It would be interesting to confirm these findings by PCR analysis. Given the importance of sialic acid for the virulence of *C. jejuni*, the absence of sialyation of *C. concisus* isolates would be intriguing as the species would lack an important virulence factor that promotes the pro-inflammatory axis.

Taken together, our findings suggest that *C. concisus* isolates exhibit heterogeneous LOS moieties. Despite many similarities to *C. jejuni*, we identified lower levels of amide linkages and phosphorylation and possibly the lack of sialic acid residues. Further investigations are necessary but our findings on LOS composition, pro-inflammatory profile and virulence imply a potentially more commensal-like nature for these *Campylobacter* species.

CHAPTER VII

DISCUSSION

Infection by members of the *Campylobacter* genus, predominantly *C. jejuni*, is one of the most common and widespread bacterial-mediated disease(s) in humans. In the last century, the incidence and prevalence of *C. jejuni* infection have dramatically increased while the impact of newly emerging *Campylobacter* species on the human host is currently unknown (Kaakoush et al., 2015). Despite being a major burden on global health, our understanding of *Campylobacter* transmission and the mechanisms of disease remain surprisingly under studied. *C. jejuni* and many other *Campylobacter* species are benign residents of the GI tract of wild animals and birds providing a reservoir for infection in farm animals, including chickens, cattle, sheep and pigs. The ubiquitous colonisation of *C. jejuni* in livestock, primarily poultry, is thought to be the most important source of human infection in the western world (Jones, 2001). The largely commensal nature of the species in the animal host represents an unsolved conundrum of *C. jejuni* as a remarkably low inoculum dose can promote disease in humans (Black et al., 1988).

To-date, most studies have focused on identifying host-pathogen interactions between *Campylobacter* and the intestinal mucosa, in particular crosstalk between the bacterium and the intestinal epithelium, an important physical and chemical barrier to all invading enteropathogens. *C. jejuni* has several advantaged features, including its spiral-shape and the flagella that provide motility through the mucus; and its polysaccharide capsule. Further, *C. jejuni* is able to disrupt the epithelial barrier function and adhere to and invade IECs initiating the activation of the innate immune system by the release of chemokines and cytokines.

A fundamental feature of acute campylobacteriosis is the extensive migration of neutrophils to the infection site. In this study we aimed to delineate the to-date undefined role of neutrophils in *C. jejuni* infection. Herein, we report for the first time that neutrophil-mediated phagocytosis of *C. jejuni* is almost exclusively serum dependent and it is the heat-labile components of the complement system that promote bacterial uptake. Furthermore, we found *C. jejuni* phagocytosis to be independent of the Ig-receptor FcR, instead our observations highlight a crucial role for complement-dependent CR1 and potential involvement of CR3. The central role of this mode of uptake seems to be exclusive for neutrophil-mediated interaction with *C. jejuni* as non-opsonised bacterial phagocytosis is well documented in macrophages and DCs (Heikema et al., 2013, Hu et al., 2006).

The mode of phagocytosis has important implications for the downstream cellular responses including the upregulation of surface receptors, cytokine generation and the fate of internalised pathogens. FcR-dependent uptake generally results in cargo delivery to the phagosome, at present the phagosome formation in response to CR engagement is less well defined.

Interestingly, we failed to observe neutrophil bactericidal killing following CR1-mediated uptake of *C. jejuni* in human neutrophils. The ability of *C. jejuni* to evade intracellular killing has been previously reported in human and murine monocytic cells (Hickey et al., 2005, Klancnik et al., 2009, Day et al., 2000). Others report bacterial clearance in murine macrophages but observed survival of *C. jejuni* in IECs (Watson and Galan, 2008). Some evidence suggests that *C. jejuni* survival in macrophages is dependent on the increased expression of oxidative stress regulators, including catalases, which could explain these opposing findings (Day et al., 2000, Pogacar et al., 2009).

It should be noted that published studies have focuses on non-opsonised bacterial uptake while the role of antibody or complement associated phagocytosis was not studied in parallel. Watson *et al.* observed the internalisation of antibody-opsonised *C. jejuni* by FcR-transfected COS-1 cells which initiated translocation to the lysosome and subsequent bacterial killing (Watson and Galan, 2008). We have found *C. jejuni* positive IgA, IgG and IgM serum titres in our cohort of healthy donors and observed minimal involvement of antibody in neutrophil-mediated phagocytosis. However, since FcR-mediated uptake is largely dependent on the antibody density it would be interesting to investigate the mode of bacterial uptake and post-phagocytic actions in the presence of immune-competent serum taken at the late-phase of *C. jejuni* infection. Given that repeated exposure to *C. jejuni* is associated with limited pathology (Havelaar et al., 2009) it is interesting to speculate that a shift to antibody-FcR mediated bacterial uptake may lead to neutrophil bacterial clearance.

The complement system was found to be crucial for *C. jejuni* phagocytosis by neutrophils and serum bactericidal activity. We observed the accumulation of complement factor C3b on the bacterial surface which was in line with CR1-mediated phagocytosis and suggests an important role for the alternative arm of complement activation. Our findings are in contrast to strategies employed by other bacterial species, including the serum-resistant *N. meningitidis* which expresses factor H binging-proteins to reduce C3b accumulation in order to evade alternative pathway-mediated killing (Schneider et al., 2006). It is tempting to hypothesise that while others specialise in the inhibition of complement activation, *C. jejuni* may employ a different route by utilising neutrophil-mediated phagocytosis *via* C3b/CR1 promoting its intracellular survival.

Under normal conditions, bacterial clearance initiates neutrophil apoptosis, a tightly regulated process which limits the destructive capacity of neutrophil toxic contents to the surrounding tissue. Further, apoptotic neutrophils can then be recognised and cleared by other phagocytic cells including macrophages, which plays a central role in the successful resolution of

inflammation (Fox et al., 2010). Some bacterial species, including *Chlamydia pneumonia* survive in neutrophils and delay neutrophil apoptosis in a LPS-dependent manner which leads to prolonged inflammation and tissue damage (van Zandbergen et al., 2004). Since our findings suggest intracellular survival, this may have a significant impact on *C. jejuni*-mediated neutrophil apoptosis. Should *C. jejuni* infection promote neutrophil lifespan, this mechanism may provide one explanation for the presence of marked neutrophil levels seen in human infection and may play a role in inflammatory post-infectious events.

Increasing evidence suggests an important role for the complement system in intestinal immunity. (Kopp et al., 2015). IECs secret complement components including C3, factor B and properdin providing the GI lumen with alternative pathway mediators (Bernet-Camard et al., 1996, Andoh et al., 1993). Interestingly, we observed *C. jejuni*-mediated alternative complement activation in serum. Additionally, we observed for the first time antibody-independent binding of the complement protein C1q to the surface of *C. jejuni*. Agarwal *et al.* reported significantly enhanced adherence and invasion of C1q-opsonised *S. pneumoniae* in human lung epithelial and endothelial cell-lines (Agarwal et al., 2013). While we did not observe a direct involvement of C1q in neutrophil-mediated phagocytosis a potential role in *C. jejuni* IEC invasion is unknown. Taken together, the complement system could present a yet undefined part in the interplay of *C. jejuni* and the intestinal mucosa and should be a subject of further investigation.

It is intriguing that despite the high infection rates associated with the bacterium, *C. jejuni* promotes a modest pro-inflammatory cytokine and chemokine response in contrast to other important GI pathogens, including *S. typhimurium* and enteropathogenic *E. coli*; instead *C. jejuni* enhances the generation of anti-inflammatory IL-10 *in-vitro* (Stephenson et al., 2014). *C. jejuni* surface glycans, in particular LOS, are thought to play a key role in *Campylobacter* infection by mediating immune evasion on the one hand and enhancing TLR4-related immunity on the other (Karlyshev et al., 2005b). We have observed *C. jejuni*-mediated cytokine response in PBMCs which were similar to that induced by *E. coli* LPS. Additionally, we present evidence for the generation of neutrophil IL-1β in response to the bacterium. Given the importance of TLR4 interaction one can speculate that the observed immune response may be related to *C. jejuni* LOS. *Campylobacter* surface glycans present extensive structural variations with implication for disease development (Karlyshev et al., 2005b). We found that in contrast to *C. jejuni*, *C. concisus* LOS lacks inflammation-associated sialic acid and LOS phosphorylation patterns which may play a role in reduced pathogenicity of these species. The potential implication of *C. concisus* LOS in cytokine secretion

as well as complement and neutrophil activation should be further investigated as it may help to delineate a more commensal or pathogen-like phenotype for this species.

In conclusion, our study suggests that *C. jejuni* induces complement activation and triggers neutrophil phagocytosis and activation while internalised bacteria fail to be cleared *in-vitro*. We propose that this phenomenon may present a novel mechanism of *Campylobacter* to modify immune recognition and may be influenced by bacterial (surface glycans) and host (immune-competence, complement) factors. This way, *C. jejuni* may disturb the fine balance between mobilising an effective immune defence in order to clear invading pathogens and preventing tissue damage by and an overactive inflammatory response.

CHAPTER VIII

BIBLIOGRAPHY

ADEREM, A. & UNDERHILL, D. M. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol,* 17**,** 593-623.

ADEREM, A. A., WRIGHT, S. D., SILVERSTEIN, S. C. & COHN, Z. A. 1985. Ligated complement receptors do not activate the arachidonic acid cascade in resident peritoneal macrophages. *J Exp Med,* 161**,** 617-22.

AGARWAL, S., RAM, S., NGAMPASUTADOL, J., GULATI, S., ZIPFEL, P. F. & RICE, P. A. 2010. Factor H facilitates adherence of *Neisseria gonorrhoeae* to complement receptor 3 on eukaryotic cells. *J Immunol,* 185**,** 4344-53.

AGARWAL, S., VASUDHEV, S., DEOLIVEIRA, R. B. & RAM, S. 2014. Inhibition of the classical pathway of complement by meningococcal capsular polysaccharides. *J Immunol,* 193**,** 1855-63.

AGARWAL, V., AHL, J., RIESBECK, K. & BLOM, A. M. 2013. An Alternative Role of C1q in Bacterial Infections: Facilitating *Streptococcus pneumoniae* Adherence and Invasion of Host Cells. *Journal Immunol,* 191**,** 4235-4245.

AHRENSTEDT, O., KNUTSON, L., NILSSON, B., NILSSON-EKDAHL, K., ODLIND, B. & HALLGREN, R. 1990. Enhanced local production of complement components in the small intestines of patients with Crohn's disease. *N Engl J Med,* 322**,** 1345-9.

AL-BANNA, N. A., RAGHUPATHY, R. & ALBERT, M. J. 2012. Induction of cytokines in different organs after intranasal inoculation of *Campylobacter jejuni* in mice. *Gut Pathog,* 4**,** 23.

AL-SAYEQH, A. F., LOUGHLIN, M. F., DILLON, E., MELLITS, K. H. & CONNERTON, I. F. 2010. *Campylobacter jejuni* activates NF-kappaB independently of TLR2, TLR4, Nod1 and Nod2 receptors. *Microb Pathog,* 49**,** 294-304.

AL-SHARIF, W. Z., SUNYER, J. O., LAMBRIS, J. D. & SMITH, L. C. 1998. Sea urchin coelomocytes specifically express a homologue of the complement component C3. *J Immunol,* 160**,** 2983-97.

ALEMKA, A., CLYNE, M., SHANAHAN, F., TOMPKINS, T., CORCIONIVOSCHI, N. & BOURKE, B. 2010. Probiotic colonization of the adherent mucus layer of HT29MTXE12 cells attenuates *Campylobacter jejuni* virulence properties. *Infect Immun,* 78**,** 2812-22.

ALEMKA, A., CORCIONIVOSCHI, N. & BOURKE, B. 2012. Defense and adaptation: the complex inter-relationship between *Campylobacter jejuni* and mucus. *Front Cell Infect Microbiol,* 2**,** 15.

ALEYD, E., VAN HOUT, M. W., GANZEVLES, S. H., HOEBEN, K. A., EVERTS, V., BAKEMA, J. E. & VAN EGMOND, M. 2014. IgA enhances NETosis and release of neutrophil extracellular traps by polymorphonuclear cells via Fcalpha receptor I. *J Immunol,* 192**,** 2374-83.

ALI, Y. M., LYNCH, N. J., HALEEM, K. S., FUJITA, T., ENDO, Y., HANSEN, S., HOLMSKOV, U., TAKAHASHI, K., STAHL, G. L., DUDLER, T., GIRIJA, U. V., WALLIS, R., KADIOGLU, A., STOVER, C. M., ANDREW, P. W. & SCHWAEBLE, W. J. 2012. The lectin pathway of complement activation is a critical component of the innate immune response to pneumococcal infection. *PLoS Pathog,* 8**,** e1002793.

ALLEN, L. A. & ADEREM, A. 1996. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J Exp Med,* 184**,** 627-37.

ALLEN, L. A., BEECHER, B. R., LYNCH, J. T., ROHNER, O. V. & WITTINE, L. M. 2005. *Helicobacter pylori* disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. *J Immunol,* 174**,** 3658-67.

ALLOS, B. M. 1997. Association between *Campylobacter* infection and Guillain-Barre syndrome. *J Infect Dis,* 176**,** S125-S128.

ANDERSEN-NISSEN, E., SMITH, K. D., STROBE, K. L., BARRETT, S. L., COOKSON, B. T., LOGAN, S. M. & ADEREM, A. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. *Proc Natl Acad Sci U S A,* 102**,** 9247-52.

ANDOH, A., FUJIYAMA, Y., BAMBA, T. & HOSODA, S. 1993. Differential cytokine regulation of complement C3, C4, and factor B synthesis in human intestinal epithelial cell line, Caco-2. *J Immunol,* 151**,** 4239-47.

ANDOH, A., FUJIYAMA, Y., SUMIYOSHI, K. & BAMBA, T. 1996. Local secretion of complement C3 in the exocrine pancreas: ductal epithelial cells as a possible biosynthetic site. *Gastroenterology,* 110**,** 1919-25.

ARLAUD, G. J., GABORIAUD, C., THIELENS, N. M., BUDAYOVA-SPANO, M., ROSSI, V. & FONTECILLA-CAMPS, J. C. 2002. Structural biology of the C1 complex of complement unveils the mechanisms of its activation and proteolytic activity. *Molecular Immunology,* 39**,** 383-94.

ASAKURA, M., SAMOSORNSUK, W., HINENOYA, A., MISAWA, N., NISHIMURA, K., MATSUHISA, A. & YAMASAKI, S. 2008. Development of a cytolethal distending toxin (cdt) gene-based species-specific multiplex PCR assay for the detection and identification of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter fetus*. *FEMS Immunol Med Microbiol,* 52**,** 260-266.

AVERHOFF, P., KOLBE, M., ZYCHLINSKY, A. & WEINRAUCH, Y. 2008. Single residue determines the specificity of neutrophil elastase for *Shigella* virulence factors. *J Mol Biol,* 377**,** 1053-66.

AVRIL, T., WAGNER, E. R., WILLISON, H. J. & CROCKER, P. R. 2006. Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on *Campylobacter jejuni* lipooligosaccharides. *Infect Immun,* 74**,** 4133-41.

AXELSSON-OLSSON, D., SVENSSON, L., OLOFSSON, J., SALOMON, P., WALDENSTROM, J., ELLSTROM, P. & OLSEN, B. 2010. Increase in acid tolerance of *Campylobacter jejuni* through coincubation with amoebae. *Appl Environ Microbiol,* 76**,** 4194-200.

BACON, D. J., SZYMANSKI, C. M., BURR, D. H., SILVER, R. P., ALM, R. A. & GUERRY, P. 2001. A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. *Mol Microbiol,* 40**,** 769-777.

BAKEMA, J. E. & VAN EGMOND, M. 2011. The human immunoglobulin A Fc receptor FcalphaRI: a multifaceted regulator of mucosal immunity. *Mucosal Immunology,* 4**,** 612-24.

BANOUB, J. H., ANNEED A. E., COHEN A. M., JOLY N. 2010. Structural investigation of bacterial lipopolysaccharides by mass spectrometry and tandem mass spectrometry. *Mass Spectrom Rev*, 29, 606-50

BARTNECK, M., KEUL, H. A., ZWADLO-KLARWASSER, G. & GROLL, J. 2010. Phagocytosis independent extracellular nanoparticle clearance by human immune cells. *Nano Lett,* 10**,** 59-63.

BAX, M., GARCIA-VALLEJO, J. J., JANG-LEE, J., NORTH, S. J., GILMARTIN, T. J., HERNANDEZ, G., CROCKER, P. R., LEFFLER, H., HEAD, S. R., HASLAM, S. M., DELL, A. & VAN KOOYK, Y. 2007. Dendritic cell maturation results in pronounced changes in glycan expression affecting recognition by siglecs and galectins. *J Immunol,* 179**,** 8216-24.

BEERY, J. T., HUGDAHL, M. B. & DOYLE, M. P. 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl Environ Microbiol,* 54**,** 2365-70.

BENNET, S. M., OHMAN, L. & SIMREN, M. 2015. Gut microbiota as potential orchestrators of irritable bowel syndrome. *Gut Liver,* 9**,** 318-31.

BERENDS, E. T., DEKKERS, J. F., NIJLAND, R., KUIPERS, A., SOPPE, J. A., VAN STRIJP, J. A. & ROOIJAKKERS, S. H. 2013. Distinct localization of the complement C5b-9 complex on Gram-positive bacteria. *Cell Microbiol,* 15**,** 1955-68.

BERENDS, E. T., KUIPERS, A., RAVESLOOT, M. M., URBANUS, R. T. & ROOIJAKKERS, S. H. 2014. Bacteria under stress by complement and coagulation. *FEMS Microbiol Rev,* 38**,** 1146-71.

BERESWILL, S., FISCHER, A., PLICKERT, R., HAAG, L. M., OTTO, B., KUHL, A. A., DASTI, J. I., ZAUTNER, A. E., MUNOZ, M., LODDENKEMPER, C., GROSS, U., GOBEL, U. B. & HEIMESAAT, M. M. 2011. Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. *PLoS One,* 6**,** e20953.

BERGER, A. 2000. Th1 and Th2 responses: what are they? *BMJ,* 321**,** 424.

BERNATOWSKA, E., JOSE, P., DAVIES, H., STEPHENSON, M. & WEBSTER, D. 1989. Interaction of *Campylobacter* species with antibody, complement and phagocytes. *Gut,* 30**,** 906-11.

BERNET-CAMARD, M. F., COCONNIER, M. H., HUDAULT, S. & SERVIN, A. L. 1996. Differential expression of complement proteins and regulatory decay accelerating factor in relation to differentiation of cultured human colon adenocarcinoma cell lines. *Gut,* 38**,** 248-53.

BIANCHI, M., HAKKIM, A., BRINKMANN, V., SILER, U., SEGER, R. A., ZYCHLINSKY, A. & REICHENBACH, J. 2009. Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood,* 114**,** 2619-22.

BLACK, R. E., LEVINE, M. M., CLEMENTS, M. L., HUGHES, T. P. & BLASER, M. J. 1988. Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis,* 157**,** 472-9.

BLASER, M. J. 1997. Epidemiologic and clinical features of *Campylobacter jejuni* infections. *J Infect Dis,* 176 Suppl 2**,** S103-5.

BLASER, M. J., SMITH, P. F., HOPKINS, J. A., HEINZER, I., BRYNER, J. H. & WANG, W. L. 1987. Pathogenesis of *Campylobacter fetus* infections: serum resistance associated with high-molecular-weight surface proteins. *J Infect Dis,* 155**,** 696-706.

BLASER, M. J., SMITH, P. F. & KOHLER, P. F. 1985. Susceptibility of *Campylobacter* isolates to the bactericidal activity of human serum. *J Infect Dis,* 151**,** 227-35.

BLASER, M. J., SMITH, P. F., REPINE, J. E. & JOINER, K. A. 1988. Pathogenesis of *Campylobacter fetus* infections. Failure of encapsulated *Campylobacter fetus* to bind C3b explains serum and phagocytosis resistance. *J Clin Invest,* 81**,** 1434-44.

BORREGAARD, N. & COWLAND, J. B. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood,* 89**,** 3503-21.

BOUWMAN, L. I., DE ZOETE, M. R., BLEUMINK-PLUYM, N. M. C., FLAVELL, R. A. & VAN PUTTEN, J. P. M. 2014. Inflammasome Activation by *Campylobacter jejuni*. *J Immunol,* 193**,** 4548-4557.

BOYANOVA, L., GERGOVA, G., SPASSOVA, Z., KOUMANOVA, R., YANEVA, P., MITOV, I., DEREJIAN, S. & KRASTEV, Z. 2004. *Campylobacter* infection in 682 bulgarian patients with acute entero*coli*tis, inflammatory bowel disease, and other chronic intestinal diseases. *Diagn Microbiol Infect Dis,* 49**,** 71-4.

BRATZ, K., GOLZ, G., JANCZYK, P., NOCKLER, K. & ALTER, T. 2015. Analysis of in vitro and in vivo effects of probiotics against *Campylobacter* spp. *Berl Munch Tierarztl Wochenschr,* 128**,** 155-62.

BRINKMANN, V., REICHARD, U., GOOSMANN, C., FAULER, B., UHLEMANN, Y., WEISS, D. S., WEINRAUCH, Y. & ZYCHLINSKY, A. 2004. Neutrophil extracellular traps kill bacteria. *Science,* 303**,** 1532-5.

BRINKMANN, V. & ZYCHLINSKY, A. 2012. Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol,* 198**,** 773-83.

BROWN, E. J. 1991. Complement receptors and phagocytosis. *Curr Opin Immunol,* 3**,** 76-82.

BUELOW, D. R., CHRISTENSEN, J. E., NEAL-MCKINNEY, J. M. & KONKEL, M. E. 2011. *Campylobacter jejuni* survival within human epithelial cells is enhanced by the secreted protein CiaI. *Mol Microbiol,* 80**,** 1296-312.

CARON, E., SELF, A. J. & HALL, A. 2000. The GTPase Rap1 controls functional activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory mediators. *Curr Biol,* 10**,** 974-8.

CEAN, A., STEF, L., SIMIZ, E., JULEAN, C., DUMITRESCU, G., VASILE, A., PET, E., DRINCEANU, D. & CORCIONIVOSCHI, N. 2015. Effect of human isolated probiotic bacteria on preventing *Campylobacter jejuni* colonization of poultry. *Foodborne Pathog Dis,* 12**,** 122-30.

CHAMPION, O. L., KARLYSHEV, A. V., SENIOR, N. J., WOODWARD, M., LA RAGIONE, R., HOWARD, S. L., WREN, B. W. & TITBALL, R. W. 2010. Insect Infection Model for *Campylobacter jejuni* Reveals That O-methyl Phosphoramidate Has Insecticidal Activity. *J Infect Dis,* 201**,** 776-782.

CHANG, L. F. & KARIN, M. 2001. Mammalian MAP kinase signalling cascades. *Nature,* 410**,** 37-40.

CHEN, J., SUN, X. T., ZENG, Z. & YU, Y. Y. 2011. *Campylobacter* enteritis in adult patients with acute diarrhea from 2005 to 2009 in Beijing, China. *Chin Med J (Engl),* 124**,** 1508-12.

CHRISTENSEN, J. E., PACHECO, S. A. & KONKEL, M. E. 2009. Identification of a *Campylobacter jejuni*-secreted protein required for maximal invasion of host cells. *Mol Microbiol,* 73**,** 650-62.

CHUANG, F. Y., SASSAROLI, M. & UNKELESS, J. C. 2000. Convergence of Fc gamma receptor IIA and Fc gamma receptor IIIB signaling pathways in human neutrophils. *J Immunol,* 164**,** 350-60.

COKER, A. O., ISOKPEHI, R. D., THOMAS, B. N., AMISU, K. O. & OBI, C. L. 2002. Human campylobacteriosis in developing countries. *Emerg Infect Dis,* 8**,** 237-44.

CORCIONIVOSCHI, N., ALVAREZ, L. A., SHARP, T. H., STRENGERT, M., ALEMKA, A., MANTELL, J., VERKADE, P., KNAUS, U. G. & BOURKE, B. 2012. Mucosal reactive oxygen species decrease virulence by disrupting *Campylobacter jejuni* phosphotyrosine signaling. *Cell Host Microbe,* 12**,** 47-59.

CRIM, S. M., GRIFFIN, P. M., TAUXE, R., MARDER, E. P., GILLISS, D., CRONQUIST, A. B., CARTTER, M., TOBIN-D'ANGELO, M., BLYTHE, D., SMITH, K., LATHROP, S., ZANSKY, S., CIESLAK, P. R., DUNN, J., HOLT, K. G., WOLPERT, B. & HENAO, O. L. 2015. Preliminary incidence and trends of infection with pathogens transmitted commonly through food - Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2014. *MMWR Morb Mortal Wkly Rep,* 64**,** 495-9.

CROCKER, P. R., PAULSON, J. C. & VARKI, A. 2007. Siglecs and their roles in the immune system. *Nat Rev Immunol,* 7**,** 255-66.

CULLEN, T. W., O'BRIEN, J. P., HENDRIXSON, D. R., GILES, D. K., HOBB, R. I., THOMPSON, S. A., BRODBELT, J. S. & TRENT, M. S. 2013. EptC of *Campylobacter jejuni* mediates phenotypes involved in host interactions and virulence. *Infect Immun,* 81**,** 430-40.

CUNNION, K. M., HAIR, P. S. & BUESCHER, E. S. 2004. Cleavage of complement C3b to iC3b on the surface of *Staphylococcus aureus* is mediated by serum complement factor I. *Infect Immun,* 72**,** 2858-63.

DAHL, M., TYBJAERG-HANSEN, A., SCHNOHR, P. & NORDESTGAARD, B. G. 2004. A population-based study of morbidity and mortality in mannose-binding lectin deficiency. *J Exp Med,* 199**,** 1391-9.

DALPKE, A., FRANK, J., PETER, M. & HEEG, M. 2006. Activation of Toll-like receptor 9 by DNA from different bacterial species. *Infect Immun,* 74**,** 940-946.

DAVEY, M. S., TAMASSIA, N., ROSSATO, M., BAZZONI, F., CALZETTI, F., BRUDEREK, K., SIRONI, M., ZIMMER, L., BOTTAZZI, B., MANTOVANI, A., BRANDAU, S., MOSER, B., EBERL, M. & CASSATELLA, M. A. 2011. Failure to detect production of IL-10 by activated human neutrophils. *Nat Immunol,* 12**,** 1017-8; author reply 1018-20.

DAY, W. A., JR., SAJECKI, J. L., PITTS, T. M. & JOENS, L. A. 2000. Role of catalase in *Campylobacter jejuni* intracellular survival. *Infect Immun,* 68**,** 6337-45.

DE CESARE, A., SHELDON, B. W., SMITH, K. S. & JAYKUS, L. A. 2003. Survival and persistence of *Campylobacter* and *Salmonella* species under various organic loads on food contact surfaces. *J Food Prot,* 66**,** 1587-94.

DE JONG, E. C., VIEIRA, P. L., KALINSKI, P., SCHUITEMAKER, J. H. N., TANAKA, Y., WIERENGA, E. A., YAZDANBAKHSH, M. & KAPSENBERG, M. L. 2002. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals. *J Immunol,* 168**,** 1704-1709.

DE SANTO, C., ARSCOTT, R., BOOTH, S., KARYDIS, I., JONES, M., ASHER, R., SALIO, M., MIDDLETON, M. & CERUNDOLO, V. 2010. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. *Nat Immunol,* 11**,** 1039-46.

DE ZOETE, M. R., KEESTRA, A. M., ROSZCZENKO, P. & VAN PUTTEN, J. P. 2010. Activation of human and chicken toll-like receptors by *Campylobacter* spp. *Infect Immun,* 78**,** 1229-38.

DES PREZ, R. M., BRYAN, C. S., HAWIGER, J. & COLLEY, D. G. 1975. Function of the classical and alternate pathways of human complement in serum treated with ethylene glycol tetraacetic acid and MgCl2-ethylene glycol tetraacetic acid. *Infect Immun,* 11**,** 1235-43.

DICKSVED, J., ELLSTROM, P., ENGSTRAND, L. & RAUTELIN, H. 2014. Susceptibility to *Campylobacter* infection is associated with the species composition of the human fecal microbiota. *MBio,* 5**,** e01212-14.

DORRELL, N., MANGAN, J. A., LAING, K. G., HINDS, J., LINTON, D., AL-GHUSEIN, H., BARRELL, B. G., PARKHILL, J., STOKER, N. G., KARLYSHEV, A. V., BUTCHER, P. D. & WREN, B. W. 2001. Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. *Genome Res,* 11**,** 1706-15.

DUFFY, G., LYNCH, O. A. & CAGNEY, C. 2008. Tracking emerging zoonotic pathogens from farm to fork. *Meat Sci,* 78**,** 34-42.

EDWARDS, L. A., NISTALA, K., MILLS, D. C., STEPHENSON, H. N., ZILBAUER, M., WREN, B. W., DORRELL, N., LINDLEY, K. J., WEDDERBURN, L. R. & BAJAJ-ELLIOTT, M. 2010. Delineation of the innate and adaptive T-cell immune outcome in the human host in response to *Campylobacter jejuni* infection. *PLoS One,* 5**,** e15398.

EHLENBERGER, A. G. & NUSSENZWEIG, V. 1977. The role of membrane receptors for C3b and C3d in phagocytosis. *J Exp Med,* 145**,** 357-71.

ELLIS-IVERSEN, J., RIDLEY, A., MORRIS, V., SOWA, A., HARRIS, J., ATTERBURY, R., SPARKS, N. & ALLEN, V. 2012. Persistent environmental reservoirs on farms as risk factors for *Campylobacter* in commercial poultry. *Epidemiol Infect,* 140**,** 916-24.

EWING, C. P., ANDREISHCHEVA, E. & GUERRY, P. 2009. Functional characterization of flagellin glycosylation in *Campylobacter jejuni* 81-176. *J Bacteriol,* 191**,** 7086-93.

FALLMAN, M., ANDERSSON, R. & ANDERSSON, T. 1993. Signaling Properties of Cr3 (Cd11b/Cd18) and Cr-1 (Cd35) in Relation to Phagocytosis of Complement-Opsonized Particles. J Immunol*,* 151**,** 330-338.

FERNANDEZ, H., VERA, F., VILLANUEVA, M. P. & GARCIA, A. 2008. Occurrence of *Campylobacter* species in healthy well-nourished and malnourished children. *Braz J Microbiol,* 39**,** 56-8.

FIGURA, N., GUGLIELMETTI, P., ZANCHI, A., PARTINI, N., ARMELLINI, D., BAYELI, P. F., BUGNOLI, M. & VERDIANI, S. 1993. Two cases of *Campylobacter* mucosalis enteritis in children. *J Clin Microbiol,* 31**,** 727-8.

FLANAGAN, R. C., NEAL-MCKINNEY, J. M., DHILLON, A. S., MILLER, W. G. & KONKEL, M. E. 2009. Examination of *Campylobacter jejuni* putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization. *Infect Immun,* 77**,** 2399-407.

FOSSATI-JIMACK, L., LING, G. S., CORTINI, A., SZAJNA, M., MALIK, T. H., MCDONALD, J. U., PICKERING, M. C., COOK, H. T., TAYLOR, P. R. & BOTTO, M. 2013. Phagocytosis is the main CR3-mediated function affected by the lupus-associated variant of CD11b in human myeloid cells. *PLoS One,* 8**,** e57082.

FOURNIER, B. M. & PARKOS, C. A. 2012. The role of neutrophils during intestinal inflammation. *Mucosal Immunol,* 5**,** 354-66.

FOX, S., LEITCH, A. E., DUFFIN, R., HASLETT, C. & ROSSI, A. G. 2010. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. *J Innate Immun,* 2**,** 216-27.

FRANCHI, L., WARNER, N., VIANI, K. & NUNEZ, G. 2009. Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev*,* 227**,** 106-28.

FUCHS, T. A., ABED, U., GOOSMANN, C., HURWITZ, R., SCHULZE, I., WAHN, V., WEINRAUCH, Y., BRINKMANN, V. & ZYCHLINSKY, A. 2007. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol,* 176**,** 231-41.

FUJIWARA, N. & KOBAYASHI, K. 2005. Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy,* 4**,** 281-6.

FUKUBA, N., ISHIHARA, S., TADA, Y., OSHIMA, N., MORIYAMA, I., YUKI, T., KAWASHIMA, K., KUSHIYAMA, Y., FUJISHIRO, H. & KINOSHITA, Y. 2014. Prevalence of irritable bowel

syndrome-like symptoms in ulcerative *coli*tis patients with clinical and endoscopic evidence of remission: prospective multicenter study. *Scand J Gastroenterol,* 49**,** 674-80.

FURTADO, P. B., HUANG, C. Y., IHYEMBE, D., HAMMOND, R. A., MARSH, H. C. & PERKINS, S. J. 2008. The partly folded back solution structure arrangement of the 30 SCR domains in human complement receptor type 1 (CR1) permits access to its C3b and C4b ligands. *J Mol Biol,* 375**,** 102-18.

GABELLONI, M. L., SABBIONE, F., JANCIC, C., BASS, J. F., KEITELMAN, I., IULA, L., OLEASTRO, M., GEFFNER, J. R. & TREVANI, A. S. 2013. NADPH oxidase derived reactive oxygen species are involved in human neutrophil IL-1 beta secretion but not in inflammasome activation. *Eur J Immunol,* 43**,** 3324-3335.

GASQUE, P. 2004. Complement: a unique innate immune sensor for danger signals. *Mol Immunol,* 41**,** 1089-1098.

GAZENDAM, R., HAMME, J. L., TOOL, A., HOUDT, M., HERBST, M., LIESE, J. G., VEERDONK, F. L., ROOS, D., VAN DEN BERG, T. K. & KUIJPERS, T. W. 2014a. Two Independent Killing Mechanisms of *Candida Albicans* by Human Neutrophils: Evidence from Innate Immunity Defects. *J Clin Immunol,* 34**,** S142-S142.

GAZENDAM, R. P., VAN HAMME, J. L., TOOL, A. T., VAN HOUDT, M., VERKUIJLEN, P. J., HERBST, M., LIESE, J. G., VAN DE VEERDONK, F. L., ROOS, D., VAN DEN BERG, T. K. & KUIJPERS, T. W. 2014b. Two independent killing mechanisms of *Candida Albicans* by human neutrophils: evidence from innate immunity defects. *Blood,* 124**,** 590-7.

GEURTSEN, J., DZIECIATKOWSKA, M., STEEGHS, L., HAMSTRA, H. J., BOLEIJ, J., BROEN, K., AKKERMAN, G., EL HASSAN, H., LI, J., RICHARDS, J. C., TOMMASSEN, J. & VAN DER LEY, P. 2009. Identification of a novel lipopolysaccharide core biosynthesis gene cluster in *Bordetella pertussis*, and influence of core structure and lipid A glucosamine substitution on endotoxic activity. *Infect Immun,* 77**,** 2602-11.

GHAREEB, K., AWAD, W. A., MOHNL, M., PORTA, R., BIARNES, M., BOHM, J. & SCHATZMAYR, G. 2012. Evaluating the efficacy of an avian-specific probiotic to reduce the colonization of *Campylobacter jejuni* in broiler chickens. *Poult Sci,* 91**,** 1825-32.

GHIRAN, I., BARBASHOV, S. F., KLICKSTEIN, L. B., TAS, S. W., JENSENIUS, J. C. & NICHOLSON-WELLER, A. 2000. Complement receptor 1/CD35 is a receptor for mannan-binding lectin. *J Exp Med,* 192**,** 1797-808.

GILBERT, M., PARKER, C. T. & MORAN, A. P. 2008. *Campylobacter jejuni* Lipooligosaccharides: Structures and Biosynthesis. *Campylobacter, 3rd Edition***,** 483-504.

GOODMAN, M. J., PEARSON, K. W., MCGHIE, D., DUTT, S. & DEODHAR, S. G. 1980. *Campylobacter* and *Giardia lamblia* causing exacerbation of inflammatory bowel disease. *Lancet,* 2**,** 1247.

GORDON, D. L., JOHNSON, G. M. & HOSTETTER, M. K. 1986. Ligand-receptor interactions in the phagocytosis of virulent *Streptococcus pneumoniae* by polymorphonuclear leukocytes. *J Infect Dis,* 154**,** 619-26.

GORDON, D. L., JOHNSON, G. M. & HOSTETTER, M. K. 1987. Characteristics of iC3b binding to human polymorphonuclear leucocytes. *Immunology,* 60**,** 553-8.

GORSUCH, W. B., CHRYSANTHOU, E., SCHWAEBLE, W. J. & STAHL, G. L. 2012. The complement system in ischemia-reperfusion injuries. *Immunobiology,* 217**,** 1026-33.

GRADEL, K. O., NIELSEN, H. L., SCHONHEYDER, H. C., EJLERTSEN, T., KRISTENSEN, B. & NIELSEN, H. 2009. Increased short- and long-term risk of inflammatory bowel disease after *Salmonella* or *Campylobacter* gastroenteritis. *Gastroenterology,* 137**,** 495-501.

GRAVES, D. T. & JIANG, Y. 1995. Chemokines, a family of chemotactic cytokines. *Crit Rev Oral Biol Med,* 6**,** 109-18.

GRESHAM, H. D., ZHELEZNYAK, A., MORMOL, J. S. & BROWN, E. J. 1990. Studies on the molecular mechanisms of human neutrophil Fc receptor-mediated phagocytosis. Evidence that a distinct pathway for activation of the respiratory burst results in reactive oxygen metabolite-dependent amplification of ingestion. *J Biol Chem,* 265**,** 7819-26.

GROMMES, J., VIJAYAN, S., DRECHSLER, M., HARTWIG, H., MORGELIN, M., DEMBINSKI, R., JACOBS, M., KOEPPEL, T. A., BINNEBOSEL, M., WEBER, C. & SOEHNLEIN, O. 2012. Simvastatin Reduces Endotoxin-Induced Acute Lung Injury by Decreasing Neutrophil Recruitment and Radical Formation. *PLoS One,* 7.

GUERRY, P. 2007. *Campylobacter* flagella: not just for motility. *Trends Microbiol,* 15**,** 456-61.

GUERRY, P., ALM, R. A., POWER, M. E., LOGAN, S. M. & TRUST, T. J. 1991. Role of two flagellin genes in *Campylobacter* motility. *J Bacteriol,* 173**,** 4757-64.

HAAG, L. M., FISCHER, A., OTTO, B., PLICKERT, R., KUHL, A. A., GOBEL, U. B., BERESWILL, S. & HEIMESAAT, M. M. 2012a. *Campylobacter jejuni* induces acute entero*coli*tis in gnotobiotic IL-10-/- mice via Toll-like-receptor-2 and -4 signaling. *PLoS One,* 7**,** e40761.

HAAG, L. M., FISCHER, A., OTTO, B., PLICKERT, R., KUHL, A. A., GOBEL, U. B., BERESWILL, S. & HEIMESAAT, M. M. 2012b. Intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogate colonization resistance against *Campylobacter jejuni* in mice. *PLoS One,* 7**,** e35988.

HALSTENSEN, T. S., HVATUM, M., SCOTT, H., FAUSA, O. & BRANDTZAEG, P. 1992. Association of subepithelial deposition of activated complement and immunoglobulin G and M response to gluten in celiac disease. *Gastroenterology,* 102**,** 751-9.

HALSTENSEN, T. S., MOLLNES, T. E., GARRED, P., FAUSA, O. & BRANDTZAEG, P. 1990. Epithelial deposition of immunoglobulin G1 and activated complement (C3b and terminal complement complex) in ulcerative *coli*tis. *Gastroenterology,* 98**,** 1264-71.

HANSEN, R., BERRY, S. H., MUKHOPADHYA, I., THOMSON, J. M., SAUNDERS, K. A., NICHOLL, C. E., BISSET, W. M., LOGANATHAN, S., MAHDI, G., KASTNER-COLE, D., BARCLAY, A. R., BISHOP, J., FLYNN, D. M., MCGROGAN, P., RUSSELL, R. K., EL-OMAR, E. M. & HOLD, G. L. 2013. The microaerophilic microbiota of de-novo paediatric inflammatory bowel disease: the BISCUIT study. *PLoS One,* 8**,** e58825.

HAVELAAR, A. H., IVARSSON, S., LOFDAHL, M. & NAUTA, M. J. 2013. Estimating the true incidence of campylobacteriosis and salmonellosis in the European Union, 2009.*Epidemiol Infect,* 141**,** 293-302.

HAVELAAR, A. H., VAN PELT, W., ANG, C. W., WAGENAAR, J. A., VAN PUTTEN, J. P. M., GROSS, U. & NEWELL, D. G. 2009. Immunity to Campylobacter: its role in risk assessment and epidemiology. *Crit Rev Microbiol,* 35**,** 1-22.

HED, J. & STENDAHL, O. 1982. Differences in the ingestion mechanisms of IgG and C3b particles in phagocytosis by neutrophils. *Immunology,* 45**,** 727-36.

HEIKEMA, A. P., BERGMAN, M. P., RICHARDS, H., CROCKER, P. R., GILBERT, M., SAMSOM, J. N., VAN WAMEL, W. J., ENDTZ, H. P. & VAN BELKUM, A. 2010. Characterization of the specific interaction between sialoadhesin and sialylated *Campylobacter jejuni* lipooligosaccharides. *Infect Immun,* 78**,** 3237-46.

HEIKEMA, A. P., KONING, R. I., DUARTE DOS SANTOS RICO, S., REMPEL, H., JACOBS, B. C., ENDTZ, H. P., VAN WAMEL, W. J. & SAMSOM, J. N. 2013. Enhanced, sialoadhesin-dependent uptake of Guillain-Barre syndrome-associated *Campylobacter jejuni* strains by human macrophages. *Infect Immun,* 81**,** 2095-103.

HENDRIXSON, D. R. & DIRITA, V. J. 2004. Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. *Mol Microbiol,* 52**,** 471-84.

HERBRINK, P., VANDENMUNCKHOF, H. A. M., BUMKENS, M., LINDEMAN, J. & VANDIJK, W. C. 1988. Human-Serum Antibody-Response in *Campylobacter-Jejuni* Enteritis as Measured by Enzyme-Linked Immunosorbent-Assay. *Eur J Clin Microbiol Infect Dis,* 7**,** 388-393.

HICKEY, T. E., MAJAM, G. & GUERRY, P. 2005. Intracellular survival of *Campylobacter jejuni* in human monocytic cells and induction of apoptotic death by cytholethal distending toxin. *Infect Immun,* 73**,** 5194-7.

HOFFMANN, S., BATZ, M. B. & MORRIS, J. G., JR. 2012. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. *J Food Prot,* 75**,** 1292-302.

HOURCADE, D. E. 2006. The role of properdin in the assembly of the alternative pathway C3 convertases of complement. *J Biol Chem,* 281**,** 2128-32.

HOWARD, S. L., JAGANNATHAN, A., SOO, E. C., HUI, J. P., AUBRY, A. J., AHMED, I., KARLYSHEV, A., KELLY, J. F., JONES, M. A., STEVENS, M. P., LOGAN, S. M. & WREN, B. W. 2009. *Campylobacter jejuni* glycosylation island important in cell charge, legionaminic acid biosynthesis, and colonization of chickens. *Infect Immun,* 77**,** 2544-56.

HU, L., BRAY, M. D., OSORIO, M. & KOPECKO, D. J. 2006. *Campylobacter jejuni* induces maturation and cytokine production in human dendritic cells. *Infect Immun,* 74**,** 2697-705.

HU, L. & HICKEY, T. E. 2005. *Campylobacter jejuni* induces secretion of proinflammatory chemokines from human intestinal epithelial cells. *Infect Immun,* 73**,** 4437-40.

HU, L. & KOPECKO, D. J. 1999. *Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells. *Infect Immun,* 67**,** 4171-82.

HUIZINGA, R., VAN DEN BERG, B., VAN RIJS, W., TIO-GILLEN, A. P., FOKKINK, W. J. R., BAKKER-JONGES, L. E., GELEIJNS, K., SAMSOM, J. N., VAN DOORN, P. A., LAMAN, J. D. & JACOBS, B. C. 2015. Innate Immunity to *Campylobacter jejuni* in Guillain-Barre Syndrome. *Annals of Neurology,* 78**,** 343-354.

HUMPHREY, S., CHALONER, G., KEMMETT, K., DAVIDSON, N., WILLIAMS, N., KIPAR, A., HUMPHREY, T. & WIGLEY, P. 2014. *Campylobacter jejuni* is not merely a commensal in commercial broiler chickens and affects bird welfare. *MBio,* 5**,** e01364-14.

HYAMS, C., CAMBERLEIN, E., COHEN, J. M., BAX, K. & BROWN, J. S. 2010. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect Immun,* 78**,** 704-15.

INDIK, Z. K., PARK, J. G., HUNTER, S. & SCHREIBER, A. D. 1995. The molecular dissection of Fc gamma receptor mediated phagocytosis. *Blood,* 86**,** 4389-99.

ISHIBASHI, Y. & ARAI, T. 1990. Roles of the complement receptor type 1 (CR1) and type 3 (CR3) on phagocytosis and subsequent phagosome-lysosome fusion in *Salmonella*-infected murine macrophages. *FEMS Microbiol Immunol,* 2**,** 89-96.

ISHIBASHI, Y. & ARAI, T. 1996. A possible mechanism for host-specific pathogenesis of *Salmonella* serovars. *Microb Pathog,* 21**,** 435-46.

ISLAM, Z., GILBERT, M., MOHAMMAD, Q. D., KLAIJ, K., LI, J. J., VAN RIJS, W., TIO-GILLEN, A. P., TALUKDER, K. A., WILLISON, H. J., VAN BELKUM, A., ENDTZ, H. P. & JACOBS, B. C. 2012. Guillain-Barre Syndrome-Related *Campylobacter jejuni* in Bangladesh: Ganglioside Mimicry and Cross-Reactive Antibodies. *PLoS One,* 7.

ISMAIL, Y., LEE, H., RIORDAN, S. M., GRIMM, M. C. & ZHANG, L. 2013. The effects of oral and enteric *Campylobacter concisus* strains on expression of TLR4, MD-2, TLR2, TLR5 and COX-2 in HT-29 cells. *PLoS One,* 8**,** e56888.

IWAKI, D., KANNO, K., TAKAHASHI, M., ENDO, Y., MATSUSHITA, M. & FUJITA, T. 2011. The role of mannose-binding lectin-associated serine protease-3 in activation of the alternative complement pathway. *J Immunol,* 187**,** 3751-8.

JACOBS, B. C., ROTHBARTH, P. H., VAN DER MECHE, F. G. A., HERBRINK, P., SCHMITZ, P. I. M., DE KLERK, M. A. & VAN DOORN, P. A. 1998. The spectrum of antecedent infections in Guillain-Barre syndrome - A case-control study. *Neurology,* 51**,** 1110-1115.

JAMES, C., THABANE, M., BORGAONKAR, M. R. & MARSHALL, J. K. 2004. Post infectious irritable bowel syndrome (PI-IBS) is transient following a foodborne outbreak of acute gastroenteritis attributed to a viral pathogen. *Gastroenterology,* 126**,** A53-A53.

JANOFF, E. N., FASCHING, C., ORENSTEIN, J. M., RUBINS, J. B., OPSTAD, N. L. & DALMASSO, A. P. 1999. Killing of *Streptococcus pneumoniae* by capsular polysaccharide-specific polymeric IgA, complement, and phagocytes. *J Clin Invest,* 104**,** 1139-47.

JARVIS, G. A. & VEDROS, N. A. 1987. Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. *Infect Immun,* 55**,** 174-80.

JIN, S. M., SONG, Y. C., EMILI, A., SHERMAN, P. M. & CHAN, V. L. 2003. JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90 alpha and triggers signalling pathways leading to the activation of NF-kappa B and p38 MAP kinase in epithelial cells. *Cell Microbiol,* 5**,** 165-174.

JOHN, C. M., LIU, M., PHILLIPS, N. J., YANG, Z., FUNK, C. R., ZIMMERMAN, L. I., GRIFFISS, J. M., STEIN, D. C. & JARVIS, G. A. 2012. Lack of lipid A pyrophosphorylation and functional lptA reduces inflammation by *Neisseria* commensals. *Infect Immun,* 80**,** 4014-26.

JOINER, K. A., SCHMETZ, M. A., GOLDMAN, R. C., LEIVE, L. & FRANK, M. M. 1984. Mechanism of bacterial resistance to complement-mediated killing: inserted C5b-9 correlates with killing for *Escherichia coli* O111B4 varying in O-antigen capsule and O-polysaccharide coverage of lipid A core oligosaccharide. *Infect Immun,* 45**,** 113-7.

JONES, H. E., STRID, J., OSMAN, M., URONEN-HANSSON, H., DIXON, G., KLEIN, N., WONG, S. Y. & CALLARD, R. E. 2008. The role of beta2 integrins and lipopolysaccharide-binding protein in the phagocytosis of dead *Neisseria meningitidis*. *Cell Microbiol,* 10**,** 1634-45.

JONES, K. 2001. The *Campylobacter* conundrum. *Trends Microbiol,* 9**,** 365-6.

JONES, M. A., TOTEMEYER, S., MASKELL, D. J., BRYANT, C. E. & BARROW, P. A. 2003. Induction of proinflammatory responses in the human monocytic cell line THP-1 by *Campylobacter jejuni*. *Infect Immun,* 71**,** 2626-33.

JOWIYA, W., BRUNNER, K., ABOUELHADID, S., HUSSAIN, H. A., NAIR, S. P., SADIQ, S., WILLIAMS, L. K., TRANTHAM, E. K., STEPHENSON, H., WREN, B. W., BAJAJ-ELLIOTT, M., COGAN, T. A., LAWS, A. P., WADE, J., DORRELL, N. & ALLAN, E. 2015. Pancreatic Amylase Is an Environmental Signal for Regulation of Biofilm Formation and Host Interaction in *Campylobacter jejuni*. *Infect Immun,* 83**,** 4884-95.

KAAKOUSH, N. O., CASTANO-RODRIGUEZ, N., MITCHELL, H. M. & MAN, S. M. 2015. Global Epidemiology of *Campylobacter* Infection. *Clinical Microbiology Reviews,* 28**,** 687-720.

KAAKOUSH, N. O. & MITCHELL, H. M. 2012. *Campylobacter concisus* - A new player in intestinal disease. *Front Cell Infect Microbiol,* 2**,** 4.

KALISCHUK, L. D. & BURET, A. G. 2010. A role for *Campylobacter jejuni*-induced enteritis in inflammatory bowel disease? *Am J Physiol Gastrointest Liver Physiol,* 298**,** G1-9.

KALISCHUK, L. D., INGLIS, G. D. & BURET, A. G. 2009. *Campylobacter jejuni* induces transcellular translocation of commensal bacteria via lipid rafts. *Gut Pathog,* 1**,** 2.

KANJI, A., JONES, M. A., MASKELL, D. J. & GRANT, A. J. 2015. *Campylobacter jejuni* PflB is required for motility and colonisation of the chicken gastrointestinal tract. *Microb Pathog,* 89**,** 93-9.

KAPLAN, G. 1977. Differences in the mode of phagocytosis with Fc and C3 receptors in macrophages. *Scand J Immunol,* 6**,** 797-807.

KARLSSON, A. & DAHLGREN, C. 2002. Assembly and activation of the neutrophil NADPH oxidase in granule membranes. *Antioxid Redox Signal,* 4**,** 49-60.

KARLYSHEV, A. V., CHAMPION, O. L., CHURCHER, C., BRISSON, J. R., JARRELL, H. C., GILBERT, M., BROCHU, D., ST MICHAEL, F., LI, J., WAKARCHUK, W. W., GOODHEAD, I., SANDERS, M., STEVENS, K., WHITE, B., PARKHILL, J., WREN, B. W. & SZYMANSKI, C. M. 2005a. Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. *Mol Microbiol,* 55**,** 90-103.

KARLYSHEV, A. V., KETLEY, J. M. & WREN, B. W. 2005b. The *Campylobacter jejuni* glycome. *FEMS Microbiol Rev,* 29**,** 377-90.

KARLYSHEV, A. V., WREN, B. W. & MORAN, A. P. 2008. *Campylobacter jejuni* Capsular Polysaccharide. *Campylobacter, 3rd Edition***,** 505-521.

KARMAKAR, M., SUN, Y., HISE, A. G., RIETSCH, A. & PEARLMAN, E. 2012. Cutting edge: IL-1beta processing during *Pseudomonas aeruginosa* infection is mediated by neutrophil serine proteases and is independent of NLRC4 and caspase-1. *J Immunol,* 189**,** 4231-5.

KASTEN, K. R., MUENZER, J. T. & CALDWELL, C. C. 2010. Neutrophils are significant producers of IL-10 during sepsis. *Biochem Biophys Res Commun,* 393**,** 28-31.

KENNEDY, A. D. & DELEO, F. R. 2009. Neutrophil apoptosis and the resolution of infection. *Immunologic Research,* 43**,** 25-61.

KEO, T., COLLINS, J., KUNWAR, P., BLASER, M. J. & IOVINE, N. M. 2011. *Campylobacter* capsule and lipooligosaccharide confer resistance to serum and cationic antimicrobials. *Virulence,* 2**,** 30-40.

KIEHLBAUCH, J. A., ALBACH, R. A., BAUM, L. L. & CHANG, K. P. 1985. Phagocytosis of *Campylobacter-Jejuni* and Its Intracellular Survival in Mononuclear Phagocytes. *Infect Immun,* 48**,** 446-451.

KIRK, K. F., NIELSEN, H. L. & NIELSEN, H. 2015. The susceptibility of *Campylobacter concisus* to the bactericidal effects of normal human serum. *APMIS,* 123**,** 269-74.

KISHORE, U., GHAI, R., GREENHOUGH, T. J., SHRIVE, A. K., BONIFATI, D. M., GADJEVA, M. G., WATERS, P., KOJOUHAROVA, M. S., CHAKRABORTY, T. & AGRAWAL, A. 2004. Structural and functional anatomy of the globular domain of complement protein C1q. *Immunol Lett,* 95**,** 113-28.

KISHORE, U., GUPTA, S. K., PERDIKOULIS, M. V., KOJOUHAROVA, M. S., URBAN, B. C. & REID, K. B. 2003. Modular organization of the carboxyl-terminal, globular head region of human C1q A, B, and C chains. *J Immunol,* 171**,** 812-20.

KLANCNIK, A., GUZEJ, B., JAMNIK, P., VUCKOVIC, D., ABRAM, M. & MOZINA, S. S. 2009. Stress response and pathogenic potential of *Campylobacter jejuni* cells exposed to starvation. *Res Microbiol,* 160**,** 345-52.

KLEBANOFF, S. J. 2005. Myeloperoxidase: friend and foe. *J Leukoc Biol,* 77**,** 598-625.

KLENA, J., ZHANG, P., SCHWARTZ, O., HULL, S. & CHEN, T. 2005. The core lipopolysaccharide of *Escherichia coli* is a ligand for the dendritic-cell-specific intercellular adhesion molecule nonintegrin CD209 receptor. *J Bacteriol,* 187**,** 1710-5.

KLICKSTEIN, L. B., BARBASHOV, S. F., LIU, T., JACK, R. M. & NICHOLSON-WELLER, A. 1997. Complement receptor type 1 (CR1, CD35) is a receptor for C1q. *Immunity,* 7**,** 345-55.

KO, Y. P., KUIPERS, A., FREITAG, C. M., JONGERIUS, I., MEDINA, E., VAN ROOIJEN, W. J., SPAAN, A. N., VAN KESSEL, K. P., HOOK, M. & ROOIJAKKERS, S. H. 2013. Phagocytosis escape by a *Staphylococcus aureus* protein that connects complement and coagulation proteins at the bacterial surface. *PLoS Pathog,* 9**,** e1003816.

KOCSIS, A., KEKESI, K. A., SZASZ, R., VEGH, B. M., BALCZER, J., DOBO, J., ZAVODSZKY, P., GAL, P. & PAL, G. 2010. Selective inhibition of the lectin pathway of complement with phage display selected peptides against mannose-binding lectin-associated serine protease (MASP)-1 and -2: significant contribution of MASP-1 to lectin pathway activation. *J Immunol,* 185**,** 4169-78.

KOLACZKOWSKA, E. & KUBES, P. 2013. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol,* 13**,** 159-75.

KONDOS, S. C., HATFALUDI, T., VOSKOBOINIK, I., TRAPANI, J. A., LAW, R. H., WHISSTOCK, J. C. & DUNSTONE, M. A. 2010. The structure and function of mammalian membrane-attack complex/perforin-like proteins. *Tissue Antigens,* 76**,** 341-51.

KONKEL, M. E., CHRISTENSEN, J. E., KEECH, A. M., MONTEVILLE, M. R., KLENA, J. D. & GARVIS, S. G. 2005. Identification of a fibronectin-binding domain within the *Campylobacter jejuni* CadF protein. *Mol Microbiol,* 57**,** 1022-35.

KONKEL, M. E., GARVIS, S. G., TIPTON, S. L., ANDERSON, D. E., JR. & CIEPLAK, W., JR. 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol Microbiol,* 24**,** 953-63.

KONKEL, M. E., KLENA, J. D., RIVERA-AMILL, V., MONTEVILLE, M. R., BISWAS, D., RAPHAEL, B. & MICKELSON, J. 2004. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *J Bacteriol,* 186**,** 3296-303.

KOPP, Z. A., JAIN, U., VAN LIMBERGEN, J. & STADNYK, A. W. 2015. Do antimicrobial peptides and complement collaborate in the intestinal mucosa? *Front Immunol,* 6**,** 17.

KRAUSE-GRUSZCZYNSKA, M., ROHDE, M., HARTIG, R., GENTH, H., SCHMIDT, G., KEO, T., KONIG, W., MILLER, W. G., KONKEL, M. E. & BACKERT, S. 2007a. Role of the small Rho GTPases Rac1 and Cdc42 in host cell invasion of *Campylobacter jejuni*. *Cell Microbiol,* 9**,** 2431-44.

KRAUSE-GRUSZCZYNSKA, M., VAN ALPHEN, L. B., OYARZABAL, O. A., ALTER, T., HANEL, I., SCHLIEPHAKE, A., KONIG, W., VAN PUTTEN, J. P., KONKEL, M. E. & BACKERT, S. 2007b. Expression patterns and role of the CadF protein in *Campylobacter jejuni* and *Campylobacter coli*. *Fems Microbiology Letters,* 274**,** 9-16.

KRUGER, P., SAFFARZADEH, M., WEBER, A. N., RIEBER, N., RADSAK, M., VON BERNUTH, H., BENARAFA, C., ROOS, D., SKOKOWA, J. & HARTL, D. 2015. Neutrophils: Between host defence, immune modulation, and tissue injury. *PLoS Pathog,* 11**,** e1004651.

KRYCH-GOLDBERG, M. & ATKINSON, J. P. 2001. Structure-function relationships of complement receptor type 1. *Immunol Rev,* 180**,** 112-22.

KUGELBERG, E., GOLLAN, B. & TANG, C. M. 2008. Mechanisms in *Neisseria meningitidis* for resistance against complement-mediated killing. *Vaccine,* 26 Suppl 8**,** I34-9.

KUIJF, M. L., SAMSOM, J. N., VAN RIJS, W., BAX, M., HUIZINGA, R., HEIKEMA, A. P., VAN DOORN, P. A., VAN BELKUM, A., VAN KOOYK, Y., BURGERS, P. C., LUIDER, T. M., ENDTZ, H. P., NIEUWENHUIS, E. E. & JACOBS, B. C. 2010. TLR4-mediated sensing of *Campylobacter jejuni* by dendritic cells is determined by sialylation. *J Immunol,* 185**,** 748-55.

LACY, P. & STOW, J. L. 2011. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood,* 118**,** 9-18.

LAMB-ROSTESKI, J. M., KALISCHUK, L. D., INGLIS, G. D. & BURET, A. G. 2008. Epidermal growth factor inhibits *Campylobacter jejuni*-induced claudin-4 disruption, loss of epithelial barrier function, and *Escherichia coli* translocation. *Infect Immun,* 76**,** 3390-8.

LATZ, E., XIAO, T. S. & STUTZ, A. 2013. Activation and regulation of the inflammasomes. *Nat Rev Immunol,* 13**,** 397-411.

LAUFER, J., OREN, R., GOLDBERG, I., HORWITZ, A., KOPOLOVIC, J., CHOWERS, Y. & PASSWELL, J. H. 2000. Cellular localization of complement C3 and C4 transcripts in intestinal specimens from patients with Crohn's disease. *Clin Exp Immunol,* 120**,** 30-7.

LEE, H., MA, R., GRIMM, M. C., RIORDAN, S. M., LAN, R., ZHONG, L., RAFTERY, M. & ZHANG, L. 2014. Examination of the Anaerobic Growth of *Campylobacter concisus* Strains. *Int J Microbiol,* 2014**,** 476047.

LEE, S. H., STARKEY, P. M. & GORDON, S. 1985. Quantitative analysis of total macrophage content in adult mouse tissues. Immunochemical studies with monoclonal antibody F4/80. *J Exp Med,* 161**,** 475-89.

LEE, W. L., HARRISON, R. E. & GRINSTEIN, S. 2003. Phagocytosis by neutrophils. *Microbes Infect,* 5**,** 1299-306.

LENGERH, A., MOGES, F., UNAKAL, C. & ANAGAW, B. 2013. Prevalence, associated risk factors and antimicrobial susceptibility pattern of *Campylobacter* species among under five diarrheic children at Gondar University Hospital, Northwest Ethiopia. *BMC Pediatr,* 13**,** 82.

LEPPER, P. M., TRIANTAFILOU, M., SCHUMANN, C., SCHNEIDER, E. M. & TRIANTAFILOU, K. 2005. Lipopolysaccharides from *Helicobacter pylori* can act as antagonists for Toll-like receptor 4. *Cell Microbiol,* 7**,** 519-28.

LERTSETHTAKARN, P., OTTEMANN, K. M. & HENDRIXSON, D. R. 2011. Motility and chemotaxis in *Campylobacter* and Helicobacter. *Annu Rev Microbiol,* 65**,** 389-410.

LI, Z., JIANG, H., XIE, W., ZHANG, Z., SMRCKA, A. V. & WU, D. 2000. Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science,* 287**,** 1046-9.

LINDEN, S. K., FLORIN, T. H. & MCGUCKIN, M. A. 2008. Mucin dynamics in intestinal bacterial infection. *PLoS One,* 3**,** e3952.

LIU, D. & NIU, Z. X. 2009. The structure, genetic polymorphisms, expression and biological functions of complement receptor type 1 (CR1/CD35). *Immunopharmacol Immunotoxicol,* 31**,** 524-35.

LIU, M., JOHN, C. M. & JARVIS, G. A. 2010. Phosphoryl moieties of lipid A from *Neisseria meningitidis* and N. gonorrhoeae lipooligosaccharides play an important role in activation of both MyD88- and TRIF-dependent TLR4-MD-2 signaling pathways. *J Immunol,* 185**,** 6974-84.

LOFGREN, R., SERRANDER, L., FORSBERG, M., WILSSON, A., WASTESON, A. & STENDAHL, O. 1999. CR3, FcgammaRIIA and FcgammaRIIIB induce activation of the respiratory burst in human neutrophils: the role of intracellular Ca(2+), phospholipase D and tyrosine phosphorylation. *Biochim Biophys Acta,* 1452**,** 46-59.

LONE, A. G., SELINGER, L. B., UWIERA, R. R., XU, Y. & INGLIS, G. D. 2013a. *Campylobacter jejuni* colonization is associated with a dysbiosis in the cecal microbiota of mice in the absence of prominent inflammation. *PLoS One,* 8**,** e75325.

LONE, A. G., SELINGER, L. B., UWIERA, R. R. E., XU, Y. & INGLIS, G. D. 2013b. *Campylobacter jejuni* Colonization Is Associated with a Dysbiosis in the Cecal Microbiota of Mice in the Absence of Prominent Inflammation. *PLoS One,* 8.

LOUWEN, R., HEIKEMA, A., VAN BELKUM, A., OTT, A., GILBERT, M., ANG, W., ENDTZ, H. P., BERGMAN, M. P. & NIEUWENHUIS, E. E. 2008. The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion. *Infect Immun,* 76**,** 4431-8.

LU, Y. C., YEH, W. C. & OHASHI, P. S. 2008. LPS/TLR4 signal transduction pathway. *Cytokine,* 42**,** 145-151.

MACAULEY, M. S., CROCKER, P. R. & PAULSON, J. C. 2014. Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol,* 14**,** 653-66.

MACCALLUM, A., HADDOCK, G. & EVEREST, P. H. 2005a. *Campylobacter jejuni* activates mitogen-activated protein kinases in Caco-2 cell monolayers and in vitro infected primary human colonic tissue. *Microbiology,* 151**,** 2765-72.

MACCALLUM, A., HARDY, S. P. & EVEREST, P. H. 2005b. *Campylobacter jejuni* inhibits the absorptive transport functions of Caco-2 cells and disrupts cellular tight junctions. *Microbiology,* 151**,** 2451-8.

MAESHIMA, N. & FERNANDEZ, R. C. 2013. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. *Front Cell Infect Microbiol,* 3**,** 3.

MAHENDRAN, V., RIORDAN, S. M., GRIMM, M. C., TRAN, T. A., MAJOR, J., KAAKOUSH, N. O., MITCHELL, H. & ZHANG, L. 2011. Prevalence of *Campylobacter* species in adult Crohn's disease and the preferential colonization sites of *Campylobacter* species in the human intestine. *PLoS One,* 6**,** e25417.

MAN, S. M. 2011. The clinical importance of emerging *Campylobacter* species. *Nat Rev Gastroenterol Hepatol,* 8**,** 669-85.

MAN, S. M., KAAKOUSH, N. O., LEACH, S. T., NAHIDI, L., LU, H. K., NORMAN, J., DAY, A. S., ZHANG, L. & MITCHELL, H. M. 2010a. Host attachment, invasion, and stimulation of proinflammatory cytokines by *Campylobacter concisus* and other non-*Campylobacter jejuni Campylobacter* species. *J Infect Dis,* 202**,** 1855-65.

MAN, S. M., ZHANG, L., DAY, A. S., LEACH, S. T., LEMBERG, D. A. & MITCHELL, H. 2010b. *Campylobacter concisus* and other *Campylobacter* species in children with newly diagnosed Crohn's disease. *Inflamm Bowel Dis,* 16**,** 1008-16.

MARSHALL, J. K., THABANE, M., GARG, A. X., CLARK, W. F., SALVADORI, M. & COLLINS, S. M. 2006. Incidence and epidemiology of irritable bowel syndrome after a large waterborne outbreak of bacterial dysentery. *Gastroenterology,* 131**,** 445-50; quiz 660.

MASON, J., ITURRIZA-GOMARA, M., O'BRIEN, S. J., NGWIRA, B. M., DOVE, W., MAIDEN, M. C. & CUNLIFFE, N. A. 2013. *Campylobacter* infection in children in Malawi is common and is frequently associated with enteric virus co-infections. *PLoS One,* 8**,** e59663.

MATSUSHITA, M., ENDO, Y., TAIRA, S., SATO, Y., FUJITA, T., ICHIKAWA, N., NAKATA, M. & MIZUOCHI, T. 1996. A novel human serum lectin with collagen- and fibrinogen-like domains that functions as an opsonin. *J Biol Chem,* 271**,** 2448-54.

MAUE, A. C., MOHAWK, K. L., GILES, D. K., POLY, F., EWING, C. P., JIAO, Y., LEE, G., MA, Z., MONTEIRO, M. A., HILL, C. L., FERDERBER, J. S., PORTER, C. K., TRENT, M. S. & GUERRY, P. 2013. The polysaccharide capsule of *Campylobacter jejuni* modulates the host immune response. *Infect Immun,* 81**,** 665-72.

MCGRATH, F. D., BROUWER, M. C., ARLAUD, G. J., DAHA, M. R., HACK, C. E. & ROOS, A. 2006. Evidence that complement protein C1q interacts with C-reactive protein through its globular head region. *J Immunol,* 176**,** 2950-7.

MCSWEEGAN, E. & WALKER, R. I. 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. *Infect Immun,* 53**,** 141-8.

MEDINA, E., GOLDMANN, O., TOPPEL, A. W. & CHHATWAL, G. S. 2003. Survival of Streptococcus *pyogenes* within host phagocytic cells: a pathogenic mechanism for persistence and systemic invasion. *J Infect Dis,* 187**,** 597-603.

MELLER, S., DI DOMIZIO, J., VOO, K. S., FRIEDRICH, H. C., CHAMILOS, G., GANGULY, D., CONRAD, C., GREGORIO, J., LE ROY, D., ROGER, T., LADBURY, J. E., HOMEY, B., WATOWICH, S., MODLIN, R. L., KONTOYIANNIS, D. P., LIU, Y. J., AROLDL, S. T. & GILLIET, M. 2015. T(H)17 cells promote microbial killing and innate immune sensing of DNA via interleukin 26. *Nat Immunol,* 16**,** 970-979.

MENTZING, L. O. 1981. Waterborne outbreaks of *Campylobacter* enteritis in central Sweden. *Lancet,* 2**,** 352-4.

MERI, S. & PANGBURN, M. K. 1990. Discrimination between Activators and Nonactivators of the Alternative Pathway of Complement - Regulation Via a Sialic-Acid Polyanion Binding-Site on Factor-H. *Proc Natl Acad Sci U S A,* 87**,** 3982-3986.

MESTAS, J. & HUGHES, C. C. W. 2004. Of mice and not men: Differences between mouse and human immunology.*J Immunol,* 172**,** 2731-2738.

MILLS, D. C., GUNDOGDU, O., ELMI, A., BAJAJ-ELLIOTT, M., TAYLOR, P. W., WREN, B. W. & DORRELL, N. 2012. Increase in *Campylobacter jejuni* invasion of intestinal epithelial cells under low-oxygen coculture conditions that reflect the in vivo environment. *Infect Immun,* 80**,** 1690-8.

MINDERHOUD, I. M., OLDENBURG, B., WISMEIJER, J. A., VAN BERGE HENEGOUWEN, G. P. & SMOUT, A. J. 2004. IBS-like symptoms in patients with inflammatory bowel disease in remission; relationships with quality of life and coping behavior. *Dig Dis Sci,* 49**,** 469-74.

MOBBERLEY-SCHUMAN, P. S. & WEISS, A. A. 2005. Influence of CR3 (CD11b/CD18) expression on phagocytosis of *Bordetella pertussis* by human neutrophils. *Infect Immun,* 73**,** 7317-23.

MOLLNES, T. E., BREKKE, O. L., FUNG, M., FURE, H., CHRISTIANSEN, D., BERGSETH, G., VIDEM, V., LAPPEGARD, K. T., KOHL, J. & LAMBRIS, J. D. 2002. Essential role of the C5a receptor in E-*coli*-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood,* 100**,** 1869-1877.

MONCADA, D. M., KAMMANADIMINTI, S. J. & CHADEE, K. 2003. Mucin and Toll-like receptors in host defense against intestinal parasites. *Trends Parasitol,* 19**,** 305-11.

MONNO, R., RENDINA, M., CECI, G., RIZZO, C., LUZZI, I., FRANCAVILLA, A., RIZZO, G. & IERARDI, E. 2004. *Campylobacter fetus* bacteremia in an immunocompromised patient: case report and review of the literature. *New Microbiol,* 27**,** 281-5.

MONTEVILLE, M. R. & KONKEL, M. E. 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. *Infect Immun,* 70**,** 6665-71.

MONTEVILLE, M. R., YOON, J. E. & KONKEL, M. E. 2003. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. *Microbiology,* 149**,** 153-65.

MORAN, A. P., ZAHRINGER, U., SEYDEL, U., SCHOLZ, D., STUTZ, P. & RIETSCHEL, E. T. 1991. Structural analysis of the lipid A component of *Campylobacter jejuni* CCUG 10936 (serotype O:2) lipopolysaccharide. Description of a lipid A containing a hybrid backbone of 2-amino-2-deoxy-D-glucose and 2,3-diamino-2,3-dideoxy-D-glucose. *Eur J Biochem,* 198**,** 459-69.

MOREIRA, L. O. & ZAMBONI, D. S. 2012. NOD1 and NOD2 Signaling in Infection and Inflammation. *Front Immunol,* 3**,** 328.

MORTENSEN, N. P., SCHIELLERUP, P., BOISEN, N., KLEIN, B. M., LOCHT, H., ABUOUN, M., NEWELL, D. & KROGFELT, K. A. 2011. The role of *Campylobacter jejuni* cytolethal distending toxin in gastroenteritis: toxin detection, antibody production, and clinical outcome. *APMIS,* 119**,** 626-634.

MUKHERJEE, P., RAMAMURTHY, T., BHATTACHARYA, M. K., RAJENDRAN, K. & MUKHOPADHYAY, A. K. 2013. *Campylobacter jejuni* in hospitalized patients with diarrhea, Kolkata, India. *Emerg Infect Dis,* 19**,** 1155-6.

MUKHOPADHYA, I., HANSEN, R., EL-OMAR, E. M. & HOLD, G. L. 2012. IBD-what role do Proteobacteria play? *Nat Rev Gastroenterol Hepatol,* 9**,** 219-30.

MUKHOPADHYA, I., THOMSON, J. M., HANSEN, R., BERRY, S. H., EL-OMAR, E. M. & HOLD, G. L. 2011. Detection of *Campylobacter concisus* and other *Campylobacter* species in colonic biopsies from adults with ulcerative *coli*tis. *PLoS One,* 6**,** e21490.

MULLER, J., MEYER, B., HANEL, I. & HOTZEL, H. 2007. Comparison of lipooligosaccharide biosynthesis genes of *Campylobacter jejuni* strains with varying abilities to colonize the chicken gut and to invade Caco-2 cells. *J Med Microbiol,* 56**,** 1589-94.

MULLER, S., SCHAFFER, T., FLOGERZI, B., SEIBOLD-SCHMID, B., SCHNIDER, J., TAKAHASHI, K., DARFEUILLE-MICHAUD, A., VAZEILLE, E., SCHOEPFER, A. M. & SEIBOLD, F. 2010. Mannan-binding lectin deficiency results in unusual antibody production and excessive experimental *coli*tis in response to mannose-expressing mild gut pathogens. *Gut,* 59**,** 1493-500.

MURPHY, H., COGAN, T. & HUMPHREY, T. 2011. Direction of neutrophil movements by Campylobacter-infected intestinal epithelium. *Microbes Infect,* 13**,** 42-8.

NACHAMKIN, I., YANG, X. H. & STERN, N. J. 1993. Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants. *Appl Environ Microbiol,* 59**,** 1269-73.

NAITO, M., FRIRDICH, E., FIELDS, J. A., PRYJMA, M., LI, J., CAMERON, A., GILBERT, M., THOMPSON, S. A. & GAYNOR, E. C. 2010. Effects of sequential *Campylobacter jejuni* 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. *J Bacteriol,* 192**,** 2182-92.

NAUSEEF, W. M. 2007. Isolation of human neutrophils from venous blood. *Methods Mol Biol,* 412**,** 15-20.

NEAL-MCKINNEY, J. M. & KONKEL, M. E. 2012. The *Campylobacter jejuni* CiaC virulence protein is secreted from the flagellum and delivered to the cytosol of host cells. *Front Cell Infect Microbiol,* 2**,** 31.

NETEA, M. G., NOLD-PETRY, C. A., NOLD, M. F., JOOSTEN, L. A., OPITZ, B., VAN DER MEER, J. H., VAN DE VEERDONK, F. L., FERWERDA, G., HEINHUIS, B., DEVESA, I., FUNK, C. J., MASON, R. J., KULLBERG, B. J., RUBARTELLI, A., VAN DER MEER, J. W. & DINARELLO, C. A. 2009. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood,* 113**,** 2324-35.

NEWMAN, A. & LAMBERT, J. R. 1980. *Campylobacter jejuni* causing flare-up in inflammatory bowel disease. *Lancet,* 2**,** 919.

NEWMAN, S. L., BECKER, S. & HALME, J. 1985. Phagocytosis by receptors for C3b (CR1), iC3b (CR3), and IgG (Fc) on human peritoneal macrophages. *J Leukoc Biol,* 38**,** 267-78.

NG, L. K., SHERBURNE, R., TAYLOR, D. E. & STILES, M. E. 1985. Morphological Forms and Viability of *Campylobacter* Species Studied by Electron-Microscopy. *J Bacteriol,* 164**,** 338-343.

NIELSEN, H., STEFFENSEN, R. & EJLERTSEN, T. 2012. Risk and Prognosis of Campylobacteriosis in Relation to Polymorphisms of Host Inflammatory Cytokine Genes. *Scand J Immunol,* 75**,** 449-454.

NIELSEN, H. L., EJLERTSEN, T., ENGBERG, J. & NIELSEN, H. 2013. High incidence of *Campylobacter concisus* in gastroenteritis in North Jutland, Denmark: a population-based study. *Clin Microbiol Infect,* 19**,** 445-50.

NIMMERJAHN, F. & RAVETCH, J. V. 2006. Fcgamma receptors: old friends and new family members. *Immunity,* 24**,** 19-28.

NONAKA, M. & KIMURA, A. 2006. Genomic view of the evolution of the complement system. *Immunogenetics,* 58**,** 701-13.

NORDENFELT, P. & TAPPER, H. 2011. Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol,* 90**,** 271-84.

NYATI, K. K. & NYATI, R. 2013. Role of *Campylobacter jejuni* Infection in the Pathogenesis of Guillain-Barre Syndrome: An Update. *Biomed Research International*.

NYATI, K. K., PRASAD, K. N., RIZWAN, A., VERMA, A. & PALIWAL, V. K. 2011. T(H)1 and T(H)2 Response to *Campylobacter jejuni* Antigen in Guillain-Barre Syndrome. *Arch Neurol,* 68**,** 445-452.

OGINO, T., OHNO, R., SEKIYA, K., KUWAE, A., MATSUZAWA, T., NONAKA, T., FUKUDA, H., IMAJOH-OHMI, S. & ABE, A. 2006. Assembly of the type III secretion apparatus of enteropathogenic *Escherichia coli*. *J Bacteriol,* 188**,** 2801-11.

OSTVIK, A. E., GRANLUND, A., GUSTAFSSON, B. I., TORP, S. H., ESPEVIK, T., MOLLNES, T. E., DAMAS, J. K. & SANDVIK, A. K. 2014. Mucosal toll-like receptor 3-dependent synthesis of complement factor B and systemic complement activation in inflammatory bowel disease. *Inflamm Bowel Dis,* 20**,** 995-1003.

OUYANG, W., KOLLS, J. K. & ZHENG, Y. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity,* 28**,** 454-67.

PACANOWSKI, J., LALANDE, V., LACOMBE, K., BOUDRAA, C., LESPRIT, P., LEGRAND, P., TRYSTRAM, D., KASSIS, N., ARLET, G., MAINARDI, J. L., DOUCET-POPULAIRE, F., GIRARD, P. M. & MEYNARD, J. L. 2008. *Campylobacter* bacteremia: clinical features and factors associated with fatal outcome. *Clin Infect Dis,* 47**,** 790-6.

PACHECO, P., WHITE, D. & SULCHEK, T. 2013. Effects of Microparticle Size and Fc Density on Macrophage Phagocytosis. *PLoS One,* 8.

PAPAYANNOPOULOS, V., METZLER, K. D., HAKKIM, A. & ZYCHLINSKY, A. 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol,* 191**,** 677-91.

PARK, B. S., SONG, D. H., KIM, H. M., CHOI, B. S., LEE, H. & LEE, J. O. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature,* 458**,** 1191-5.

PARKER, C. T., HORN, S. T., GILBERT, M., MILLER, W. G., WOODWARD, D. L. & MANDRELL, R. E. 2005. Comparison of *Campylobacter jejuni* lipooligosaccharide biosynthesis loci from a variety of sources. *J Clin Microbiol,* 43**,** 2771-81.

PARKHILL, J., WREN, B. W., MUNGALL, K., KETLEY, J. M., CHURCHER, C., BASHAM, D., CHILLINGWORTH, T., DAVIES, R. M., FELTWELL, T., HOLROYD, S., JAGELS, K., KARLYSHEV, A. V., MOULE, S., PALLEN, M. J., PENN, C. W., QUAIL, M. A., RAJANDREAM, M. A., RUTHERFORD, K. M., VAN VLIET, A. H., WHITEHEAD, S. & BARRELL, B. G. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature,* 403**,** 665-8.

PENNER, J. L. 1988. The genus *Campylobacter*: a decade of progress. *Clin Microbiol Rev,* 1**,** 157-72.

PHAM, T. A. N. & LAW, T. D. 2014. Emerging insights on intestinal dysbiosis during bacterial infections. *Curr Opin Microbiol,* 17**,** 67-74.

PLATTS-MILLS, J. A. & KOSEK, M. 2014. Update on the burden of *Campylobacter* in developing countries. *Curr Opin Infect Dis,* 27**,** 444-50.

POGACAR, M. S., MIHALJEVIC, R. R., KLANCNIK, A., BRUMUINI, G., ABRAM, M. & MOZINA, S. S. 2009. Survival of stress exposed *Campylobacter jejuni* in the murine macrophage J774 cell line. *Int J Food Microbiol,* 129**,** 68-73.

POLY, F. & GUERRY, P. 2008. Pathogenesis of *Campylobacter*. *Curr Opin Gastroenterol,* 24**,** 27-31.

PORTEU, F., MIR, A. & HALBWACHS-MECARELLI, L. 1987. Modulation of neutrophil expression of C3b receptors (CR1) by soluble monomeric human C3b. *Eur J Immunol,* 17**,** 629-35.

RATHINAM, V. A., APPLEDORN, D. M., HOAG, K. A., AMALFITANO, A. & MANSFIELD, L. S. 2009a. *Campylobacter jejuni*-induced activation of dendritic cells involves cooperative signaling through Toll-like receptor 4 (TLR4)-MyD88 and TLR4-TRIF axes. *Infect Immun,* 77**,** 2499-507.

RATHINAM, V. A. K., APPLEDORN, D. M., HOAG, K. A., AMALFITANO, A. & MANSFIELD, L. S. 2009b. *Campylobacter jejuni*-Induced Activation of Dendritic Cells Involves Cooperative Signaling through Toll-Like Receptor 4 (TLR4)-MyD88 and TLR4-TRIF Axes. *Infect Immun,* 77**,** 2499-2507.

RETI, K. L., TYMENSEN, L. D., DAVIS, S. P., AMREIN, M. W. & BURET, A. G. 2015. *Campylobacter jejuni* Increases Flagellar Expression and Adhesion of Noninvasive *Escherichia coli*: Effects on Enterocytic Toll-Like Receptor 4 and CXCL-8 Expression. *Infect Immun,* 83**,** 4571-81.

RICKLIN, D. & LAMBRIS, J. D. 2007. Complement-targeted therapeutics. *Nat Biotechnol,* 25**,** 1265-75.

RIORDAN, S. M., MCIVER, C. J., WAKEFIELD, D., ANDREOPOULOS, P. C., DUNCOMBE, V. M., BOLIN, T. D. & THOMAS, M. C. 1997. Local and systemic complement activity in small intestinal bacterial overgrowth. *Dig Dis Sci,* 42**,** 1128-36.

RIVERA-AMILL, V., KIM, B. J., SESHU, J. & KONKEL, M. E. 2001. Secretion of the virulence-associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. *J Infect Dis,* 183**,** 1607-16.

ROOIJAKKERS, S. H. & VAN STRIJP, J. A. 2007. Bacterial complement evasion. *Mol Immunol,* 44**,** 23-32.

ROOIJAKKERS, S. H., VAN WAMEL, W. J., RUYKEN, M., VAN KESSEL, K. P. & VAN STRIJP, J. A. 2005. Anti-opsonic properties of staphylokinase. *Microbes Infect,* 7**,** 476-84.

ROSE, A., KAY, E., WREN, B. W. & DALLMAN, M. J. 2012. The *Campylobacter jejuni* NCTC11168 capsule prevents excessive cytokine production by dendritic cells. *Med Microbiol Immunlo,* 201**,** 137-144.

ROTIMI, V. O., EGWARI, L. & AKANDE, B. 1990. Acidity and intestinal bacteria: an in-vitro assessment of the bactericidal activity of hydrochloric acid on intestinal pathogens. *Afr J Med Med Sci,* 19**,** 275-80.

ROUMENINA, L. T., POPOV, K. T., BUREEVA, S. V., KOJOUHAROVA, M., GADJEVA, M., RABHERU, S., THAKRAR, R., KAPLUN, A. & KISHORE, U. 2008. Interaction of the globular domain of human C1q with *Salmonella typhimurium* lipopolysaccharide. *Biochimica Et Biophysica Acta-Proteins and Proteomics,* 1784**,** 1271-1276.

ROUND, J. L. & MAZMANIAN, S. K. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol,* 9**,** 313-23.

RUBIN-BEJERANO, I., FRASER, I., GRISAFI, P. & FINK, G. R. 2003. Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida Albicans*. *Proc Natl Acad Sci U S A,* 100**,** 11007-12.

RUDDY, M. J., SHEN, F., SMITH, J. B., SHARMA, A. & GAFFEN, S. L. 2004. Interleukin-17 regulates expression of the CXC chemokine LIX/CXCL5 in osteoblasts: implications for inflammation and neutrophil recruitment. *J Leukoc Biol,* 76**,** 135-144.

RUIZPALACIOS, G. M., CALVA, J. J., PICKERING, L. K., LOPEZVIDAL, Y., VOLKOW, P., PEZZAROSSI, H. & WEST, M. S. 1990. Protection of Breast-Fed Infants against *Campylobacter* Diarrhea by Antibodies in Human-Milk.*J Pediatr,* 116**,** 707-713.

SADIK, C. D., KIM, N. D. & LUSTER, A. D. 2011. Neutrophils cascading their way to inflammation. *Trends Immunol,* 32**,** 452-60.

SAMUELSON, D. R., EUCKER, T. P., BELL, J. A., DYBAS, L., MANSFIELD, L. S. & KONKEL, M. E. 2013. The *Campylobacter jejuni* CiaD effector protein activates MAP kinase signaling pathways and is required for the development of disease. *Cell Communication and Signaling,* 11.

SAMUELSON, D. R. & KONKEL, M. E. 2013. Serine phosphorylation of cortactin is required for maximal host cell invasion by *Campylobacter jejuni*. *Cell Commun Signal,* 11.

SCHNEIDER, M. C., EXLEY, R. M., CHAN, H., FEAVERS, I., KANG, Y. H., SIM, R. B. & TANG, C. M. 2006. Functional significance of factor H binding to *Neisseria meningitidis*. *J Immunol,* 176**,** 7566-75.

SCHROEDER, G. N. & HILBI, H. 2008. Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion. *Clin Microbiol Rev,* 21**,** 134-56.

SCHWAEBLE, W. J., LYNCH, N. J., CLARK, J. E., MARBER, M., SAMANI, N. J., ALI, Y. M., DUDLER, T., PARENT, B., LHOTTA, K., WALLIS, R., FARRAR, C. A., SACKS, S., LEE, H., ZHANG, M., IWAKI, D., TAKAHASHI, M., FUJITA, T., TEDFORD, C. E. & STOVER, C. M. 2011. Targeting of mannan-binding lectin-associated serine protease-2 confers protection from myocardial and gastrointestinal ischemia/reperfusion injury. *Proc Natl Acad Sci U S A,* 108**,** 7523-8.

SCHWARTZ, J. T., BARKER, J. H., LONG, M. E., KAUFMAN, J., MCCRACKEN, J. & ALLEN, L. A. 2012. Natural IgM mediates complement-dependent uptake of *Francisella tularensis* by human neutrophils via complement receptors 1 and 3 in nonimmune serum. *J Immunol,* 189**,** 3064-77.

SCOTT, N. E., NOTHAFT, H., EDWARDS, A. V., LABBATE, M., DJORDJEVIC, S. P., LARSEN, M. R., SZYMANSKI, C. M. & CORDWELL, S. J. 2012. Modification of the *Campylobacter jejuni* N-linked glycan by EptC protein-mediated addition of phosphoethanolamine. *J Biol Chem,* 287**,** 29384-96.

SEBALD, M. & VERON, M. 1963. [Base DNA Content and Classification of Vibrios]. *Ann Inst Pasteur (Paris),* 105**,** 897-910.

SEGAL, A. W. 2005. How neutrophils kill microbes. *Annu Rev Immunol,* 23**,** 197-223.

SEGAL, A. W., DORLING, J. & COADE, S. 1980. Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. *J Cell Biol,* 85**,** 42-59.

SEKINE, H., TAKAHASHI, M., IWAKI, D. & FUJITA, T. 2013. The role of MASP-1/3 in complement activation. *Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury 2, Pts a and B,* 735**,** 41-53.

SEMCHENKO, E. A., DAY, C. J., WILSON, J. C., GRICE, I. D., MORAN, A. P. & KOROLIK, V. 2010. Temperature-dependent phenotypic variation of *Campylobacter jejuni* lipooligosaccharides. *BMC Microbiol,* 10.

SERRANDER, L., SKARMAN, P., RASMUSSEN, B., WITKE, W., LEW, D. P., KRAUSE, K. H., STENDAHL, O. & NUSSE, O. 2000. Selective inhibition of IgG-mediated phagocytosis in gelsolin-deficient murine neutrophils. *J Immunol,* 165**,** 2451-7.

SEYA, T., OKADA, M., NISHINO, H. & ATKINSON, J. P. 1990. Regulation of proteolytic activity of complement factor I by pH: C3b/C4b receptor (CR1) and membrane cofactor protein (MCP) have different pH optima for factor I-mediated cleavage of C3b. *J Biochem,* 107**,** 310-5.

SHEPPARD, S. K. & MAIDEN, M. C. 2015. The evolution of *Campylobacter jejuni* and *Campylobacter coli*. *Cold Spring Harb Perspect Biol,* 7**,** a018119.

SHIGEMATSU, M., UMEDA, A., FUJIMOTO, S. & AMAKO, K. 1998. Spirochaete-like swimming mode of *Campylobacter jejuni* in a viscous environment. *J Med Microbiol,* 47**,** 521-6.

SIEGAL, D., SYED, F., HAMID, N. & CUNHA, B. A. 2005. *Campylobacter jejuni* pan*coli*tis mimicking idiopathic ulcerative *coli*tis. *Heart Lung,* 34**,** 288-90.

SIEMER, B. L., NIELSEN, E. M. & ON, S. L. 2005. Identification and molecular epidemiology of *Campylobacter coli* isolates from human gastroenteritis, food, and animal sources by amplified fragment length polymorphism analysis and Penner serotyping. *Appl Environ Microbiol,* 71**,** 1953-8.

SILVA, J., LEITE, D., FERNANDES, M., MENA, C., GIBBS, P. A. & TEIXEIRA, P. 2011. *Campylobacter* spp. as a Foodborne Pathogen: A Review. *Front Microbiol,* 2**,** 200.

SKARP, C. P., HANNINEN, M. L. & RAUTELIN, H. I. 2015. Campylobacteriosis: the role of poultry-meat. *Clin Microbiol Infect*.

SMITH, K. D., ANDERSEN-NISSEN, E., HAYASHI, F., STROBE, K., BERGMAN, M. A., BARRETT, S. L., COOKSON, B. T. & ADEREM, A. 2003. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat Immunol,* 4**,** 1247-53.

SMITH, P. D., SMYTHIES, L. E., SHEN, R., GREENWELL-WILD, T., GLIOZZI, M. & WAHL, S. M. 2011. Intestinal macrophages and response to microbial encroachment. *Mucosal Immunol,* 4**,** 31-42.

SOLTIS, R. D., HASZ, D., MORRIS, M. J. & WILSON, I. D. 1979a. The effect of heat inactivation of serum on the detection of immune complexes. *J Clin Lab Immunol,* 1**,** 321-7.

SOLTIS, R. D., HASZ, D., MORRIS, M. J. & WILSON, I. D. 1979b. Studies on the nature of heat-labile anti-complementary activity in normal human serum. *Clin Exp Immunol,* 37**,** 310-22.

SOMMERLAD, S. M. & HENDRIXSON, D. R. 2007. Analysis of the roles of FlgP and FlgQ in flagellar motility of *Campylobacter jejuni*. *J Bacteriol,* 189**,** 179-86.

SOUTHGATE, E. L., HE, R. L., GAO, J. L., MURPHY, P. M., NANAMORI, M. & YE, R. D. 2008. Identification of formyl peptides from *Listeria monocytogenes* and *Staphylococcus aureus* as potent chemoattractants for mouse neutrophils. *J Immunol,* 181**,** 1429-37.

SPILLER, R. & CAMPBELL, E. 2006. Post-infectious irritable bowel syndrome. *Curr Opin Gastroenterol,* 22**,** 13-17.

SPILLER, R. & GARSED, K. 2009. Postinfectious Irritable Bowel Syndrome. *Gastroenterology,* 136**,** 1979-1988.

SPILLER, R. C., JENKINS, D., THORNLEY, J. P., HEBDEN, J. M., WRIGHT, T., SKINNER, M. & NEAL, K. R. 2000. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute *Campylobacter* enteritis and in post-dysenteric irritable bowel syndrome. *Gut,* 47**,** 804-811.

ST MICHAEL, F., SZYMANSKI, C. M., LI, J., CHAN, K. H., KHIEU, N. H., LAROCQUE, S., WAKARCHUK, W. W., BRISSON, J. R. & MONTEIRO, M. A. 2002. The structures of the lipooligosaccharide and capsule polysaccharide of *Campylobacter jejuni* genome sequenced strain NCTC 11168. *Eur J Biochem,* 269**,** 5119-36.

STAHL, M., RIES, J., VERMEULEN, J., YANG, H., SHAM, H. P., CROWLEY, S. M., BADAYEVA, Y., TURVEY, S. E., GAYNOR, E. C., LI, X. X. & VALLANCE, B. A. 2014. A Novel Mouse Model of *Campylobacter jejuni* Gastroenteritis Reveals Key Pro-inflammatory and Tissue Protective Roles for Toll-like Receptor Signaling during Infection. *PLoS Pathog,* 10.

STEPHENSON, H. N., JOHN, C. M., NAZ, N., GUNDOGDU, O., DORRELL, N., WREN, B. W., JARVIS, G. A. & BAJAJ-ELLIOTT, M. 2013. *Campylobacter jejuni* lipooligosaccharide sialylation, phosphorylation, and amide/ester linkage modifications fine-tune human Toll-like receptor 4 activation. *J Biol Chem,* 288**,** 19661-72.

STEPHENSON, H. N., MILLS, D. C., JONES, H., MILIORIS, E., COPLAND, A., DORRELL, N., WREN, B. W., CROCKER, P. R., ESCORS, D. & BAJAJ-ELLIOTT, M. 2014. Pseudaminic acid on *Campylobacter jejuni* flagella modulates dendritic cell IL-10 expression via Siglec-10 receptor: a novel flagellin-host interaction. *J Infect Dis,* 210**,** 1487-98.

SUGIHARA, T., KOBORI, A., IMAEDA, H., TSUJIKAWA, T., AMAGASE, K., TAKEUCHI, K., FUJIYAMA, Y. & ANDOH, A. 2010. The increased mucosal mRNA expressions of complement C3 and interleukin-17 in inflammatory bowel disease. *Clin Exp Immunol,* 160**,** 386-93.

SUHONEN, J., HARTIALA, K., TUOMINEN-GUSTAFSSON, H. & VILJANEN, M. K. 2000. *Borrelia burgdorferi*--induced oxidative burst, calcium mobilization, and phagocytosis of human neutrophils are complement dependent. *J Infect Dis,* 181**,** 195-202.

SUN, X. & JOBIN, C. 2014. Nucleotide-binding oligomerization domain-containing protein 2 controls host response to *Campylobacter jejuni* in Il10-/- mice. *J Infect Dis,* 210**,** 1145-54.

SUN, X., LIU, B., SARTOR, R. B. & JOBIN, C. 2013. Phosphatidylinositol 3-kinase-gamma signaling promotes *Campylobacter jejuni*-induced *coli*tis through neutrophil recruitment in mice. *J Immunol,* 190**,** 357-65.

SUN, X. L., LIU, B., SARTOR, R. B. & JOBIN, C. 2012a. PI3K gamma Signaling and Neutrophil Infiltration Mediate *Campylobacter jejuni*-Induced *Coli*tis in Mice. *Gastroenterology,* 142**,** S20-S21.

SUN, X. L., THREADGILL, D. & JOBIN, C. 2012b. *Campylobacter jejuni* Induces *Coli*tis Through Activation of Mammalian Target of Rapamycin Signaling. *Gastroenterology,* 142**,** 86-U234.

SUTTERWALA, F. S., ROSENTHAL, L. A. & MOSSER, D. M. 1996. Cooperation between CR1 (CD35) and CR3 (CD11b/CD18) in the binding of complement-opsonized particles. *J Leukoc Biol,* 59**,** 883-890.

SZYMANSKI, C. M., MICHAEL, F. S., JARRELL, H. C., LI, J., GILBERT, M., LAROCQUE, S., VINOGRADOV, E. & BRISSON, J. R. 2003. Detection of conserved N-linked glycans and phase-variable lipooligosaccharides and capsules from *Campylobacter* cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy. *J Biol Chem,* 278**,** 24509-20.

SZYMANSKI, C. M. & WREN, B. W. 2005. Protein glycosylation in bacterial mucosal pathogens. *Nat Rev Microbiol,* 3**,** 225-37.

TAKAHASHI, M., IWAKI, D., KANNO, K., ISHIDA, Y., XIONG, J., MATSUSHITA, M., ENDO, Y., MIURA, S., ISHII, N., SUGAMURA, K. & FUJITA, T. 2008. Mannose-binding lectin (MBL)-associated serine protease (MASP)-1 contributes to activation of the lectin complement pathway. *J Immunol,* 180**,** 6132-8.

TAKEDA, K. & AKIRA, S. 2005. Toll-like receptors in innate immunity. *Int Immunol,* 17**,** 1-14.

TAM, C. C., O'BRIEN, S. J., PETERSEN, I., ISLAM, A., HAYWARD, A. & RODRIGUES, L. C. 2007. Guillain-Barre Syndrome and Preceding Infection with *Campylobacter*, Influenza and Epstein-Barr Virus in the General Practice Research Database. *PLoS One,* 2.

TAMASSIA, N., CASSATELLA, M. A. & BAZZONI, F. 2014. Fast and accurate quantitative analysis of cytokine gene expression in human neutrophils. *Methods Mol Biol,* 1124**,** 451-67.

TAPPER, H. & GRINSTEIN, S. 1997. Fc receptor-triggered insertion of secretory granules into the plasma membrane of human neutrophils - Selective retrieval during phagocytosis. *J Immunol,* 159**,** 409-418.

TAS, S. W., KLICKSTEIN, L. B., BARBASHOV, S. F. & NICHOLSON-WELLER, A. 1999. C1q and C4b bind simultaneously to CR1 and additively support erythrocyte adhesion. *J Immunol,* 163**,** 5056-63.

TECCHIO, C., MICHELETTI, A. & CASSATELLA, M. A. 2014. Neutrophil-derived cytokines: facts beyond expression. *Front Immunol,* 5**,** 1-7.

TEGLA, C. A., CUDRICI, C., PATEL, S., TRIPPE, R., 3RD, RUS, V., NICULESCU, F. & RUS, H. 2011. Membrane attack by complement: the assembly and biology of terminal complement complexes.*Immunol Res,* 51**,** 45-60.

THOMPSON-CHAGOYAN, O. C., MALDONADO, J. & GIL, A. 2005. Aetiology of inflammatory bowel disease (IBD): role of intestinal microbiota and gut-associated lymphoid tissue immune response. *Clin Nutr,* 24**,** 339-52.

THURMAN, J. M. & HOLERS, V. M. 2006. The central role of the alternative complement pathway in human disease. *J Immunol,* 176**,** 1305-10.

TRIBBLE, D. R., BAQAR, S., SCOTT, D. A., OPLINGER, M. L., TRESPALACIOS, F., ROLLINS, D., WALKER, R. I., CLEMENTS, J. D., WALZ, S., GIBBS, P., BURG, E. F., MORAN, A. P., APPLEBEE, L. & BOURGEOIS, A. L. 2010. Assessment of the Duration of Protection in *Campylobacter jejuni* Experimental Infection in Humans. *Infect Immun,* 78**,** 1750-1759.

UNDERHILL, D. M. & OZINSKY, A. 2002. Phagocytosis of microbes: complexity in action. *Annu Rev Immunol,* 20**,** 825-52.

UNEMO, M., ASPHOLM-HURTIG, M., ILVER, D., BERGSTROM, J., BOREN, T., DANIELSSON, D. & TENEBERG, S. 2005. The sialic acid binding SabA adhesin of *Helicobacter pylori* is essential for nonopsonic activation of human neutrophils. *J Biol Chem,* 280**,** 15390-7.

URBAN, C. F., LOURIDO, S. & ZYCHLINSKY, A. 2006. How do microbes evade neutrophil killing? *Cell Microbiol,* 8**,** 1687-96.

VAN ALPHEN, L. B., WENZEL, C. Q., RICHARDS, M. R., FODOR, C., ASHMUS, R. A., STAHL, M., KARLYSHEV, A. V., WREN, B. W., STINTZI, A., MILLER, W. G., LOWARY, T. L. & SZYMANSKI, C. M. 2014. Biological roles of the O-methyl phosphoramidate capsule modification in *Campylobacter jejuni*. *PLoS One,* 9**,** e87051.

VAN EGMOND, M., HANNEKE VAN VUUREN, A. J. & VAN DE WINKEL, J. G. 1999a. The human Fc receptor for IgA (Fc alpha RI, CD89) on transgenic peritoneal macrophages triggers phagocytosis and tumor cell lysis. *Immunol Lett,* 68**,** 83-7.

VAN EGMOND, M., VAN VUUREN, A. J., MORTON, H. C., VAN SPRIEL, A. B., SHEN, L., HOFHUIS, F. M., SAITO, T., MAYADAS, T. N., VERBEEK, J. S. & VAN DE WINKEL, J. G. 1999b. Human immunoglobulin A receptor (FcalphaRI, CD89) function in transgenic mice requires both FcR gamma chain and CR3 (CD11b/CD18). *Blood,* 93**,** 4387-94.

VAN ETTERIJCK, R., BREYNAERT, J., REVETS, H., DEVREKER, T., VANDENPLAS, Y., VANDAMME, P. & LAUWERS, S. 1996. Isolation of *Campylobacter concisus* from feces of children with and without diarrhea. *J Clin Microbiol,* 34**,** 2304-6.

VAN KESSEL, K. P., BESTEBROER, J. & VAN STRIJP, J. A. 2014. Neutrophil-Mediated Phagocytosis of *Staphylococcus aureus*. *Front Immunol,* 5**,** 467.

VAN SPREEUWEL, J. P., DUURSMA, G. C., MEIJER, C. J., BAX, R., ROSEKRANS, P. C. & LINDEMAN, J. 1985. *Campylobacter* colitis: histological immunohistochemical and ultrastructural findings. *Gut,* 26**,** 945-51.

VAN ZANDBERGEN, G., GIEFFERS, J., KOTHE, H., RUPP, J., BOLLINGER, A., AGA, E., KLINGER, M., BRADE, H., DALHOFF, K., MAASS, M., SOLBACH, W. & LASKAY, T. 2004. *Chlamydia pneumoniae* multiply in neutrophil granulocytes and delay their spontaneous apoptosis. *J Immunol,* 172**,** 1768-76.

VANDAMME, P., FALSEN, E., ROSSAU, R., HOSTE, B., SEGERS, P., TYTGAT, R. & DE LEY, J. 1991. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol,* 41**,** 88-103.

VANDAMME, P., HARRINGTON, C. S., JALAVA, K. & ON, S. L. 2000. Misidentifying helicobacters: the *Helicobacter cinaedi* example. *J Clin Microbiol,* 38**,** 2261-6.

VEERHUIS, R., VAN BREEMEN, M. J., HOOZEMANS, J. M., MORBIN, M., OULADHADJ, J., TAGLIAVINI, F. & EIKELENBOOM, P. 2003. Amyloid beta plaque-associated proteins C1q and SAP enhance the Abeta1-42 peptide-induced cytokine secretion by adult human microglia in vitro. *Acta Neuropathol,* 105**,** 135-44.

VEGGE, C. S., BRONDSTED, L., LI, Y. P., BANG, D. D. & INGMER, H. 2009. Energy taxis drives *Campylobacter jejuni* toward the most favorable conditions for growth. *Appl Environ Microbiol,* 75**,** 5308-14.

VORUP-JENSEN, T., PETERSEN, S. V., HANSEN, A. G., POULSEN, K., SCHWAEBLE, W., SIM, R. B., REID, K. B., DAVIS, S. J., THIEL, S. & JENSENIUS, J. C. 2000. Distinct pathways of mannan-binding lectin (MBL)- and C1-complex autoactivation revealed by reconstitution of MBL with recombinant MBL-associated serine protease-2. *J Immunol,* 165**,** 2093-100.

VUCIC, S., KIERNAN, M. C. & CORNBLATH, D. R. 2009. Guillain-Barre syndrome: An update. *Neurol Clin Neurosci,* 16**,** 733-741.

WAGENAAR, J. A., VAN BERGEN, M. A., BLASER, M. J., TAUXE, R. V., NEWELL, D. G. & VAN PUTTEN, J. P. 2014. *Campylobacter fetus* infections in humans: exposure and disease. *Clin Infect Dis,* 58**,** 1579-86.

WALAN, A., DAHLGREN, C., KIHLSTROM, E., STENDAHL, O. & LOCK, R. 1992. Phagocyte killing of *Campylobacter jejuni* in relation to oxidative activation. *APMIS,* 100**,** 424-30.

WALPORT, M. J. 2001. Complement. First of two parts. *N Engl J Med,* 344**,** 1058-66.

WALZ, S. E., BAQAR, S., BEECHAM, H. J., ECHEVERRIA, P., LEBRON, C., MCCARTHY, M., KUSCHNER, R., BOWLING, S., BOURGEOIS, A. L. & SCOTT, D. A. 2001. Pre-exposure anti-*Campylobacter jejuni* immunoglobulin A levels associated with reduced risk of *Campylobacter* diarrhea in adults traveling to Thailand. *Am J Trop Med Hyg,* 65**,** 652-656.

WANG, L. H., FANG, X. C. & PAN, G. Z. 2004. Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis (vol 53, pg 1096, 2004). *Gut,* 53**,** 1390-1390.

WASSENAAR, T. M. & BLASER, M. J. 1999. Pathophysiology of *Campylobacter jejuni* infections of humans. *Microbes Infect,* 1**,** 1023-33.

WASSENAAR, T. M., VAN DER ZEIJST, B. A., AYLING, R. & NEWELL, D. G. 1993. Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. *J Gen Microbiol,* 139 Pt 6**,** 1171-5.

WATERMAN, S. R. & SMALL, P. L. 1998. Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. *Appl Environ Microbiol,* 64**,** 3882-6.

WATSON, R. O. & GALAN, J. E. 2008. *Campylobacter jejuni* survives within epithelial cells by avoiding delivery to lysosomes. *PLoS Pathog,* 4**,** e14.

WENISCH, C., PARSCHALK, B., PATRUTA, S., BRUSTBAUER, R. & GRANINGER, W. 1999. Effect of polyclonal immunoglobulins on neutrophil phagocytic capacity and reactive oxygen production in patients with gram-negative septicemia. *Infection,* 27**,** 183-6.

WINTERBOURN, C. C. & KETTLE, A. J. 2013. Redox reactions and microbial killing in the neutrophil phagosome. *Antioxid Redox Signal,* 18**,** 642-60.

WITKO-SARSAT, V., RIEU, P., DESCAMPS-LATSCHA, B., LESAVRE, P. & HALBWACHS-MECARELLI, L. 2000. Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest,* 80**,** 617-53.

WRIGHT, S. D. & SILVERSTEIN, S. C. 1983. Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J Exp Med,* 158**,** 2016-23.

YAMAMOTO, M., SATO, S., HEMMI, H., HOSHINO, K., KAISHO, T., SANJO, H., TAKEUCHI, O., SUGIYAMA, M., OKABE, M., TAKEDA, K. & AKIRA, S. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science,* 301**,** 640-643.

YAO, R., BURR, D. H. & GUERRY, P. 1997. CheY-mediated modulation of *Campylobacter jejuni* virulence. *Mol Microbiol,* 23**,** 1021-31.

YOUNG, K. T., DAVIS, L. M. & DIRITA, V. J. 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev Microbiol,* 5**,** 665-79.

YUSTE, J., SEN, A., TRUEDSSON, L., JONSSON, G., HYAMS, C., COHEN, J. M., CAMBERLEIN, E., SRISKANDAN, S. & BROWN, J. S. 2010. Impaired opsonization with complement and phagocytosis of *Streptococcus pyogenes* in sera from subjects with inherited C2 deficiency. *Microbes Infect,* 12**,** 626-34.

ZHANG, L., BUDIMAN, V., DAY, A. S., MITCHELL, H., LEMBERG, D. A., RIORDAN, S. M., GRIMM, M., LEACH, S. T. & ISMAIL, Y. 2010. Isolation and detection of *Campylobacter concisus* from saliva of healthy individuals and patients with inflammatory bowel disease. *J Clin Microbiol,* 48**,** 2965-7.

ZHANG, L., MAN, S. M., DAY, A. S., LEACH, S. T., LEMBERG, D. A., DUTT, S., STORMON, M., OTLEY, A., O'LOUGHLIN, E. V., MAGOFFIN, A., NG, P. H. & MITCHELL, H. 2009. Detection and

isolation of *Campylobacter* species other than C. jejuni from children with Crohn's disease. *J Clin Microbiol,* 47**,** 453-5.

ZHENG, J., MENG, J., ZHAO, S., SINGH, R. & SONG, W. 2008. *Campylobacter*-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires *Campylobacter*-secreted cytolethal distending toxin- and Toll-like receptor-mediated activation of NF-kappaB. *Infect Immun,* 76**,** 4498-508.

ZILBAUER, M., DORRELL, N., ELMI, A., LINDLEY, K. J., SCHULLER, S., JONES, H. E., KLEIN, N. J., NUNEZ, G., WREN, B. W. & BAJAJ-ELLIOTT, M. 2007. A major role for intestinal epithelial nucleotide oligomerization domain 1 (NOD1) in eliciting host bactericidal immune responses to *Campylobacter jejuni*. *Cell Microbiol,* 9**,** 2404-16.