

**Interactions between *Neisseria meningitidis*
and galectin-3**

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Abstract

Galectins are beta-galactoside animal lectins which possess similar carbohydrate recognition domains and have important roles in the development and regulation of innate and adaptive immunity. Galectins have been previously shown to modulate inflammatory responses to bacterial infections, however nothing is known about the functions of different galectins during meningococcal disease.

Here I describe the interaction between galectins, in particular galectin-3, and *Neisseria meningitidis*, a commensal of the human respiratory tract that occasionally causes septicaemia and meningitis. Immunohistochemical analyses showed that expression of galectin-3, but not galectin-1 and galectin-8, is increased in tissues of mice following challenge with *N. meningitidis*; furthermore, galectin-3 staining co-localises with the bacterium in tissues from patients with meningococcal disease.

The molecular basis of interaction between *N. meningitidis* and exogenous galectin-3 was investigated by identifying both the bacterial structures and the regions of galectin-3 involved. Binding of galectin-3 to *N. meningitidis* was shown to be dependent on bacterial lipopolysaccharide (LPS), while inhibition assays with lactose and the use of truncated versions of galectin-3 demonstrated that the C-terminal carbohydrate recognition domain is necessary, but not sufficient, for the interaction. Furthermore, the biological consequences of interaction of bacteria with galectin-3 were investigated. Exogenous galectin-3 did not affect adhesion of *N. meningitidis* to epithelial cells, but increased the attachment of bacteria to phagocytic cells; silencing of galectin-3 expression in phagocytes did not alter the interaction of *N. meningitidis* with cells.

In summary, galectin-3 binds to *N. meningitidis* via LPS and affects meningococcal interaction with phagocytic cells. The potential role of galectin-3 in pathogen recognition is discussed.

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Abbreviations

A ₂₆₀	Optical density at 260 nM
A ₂₈₀	Optical density at 280 nM
AP	Alkaline phosphatase
APS	Ammonium persulphate
Arg	c-Abl related gene
Arg	Arginine
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BHI	Brain heart infusion
CAMP	Cationic antimicrobial peptide
CCD	Cytochalasin D
cDNA	Complementary DNA
CD13	Aminopeptidase N
CEACAM	Carcinoembryonic antigen related cell adhesion molecule
C4BP	Factor 4b-binding protein
CFU	Colony forming unit
CK	Casein kinase
CLR	C-type lectin receptor
CMP-NANA	Cytidine-5'-monophospho-N-acetylneuraminic acid
CNS	Central nervous system
CRD	Carbohydrate-recognition domain
CSF	Cerebrospinal fluid
DAB	Diaminobenzidine
DAMP	Danger associated molecular pattern
DAPI	4,6-Diamino-2-phenylindole
DC	Dendritic cell
DC-SIGN	Dendritic cell-ICAM3 grabbing non integrin
ddH ₂ O	Double distilled water
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ETs	Electrophoretic types
FACS	Fluorescence activating cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
Gal	Galactose
GalNAc	N-acetyl-galactosamine
Glc	Glucose
GlcNAc	N-acetyl-glucosamine
Gln	Glutamine
Gly	Glycine
GM-CSF	Granulocyte-macrophage colony stimulation factor

Hep	Heptose
HEPES	4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HMGB1	High mobility group protein 1
HRP	Horseradish peroxidase
HSPGs	Heparan sulphate proteoglycans
IAA	Iodoacetamide
ICAM	Intracellular adhesion molecule
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
K_d	Dissociation constant
KDa	Kilo Daltons
Kdo	3-deoxy-D-manno-2-octulosonic acid
LacNAc	N-acetyl-lactosamine
LBP	LPS-binding protein
LNnT	Lacto-N-neotetraose
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MARCO	Macrophage receptor with collagenous structure
MBL	Mannose binding lectin
M-CSF	Macrophage colony stimulation factor
MLEE	Multi Locus Enzyme electrophoresis
MLST	Multi Locus Sequence Typing
MMP	Metalloproteinase
MOI	Multiplicity of infection
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
N-CAM	Human neural cell adhesion molecule
ND	N-terminal domain
NFkB	Nuclear factor-kappaB
NO	Nitric oxide
O.D.	Optical density
OMP	Outer membrane protein
pAb	Polyclonal antibody
PAMP	Pathogen associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PEA	Phosphoethanolamine
PFA	Paraformaldehyde
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
PS	Phosphatidyl serine
PVDF	Polyvinylidene
qRT-PCR	Semi-quantitative reverse transcriptase polymerase chain reaction
rpm	Revolutions per minute

ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Siglec	Sialic acid binding Ig-like lectin
siRNA	Small interfering RNA
SR	Scavenger receptor
ssRNA	Single stranded RNA
STs	Sequence types
Sufu	Suppressor of fused
TCR	T-cell receptor
TAE	Tris-acetate
TEMED	Tetramethylethylenediamine
Tfp	Type four pili
Th	T-helper
TIM-3	T-cell immunoglobulin and mucin domain containing molecule 3
TLR	Toll like receptor
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultra violet
VDAC	Voltage dependent anion channel
v/v	Volume/volume
w/v	Weight/volume

CHAPTER 1: Introduction

1.1 History of meningococcal disease

Neisseria meningitidis is a human specific, Gram negative coccus which colonises approximately 10% of the healthy population, with the human nasopharynx its sole reservoir (Cartwright *et al.* 1995). This bacterium is one of the leading causes of bacterial meningitis worldwide (Hill *et al.* 2010).

Meningococcal disease was probably first described by Vieusseux in 1805 in Geneva, who reported a “non-contagious malignant cerebral fever” which was fatal in a few hours and attributed to a “special constitution of the air” (Vieusseux 1805). After Vieusseux’s description, there were several reports of a disease with similar symptoms on both sides of the Atlantic during the 19th and 20th centuries. The meningococcus was isolated in the 19th century from the cerebrospinal fluid (CSF) of patients with meningitis by Weichselbaum (Weichselbaum 1887). The mortality rate for untreated meningococcal disease was between 75-80 % (Swartz 2004), and was only reduced following the production of a meningococcal antiserum (Flexner and Jobling 1908). The serum was initially prepared by injecting live and killed bacteria into horses, and the first trial in humans was performed in 1908; overall mortality rates fell from 80% to 30% with treatment (Flexner and Jobling 1908). In the 1930s, the mortality associated with meningococcal disease decreased further with the introduction of sulphonamides (Feldman 1972). Furthermore, preventive measures against *N. meningitidis* have been available since the development of the first vaccine in 1969 (Goldschneider *et al.* 1969b). Despite these advances, *N. meningitidis* infection continues to be a major public health problem worldwide.

1.2 Classification of *N. meningitidis*: genetic and serological typing

To date, 13 serogroups of *N. meningitidis* have been identified, on the basis of antigenically distinct polysaccharide capsules (Hill *et al.* 2010). Strains can also be distinguished by variation in other antigens including surface proteins and lipopolysaccharide (LPS) (Kuipers *et al.* 2001). For example, differences in the structure of meningococcal LPS determine immunotypes. Immunotypes L8, L9, L10 and L11 are generally expressed by serogroup A strains, whilst immunotypes L1 through to L9 are associated with serogroup B and C strains, the most prevalent being L3, 7, 9 (Verheul *et al.* 1993).

As surface antigens of *N. meningitidis* can alter through antigenic or phase variation (Martin *et al.* 2004), alternative typing techniques have been implemented. *N. meningitidis* can be divided into clonal complexes by multi-locus enzyme electrophoresis (MLEE) or multi-locus sequence typing (MLST) (Caugant *et al.* 1986; Maiden *et al.* 1998). MLEE is based on the electrophoretic mobility of abundant metabolic enzymes. However, MLEE is not used routinely because of a lack of reproducibility (Tibayrenc 2009; Harrison *et al.* 2011). In contrast, MLST is based on differences in the nucleotide sequence of seven genes encoding housekeeping functions (Maiden *et al.* 1998). Disease causing strains can be grouped into clonal complexes based on their genetic similarity, either as electrophoretic (ETs) or sequence types (STs).

1.3 Epidemiology

There are approximately 1.2 million cases of invasive meningococcal disease each year around the world, with around 135,000 deaths (Bai *et al.* 2011). Meningococcal disease

affects individuals of all ages, and the serogroups commonly associated with disease are A, B, C, W135, X and Y ((Panatto *et al.* 2011) and Fig. 1.1).

Certain serogroups are associated with particular epidemiological features. Strains of serogroups B and C cause the majority of infections in industrialized countries, while serogroups A and C strains dominate in third world countries (Stephens *et al.* 2007). Most of the disease in Africa is caused by serogroup A meningococci (Halperin *et al.* 2011). Historically, serogroups A and C have caused epidemic disease and pandemics in Africa, Asia and South America (el-Akkad 1969); serogroups X and W-135 have also caused outbreaks in Africa (Trotter and Greenwood 2007). In particular, sub-Saharan Africa has been designated the “meningitis belt”, first described in 1963, as it contains the countries from Senegal to Ethiopia (Molesworth *et al.* 2002). The epidemiology of meningococcal disease in this area is characterised by seasonal and epidemic waves (Tall *et al.* 2012). Most epidemics of meningitis have been caused by serogroup A strains, although serogroups W135 and X have caused disease in the last decade (Boisier *et al.* 2006; Mueller *et al.* 2006; Collard *et al.* 2010). Major serogroup B epidemics occurred during the second half of the 20th century in Iceland, Norway, Turkey and Cuba as well as other countries (Racloz and Luiz 2010). However, serogroup B still causes the overwhelming majority of endemic meningococcal disease in Europe and the USA, and can cause prolonged epidemics (Cohn *et al.* 2010). In addition, an increase in serogroup Y disease was also reported in Europe and the USA during the last decade (Hedberg *et al.* 2011).



From the "Canada communicable disease report", 2009

Figure 1.1 Meningococcal serogroups associated with disease in different countries.

Disease caused by serogroups B, C and Y are frequent in the Americas, Europe and Australia, while disease caused by serogroup A is more frequent in Africa and Asia. Epidemics of serogroup W-135 disease can occur in Africa.

1.4 Pathogenesis of meningococcal infection

N. meningitidis is an inhabitant of the human nasopharynx, and occasionally crosses the mucosal barrier and enters the bloodstream (Perrin *et al.* 2002). Colonisation of and passage through the epithelial barrier are crucial first steps in the invasion process, and type four pili (Tfp) and outer membrane proteins (OMPs) are involved in initial meningococcal adhesion (Corbett *et al.* 2004). Tfp are filamentous glycosylated structures extending from the bacterial surface, which traverse the capsular polysaccharide. They have been proposed to bind membrane co-factor protein, CD46 (Kallstrom *et al.* 1997). Meningococcal attachment can trigger changes in cortical actin of epithelial cells, leading to neisserial uptake (Rytönen *et al.* 2004). Further contact with the host cells is established through the class 5 OMPs, Opa and Opc. Opa binds to carcinoembryonic antigen CD66, while Opc binds to heparan sulphate proteoglycan receptors (HSPGs), resulting in bacterial engulfment by epithelial cells and transcellular traversal (Virji *et al.* 1994). Interestingly, passage into the bloodstream is reduced by the presence of capsule or sialylated LPS, factors that are crucial for survival of meningococci in the bloodstream (Vogel *et al.* 1996). During carriage and invasion, the expression of class 5 OMPs, pili, LPS and capsule is subject to phase switching and/or antigenic variation (Nassif and So 1995; Hammerschmidt *et al.* 1996).

N. meningitidis can enter non-ciliated respiratory epithelial cells and reach the submucosa, or directly invade damaged epithelial surfaces (Capecchi *et al.* 2005). At this point, binding of pili and Opa proteins to host receptors is responsible for signal transduction in host cells, resulting in maturation of lysosomes and facilitating bacterial invasion (Hauck and Meyer 2003). Subsequently, bacteria reach the systemic circulation where they replicate and survive through expression of bacterial virulence factors and subverting host immunity. Within the bloodstream, meningococci induce a marked inflammatory response,

activating the complement and coagulation cascades. This leads to fever with or without septic shock (Tzeng and Stephens 2000).

A key inducer of the inflammatory response is LPS, which results in meningococcal septic shock (Plant *et al.* 2006; Fransen *et al.* 2010) and interacts with Toll like receptor-4 (TLR-4) leading to the production of pro-inflammatory cytokines (Zimmer *et al.* 2007). Induction of cytokine expression in the vasculature results in endothelial damage and capillary leakage (de Kleijn *et al.* 1998), leading to necrosis of tissues and multiple organ failure (Brandtzaeg *et al.* 1989a). There is a clear correlation between circulating levels of LPS and the severity of disease (Brandtzaeg *et al.* 1989a). In general, bacteraemia is not always followed by meningitis, but the vasculature is still considered the primary route of infection to the central nervous system (CNS) (Mikaty *et al.* 2009). In some cases, bacteria reach the meninges leading to meningitis. The blood CSF barrier is formed by two structures: the choroid plexus, situated in the ventricles and composed of cuboidal epithelial cells joined by tight junctions, and the capillary endothelium (Fan *et al.* 2012). Adhesion of bacteria to the endothelium is dependent on the flow rate and shear stresses imposed by the blood flow (Soyer and Dumenil 2011). The route of bacterial passage across this barrier is unclear, but pili and other adhesins may have similar functions here as with epithelial cells. However, although pilus-mediated adhesion of *N. meningitidis* to epithelial and endothelial cells is followed by the formation of an identical cortical plaque (which is a molecular structure formed by actin, adhesion molecules and membrane receptors crucial for adherence), different mechanisms for bacterial interaction have been proposed for these cell types (Lecuyer *et al.* 2012). How pilated bacteria cross endothelial cells to enter the meninges is unclear (Nassif *et al.* 1999), but one hypothesis is that intracellular signalling induced by Tfp leads to the disruption of intercellular junctions at the site of bacterium-cell

interaction, enabling bacterial passage (Coureuil *et al.* 2009). Once in the subarachnoid space, meningococci promote the production of pro-inflammatory cytokines resulting in meningitis, oedema, apoptosis and ischaemia (Rosenstein *et al.* 2001).

1.5 Host susceptibility to meningococcal infection

The factors that contribute to meningococcal disease and epidemics are not fully understood. However, they include the immune status and susceptibility of the host, bacterial characteristics and environmental factors. The lack of protective bactericidal antibodies in individuals is an important factor, but host genetic polymorphisms also contribute (Goldschneider *et al.* 1969b; Brouwer *et al.* 2009). Specifically, polymorphisms in genes encoding the Fcγ-receptor II (CD32), Fcγ-receptor III (CD16), mannose binding lectin (MBL), factor H and TLR-4 have been associated with meningococcal disease (Emonts *et al.* 2003; Brouwer *et al.* 2009). Genome wide association studies highlighted that genetic variation in regulators of complement activation, including factor H, contributes to host susceptibility to meningococcal disease (Davila *et al.* 2010).

A functional complement system is necessary to prevent meningococcal disease; individuals lacking components of the membrane attack complex (MAC) have an increased risk of developing disseminated meningococcal infection compared to normal individuals (Kuijpers *et al.* 2010). Deficiencies or dysfunction of properdin are associated with invasive or severe meningococcal infection (Nielsen *et al.* 1989), while factor D deficiency also predisposes to meningococcal infection (Sprong *et al.* 2006).

Deficiency of MBL has been associated with *N. meningitidis* infection, although only in early childhood (Emonts *et al.* 2003; Faber *et al.* 2007), and clinical studies have shown that

MBL deficiency can be associated with bacterial infection in patients with neutropenia and meningococcal sepsis (Eisen and Minchinton 2003).

1.6 *Neisseria meningitidis* virulence factors

N. meningitidis is able to evade immune defences by varying its surface structures or by mimicking host molecules (Hill *et al.* 2010), and expresses numerous virulence factors that promote bacterial survival in the human host (Fig. 1.2).

1.6.1 Polysaccharide capsule

The capsule polysaccharide has an important role in the interactions between the pathogen and the host. Capsules of serogroup B, C, Y and W-135 strains (which cause the most cases of disease) contain sialic acid (5-N-acetyl-neuraminic acid, NANA). The capsule biosynthesis genes are clustered in a chromosomal locus called the *cps*. The genes *siaA*, *siaB* and *siaC* are responsible for the synthesis of sialic acid; *siaD* encodes for a poly sialyl transferase involved in capsule polymerisation (Edwards *et al.* 1994).

The serogroup B capsule is formed by an α -(2-8)-linked sialic acid homopolymer which has identical structure to a modification on a human neural cell adhesion molecule, N-CAM1 (Finne *et al.* 1987) and is a clear example of molecular mimicry which enables meningococci to avoid recognition by the host immune response. In addition, the serogroup B capsule is chemically similar to the capsule of K1 strains of *Escherichia coli* (Frosch *et al.* 1991). The capsular polysaccharide promotes resistance against antibody/complement mediated killing and inhibits phagocytosis (Kahler *et al.* 1998). In particular, high levels of

capsule expression can inhibit complement mediated lysis in the presence of bactericidal antibodies that are specific for PorA or are raised against the capsule itself (Uria *et al.* 2008). Furthermore, the meningococcal capsule promotes bacterial survival in intracellular compartments, possibly by protecting bacteria against cationic antimicrobial peptides (CAMPs) (Spinosa *et al.* 2007).

Interestingly, meningococci which are isolated from carriers can be capsulated but are more often non-encapsulated, while meningococci which are isolated from blood or CSF of patients are almost always encapsulated (Andersen 1989).

1.6.2 Lipopolysaccharide (LPS)

Meningococcal LPS consists of a variable, surface exposed saccharide region and a more conserved lipid A portion that anchors the molecule in the bacterial outer membrane. Meningococcal LPS is subject to variation of terminal structures and for example its sialylation depends on the growth conditions including the presence of cytidine-5'-monophospho-N-acetyl-neuraminic acid (CMP-NANA) (Schmiel *et al.* 2011). The surface exposed polysaccharide region consists of up to 10 monosaccharides but lacks an O-antigen, typical of the LPS of some Gram negative bacteria (Samuel and Reeves 2003).

The 12 immunotypes of meningococcal LPS are distinguished on the basis of a combination of sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) analysis and the use of rabbit antisera and monoclonal antibodies (Tsai *et al.* 1987). Strains can express more than one immunotype-specific epitope, defining the classification of the strains as L3,7,9, L2,4 or L1,8 for example (Verheul *et al.* 1994). However, there are no monoclonal antibodies available for several other immunotypes.

Lipid A consists of two β -1-6 linked glucosamine residues containing α -glycosidic and non-glycosidic phosphoryl groups at the 1 and 4 positions, and (R)-3-hydroxy fatty acids in ester and amide linkages (Alexander and Rietschel 2001). Meningococcal lipid A is linked to a conserved inner core region consisting of two heptose (HepI and HepII) residues, attached via a 3-deoxy-D-manno-2-octulosonic acid (Kdo) residue (Fig. 1.3). Meningococcal LPS is very similar to LPS of the closely related pathogen *Neisseria gonorrhoeae* that only differs in its β -chain which contains a different number of saccharide residues. The Lacto-N-neo-tetraose (LNnT) is present in the LPS from both species, and is attached to HepI via a glucose (Glc) in meningococcal LPS, while in the gonococcus it is attached to HepI via a N-acetyl-galactosamine (GalNAc) and a galactose (Gal) residue (Fig. 1.3, A and B).

Immunotypes differ in their monosaccharide composition, type of linkage between sugar residues, amount and location of phospho-ethanolamine (PEA) groups and degree of acetylation. Lipid A can be modified with one or multiple PEA groups at the 4' position by the PEA transferase LptA (Cox *et al.* 2003). HepII is invariably substituted by an N-acetyl glucosamine (GlcNAc) residue and PEA groups can be attached to HepII by the PEA transferases Lpt6 and Lpt3 (Mackinnon *et al.* 2002; Wright *et al.* 2004). Addition of a glucose residue can occur at the 3 position by the LgtG α 1,3-glycosyl transferase (Banerjee *et al.* 1998), (Fig. 1.3).

While the inner core structure is common to most immunotypes, the LNnT epitope is only found in L2, L3, L4, L5, L7, L8 and L9 immunotype LPS (Tsai *et al.* 1998). The L3,7,9 immunotype strains can switch to L8 immunotype strains by phase variation of the *lgtABE* locus; this results in the termination of the α -chain after the first Gal residue (Jennings *et al.* 1995b; Jennings *et al.* 1999). LNnT is also present in host paragloboside, a precursor of

group blood antigens and some human sialylated glycosphingolipids (gangliosides) (Macher and Klock 1980).

The LNnT can be sialylated on the terminal Gal residue, and this can modify epitopes recognised by monoclonal antibodies and have an impact on the interaction with the immune system. The Lst α -2,3 sialyl transferase catalyses the linkage of sialic acid to the LNnT epitope, using CMP-NANA synthesised by the meningococcus or obtained from the environment (Tsai 2001).

LPS sialylation modulates susceptibility to bactericidal antibodies, phagocytosis and down regulates complement activation (Vogel *et al.* 1997). Furthermore, meningococcal adhesion and invasion of epithelial cells is inhibited by LPS sialylation (Virji *et al.* 1994) and resistance to killing by polymorphonuclear leukocytes (PMNs, neutrophils) correlates with the extent of LPS sialylation as well as encapsulation (Estabrook *et al.* 1992).

Meningococcal LPS has a pivotal role in septicaemic disease and septic shock, by inducing expression of cytokines (with Tumour necrosis factor- α , IL-6 and IL-1 being the most abundant), activating the complement system and increasing plasminogen activator inhibitor levels (Hellerud *et al.* 2008). Once infection is established, this marked host inflammatory response to LPS contributes to the mortality and morbidity associated with meningococcal disease (Brandtzaeg *et al.* 1989b; Brandtzaeg *et al.* 2001).

LPS, as well as intact or degraded bacteria and outer membrane vesicles, are highly biologically potent. LPS activates immune cells via TLR-4 and this depends on the lipid A moiety of LPS (Jerala 2007). LPS binding protein (LBP) and CD14 facilitate engagement of the co-receptor MD2 (Fitzgerald *et al.* 2004), forming a complex on macrophages, monocytes and neutrophils (Jerala 2007). The clustering of TLR-4 and recruitment of adaptor proteins triggers intracellular signalling, leading to the activation of nuclear factor kappaB (NFkB) and

other transcription factors that induce cytokine production. NF κ B migrates to the nucleus and initiates the transcription of genes encoding various inflammatory mediators such as IL-1 β and TNF- α ((Akira and Takeda 2004) and Fig. 1.4).

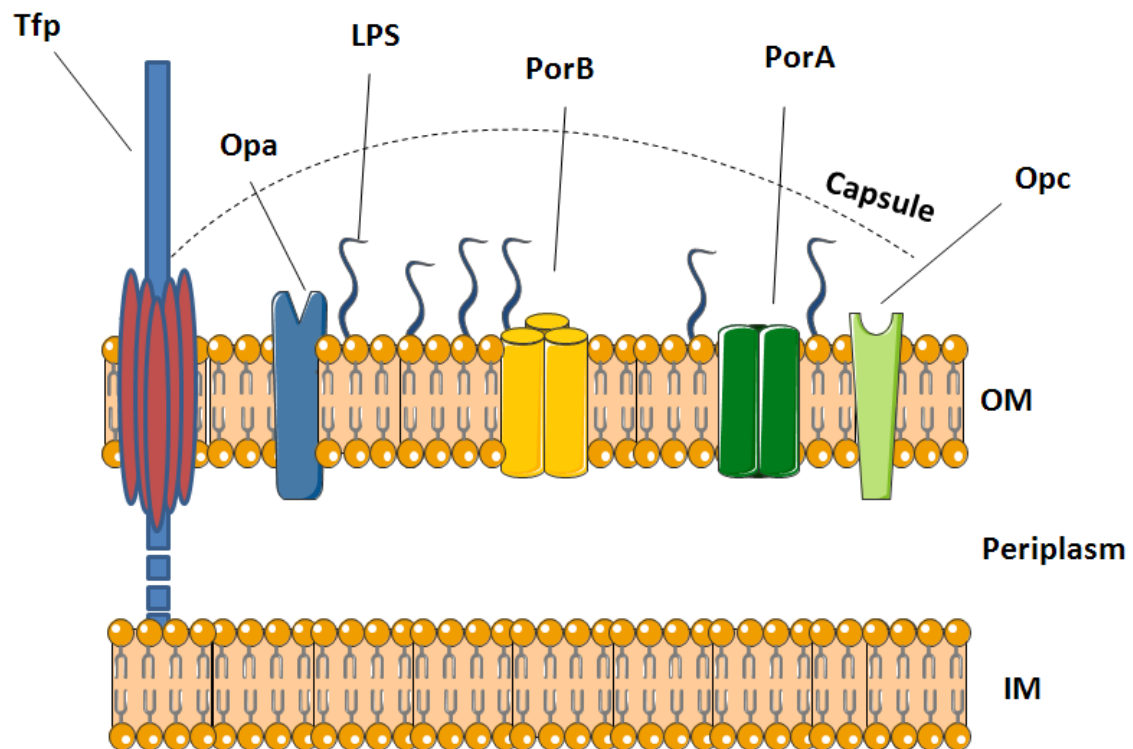
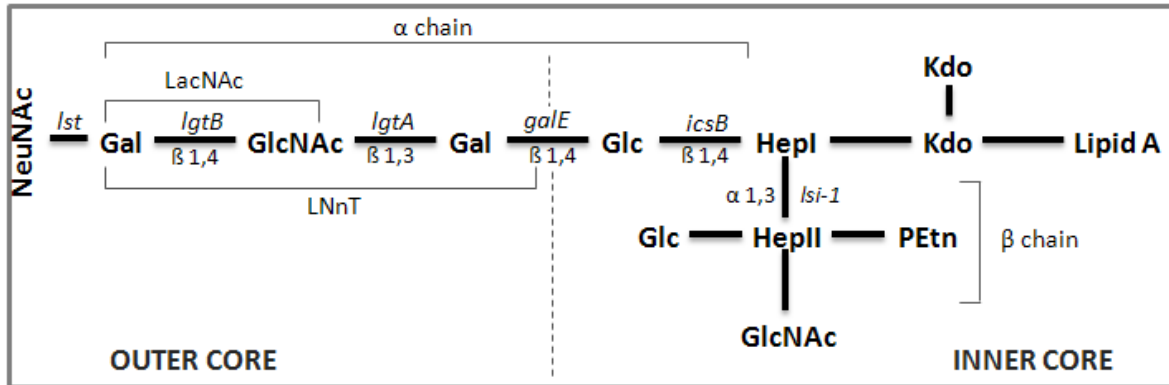


Figure 1.2 Major meningococcal surface structures

Schematic representation of the meningococcal outer membrane containing the principal surface exposed proteins responsible for virulence of the bacterium. The periplasm is between the outer and inner membranes (OM and IM) and contains the peptidoglycan cell wall. OMPs include the two porins PorA and PorB, opacity proteins (Opa and Opc). The Tfp extends beyond the other protein structures, traverses the OM and reaches the IM. LPS is indicated.

A



B

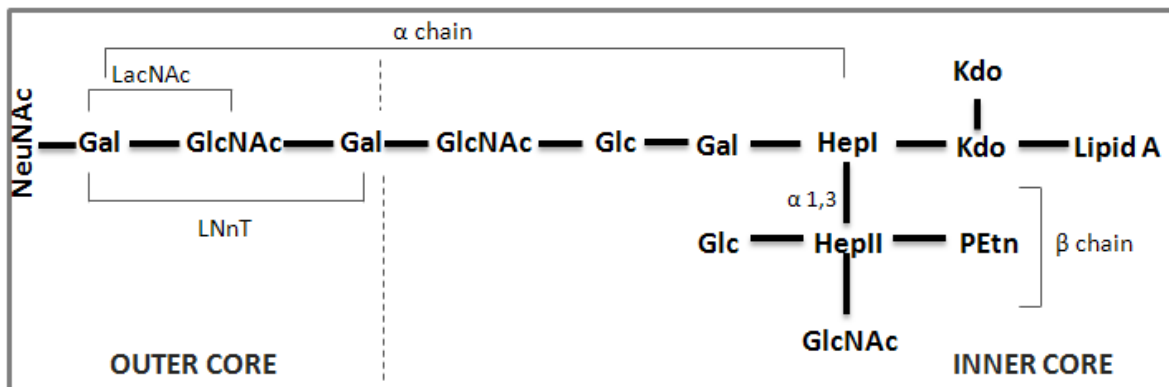
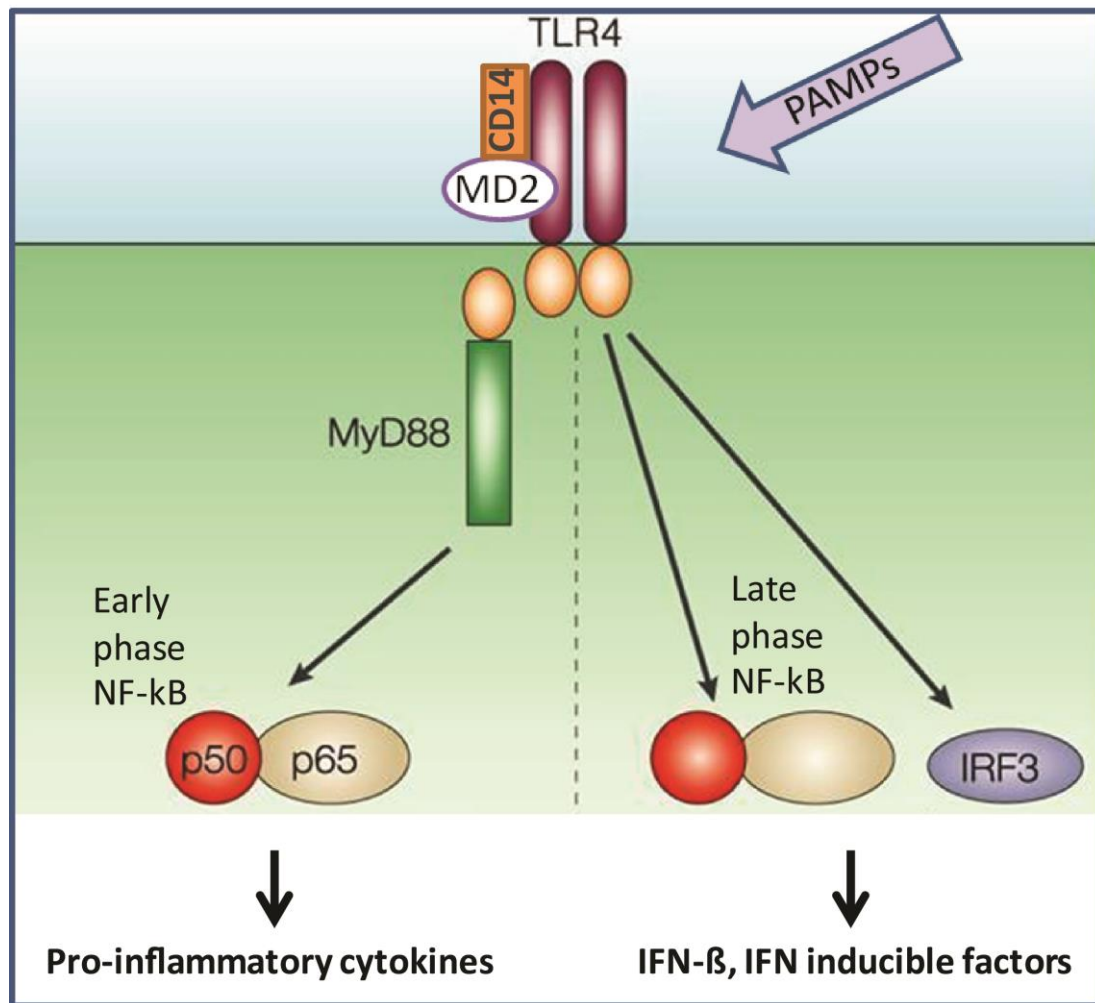


Figure 1.3 Schematic structure of meningococcal and gonococcal LPS.

(A) Structure of meningococcal LPS, immunotype L3,7,9. Genes encoding enzymes responsible for the addition of sugars and the glycosidic linkages (*Ist*, *LgtB*, *LgtA*, *icsB*) and epimerases (*galE*) are indicated. (B) Structure of gonococcal LPS. Key: NeuNAc, N-acetylneuraminic acid; Kdo, 3-deoxy-D-manno-2-octulosonic acid; Hep, heptose; Glc, glucose; GlcNAc, N-acetyl-glucosamine; Gal, galactose; PEtn, phospho-ethanolamine; N-acetyl-lactosamine (LacNAc); Lacto-N-neo-tetraose (LNnT).



Modified from Akira et. al 2004

Figure 1.4 NF-κB signalling pathways dependent on TLR-4 activation.

Recognition of pathogen associated molecular patterns (PAMPs) by TLR-4 triggers dimerisation, interaction with the co-factor MD-2 and the binding to CD14. The MyD88 signalling pathway involves the early phase NFκB activation which leads to the production of pro-inflammatory cytokines and the late phase NFκB activation, which leads to production of Interferon-β (IFN-β) and expression of IFN-inducible genes.

1.6.3 Type IV pili

An important step in the pathogenesis of *N. meningitidis* infection is the adhesion of bacteria to epithelial cells in the nasopharynx. This initial step of colonisation is mediated by type IV pili (Tfp) (Hill *et al.* 2010). Pili are dynamic organelles that undergo cycles of extension and retraction (Carbonnelle *et al.* 2006); they are filamentous protein structures on the surface of bacteria and are composed of repeating pilin protein subunits of approximately 17 to 21 kDa each (Parge *et al.* 1995). Neisserial pilins belong to typeIV pilins that are found in diverse bacterial genera such as *Moraxella*, *Pseudomonas* and *Vibrio* (Nassif and So 1995). The major pilin protein in *N. meningitidis* is PilE, which multimerises and forms an α -helical fibre with other “minor” pilins such as PilV and PilX (Cehovin *et al.* 2010). The PilC proteins are other components of the Tfp machinery which are required for adhesion (Nassif *et al.* 1994). Pilin is essential for binding to epithelial cells and endothelial cells (Virji *et al.* 1991; Nassif *et al.* 1993), while pilus-mediated adhesion and filament retraction contribute to host-cell signaling and cytoskeletal rearrangements (Lambotin *et al.* 2005).

Studies conducted with *N. gonorrhoeae*, revealed that *pilE* undergoes antigenic variation (Koomey *et al.* 1987; Criss *et al.* 2005). Furthermore, neisserial pilins can undergo glycosylation, a post-translational modification which is subject to phase variation (Power *et al.* 2003). Glycosylation is not required for pilus formation, but can impair the aggregation of pili into bundles (Marceau and Nassif 1999). Tfp can also be modified with phosphocholine, PEA or phosphoglycerol, modifications that can cause the release of bacteria from aggregates, and could enhance dissemination in the host, favouring transmigration through epithelial cells (Chamot-Rooke *et al.* 2011).

1.6.4 Opa and Opc proteins

Opa and Opc are neisserial OMPs that facilitate the interaction of bacteria with epithelial and a variety of immune cells. Expression of Opa and Opc confers “opacity” to colonies of *N. meningitidis* when grown *in vitro*. This is noticeable in acapsulate strains, and reflects the contribution of Opc and Opa to cell adhesion (Virji *et al.* 1995). Opa and Opc bind at least two negatively charged molecules, HSPGs and sialic acids, but still exhibit receptor specificity (de Vries *et al.* 1998). Opa protein expression undergoes phase variation at the transcriptional level (Belland *et al.* 1997); therefore a bacterial population can include bacteria expressing none, one or multiple Opas (Jerse *et al.* 1994; Sadarangani *et al.* 2011). Opa proteins have different structures and rearrangement of their domains results in interactions with different carcinoembryonic antigen-related cell adhesion molecules (CEACAMs). CEACAMs are members of the immunoglobulin super-family and include several important receptors such as CEACAM1, CEACAM3 and CEA (Sadarangani *et al.* 2011).

The majority of Opas target CEACAM1 on epithelial and phagocytic cells (Pantelic *et al.* 2004; Kuespert *et al.* 2011). Interestingly, expression of some CAECAMs is restricted to particular cells or tissues, and this may influence bacterial tropism. For instance, binding of Opa to CEACAM1 on neutrophils leads to phagocytosis and elimination of meningococci (Kuespert *et al.* 2011). Opc mediates adherence of non-piliated meningococci, probably through interaction with cell surface proteoglycans (de Vries *et al.* 1998). However, the precise proteoglycan receptors recognised by Opc have not been identified. Furthermore, Opc contributes to bacterial adhesion to endothelial cells by binding vitronectin in the extracellular matrix (ECM) (Virji *et al.* 1992b; Sa *et al.* 2010), which forms a molecular bridge with integrins on the cell surface.

1.6.5 Porins

Porins are the most abundant OMPs in pathogenic *Neisseria* (Massari *et al.* 2003); in the gonococcus they are termed Por IA or IB, and in the meningococcus they are termed PorA (class 1 protein) and PorB (class 2 and 3 proteins) (Feavers and Maiden 1998; Nassif *et al.* 1999). There is structural and functional homology amongst neisserial porins and those of other Gram negative bacteria, with each porin monomer forming a β -barrel fold and a pore for the passage of ions and/or solutes (Murakami *et al.* 1989).

Neisserial porins translocate into the plasma membrane of eukaryotic cells, forming voltage gated ion channels and causing transient changes in membrane potential and interfering with cell signalling (Kozjak-Pavlovic *et al.* 2009). PorB is a non-selective porin that binds ATP molecules through a non-covalent interaction (Rudel *et al.* 1996). It allows passage of sugars and interacts directly with the host mitochondrial voltage-dependent anion channel (VDAC) (Minetti *et al.* 1997). Because both VDAC and the mitochondrial membrane potential control apoptosis, PorB might influence the initiation of apoptosis (Massari *et al.* 2010).

An important contribution of porins to pathogenesis is their ability to modify immune responses; for example, TLR-2 binds PorB, probably even when TLR-2 is dimerised with TLR-1 (Massari *et al.* 2006). However, the exact mechanism of interaction or the precise binding sites have not been elucidated, probably due to the variation in porin sequence, form and structure (Tanabe *et al.* 2010).

1.7 Host immunity against *N. meningitidis*

Host defence against meningococci is determined by humoral and cellular responses belonging to both the innate and adaptive immune systems. It is well known that the complement system plays a crucial role in protection against invasive meningococcal disease, as complement deficiencies are associated with marked susceptibility to meningococcal disease (Densen 1991; Mathew and Overturf 2006). Furthermore, presence of bactericidal antibodies against *N. meningitidis* can prevent the development of infection (Goldschneider *et al.* 1969a). However, the production of specific antibodies occurs only after one week of *N. meningitidis* colonisation, so that the initial defence is dependent mainly on innate immune responses (van Deuren *et al.* 2000). The sites where *Neisseria* are commonly found, such as the nasopharynx and the upper respiratory tract, may be deficient in the components of the complement system and are populated by immune cells such as resident phagocytes and T cells (Cooper and Moticka 1989; Fokkens and Scheeren 2000). Therefore, understanding the basis of the interactions between innate immune cells and the meningococcus is of particular importance and is the main subject of this thesis.

Indeed, the immune cells which reside within epithelia contribute to the development of immunity and are the first line of defence against bacterial invasion. Immunosurveillance mechanisms are particularly active at this site and resident cells such as macrophages are responsible for a non-specific immune response initiated by initial contact with the meningococcus (Fokkens and Scheeren 2000). Bacterial pathogen associated molecular patterns (PAMPs) trigger a series of events leading to activation of resident macrophages and recruitment of neutrophils, monocytes, macrophages and dendritic cells (DCs) to the site of infection (Miller *et al.* 2005). In addition, meningococci provoke the

production of pro-inflammatory cytokines by immune cells, leading to stimulation of subsequent adaptive immune responses (Koedel 2009). Meningococci can evade some of these initial immune defences and reach the bloodstream where they are able to replicate and cause disease (Brandtzaeg and van Deuren 2002; Namork and Brandtzaeg 2002; Colicchio *et al.* 2009).

1.7.1 Neutrophils in meningococcal disease

Neutrophils are the most abundant population of circulating leukocytes and mediate the early phase of inflammatory responses (Peters *et al.* 2003). During *N. meningitidis* infection, there is considerable activation of neutrophils (Heyderman *et al.* 1999). This is dependent on the formation of platelet-neutrophil complexes which contain activated neutrophils and seem to be rapidly lost from the circulation during meningococcal disease (Peters *et al.* 2003). Furthermore, neutrophils are the predominant cell type in the CSF of patients with meningococcal meningitis (Arevalo *et al.* 1989).

The importance of neutrophils in meningococcal disease has been demonstrated by depleting neutrophils in an infant rat model, a phenomenon that led to 100% mortality after challenge with *N. meningitidis*, even after vaccination (Peters *et al.* 2001). The principal mechanism neutrophils employ to destroy pathogens is through localised granule exocytosis and extracellular production of reactive oxygen species (ROS)(Criss and Seifert 2012). Neutrophils also produce extracellular traps which capture and inactivate bacteria that are distant from the neutrophil surface (Brinkmann *et al.* 2004; Young *et al.* 2011). The meningococcus has evolved mechanisms to overcome oxidative stress by encoding several proteins that detoxify ROS such as superoxide dismutase, catalase and cytochrome

cperoxidase (Seib *et al.* 2004). In addition, DNA repair systems have been recently shown to be responsible for correcting DNA damaged by oxidative stress (Nagorska *et al.* 2012).

Moreover, resistance of *N. meningitidis* to phagocytosis has been widely described. It is well established that capsule prevents opsono-phagocytosis and killing by the complement system and is subsequently required for survival in the extracellular fluids (Read *et al.* 1996). However, other components of the *N. meningitidis* outer membrane can be involved in interaction with immune cells. Opa and Opc proteins are involved in the interaction of unencapsulated *N. meningitidis* with neutrophils. These adhesins contribute to non-opsonic phagocytosis of *N. meningitidis* by neutrophils (Estabrook *et al.* 1998), but the precise mechanisms have not been elucidated. Furthermore, sialylated LPS limits complement mediated phagocytosis and impairs resistance to immune mechanisms of the host (Kahler *et al.* 1998). Finally, it has been proposed that the acquisition of glutamate by meningococcus promotes survival of bacteria in association with neutrophils and enhances the ability of the bacterium to cause bloodstream disease. Specifically, glutamate is converted by bacteria into glutathione which regulates the redox potential of bacteria, thus protecting *N. meningitidis* from ROS production (Tala *et al.* 2011).

1.7.2 Dendritic cells in meningococcal disease

Dendritic cells have an important role in the activation and regulation of the human immune system; DCs can open tight junctions between adjacent epithelial cells and capture bacteria directly by recognising and phagocytosing pathogens (Salter and Watkins 2006). Contact with micro-organisms induces a maturation process that leads to down regulation of pathogen recognition receptors and up regulation of antigen presentation molecules

(Banchereau and Steinman 1998). During maturation, DCs migrate to secondary lymphoid tissues where they activate T cells. *N. meningitidis* is known to bind the DC-specific intracellular adhesion molecule 3 (ICAM-3) grabbing non integrin (DC-SIGN), a type II transmembrane receptor with a single extracellular C-type lectin domain. DC-SIGN binds mannose structures and fucose containing glycoconjugates (Feinberg *et al.* 2001; Appelmelk *et al.* 2003) and recognises truncated meningococcal LPS, mediates bacterial uptake, thus triggering a T-helper 1 (Th1) immune response (Steeghs *et al.* 2006).

1.7.3 Monocytes and macrophages in meningococcal disease

Monocytes are a heterogeneous population of leukocytes present in the blood, spleen and bone marrow (Auffray *et al.* 2009; Swirski *et al.* 2009) that migrate to tissues during infection and inflammation (Geissmann *et al.* 2010; Shi and Pamer 2011). Monocytes have long been considered as a developmental intermediate between bone marrow precursors and tissue macrophages and DCs (Serbina *et al.* 2008). However, it is established now that DCs and tissue macrophages originate from distinct cells termed “inflammatory” monocytes (Geissmann *et al.* 2008). “Inflammatory” monocytes are characterised by specific effector functions and express a wide range of scavenger receptors (SRs) that recognise micro-organisms, toxic molecules and dying cells. Therefore monocytes provide a systemic reservoir of myeloid precursors of macrophages and DCs, and also link inflammation and adaptive immunity during infection.

The interaction of *N. meningitidis* with monocytes has been studied (McNeil *et al.* 1994). In particular, the importance of the expression of opacity proteins for bacterial adherence with monocytes and down regulation of the polysaccharide capsule has been

assessed (Unkmeir *et al.* 2002). Serogroup B *N. meningitidis* has been observed within monocytes during experimental infection of human nasopharyngeal mucosa (Read *et al.* 1995).

Monocytes are able to kill meningococci, but a small population of bacteria can be detected inside the cells several hours after challenge (Spinosa *et al.* 2007). This suggests that bacteria could be carried within the cells to distal tissues, although this hypothesis remains unproven.

Macrophages are phagocytic cells present within the tissues and are responsible for the initiation and resolution of inflammation. Resident macrophages are sentinel cells detecting tissue damage and changes in local homeostasis following infection and injury (Liddiard *et al.* 2011). Their functions include clearance of apoptotic cells, tissue remodelling and repair, pathogen recognition and immune activation, and linking innate and adaptive immunity. Activated macrophages kill phagocytosed microbes through the action of lytic enzymes, ROS and nitric oxide (NO), and by releasing CAMPs into the extracellular space (Hancock 2001).

Both monocytes and macrophages are “plastic” cells, as they assume diverse characteristics based on their stage of cellular differentiation, tissue distribution and response to endogenous and exogenous stimuli (Geissmann *et al.* 2010). Their “plasticity” can be induced by cytokines *in vitro*. For example, exposure of human and murine monocytes to granulocyte-stimulating factor (GM-CSF) and IL-4 induces differentiation into DCs, while exposure to macrophage colony-stimulating factor (M-CSF) induces differentiation into macrophages (Manzanero 2012). Furthermore, addition of LPS or IFN- γ and M-CSF to macrophages induces differentiation into M1-like macrophages (classically activated) whereas addition of IL-4 induces differentiation into M2-like macrophages

(alternatively activated). This type of differentiation is called polarisation and determines the functions. M1-like macrophages exhibit microbicidal properties and promote IL-12-mediated, Th1 associated responses, while M2-like macrophages play a role in the resolution of inflammation and support Th2 response (Goerdts *et al.* 1999; Gordon 2003). Macrophages express several surface molecules which recognise micro-organisms, such as SRs which bind conserved motifs on pathogen surfaces (Pluddemann *et al.* 2009). In particular SR-A is involved in non-opsonic phagocytosis of the meningococcus by bone marrow derived macrophages (Peiser *et al.* 2002).

Meningococci are found within macrophages during invasive infection (Read *et al.* 1996), and uptake of *N.meningitidis* by these cells can occur without opsonisation. Once internalised by macrophages, meningococci are rapidly killed in phagolysosomal compartments whether or not they are encapsulated (Read *et al.* 1996). Similar to other Gram negative bacterial infections, cytokine release by macrophages is dependent on the presence of LPS and TLR-4 (Zughaier 2011).

1.8 Immune recognition

The innate immune system has evolved to specifically recognise and eliminate potentially dangerous micro-organisms. Cells responsible for this response sense PAMPs through specific receptors called pattern recognition receptors (PRRs) to elicit immune cell signalling (Fig. 1.4).

Cell surface TLRs are PRRs that recognise PAMPs on the bacterial surface, such as the LPS of Gram negative bacteria (TLR-4), lipoteichoic acid of Gram positive bacteria, bacterial lipoproteins (TLR-1/TLR-2 and TLR-2/TLR-6), and flagellin (TLR-5). Endosomal TLRs mainly

detect nucleic acids, such as double stranded RNA (dsRNA) (TLR-3), single stranded RNA (ssRNA) (TLR-7) and dsDNA (TLR-9). Activation of TLRs leads to specific signalling cascades resulting in antimicrobial and inflammatory responses that are dependent on the receptors activated (Akira *et al.* 2001).

Transmembrane PRRs include TLRs and C-type lectin receptors (CLRs). CLRs are expressed by immune cells and can distinguish between different pathogens, consequently eliciting signalling pathways to initiate appropriate immune responses. Examples of CLRs are DC-SIGN, MBL (which is specific for terminal GalNAc structures), and Dectin-1 (which recognises β -glucans and mannans in fungal cell walls) (Saijo and Iwakura 2011).

PRRs can also recognise endogenous ligands termed damage-associated molecular patterns (DAMPs), which are released by host cells upon tissue damage and acute inflammation or tumour growth (Matzinger 2002; Kono and Rock 2008). DAMPs have gained major attention for anti-cancer therapy, as cancer cells which die following UV treatment release molecules which improve immune responses against the cancer cells (Garg *et al.* 2010).

The list of DAMPs includes heat shock proteins, high mobility group protein-1 (HMGB-1), IL-1 α , defensins and annexins (Rubartelli and Lotze 2007). In addition, as glycan structures are present on host and bacterial cells, these molecules may play a role in the communication between microbes and the host. Lectins, which act as receptors that can be secreted or expressed on the surface of immune cells, include sialic acid-binding immunoglobulin domains (siglecs) and galectins. Siglecs are surface receptors which bind sialic acid containing glycans (Dam and Brewer 2010); galectins are described in the next section.

1.9 Galectins in innate immunity

Galectins are a family of animal lectins that recognise β -galactoside structures (Barondes *et al.* 1994), share sequence similarity in a carbohydrate recognition domain (CRD) of approximately 130 amino-acids and lack a signal peptide. Galectins can be exported by an atypical secretory pathway and function mainly extracellularly where they recognise glycans and mediate inter-cellular communication (Sato *et al.* 2009).

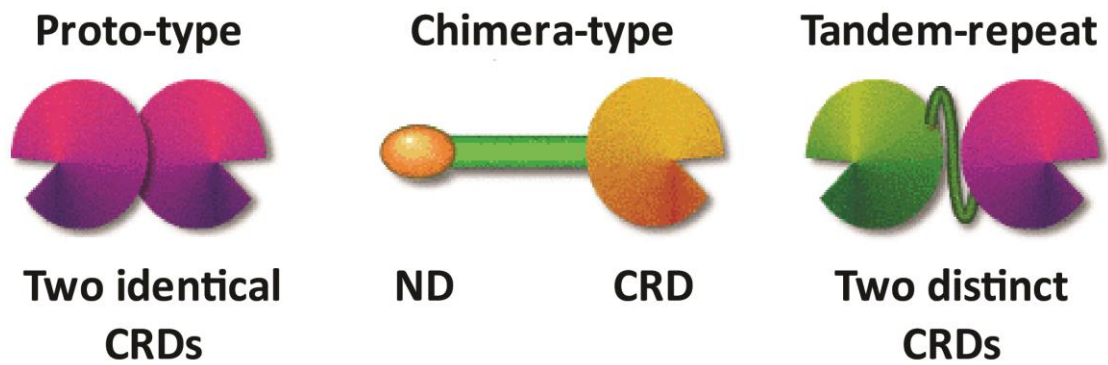
To date, 15 galectins have been identified in mammals and these are divided into two subtypes; proto-type galectins have a single CRD (galectin-1, -2, -3, -5, -7,-10,-11, -13, -14 and 15) while tandem-repeat galectins have two CRDs joined by a short linker (galectin -4, -6, -8, -9 and -12) ((Liu and Hsu 2007) and Fig. 1.5). Among the single CRD type galectins, galectin-3 is unique as it is a chimeric galectin which also contains an N-terminal domain (ND) of approximately 120 amino acids that allows the formation of pentamers (Ahmad *et al.* 2004a).

Most galectins are soluble proteins, although some galectins including galectin-3 have been proposed to possess a transmembrane domain and can be expressed as a soluble protein or a transmembrane protein (Gorski *et al.* 2002). The minimal saccharide unit recognised by galectins is a galactose residue linked to an adjacent monosaccharide in the β -configuration (*i.e.* $\alpha\beta$ galactoside). In particular, glycans that contain polylactosamine chains ((Gal β 1, 4GlcNAc) $_n$) such as laminin, fibronectin or lysosome associated membrane proteins are endogenous galectin ligands (Inohara and Raz 1994; Ochieng *et al.* 1998b; Barboni *et al.* 1999).

Although all galectins recognise β -galactosides, specific glycan structures are necessary for efficient binding, and the affinity of each galectin for its ligand is dependent on substitutions of the β -galactoside (Rapoport *et al.* 2010). In addition, differences in the

biological effect of galectin-1 and galectin-3 result from diversity in the tertiary structure of the protein rather than ligand binding properties (Seetharaman *et al.* 1998).

The roles of galectins in the development and regulation of innate and adaptive immune homeostasis have been recently reviewed (Rabinovich and Toscano 2009). Galectins are synthesised and accumulate in the cytoplasm of immune cells, as well as fibroblasts, keratinocytes, endothelial cells and epithelial cells (Cerliani *et al.* 2011). Once host cells are damaged following pathogen invasion, galectins are released and function as lectins. However, galectins are also actively secreted during cell differentiation or activation, and can trigger cell signalling, mediate cell-cell interaction and induce migration of monocytes, macrophages and endothelial cells (Dumic *et al.* 2006).



Modified from www.glycoforum.gr.jp, Hirabayashi

Figure 1.5 Schematic structure of galectins from different groups.

Proto-type galectins are formed by two identical CRD (galectin-1, -2, -3, -5, -7, -10, -11, -13, -14 and -15), the chimera-type galectins by a CRD connected to an ND (galectin-3) and the tandem-repeat galectins have two distinct CRDs joined by a short linker (galectin -4, -6, -8, -9 and -12).

1.10 Galectin-3

Galectin-3 is the sole chimera-type galectin as it contains an ND consisting of multiple repeats of a sequence rich in proline (Pro), glycine (Gly) and tyrosine (Tyr) residues (Fig. 1.5 and 1.6). Upon ligand binding, the conformation of galectin-3 changes in a concentration-dependent manner, and galectin-3 oligomerises through its N-terminal regulatory domain; this oligomerisation results in the formation of a galectin-3 complex with multiple CRDs. Multimerisation is reversible and can occur following cleavage by certain proteases including metalloproteinases (MMPs) and bacterial collagenase IV. Truncated galectin-3 can still bind its ligands but has limited functions (Kopitz *et al.* 2001).

Galectin-3 can also be phosphorylated (Mazurek *et al.* 2000) and this form is found in the cytosolic and nuclear fractions of cultured fibroblasts (Tsay *et al.* 1999) and epithelial cells (Mazurek *et al.* 2005). Phosphorylation of the N-terminal regulatory domain significantly reduces its affinity for ligands, possibly by affecting the ability of the protein to multimerise (Mazurek *et al.* 2000).

Galectin-3 has marked preference for polylactosamine structures located at accessible sites on glycoconjugates (Togayachi *et al.* 2007). The affinity of the interaction increases in proportion to the number of polylactosamine units present on the glycan chain.

Galectin-3 was initially identified as a cell surface component of macrophages, even though it is not a conventional membrane protein (Bornstein and Sage 2002). Like the other galectins, galectin-3 does not contain obvious secretion sequences but it can be released and has different functions, depending on its localisation (Liu and Hsu 2007).

1.10.1 Intracellular functions of galectin-3

Intracellular galectin-3 is located in the nucleus and the cytoplasm, with the relative distribution influenced by the cell type and specific experimental conditions (Haudek *et al.* 2010). The cytoplasmic fraction contains only phosphorylated galectin-3, while the nuclear fraction has the phosphorylated and non-phosphorylated protein (Tsay *et al.* 1999).

Galectin-3 has important roles in the regulation of cell cycle and apoptosis. Intracellular galectin-3 can inhibit apoptosis (Liu and Hsu 2007) which is likely to be dependent on its phosphorylation (Yoshii *et al.* 2002). Initially, galectin-3 phosphorylation was shown to occur on residues Ser⁶ and Ser¹²; more recently, phosphorylation has been shown to occur on residues Tyr⁷⁹, Tyr¹⁰⁷ and Tyr¹¹⁸ of the collagen-like sequence rich in Gly, Pro, Tyr and glutamine (Gln) residues through the action of c-Abl and Arg (c-Abl related gene) kinases ((Balan *et al.* 2010; Balan *et al.* 2012) and Fig. 1.6).

Galectin-3 and Bcl-2 proteins share high sequence identity and are rich in Pro, Gly and alanine (Ala, A) in their N-terminal regions. Moreover, they both contain a sequence of asparagine (Asn, N), tryptophan (Trp, W), Gly (G) and arginine (Arg, R) in their CRD, a quartet conserved in the Bcl-2 family proteins that is critical for its anti-apoptotic activity (Kim *et al.* 1999; Yang and Liu 2003). Galectin-3 directly interacts with Bcl-2 in the cytoplasm, mimicking the ability of the Bcl-2 family members to form heterodimers (Haudek *et al.* 2010). Moreover, intracellular galectin-3 also regulates cell cycle and differentiation. Cells overexpressing galectin-3 undergo G1 arrest without detectable cell death (Kim *et al.* 1999). The NWGR motif is essential for regulation of cell cycle activity, as substitution of the Gly with Ala leads to loss of this function (Akahani *et al.* 1997).

Galectin-3 can bind nucling, another pro-apoptotic protein in the cytoplasm, and synexin, a calcium- and phospholipid-binding protein (Haudek *et al.* 2010). Another ligand

for galectin-3 in the cytoplasm is the transcription factor Suppressor of fused (Sufu), which serves as a shuttle between the cytoplasm and the nucleus (Paces-Fessy *et al.* 2004). Therefore, galectin-3 has been proposed to play a role in the nuclear versus cytoplasmic distribution of Sufu, thus affecting its transcriptional activity (Haudek *et al.* 2010).

A

MADNF**S**LHDAL**S**GSGNPNPQGWP GAWGNQPAGAGGYPGASY**P**GAY**P**GQ
 APP**P**GAY**P**GQAPP**P**GAY**P**GAP**P**GAY**P**GAPAVYPGPPSG**P**GAY**P**SSGQPSATGAY
 PATGPYGA**P**AGPLIVPYNLPLPGGVPR**M**LITILGTVKPNANRIALDFQ**R**GND
 VAFHFNPRFNENRRVIVC**N**TKLDNNWGREERQSVFPFESGKPFKIQLVE**P**
 DHFKVAVNDAHLLQYNHRVKKLNEISKLGISGDIDLTSASY**T**MI

B

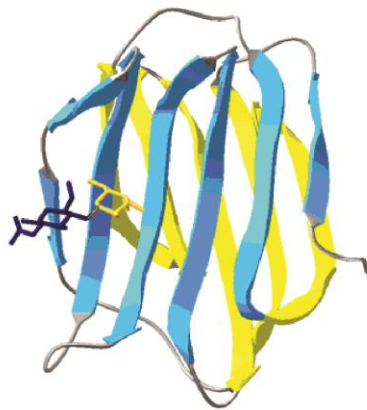


Figure 1.6. Protein sequence of galectin-3 and structure of the CRD.

(A) Galectin-3 amino acid sequence showing the ND (black) and the CRD (red). The repeats of Pro-Gly-Ala-Tyr-Pro-Gly (green) in the ND, Ser⁵ and Ser¹¹ phosphorylation sites (blue) and the Met¹²⁷ cleavage site (in bold) are indicated. (B) Representation of the CRD of galectin-3 complexed with LacNAc (dark blue and yellow). β -strands are shown in light blue and yellow (Protein Data Bank structure, www.rcsb.org/pdb/explore.do?structureId=1KJL).

1.10.2 Extracellular functions of galectin-3

Release of galectin-3 can be due to cell necrosis (passive release) or through an active process from live cells (active release) (Hughes 1999). It has been suggested that the release of galectin-3 is tightly regulated by cell differentiation (van Stijn *et al.* 2009). Galectin-3 is up-regulated during inflammation; for example, high concentrations of the protein are found in bronchoalveolar lavage fluids (> 50 µg/ml) following *Streptococcus pneumoniae* infection (Henderson and Sethi 2009). Moreover, this lectin has been used as a marker of inflammation for microglia (Venkatesan *et al.* 2010), and contributes to brain damage in pneumococcal meningitis, where immune cells are responsible for galectin-3 expression (Bellac *et al.* 2007).

Once galectin-3 is secreted it binds to β -galactoside containing glycoconjugates on cell surfaces and undergoes oligomerisation. One of the important mechanisms by which galectins regulate their functions is through their ability to create galectin-glycoprotein complexes. As galectin-3 is multivalent, it can cross link specific ligands on the cell surface, induce clustering on the surface (called lattice) and trigger responses that can lead to cytokine production (Rabinovich *et al.* 2002).

Extracellular galectin-3 binds to ECM proteins such as fibronectin, laminin, collagen IV, elastin, hensin, tanascin-C and -R (Dumic *et al.* 2006). Furthermore it binds to certain integrins present on the surface of macrophages, such as CD11b (α subunit of α M β 1 integrin, CD11b/18, Mac1 antigen) (Dong and Hughes 1997), and the α 3 β 1 integrin on epithelial cells, triggering signalling that leads to the formation of lamellipodia (Saravanan *et al.* 2009). Moreover, galectin-3 associates with the heavy chain of CD98, a membrane glycoprotein present on monocytes/macrophages and activated T cells, and mediates CD98 dimerisation (Dong and Hughes 1996). CD98 is required for activation of an alternative

macrophage phenotype once bound to galectin-3, suggesting that galectin-3 is involved in the resolution of inflammation (MacKinnon *et al.* 2008). Galectin-3 also binds aminopeptidase N (CD13), a metalloproteinase expressed by monocytes. Galectin-3 binding to CD13 has been suggested to influence homotypic aggregation, a process that leads to cellular activation and maturation in tumour cell invasion and metastasis (Mina-Osorio *et al.* 2007). In contrast to intracellular galectin-3, extracellular galectin-3 can induce apoptosis of T cells, through caspase activation, leading to an immunosuppressive effect (Stillman *et al.* 2006).

Extracellular galectin-3 is also involved in inflammatory responses, contributing to autoimmune diseases and responses to infection (Dhirapong *et al.* 2009). Galectin-3 knockout murine macrophages are more sensitive to apoptotic stimuli and exhibit reduced pro-inflammatory responses in terms of leukocyte recruitment (Nieminen *et al.* 2008). Moreover, mice lacking galectin-3 excessively produce inflammatory cytokines and NO and are more susceptible to LPS induced shock, but at the same time impair *Salmonella* replication. These data suggested that galectin-3 protect against endotoxic shock but might be detrimental by favouring early *Salmonella* invasion (Li *et al.* 2008).

Galectins are potential opsonins, as they can cross-link ligands on cells or apoptotic bodies to cells. For example, galectin-3 can bind apoptotic neutrophils and facilitate their uptake by macrophages (Karlsson *et al.* 2009). In addition, galectin-3 is able to activate the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase of human neutrophils (Yamaoka *et al.* 1995; Karlsson *et al.* 1998).

1.11 Galectin-3 binding to micro-organisms

Although galectin-3 was initially thought to bind only endogenous glycans, it is becoming increasingly recognised that it also binds glycans on pathogenic micro-organisms, and mediates recognition and effector functions in innate responses to infection (Sato and Nieminen 2004; Nieminen *et al.* 2008). Several pathogens express glycoconjugates that contain β -galactosides similar to those present on host cells. For example, *N. meningitidis*, *N. gonorrhoeae* and *H. Influenzae* can express polylactosamine (Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc) in their LPS. Therefore galectin-3 on macrophages and epithelial cells might recognise these glycans on pathogens. Differences in the LPS structures of Gram negative bacteria affect interactions with galectin-3 (Vasta 2009). Variation in LPS is most marked in the O-antigen, a polysaccharide chain that extends from core saccharides (Wang *et al.* 2010). Galectin-3 binds LPS from *Klebsiella pneumoniae* (which has a β -galactoside-containing O-antigen) through its CRD (Mey *et al.* 1996). In contrast, LPS from *Salmonella minnesota* R7 (Rd mutant), which is devoid of β -galactosides, is recognised by the ND of galectin-3 binding to the lipidA/inner core portion of LPS (Mey *et al.* 1996). Furthermore, galectin-3 binds to the gonococcus via LPS, with highest affinity for glycoforms terminating in LacNAc (John *et al.* 2002).

Galectin-3 can bind glycans expressed by several parasites, including *Leishmania major*, *Schistosoma mansoni* and *Trypanosoma cruzi* (Pelletier and Sato 2002; van den Berg *et al.* 2004; Silva-Monteiro *et al.* 2007), and fungi such as *Candida albicans* (Fradin *et al.* 2000; Kohatsu *et al.* 2006). Importantly, galectin-3 recognition of *Candida* results in significant reduction of fungal viability (Fradin *et al.* 2000).

In addition, galectin-3/pathogen interactions may mediate pathogen adhesion to cells and therefore facilitate infection. Consistent with this, galectin-3 binding to the O-

antigen of *Helicobacter pylori* promotes attachment of bacteria to cells (Fowler *et al.* 2006b). Moreover, a recent study showed that galectin-3 enhances neutrophil activation by increasing the ability of LPS to bind neutrophils (Fermino *et al.* 2011).

In general galectin-3 may provide direct innate immune functions during infection, as demonstrated by its protective role in pneumococcal pneumonia, where it augments neutrophil activity (Farnworth *et al.* 2008). Furthermore, galectin-3 can enhance PMN recruitment to *S. pneumoniae* *in vivo* and act as a soluble adhesion molecule for neutrophils on endothelial cells (Nieminen *et al.* 2008).

1.12 Other galectins examined in the study

1.12.1 Galectin-1

Galectin-1 is a proto-type galectin, widely distributed in different tissues including epithelial cells, endothelial cells, trophoblasts, activated T cells, macrophages, activated B cells and DCs (Camby *et al.* 2006). In addition, its expression has been shown to be modulated in several pathological conditions, such as cancer, infection or autoimmune disease, and it has been proposed to control leukocyte trafficking, immune tolerance and homeostasis (Cooper *et al.* 2010).

In general, galectin-1 has an anti-inflammatory effect by impairing signalling events that lead to leukocyte infiltration, migration and recruitment (Kiss *et al.* 2007). It also has pro- or anti- apoptotic effects on T cells depending on their developmental stage and activation, and the local cytokine environment (Pace *et al.* 1999).

Galectin-1 promotes HIV infection either by enhancing binding of the virus to cells, or acting as a soluble receptor to facilitate uptake by macrophages. Virus attachment and host cell fusion occurs by binding N-linked oligosaccharides on the virion envelope (St-Pierre *et al.* 2011).

1.12.2 Galectin-4

Galectin-4 is a tandem-repeat galectin first identified in the luminal epithelium of the intestinal tract of mammals (Wasano and Hirakawa 1995; Wooters *et al.* 2005; Paclik *et al.* 2008), and in the rabbit bladder epithelium (Bhavanandan *et al.* 2001). However, galectin-4 expression is also detected in the lungs, testes, breast, liver and placenta by reverse transcriptase polymerase chain reaction (RT-PCR) (Markova *et al.* 2006). It is mainly present

intracellularly and is a tumor suppressor which inhibits cell proliferation by down-regulating Wnt signalling (Satelli *et al.* 2011). The CRD of galectin-4 has also been implicated in inflammatory bowel disease (Paclik *et al.* 2008), and linked to cancer progression (Satelli *et al.* 2011). Human galectin-4 consists of two non-identical CRDs of 133 aminoacids each connected by a linker peptide of 17 aminoacids (Huflejt and Leffler 2004). The carbohydrate binding specificities of each CRD are distinct and exhibit preference for different ligands (Wasano and Hirakawa 1999; Wu *et al.* 2002). The two domains share 40% amino acid identity, while the proline and glycine rich linker is sensitive to proteases (Huflejt and Leffler 2004).

1.12.3 Galectin-7

Galectin-7 is a proto-type galectin that can dimerise in solution (Morris *et al.* 2004). It contributes to epithelial cell migration and the establishment of epithelial layers, and is a potential marker of keratinocyte differentiation (Madsen *et al.* 1995; Timmons *et al.* 1999; Cao *et al.* 2002; Cao *et al.* 2003).

More recently, galectin-7 has been shown to have a role in apoptosis; it is over-expressed in colorectal cancer cells undergoing p53-dependent apoptosis, and has been proposed to aid the elimination of neoplastic cells (Demers *et al.* 2010).

Galectin-7, like the other galectins, binds a large range of ligands. Thermodynamic studies demonstrated that galectin-7 possesses weaker affinities for carbohydrates with LacNAc epitopes compared with galectin-1 (Brewer 2004). Galectin-7 specific ligands *in vivo* have not been identified yet, but clear differences in its carbohydrate binding specificity

have been observed; galectin-7 binds non-reducing terminal and internal LacNAc residues of poly-N-lactosamine chains often found on glycoprotein receptors (Morris *et al.* 2004).

1.12.4 Galectin-8

Galectin-8 is a tandem-repeat galectin of 34 kDa, composed of two CRDs connected by a linker peptide. The N- and C- terminal domains share approximately 35% amino acid identity and contain sequence motifs that are conserved among CRDs from most galectins (Dumic *et al.* 2006). Galectin-8 can be secreted or present in the cytoplasm particularly in association with Golgi or trans-Golgi membranes and mitochondria (Zick *et al.* 2004). Galectin-8 is expressed in many tissues including the liver, lung, spleen and kidneys, while low levels of the protein are detected in the colon, intestine, and thymus (Bidon *et al.* 2001).

The physiological role of galectin-8 is not completely understood, but multiple functions, such as cell adhesion, growth and differentiation, have been proposed. For example, it has an inhibitory effect on cell adhesion by binding several integrins on human cells (Hadari *et al.* 2000), and mediates adhesion of peripheral blood neutrophils to the endothelium (Nishi *et al.* 2003).

1.12.5 Galectin-9

Galectin-9 is another member of the tandem-repeat galectins, and has been shown to induce apoptosis in monocytic and myeloid cell lines through carbohydrate dependent mechanisms (Kuroda *et al.* 2010). Galectin-9 has been found in both immature and mature DCs and induces transcription of pro-inflammatory cytokines such as IL-1 α , IL-1 β and IFN- γ

(Dai *et al.* 2005). DC maturation induced by galectin-9 probably depends on signalling by p38 and is only partially inhibited by lactose (Dai *et al.* 2005).

In vivo, proteolytic cleavage within the linker region of galectin-9 by elastase matrix metalloproteinase-3 as well as insertional mutations can generate two independent functional CRDs (Lahm *et al.* 2000; Nishi *et al.* 2005). Consistent with this, studies on the two separate domains have shown that they have distinct properties. The N-terminal CRD of human galectin-9 exhibits affinity for poly-N-acetyl-lactosamine (poly-LacNac) and selectivity for N glycans present on cells without 2,3 sialylation (Solis *et al.* 2010). In contrast, the the C-terminal CRD has high affinity for single LacNac molecules and α -2,3-sialylated oligosaccharides (Yoshida *et al.* 2010). The N- and C- terminal CRDs of galectin-9 also contribute differently to its multiple functions. The N-terminal CRD is a potent activator of innate immune cells, while the C-terminal CRD is mainly involved in adaptive immunity by inducing T-cell death (Li *et al.* 2011).

Galectin-9 also binds the T cell immunoglobulin and mucin domain containing molecule 3 (TIM-3) which is expressed on immune cells and promotes tissue inflammation (Jayaraman *et al.* 2010).

1.13 Aims of the thesis

This project has three main objectives.

Galectins have multiple roles in fundamental biological processes such as cell growth and differentiation, and modulate inflammation and immune responses to parasitic and bacterial infections. They are capable of recognizing host cell ligands as well as structures present on the surface of micro-organisms. *N. meningitidis* infection is characterised by a marked inflammatory response at the mucosal surface and in the systemic circulation. As galectins are expressed and produced by immune cells, and these cells are encountered by *N. meningitidis* at mucosal sites or in the bloodstream during bacterial colonisation and disease, the first goal of this project was to investigate the distribution of galectins in tissues and their expression by cells during meningococcal infection.

Most of the literature about galectins focuses on the chimera-type galectin-3, which is secreted by or expressed on the surface of monocytes, macrophages and epithelial cells, and involved in many immune processes. Galectin-3 has been shown to bind the LPS of several bacteria including *E. coli*, *K. pneumoniae* and *N. gonorrhoeae*. Meningococcal and gonococcal LPS have similar structures which differ only in certain substitutions of the oligosaccharide portion. Therefore, the second goal of my thesis was to investigate whether galectin-3 would bind *N. meningitidis*.

Current knowledge on the interactions of galectins with bacteria is limited to the function of the intracellular proteins and little is known about the roles of extracellular proteins during infection. Moreover, the function of galectin-3 in meningococcal disease has never been examined. Galectin-3 is present in the intracellular environment but also in the

extracellular space, and it is possible that *N. meningitidis*, as an extracellular bacterium, encounters galectin-3 during invasion of the host. Indeed, bacteria are exposed to epithelial cells at the site of colonisation, resident macrophages in the submucosa, and circulating monocytes, recruited macrophages and PMNs in the systemic circulation. Therefore, taking into account that galectin-3 is produced by these cells, the final aim of my project was to investigate whether galectin-3 had an influence on bacterial interactions with host cells.

CHAPTER 2: Materials and Methods

2.1 Bacterial strains and growth conditions

N. meningitidis was grown on Brain Heart Infusion (BHI) agar (1.5% wt/vol, Oxoid) supplemented with 50 ml of defibrinated horse blood (previously autoclaved with 1 liter BHI broth) at 37°C in the presence of 5% CO₂ for 16-18 hours. Solid media was inoculated from frozen stocks of bacteria stored at -80°C in media with 15% glycerol. Bacteria were enumerated in a suspension of phosphate buffer saline (PBS) by measuring the optical density (O.D.) at 260 nm (A_{260}). MC58 is a serogroup B isolate of *N. meningitidis*. The strains used in this study are described in Table 2.1.

2.2 Cell culture

THP-1 and Detroit 562 human cells line were purchased from American Type Culture Collection (ATCC). THP-1 cells are a human acute monocytic leukemia cell line established from the peripheral blood of a 1-year-old boy with acute monocytic leukemia (Tsuchiya *et al.* 1980). THP-1 cells were grown in RPMI 1640 medium (Gibco, Invitrogen) with 2 mM glutamine, supplemented with 10% fetal calf serum (FCS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 14 mM D-glucose, 0.05 mM beta-mercaptoethanol. THP-1 monocytic cells (1×10^6) were differentiated into macrophages in 24 well-plates containing 1 ml RPMI 1640 medium with 100 ng/ml 12-phorbol 13-myristate acetate (PMA) for 72 hours.

Detroit 562 is a human pharyngeal cell line which was maintained in Dulbecco's modified Eagle's medium (Gibco, Invitrogen) supplemented with 10% FCS, 2 mM GlutamaxTM (Invitrogen), 1 mM sodium pyruvate and lactalbumin hydrolysate (0.1%). Cells were cultured to a density of $1-5 \times 10^5$ cells/ml and maintained at 37°C with 5% CO₂.

2.3 Galectins and antibodies

The lectins used in this work were either purchased as recombinant proteins from R&D Systems (human galectin-3), or purified label free or biotinylated after production by affinity chromatography on lactosylated Sepharose 4B and kindly provided by Professor Hans-Joachim Gabius, University of Munich (Table 2). LPS was removed by a further chromatographic step as previously described (Sarter *et al.* 2009). Purity was ascertained by one- and two-dimensional gel electrophoreses and mass spectrometry, which was also performed after proteolytic truncation with collagenase D at the Tyr¹⁰⁶/Ile¹⁰⁷ and Glu²²⁹/Ile²³⁰ peptide bonds of galectin-3; biotinylation was carried out under activity preserving conditions as described (Kubler *et al.* 2008). Activity controls were run by solid-phase and cell assays (Andre *et al.* 2007). Polyclonal antibodies against full length galectin-1, galectin-3 and galectin-4 were provided by Professor Gabius and were made and checked for specificity and absence of cross-reactivity to other galectins as previously described (Kaltner *et al.* 2002; Lohr *et al.* 2007; Lohr *et al.* 2008). Rabbit poly-clonal anti-human galectin-3 antibody was purchased from Santa Cruz Biotechnology.

Table 2.1 Meningococcal strains used in this study.

Strain	Description	Immunotype	References
MC58	Serogroup B clinical isolate	L3,7,9	(Plested <i>et al.</i> 1999; Tettelin <i>et al.</i> 2000)
F8238	Serogroup A clinical isolate	L10	(Maslanka <i>et al.</i> 1997)
Z2491	Serogroup A clinical isolate	L9	(Parkhill <i>et al.</i> 2000)
H44/76	Serogroup B clinical isolate	L3	(Holten 1979; Plested <i>et al.</i> 1999)
8013	Serogroup C clinical isolate	L3,7,9	(Rusniok <i>et al.</i> 2009)
FAM18	Serogroup C clinical isolate	L3,7,9	(Bentley <i>et al.</i> 2007)
MC58 Δ <i>siaC</i>	Insertionally inactivated <i>siaC</i>	-	(Exley <i>et al.</i> 2005)
MC58 Δ <i>siaD</i>	Insertionally inactivated <i>siaD</i>	-	(Exley <i>et al.</i> 2005)
MC58 Δ <i>lst</i>	Insertionally inactivated <i>lst</i>	-	(Exley <i>et al.</i> 2005)
MC58 Δ <i>lgtA</i>	Truncated LPS mutant	-	(Jennings <i>et al.</i> 1995b)
MC58 Δ <i>lgtB</i>	Truncated LPS mutant	-	(Jennings <i>et al.</i> 1995b)
MC58 Δ <i>galE</i>	Truncated LPS mutant	-	(Jennings <i>et al.</i> 1995b)
MC58 Δ <i>si-1</i>	Truncated LPS mutant	-	(Jennings <i>et al.</i> 1995b)
MC58 Δ <i>icsB</i>	Truncated LPS mutant	-	(van der Ley <i>et al.</i> 1997)

Table 2.2 Galectins used in this study.

Compound	Form	Species
Gal-3 full length	Label free, Santa cruz Biotechnology	Human
Gal-3 CRD	Purified label free	Human
Phosphorylated gal-3	Purified label free and modified with CK-1	Human
Gal-3 full length	Purified label free and biotinylated	Mouse
Gal-3 CRD	Purified label free and biotinylated	Mouse
Gal-3 full length	Purified label free	Chicken
Gal-3 CRD	Purified label free	Chicken
Gal-1	Purified label free	Human
Gal-1 treated with DTT (Gal-1 DTT)	Purified label free	Human
Gal-1 treated with IAA (Gal-1 IAA)	Purified label free	Human
Gal-1 C2S	Purified label free and mutated at the dimer interface	Human
Gal-4	Purified label free	Human
Gal-7	Purified label free and biotinylated	Human
Gal-8 full length	Purified label free	Human
Gal-8 ND	Purified label free	Human
Gal-9	Purified label free	Human
Gal-9 ND	Purified label free	Human

2.4 Challenge of mice with live *Neisseria meningitidis*

Groups of ten 6-week old female BALB/c mice (Harlan) were housed under pathogen-free conditions, acclimatised for one week and randomly distributed in two groups. Animals were challenged by intraperitoneal injection of *N. meningitidis* MC58. Bacteria were grown overnight on BHI plates and then harvested into PBS. The number of colony forming unit (CFU) was estimated by measuring the A_{260} of a lysate of the suspension in 200 mM sodium hydroxide (NaOH) and 1% (w/v) SDS solution (P2 lysis buffer Qiagen), and the number of viable bacteria was confirmed by plating to solid media. Each animal in the challenge group received 1×10^6 CFU of MC58 in 0.5 ml of BHI medium containing 0.5% (vol/vol) iron dextran (Sigma, Poole, United Kingdom). Control groups consisted of mice given BHI medium with iron dextran alone (*i.e.* uninfected). After 24 or 48 hrs, animals were sacrificed and organs collected, fixed in 10% formalin for one week and embedded in paraffin. Sections of five micrometer thickness were cut from each block for analysis. All animal experiments were carried out under protocols reviewed and approved by the Home Office, United Kingdom. Challenge of mice was performed by Dr. Yanwen Li (Imperial College).

2.5 Immunohistochemistry

The presence of galectin-1, -3, and -4 was assessed in different organs from mice challenged with *N. meningitidis* strain MC58 or uninfected animals, and the presence of galectin-3 was assessed in spleen tissue sections from patients with systemic meningococcal infection and uninfected patients. Tissues were obtained

during a study performed in 1991-1992 and kindly supplied by Professor S. Lucas, St. Thomas Hospital (Lucas *et al.* 1993). Paraffin-embedded sections were deparaffinised in xylene, and rehydrated in serially diluted ethanol (100%, 95%, 75%, 50% and 0%). Following washes with distilled water for 5 min, sections were incubated with 3% methanol for 10 min to quench endogenous peroxidase. For antigen retrieval, specimens were boiled in 10 mM sodium citrate buffer under full vapour pressure for 2 min, and washed in distilled water and in PBS/0.1% Tween-20. Following incubation with 150 µl of protein block (Dako) for 5 min, samples were incubated overnight at 4°C with 200 µl of rabbit polyclonal anti-galectin-3 antibody (2 µg/ml) or 200 µl of a 1/500 dilution of mouse polyclonal antibody against *N. meningitidis* (Fitzgerald Industries International). The following day, sections were washed with PBS/0.1% Tween-20 and incubated with 100 µl of goat anti-rabbit or mouse antibody conjugated with horseradish peroxidase (HRP, Dako) or alkaline phosphatase (AP, Dako) for 30 min. After washing with PBS/0.1% Tween-20, galectins in the tissues were visualised by incubation in diaminobenzidine (DAB) for HRP or permanent red for AP. Finally, tissue sections were counterstained with Mayer's hematoxylin, mounted and analysed with a light microscope. Immunohistochemical analysis was performed with the help of Dr. Yanwen Li (Imperial College).

2.6 Biochemical procedures

2.6.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For whole cell lysates, bacteria were grown for 16 hours on BHI agar, harvested and resuspended in 400 µl of PBS for quantification (A_{260}). 1×10^9 bacteria were re-suspended in 50 µl of dH₂O and combined with an equal volume of 2x SDS sample buffer (50 mM Tris-HCl, 2% SDS, 100 µM β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). Prior to loading, samples were boiled for 10 minutes, centrifuged briefly and 10-20 µl analysed by SDS-PAGE. For eukaryotic cells, aliquots of 1×10^6 adherent cells were removed with trypsin and re-suspended in dH₂O. Cell suspensions were then centrifuged (1500 rpm for 8 minutes) and suspended in 25 µl of dH₂O combined with an equal volume of SDS-PAGE sample buffer. SDS-PAGE analysis of proteins was performed according to the method described by Sambrook (Sambrook and Gething 1989) and 12% polyacrylamide gels prepared according to the recipes shown in Table 2.3. Protein samples were separated under reducing conditions by 12% SDS-PAGE at 120 V for 2 hours and protein standard Precision Plus All Blue markers (BioRad) was used as a marker (size from 10 kDa to 250 kDa).

Table 2.3 Polyacrylamide gel recipes for 10 ml solutions.

Separating Gel		Stacking gel	
ddH ₂ O	1.32 ml	ddH ₂ O	1.21 ml
1.5 M Tris pH 8.8	1 ml	1.5 M Tris pH 6.8	0.5 ml
10% SDS	40 µl	10% SDS	20 µl
40% Acrylamide	1.6 ml	40% Acrylamide	0.25 ml
10% ammonium persulphate	40 µl	10% ammonium persulphate	20 µl
TEMED	1.6 µl	TEMED	2 µl

2.6.2 Preparation of lipopolysaccharides from *N. meningitidis*

N. meningitidis strains were harvested after overnight growth on BHI agar plates at 37°C in the presence of 5% CO₂ and re-suspended in PBS. The number of bacteria was estimated by measuring the A₂₆₀ of an aliquot of the bacterial suspension lysed in P2 lysis buffer (1% SDS, 0.1% sodium hydroxide) using a UVVIS spectrophotometer (Schimadzu UK). A total of 1x10⁹CFU were centrifuged (13000 rpm for 5 minutes) and pellets were re-suspended in 50 µl of LPS buffer (3% SDS, 3%beta-mercaptoethanol, 5 mM dithiothreitol (DTT), 23% glycerol, 30 mM Tris pH 8, 0.05% bromophenol blue), then boiled for 10 minutes and incubated overnight at 37° following addition of 1 mg/ml of proteinase K solution (Qiagen).

2.6.3 Electrophoresis of lipopolysaccharide

LPS samples were analysed by separation in SDS-sulphate polyacrylamide gels formed by a separating gel (3M Tris, 0.3% SDS, pH 8.45, 20 mM Urea, 40% acrylamide, 10%, tetramethylethylenediamine, TEMED) and a stacking gel (0.5 M Tris pH 6.8, 40% Acrylamide bis, 10% SDS, 10% ammonium persulphate, TEMED). This system utilises two specific buffers for electrophoresis *i.e.* ananode buffer (0.2 M Tris, pH 8.9) and cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.25). Electrophoresis was performed with the BioRad Mini-Protean II gel apparatus (BioRad). Samples were boiled 10 minutes and then loaded into the wells. Electrophoresis was carried out at 30 V until the dye reached the separating gel and 105V afterwards. LPS was visualised by silver staining (GE Heathcare). Immunotype L3,7,9 LPS was detected using anti-L3,7,9 antibody (dilution 1:1000 NIBSC). Binding of a secondary

antibody (1:1000, anti-Mouse immunoglobulin conjugated to HRP; DakoCytomation) was detected using the enhanced chemiluminescence (ECL) system (Amersham Biosciences).

2.6.4 Western blot analysis

Protein or LPS samples were separated under reducing conditions by 12% SDS-PAGE, then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) for 1 hr in cold transfer buffer (25 mM Tris, 190 mM glycine). After blocking membranes overnight in 5% milk in PBS, galectin-3 was detected by incubation of the membrane with rabbit anti-galectin-3poly-clonal antibodies (pAbs) (Santa Cruz Biotechnology, 300 ng/ml) or anti-actin (Sigma) for 1 hour at room temperature. Binding of the primary antibody was detected using an anti-rabbit-IgG HRP-conjugated secondary antibody (1:1000 dilution; Santa Cruz Blothechnology), and ECL detection kit (GE Healthcare).

Immunotype L3,7,9 was detected using α -L3,7,9 antibody (dilution 1:1000 NIBSC). Binding of a secondary antibody (1:1000, anti-Mouse immunoglobulin conjugated to HRP, DakoCytomation) was detected using the ECL system (Amersham Biosciences). For preparation of samples, cells and supernatants were collected at different time points and cells were removed from wells using 0.5% trypsin/0.2% ethylenediaminetetraacetic acid (EDTA) or scraping, then washed with PBS and lysed at 100°C in SDS-PAGE buffer.

Table 2.4. Antibodies used for western blot analysis.

Antibodies	Source	Concentration
anti-L3,7,9	NIBSC	4.5 ng/ml
anti-hGal-3	Rabbit poly-clonal (SCB)	300 ng/ml
anti-hGal-3	Rabbit poly-clonal (Prof. Gabius)	1 µg/ml
anti-rabbit IgG HRP	SCB	1:1000
anti-actin	Sigma	1:1000

2.7 RNA procedures

2.7.1 RNA isolation

RNA was isolated from 1×10^6 THP-1 or Detroit-562 cells cultured in 24-well plates as described in section 2.2. Supernatants were removed and cells washed twice with PBS, followed by addition of 200 μ l of trypsin to detach cells. After detachment, fresh culture medium was added and cells were centrifuged 10 minutes at 300xg. Lysis buffer (Qiagen) was used to disrupt the cells and RNA was isolated using the RNeasy Miniprep Kit (Qiagen) according to the manufacturer's protocol. To determine the integrity of the RNA, samples were analysed by agarose gel electrophoresis; approximately 50 ng of RNA were combined with a 1:6 solution of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and separated on 1% w/v agarose (Bioline, UK) gels in 1% Tris Acetate EDTA (TAE) buffer (0.4 M Tris, 0.01 M EDTA- Na_2 , 0.2 M acetic acid). RNA samples were stained with SYBR Safe (Invitrogen) and visualised with a UV transilluminator (Syngene GeneFlash, UK). The purity of RNA was analysed by measuring the A_{260} and A_{280} of samples, and RNA was stored at -80°C .

2.7.2 Reverse transcription

Elimination of genomic DNA present in RNA preparations and reverse transcription of RNA into complementary DNA (cDNA) were achieved using the Quanti Tect Reverse Transcription kit (Qiagen, UK) and following the manufacturer's protocol. The kit provides a combined procedure for both reverse transcription and

genomic DNA elimination. Genomic DNA was removed by treatment with DNase I (Qiagen) and cDNA samples were stored at -20° C.

2.7.3 Semi-quantitative Real Time PCR (qRT-PCR)

Transcript levels were measured by qRT-PCR using QuantiFast with SYBR green detection (Qiagen) on a Rotor-Gene 3000 thermal cycler (Corbett Research), using 1 µl of cDNA samples corresponding to about 400 ng of cDNA. The following two-step real time cycling program was chosen for amplification: initial activation step for 5 minutes at 95°C, 40 cycles of denaturation at 95°C for 10 seconds and combined annealing/extension at 60°C for 30 seconds. Fluorescence was acquired in the combined annealing/extension step. Results for the transcription of galectin-3 were normalised to levels for the housekeeping gene *β-actin*. Primers for human galectin-3 (Forward 5'-TGTGCCTTATAACCTGCCTTT-3', Reverse 5'-TTAAAGTGGAAGGCAACATCA-3') and human *β-actin* (Forward 5'-GCACTCTTCCAGCCTTCCTCC-3', Reverse 5'-GAGCCGCCGATCCACACG-3') were designed using the Primer3 website (<http://frodo.wi.mit.edu/>) and specificity checked performing a BLAST search (National Centre for Biotechnology Information, NCBI). Primers were designed so that one half hybridises to the 3' end of one exon and the other half to the 5' end of the adjacent exon, to allow annealing of those to the cDNA synthesised from the spliced mRNA and not to the genomic DNA, eliminating detection of contaminating DNA. Data were analysed using the Comparative Quantitation method by Rotor-Gene software (version 6.0; Corbett Research). Relative quantification determines the ratio between the amount of a target and the amount of a reference nucleic acid, the housekeeping gene. Controls included reactions with no template, and samples of RNA that had not been treated with reverse transcriptase; qRT-PCR was performed in triplicate on cDNA samples derived from three independent assays.

2.7.4 *Small interfering RNA procedures*

The Amaxa transfection is an electroporation method based on the use of an electroporation machinery designed for mammalian cells and built by Amaxa (AAD-1001, Amaxa Inc, Gaithersburg, USA). THP-1 cells were grown to confluency and harvested as in section 2.2. A total of 1×10^6 cells were required for each electroporation reaction. These cells were centrifuged at 1500 rpm for 8 minutes at room temperature and supernatants removed. Cells were transfected with an On Target plus SMARTpool of a four small interfering-RNAs (siRNA) designed specifically for galectin-3 (Thermo Scientific Dharmacon).

The RNA sequences targeted are: GGAGAGUCAUUGUUUGCAA, GUACAAUCAUCGGGUUAAA, GGCCACUGAUUGUGCCUUA, GGUGAAGCCCAAUGCAAA. Cells were transfected with 3 μ g of siRNA using Nucleofector solution V (Dharmacon) according to the manufacturer's instructions. Negative controls were carried out using buffer only. Cells were transferred to a 2 mm electroporation cuvette (Amaxa Inc, Gaithersburg, USA) and electroporation was carried out with a Nucleofector machine (VCA-1003, Amaxa) using the program V-01* / V-001** (suggested by the manufacturer for high efficiency THP-1 cell transfection). Following electroporation, cells were re-suspended in culture medium supplemented with PMA for 24 hours to allow attachment. The medium was then replaced to remove dead cells and the cells were cultured for a further 48 hours to allow differentiation into macrophages. To confirm the silencing of galectin-3, western blot analysis was performed 72 hours after the initial transfection. Only cells with no detectable galectin-3 expression were used for experiments.

2.8 Preparation of human peripheral blood monocytes

Human peripheral blood was extracted from healthy volunteers in accordance with local ethical research committee guidelines. Monocytes were isolated by adherence after Ficoll-Hipaque purification of peripheral blood mononuclear cells (PBMCs) (Bennett and Breit 1994). Blood was collected into EDTA-K₂ coated tubes to avoid coagulation and subjected to density centrifugation on an equal volume of Histopaque 1077 gradient (density 1.077 g/ml, Sigma Aldrich) at 800 $\times g$ for 30 minutes. After centrifugation, plasma was removed and the layer containing PBMCs was gently collected and transferred to a 50 ml tube. 25 ml of PBS were added to PBMCs and samples were then centrifuged at 200 $\times g$ for 10 minutes. The cells were washed twice with by a final step of 300 $\times g$ for 10 minutes. Cells were enumerated between the second and the third wash, to determine number of cells to culture in 24 well plates. Approximately 10 % of the cells were monocytes present in a mixed population of lymphocytes and monocytes. Finally, cell pellets werere-suspended in 1 ml of pre-warmed culture medium, gently washed and then seeded at 3×10^5 cells/ml in 24 well plates in RPMI medium without FCS and allowed to adhere for 3 hours at 37°C, after which non adherent B and T cells were removed by extensive and thorough washing. Cell viability was estimated using the Trypan blue exclusion method and was higher than 95% on all occasions. Purity of monocytes was assessed using fluorescence microscopy by analysing nuclear shape (4,6-diamino-2-phenylindole (DAPI) staining) or by florescence ativating cell sorting (FACS) analysis, measuring surface expression of the CD14 receptoring an anti-human CD14 mouse monoclonal antibody (Abcam).

2.9 Whole Cell Enzyme Linked Immunosorbent Assay (ELISA)

Wild-type THP-1 and siRNA galectin-3 cells differentiated with PMA were infected with *N. meningitidis* for 4 hours. Cell supernatants were collected and initially centrifuged at 14000 rpm to remove bacteria and particulate material; supernatants were transferred to 0.22 μ m filter columns (Sigma-Aldrich, Corning Costar) and centrifuged twice at 2000 rpm to eliminate residual bacteria. Samples were used immediately after filter sterilisation or stored at -20° C. The Human TNF- α /TNFSF1A Quantikine kit (R&D Systems) was used to measure the concentration of TNF- α in supernatants and analysis was performed according to manufacturer's guidelines. Recombinant TNF- α protein (R&D Systems) was used as a standard at different concentrations. Standards, controls and supernatants were analysed in duplicate and the O.D. of controls containing only washing buffer was subtracted from the mean of the sample readings. The log of the TNF- α concentration versus the O.D. was plotted to build a standard curve and concentrations in the samples was calculated using the standard curve. The O.D. was measured using a micro-plate reader set at 450 nm (Multiscan Ascent, Thermo Life Sciences).

2.10 Flow cytometry

For analysis by flow cytometry, bacteria were grown overnight on BHI agar then harvested into PBS. 2×10^8 CFU were fixed in 3% paraformaldehyde (PFA) for 2 hours at room temperature, washed three times in PBS, and stored at -80°C in PBS/15% glycerol. To measure human cell surface receptors THP-1, cells were removed from wells of tissue culture plates by scraping, washed in PBS and 2.5×10^5 cells were used for analysis. To measure galectin binding, 2×10^7 bacteria were incubated with 30 μl of label free or biotinylated galectin (Andre *et al.* 2007) at different concentrations for 1 hour at 37°C . When required, lactose or sucrose were added at a concentration of 100 or 300 mM. After two washes with PBS/Tween 0.1%, binding was detected by incubation with a rabbit anti-human galectin antibody (Lohr *et al.* 2008) for 45 min at 4°C or a mouse anti-biotin antibody (1 $\mu\text{g}/\text{ml}$, Invitrogen). The cells were washed twice in PBS/Tween 0.1%, then resuspended in 50 μl of PBS with a poly-clonal donkey anti-rabbit IgG-Cy2 conjugate (1/200 dilution in PBS; Jackson ImmunoResearch Laboratories) or a poly-clonal donkey anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (1/200 dilution; Jackson ImmunoResearch Laboratories) and incubated for 30 min on ice. Galectin binding was analysed using a flow cytometer (Calibur FACSscan; BD Biosciences), and at least 4×10^4 events were recorded. Galectin-3 binding was expressed by calculating the Fluorescence Index (% positive bacteria gated multiplied by the geometric mean fluorescence).

Table 2.5 Antibodies used for flow cytometry.

Antibodies	Source	Concentration
anti-hGal-1	Rabbit poly-clonal (Prof. Gabius)	2 µg/ml
anti-hGal-3	Rabbit poly-clonal (Prof. Gabius)	2 µg/ml
anti-hGal-4	Rabbit poly-clonal (Prof. Gabius)	2 µg/ml
anti-hGal-8	Rabbit poly-clonal (Prof. Gabius)	2 µg/ml
anti-hGal-9	Rabbit poly-clonal (Prof Gabius)	2 µg/ml
anti-hGal-3	Rabbit poly-clonal (SCB)	4 µg/ml
anti-biotin	Mousemono-clonal (Sigma)	1 µg/ml
anti-rabbit IgG-Cy2	Donkey poly-clonal (Jackson Immunoreasearch laboratories)	1:200
anti-mouse IgG-FITC	Donkey poly-clonal (Jackson Immunoresearch Lboratories)	1:200

2.11 Fluorescence and confocal microscopy

For fluorescence and confocal microscopy Detroit cells, human primary monocytes and THP-1 cells were seeded onto glass coverslips and infected with MC58 at a multiplicity of infection (MOI) of 30 or 50 for 1-4 hours at 37°C. Samples were washed twice with PBS, fixed for 20 minutes at room temperature in 3% PFA in PBS, and then washed three times in PBS. For labelling, bacterial cells were incubated for 1 hour with an anti-LPS antibody (α -L3,7,9) and/or an anti-galectin-3 antibody (Santa Cruz Biotechnology) diluted in PBS/horse serum 1%, after which the cells were washed twice in PBS, then incubated for 30 minutes with an anti-mouse Alexa Fluor-488-conjugated antibody (Invitrogen) to label bacteria and an anti-human Alexa Fluor-555-conjugated antibody (Invitrogen) to label the cells. A DAPI staining was also performed to visualise the cellular nuclei, adding the dye (300 nM) with the secondary antibodies. After labelling, cells were washed twice in PBS and then ddH₂O, mounted in Aqua Poly/Mount (Polyscience, Inc), and analysed by fluorescence microscopy (BX40, Olympus) or by laser-scanning confocal microscopy (Zeiss Axiovert LSM510). Images were processed using the ImageJ program (Image Processing and Analysis in Java).

Table 2.6 Antibodies used for fluorescence and confocal microscopy.

Antibodies	Source	Concentration
anti-L3,7,9	Mouse (NIBSC)	90 ng/ml
anti-hAlexa555	Donkey (Invitrogen)	1:200
anti-mAlexa488	Donkey (Invitrogen)	1:200
anti-hGal-3	Rabbit (SCB)	1:50

2.12 Adhesion and internalisation assays

For adhesion and internalisation assays Detroit 562 cells, THP-1 cells with PMA and human primary monocytes from healthy volunteers were used. were seeded in 24 well plates at a concentration 5×10^5 cells per well, and 2×10^5 cells per well on coverslips for microscopy. Bacteria were harvested following overnight culture on solid media and re-suspended in 400 μ l of PBS. The concentration of the suspension was calculated measuring the A_{260} and adjusted in culture media to give the desired MOI. THP-1 and Detroit 562 were infected at an MOI of 30 for 1 hr at 37°C, while human primary monocytes were infected at a MOI of 50 for 90 minutes at 37°C; when required, bacteria were pre-incubated with 30 μ l of recombinant galectin-3 (3.3 μ M) or PBS for 1 hr at 37°C. For adhesion assays, cells were washed extensively with PBS to eliminate non-adherent bacteria then lysed with 1% saponin for 10 min at 37°C to recover cell-associated bacteria. For internalisation assays, cells were washed with PBS to eliminate non-adherent bacteria and exposed to gentamicin (100 μ g/ml) for 15 min to kill extracellular bacteria. Cells were washed extensively with PBS after gentamicin treatment to remove antibiotic and dead extracellular or non-bound bacteria, and subsequently lysed with saponin 1% or re-incubated in medium for 1 or 2 hours. In both adhesion and internalisation assays serial dilutions of bacteria were plated to solid media following cell lysis or gentamicin treatment, and CFU were counted the following day. Bacteria recovered from cells without gentamicin treatment represented the total number of bacteria associated with cells; bacteria recovered from cells treated with gentamicin represented the number of internalised bacteria. Bacterial interaction with cells was calculated by measuring the percentage of cell associated bacteria relative to the

initial inocula used for challenge; bacterial internalisation was calculated by measuring the percentage of bacteria recovered after gentamicin treatment relative to the initial inoculum. In some assays results were normalised to the percentage of bacteria associated to cells when pre-incubated with PBS (indicated in figures as 100%).

2.13 Statistical analysis

Statistical significance was determined by using the Student's Two-tailed unpaired t-test or One-tailed paired Student's t-test with the Graph Pad Prism v5 (GraphPad Software, California, USA). In both tests, Gaussian distributions were assumed. Statistical significance is indicated with asterisks where $*$ = $p<0.05$, $**$ = $p<0.01$ and $***$ = $p<0.001$. Exact p values are shown in the figure legends.

CHAPTER 3: Expression of galectins during meningococcal infection

3.1 Galectins during meningococcal infection

In this chapter studies were performed to determine whether infection with *N. meningitidis* leads to changes in the presence of galectins, a family of proteins modulated during bacterial infection and known to regulate leukocyte functions and inflammatory responses (Vasta 2009). A variety of cellular and *in vivo* models were employed to address this question. Although *N. meningitidis* is a human specific pathogen, rodent models have been used to represent certain stages of meningococcal disease, especially bloodstream disseminated infection (Li *et al.* 2006; Sjolinder *et al.* 2008). Therefore, initial studies were undertaken using tissues from mice that had been challenged with bacteria, and results compared with tissue from non-infected mice. Tissues obtained from patients with proven meningococcal disease were also examined.

Particular emphasis was focused on galectin-3, the only chimera-type galectin, which is constitutively expressed in various human and murine tissues, and up-regulated in inflammatory conditions such as asthma, tissue damage and infection (Liu *et al.* 1995; Bellac *et al.* 2007; Oliveira *et al.* 2007). Galectins are expressed by a range of cell types, including phagocytes and epithelial cells, and given that *N. meningitidis* interacts with both these cell types in the nasopharyngeal mucosa, blood and brain, the presence of galectin-3 was also examined in phagocytic and epithelial cell lines.

3.2 Expression of galectins in mice infected with *N. meningitidis*

In the first instance, the presence of galectins was examined in a murine model of systemic meningococcal infection. This and other models of bacteraemia have been developed for studying meningococcal infection, even though *N. meningitidis* is a human-specific pathogen (Szatanik *et al.* 2011). In particular, a murine model has been used in previous studies to examine the virulence of *N. meningitidis* strains (Li *et al.* 2004; Exley *et al.* 2005; Tala *et al.* 2011), and to evaluate the efficacy of vaccine candidates (Li *et al.* 2009). Mice receive intraperitoneal iron dextran as an adjuvant to enable bacterial replication in a host which does not naturally provide an available iron source for the meningococcus (Szatanik *et al.* 2011).

We analysed the expression of representative galectins belonging to each group of galectins in tissues during meningococcal infection: the proto-type galectin-1, the tandem repeat type galectin-4, and the chimera type galectin-3. Galectin-1 is found on the extracellular side of cell membranes and in the extracellular matrices of various normal and neoplastic tissues (Cooper and Barondes 1990); in particular, galectin-1 is widely distributed in the nervous system, human colon and in skeletal muscle (Kami and Senba 2005; Camby *et al.* 2006; Demetter *et al.* 2008). Less is known about the distribution of galectin-4 in tissues, although it has been identified in murine and porcine intestine (Rechreche *et al.* 1997; Rumilla *et al.* 2006).

Human and murine galectin-3 share 78% of amino acid identity and are widely distributed in tissues of both species. In mice, galectin-3 is ubiquitously expressed, while in humans, galectin-3 is present in intestinal epithelial cells, kidneys, lungs, thymus and the pancreas (Flotte *et al.* 1983; Bao and Hughes 1995; Kasper and Hughes 1996; Villa-Verde *et*

al. 2002); galectin-3 is mostly found in neutrophils, eosinophils, basophils, Langerhans cells, monocytes and macrophages from the various tissues.

Using antibodies raised against human galectins that cross-react with murine galectins (Kaltner *et al.* 2002; Lohr *et al.* 2008), the presence of galectins was analysed in spleen tissue 24 hours after challenge of mice with the serogroup B *N. meningitidis* isolate MC58 and findings were compared to those obtained with tissues from uninfected mice. Immunohistochemical analysis was performed with the help of Dr. Yanwen Li (Imperial College). Galectin-1 and galectin-4 were not detected in the tissues (Fig. 3.1 B and D) or present only at low levels (Fig. 3.1 A and E), and there was no obvious difference between tissues from infected and un-infected mice. In contrast, although weak galectin-3 staining was present especially in the spleens from uninfected mice (Fig. 3.1 C), a marked increased staining was observed in a large number of cells in the spleens of mice challenged with *N. meningitides* (Fig 3.1 F). The increase in galectin-3 staining coincided with the presence of *N. meningitidis* in the spleen tissue (not shown). Therefore these data indicate that the level of galectin-3 increases during meningococcal infection in the spleen, an organ that contains predominantly immune cells such B and T cells, resident and infiltrating macrophages or monocytes (Weih and Caamano 2003).

Galectin-3 is expressed ubiquitously and actively secreted by inflammatory macrophages, mast cells and epithelial cells (Sato *et al.* 1993; Sato and Hughes 1994; Craig *et al.* 1995). We therefore extended our study to examine the expression of galectin-3 in further murine tissues following infection with *N. meningitidis*. Meningococcal disease leads to myocardial depression, pneumonia, and bacteria have been detected in the liver and in pancreas (Danielsson *et al.* 1971). Therefore, the expression of galectin-3 was examined in samples of cardiac, lung and liver tissue from infected animals. As shown in Fig. 3.2, intense

staining, with a large number of cells positive for galectin-3 was observed in all tissues examined. In particular, positive signal was rarely seen in myocardial tissue from control mice, while intense signals that might correspond to infiltrating PMNs and macrophages were observed in cardiac tissue from infected mice. In the liver, some Kupffer cells were positive for galectin-3 in control tissues, while almost all Kupffer cells stained intensely following infection. In addition, weak signals for galectin-3 were detected in the pancreas of control mice, while intense staining was observed in infected mice. Therefore, meningococcal infection is associated with a marked increase in detectable galectin-3 in all tissues examined.

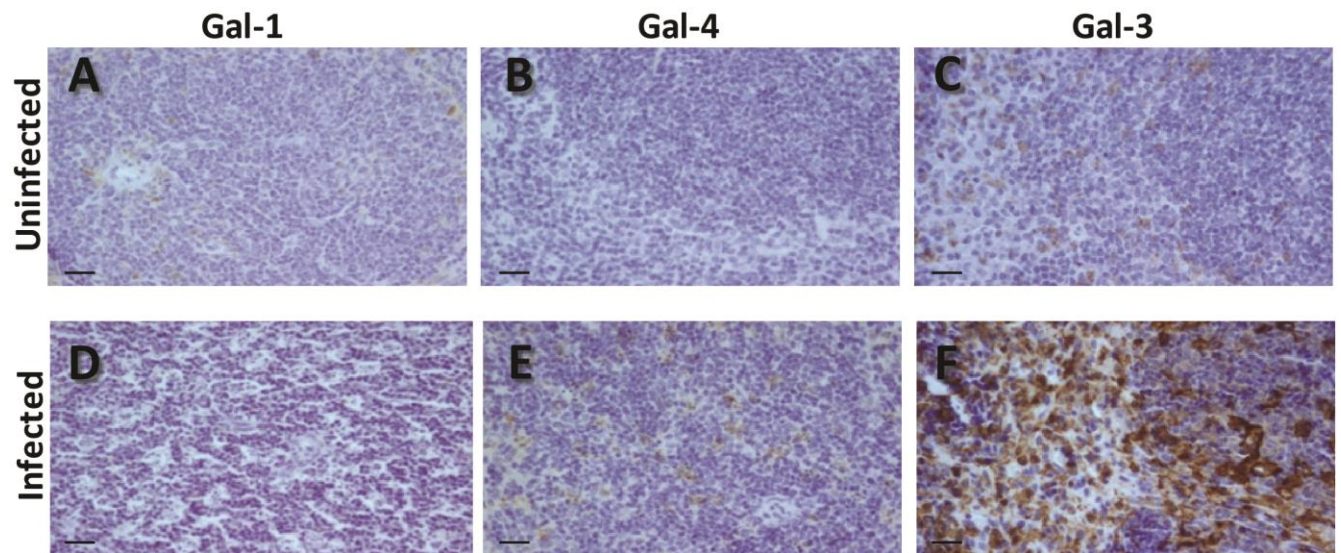


Figure 3.1 Expression of galectins in the spleens of mice infected with *N. meningitidis*.

Detection of galectin-1 (A, D), galectin-4 (B, E) and galectin-3 (C, F) in spleens of mice 24 hrs after infection with *N. meningitidis* serogroup B strain MC58 in iron/dextran (infected) and in spleens of mice challenged with iron/dextran (uninfected). Galectins were detected with rabbit anti-galectin-1, -3 and -4 antibodies. Galectin-1 and galectin-4 were either not detected (B and D) or present at low levels (A and E) in uninfected and infected tissues. Galectin-3 (brown staining) was detected in a few cells of control tissues (C), while marked staining was observed in tissues from infected mice (F). Scale bars correspond to 10 μ m.

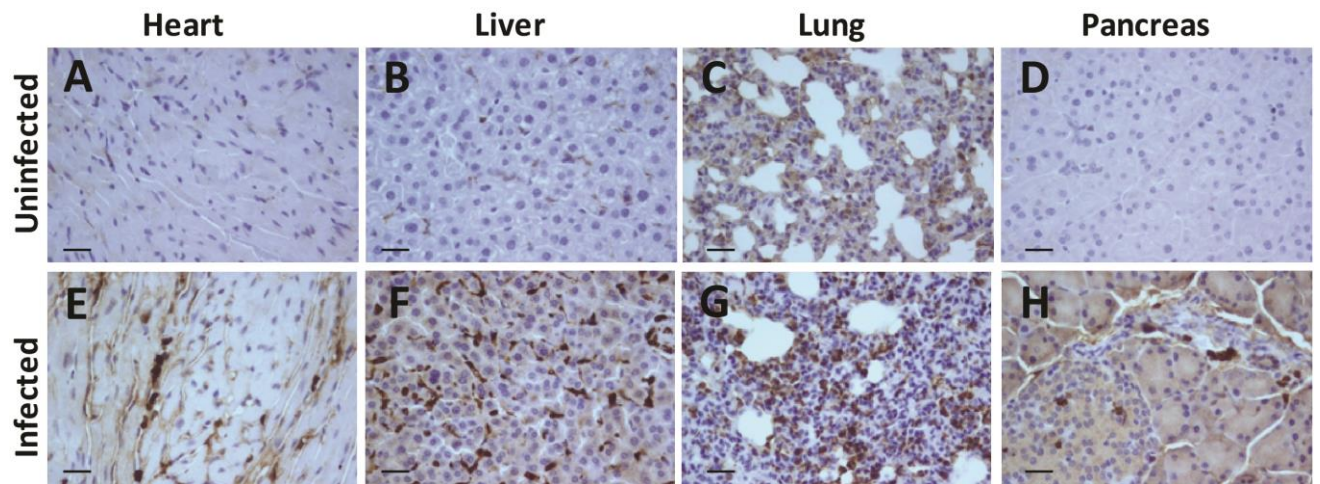


Figure 3.2 Expression of galectin-3 in organs from mice infected with *N. meningitidis*.

Detection of galectin-3 in the heart, liver, lung and pancreas from mice 24 hrs after infection with *N. meningitidis* serogroup B strain MC58 in iron/dextran (infected) or iron/dextran only (uninfected). Galectin-3 was present at low levels in control mice (A, B, C, D) with the signal increased (brown staining) in infected tissues (E, F, G, H). Scale bars correspond to 10 μ m.

3.3 Expression of galectin-3 in the brain from mice infected with *N. meningitidis*

N. meningitidis is a major cause of bacterial meningitis, which is inflammation of the meninges that surround the brain and the spinal cord (Tunkel and Scheld 1993; Fitch and van de Beek 2008). Therefore the presence of galectin-3 in the CNS of infected mice was examined in sections of cerebellum, meninges and choroid plexus (Fig. 3.3). Galectin-3 was not detected in the cerebellum or meninges of uninfected mice, but was found in the choroid plexus, where it is present in the cuboidal epithelial cells (Fig. 3.3, A, C, E). While galectin-3 expression did not change appreciably in the choroid plexus upon infection (Fig. 3.3, F), it was markedly increased in both the cerebellum and meninges (Fig. 3.3 B, and D).

N. meningitidis infection causes a severe inflammation of brain tissue (Fowler *et al.* 2006b) and the acute inflammatory response during bacterial meningitis is characterised by the recruitment of neutrophils from the bloodstream to the CSF, followed by mononuclear cells and activation of local microglia (Hoffman and Weber 2009). Galectin-3 up-regulation observed in some sections of the brain may reflect this inflammatory response, although the specific cell types expressing the lectin were not analysed in this study using specific antibodies.

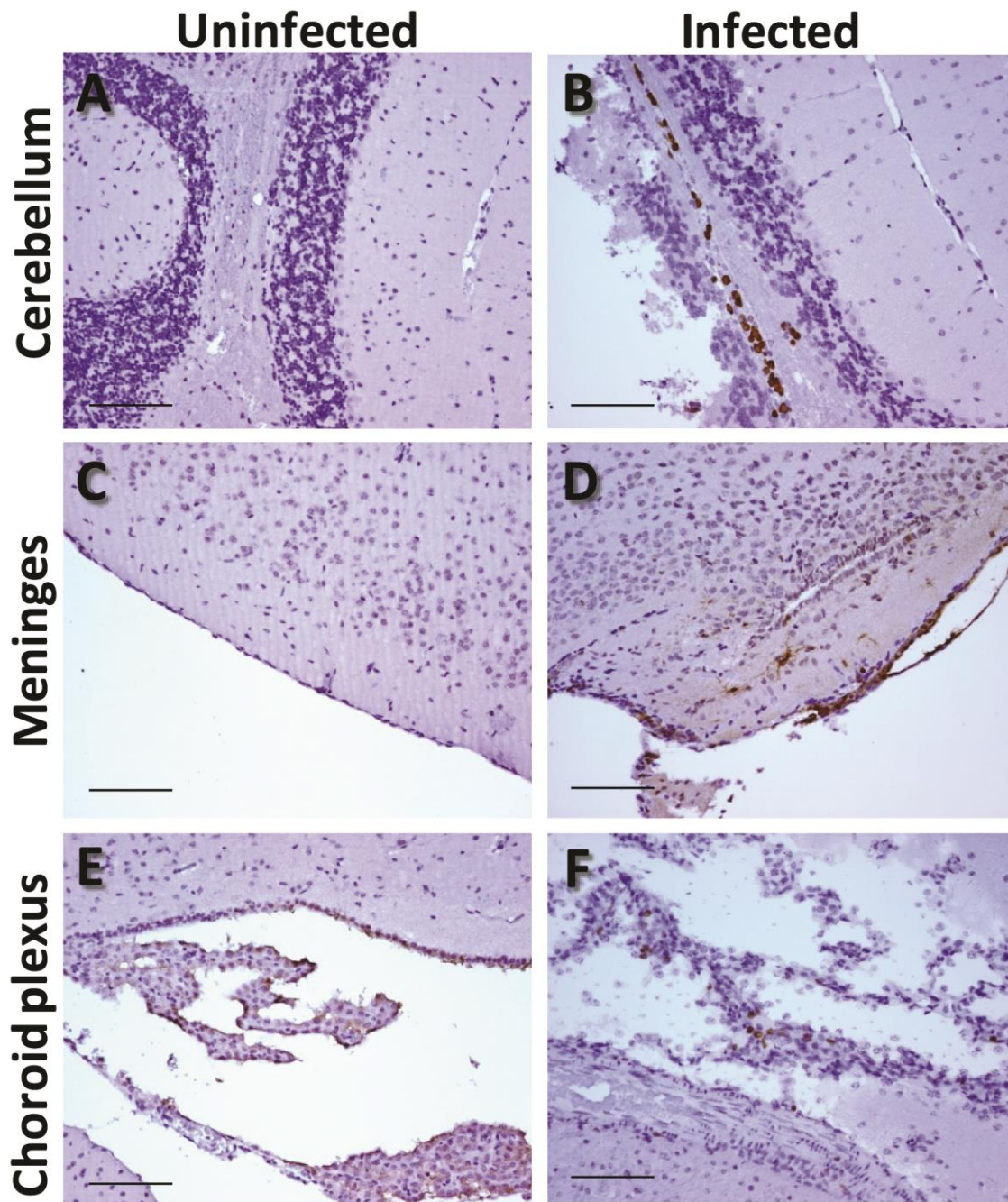


Figure 3.3 Galectin-3 in the brain of mice following infection with *N. meningitidis*.

Detection of galectin-3 in murine brain sections following 24 hours infection with *N. meningitidis* serogroup B strain MC58 in iron/dextran (infected) or with iron/dextran only (uninfected). Galectin-3 is not present in cerebellum (A) and meninges (C) from control mice. Brown staining corresponding to galectin-3 is observed in the cerebellum of infected mice (B), while diffuse signal is observed in infected

meninges. Some galectin-3 is observed in uninfected and infected choroid plexus (E and F). Scale bars correspond to 50 μ m.

3.4 Detection of galectin-3 in tissues from patients with meningococcal infection

Having detected the presence of galectin-3 in different organs of mice infected with *N. meningitidis*, we proceeded with immunohistochemical analyses of galectin-3 expression in human tissues during meningococcal infection.

Initially spleens from patients with meningococcal disease were examined (Fig. 3.4, B, C, E). Tissues were obtained from a post-mortem study in Cote d'Ivoire in 1993 and kindly supplied by Professor Lucas, St. Thomas Hospital (Lucas *et al.* 1993). The staining for galectin-3 was performed on two different patients. Meningococcal infection was defined by immunohistochemistry (IHC) using antibodies to detect *N. meningitidis* and by PCR using DNA extracted from the tissue (not shown, performed by Yanwen Li) as a microbiological diagnosis was not available. Splenic tissue from a patient who died from a non-infection condition (car accident) was used as a control (Fig. 3.4 A and D). Similar to murine tissue, intense galectin-3 staining was observed in tissues from the patient but not in tissue from the control subject. Closer inspection revealed that galectin-3 is present in low levels in control tissues (Fig. 3.4, D), but is markedly increased in samples from the patient (Fig. 3.4, E), with accumulation of the protein evident both within cells and extracellularly. The presence of galectin-3 in the extracellular space in both murine and human tissues indicates release of the protein during meningococcal infection, where it is accessible to interact with extracellular bacteria. Therefore we performed immunohistochemical analysis of human spleen tissue from patients to detect galectin-3 and bacterial colonies (using an antibody

specific for *N. meningitidis*) in serial sections. There was clear co-localisation of galectin-3 (Fig. 3.5 A) with bacteria (Fig. 3.5 B) within tissues, seen both at lower and higher magnification.

To examine the specificity of galectin expression in human tissue in response to *N. meningitidis* infection, we also analysed the presence of galectin-7 (proto-type galectin) using an anti-galectin-7 antibody that recognises the human version of this protein by IHC. Galectin-7 expression was not detected in the infected tissues, although non-infected control tissues were not examined (Fig. 3.4 C).

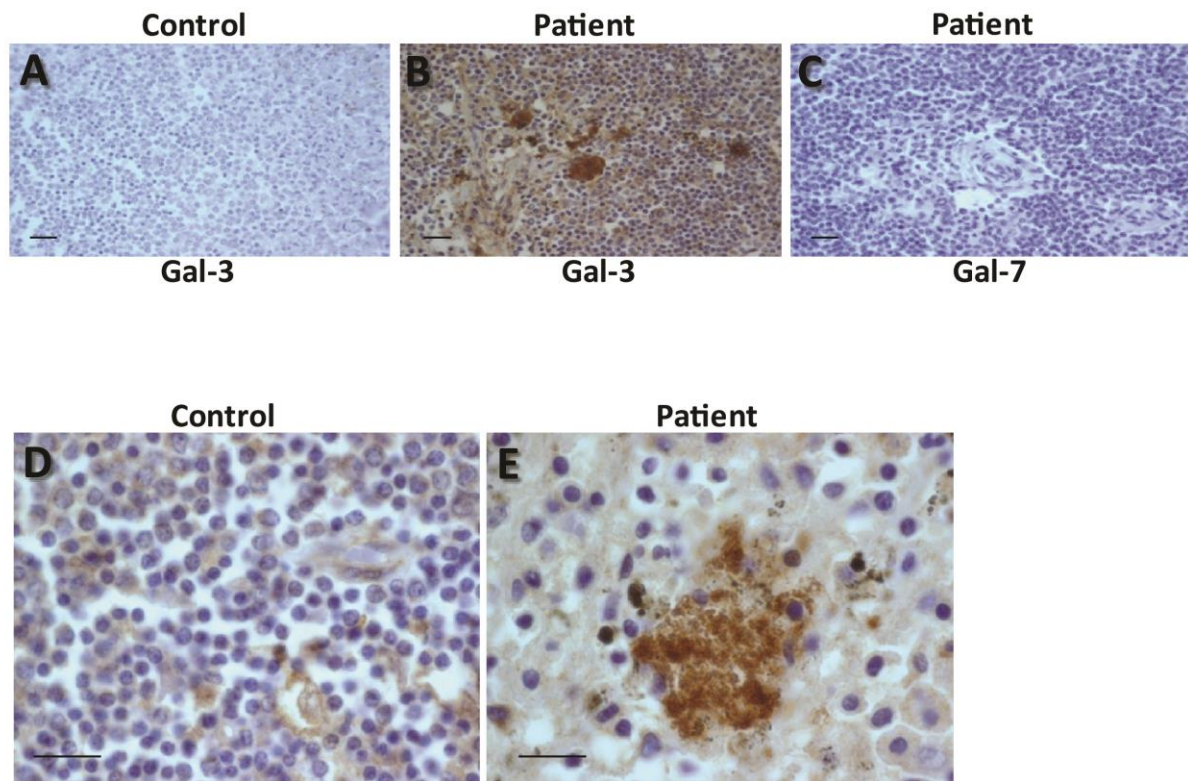


Figure 3.4 Galectin-3 expression in the spleen from patients with meningococcal disease.

Sections of spleens were stained for the presence of galectin-3 (brown staining) or galectin-7. Galectin-3 was detected in the patient (B and E) but only low levels were present in control tissue (A and D); galectin-7 was not detected (C). Higher magnification reveals diffuse staining corresponding to extracellular galectin-3 that surrounds cells, and intracellular galectin-3 contained in immune cells. Images are from one patient. Scale bars correspond to 10 μ m.

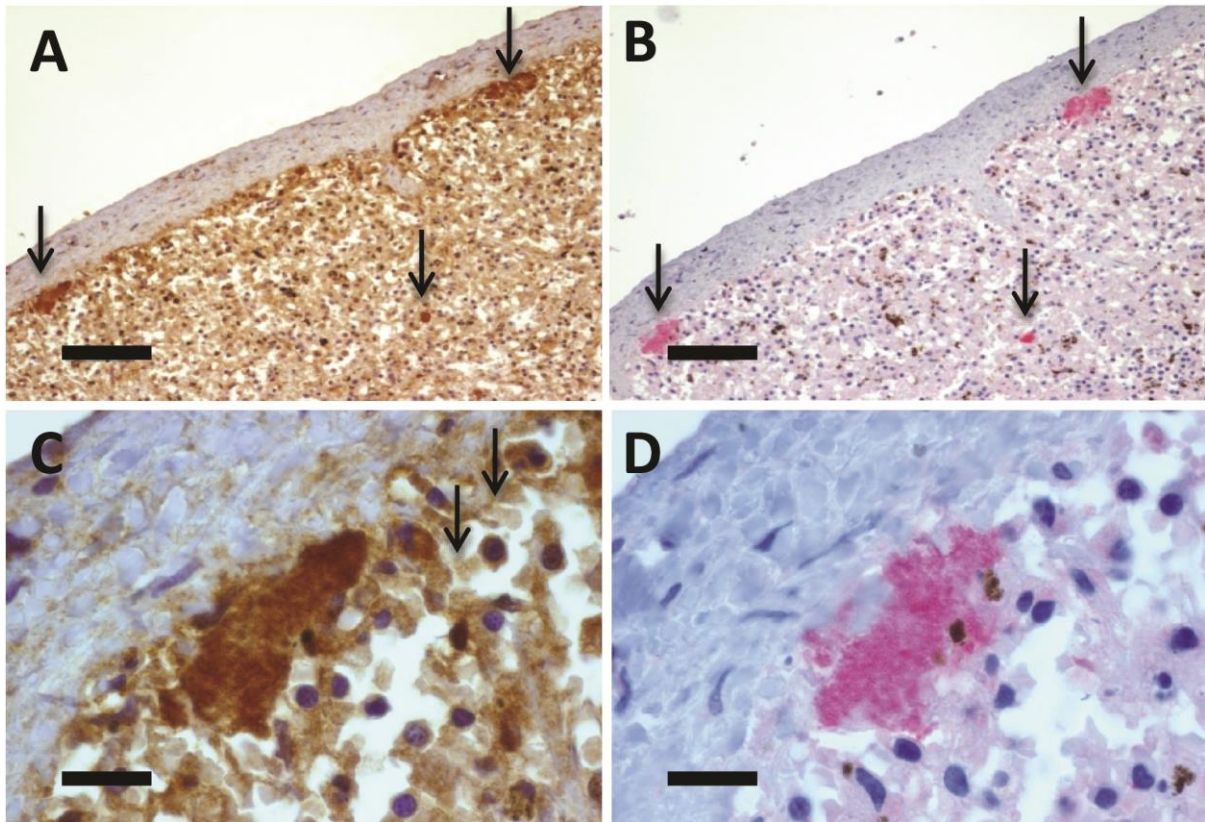


Figure 3.5 Co-localisation of galectin-3 and *N. meningitidis* in the spleen from patients with meningococcal infection.

Immunohistochemical staining of spleen tissue showing co-localisation of bacterial colonies and galectin-3. Serial sections of spleen tissue were stained with a rabbit anti-human galectin-3 antibody (brown in A and C) or a polyclonal antibody against *N. meningitidis* (red in B and D). Intense accumulation of brown staining is seen in the spleen at different locations (A and C, arrows). Red staining corresponding to *N. meningitidis* (B and D, arrows) occurs at the same location as the accumulations of galectin-3. The higher magnification confirmed this co-localisation, and shows galectin-3 in individual cells and extracellularly in a pattern similar to that observed in infected murine tissues. Scale bars correspond to 50 μ m in A and B, and to 10 μ m in C and D.

3.5 Expression of galectin-3 in human phagocytes

In summary, there was a clear increase in galectin-3 staining in both murine and human tissue following infection with *N. meningitidis*; the staining occurred both intracellularly and extracellularly, and co-localised with bacterial colonies. However, although it is known that galectin-3 is expressed by immune cells, and that these types of cells are usually present during infection, it was not possible to evaluate whether staining was due to increased galectin-3 expression by resident cells (triggered by meningococcal infection) or recruitment of galectin-3 expressing cells to the infected tissue. Intense staining was seen in the spleen, which is replete with phagocytic cells, and in the meninges which are infiltrated with phagocytes during meningococcal infection (Nielsen *et al.* 1988; Arevalo *et al.* 1989). In addition, galectin-3 expression by macrophages and monocytes *in vivo* has been previously reported (Reichert and Rotshenker 1999; MacKinnon *et al.* 2008). Therefore we analysed the expression of galectin-3 by phagocytes such as monocytes and macrophages *in vitro* using a human monocytic cell line (THP-1) and human primary monocytes obtained from whole blood. The macrophage-like cell line THP-1 has been used extensively as a representative model of human phagocytes (Borrmann *et al.* 2011; Estrella *et al.* 2011; Qin 2011; Zughaier 2011). Human primary monocytes obtained from whole blood were separated by adherence (Bennett and Breit 1994), and identified by nuclear staining and the presence of CD14 on the cell surface by flow cytometry (data not shown). CD14 is a monocyte receptor and is up-regulated upon activation and differentiation (Ziegler-Heitbrock 2007). CD14 positive monocyte derived progenitor cells can be isolated from PBMCs and are characterised by slow proliferation in absence of growth factors and by the expression of surface markers including CD14, CD35 and CD45 (Zhao *et al.* 2003).

Furthermore, they are able to differentiate into different cell lineages including osteoclasts, chondrocytes, myocytes and endothelial cells (Kuwana *et al.* 2003).

Galectin-3 was previously known as Mac-2 and used as a cell surface antigen marker for mature macrophages, while levels of galectin-3 in THP-1 cells are known to be enhanced upon PMA activation (Kim *et al.* 2003). THP-1 cells were differentiated into macrophages by exposing them to PMA for 72 hours, and western blot analysis performed on cell lysates of undifferentiated and differentiated cells; cellular actin was detected as a control. There was a clear increase in the amount of galectin-3 in differentiated cells compared to undifferentiated cells, consistent with previous results ((Kim *et al.* 2003), Fig. 3.6 A). Next, galectin-3 mRNA and protein levels were analysed in differentiated THP-1 macrophages infected with *N. meningitidis* isolate MC58 at an MOI of 30 for 3, 7 and 24 hours to examine whether bacterial infection modulates galectin-3 expression. Western blot analysis and qRT-PCR analysis of galectin-3 mRNA level were compared with the expression of the housekeeping gene, *β-actin*. As shown in Fig. 3.6 B, the level of galectin-3 mRNA was similar in uninfected and infected cells at all the time points examined, indicating that there was no modulation of galectin-3 mRNA level upon infection with *N. meningitidis*. In addition, similar levels of protein were observed in all samples at each time point, suggesting that endogenous galectin-3 is not affected by *N. meningitidis* infection (Fig. 3.6 C). Galectin-3 was not detected in supernatants of THP-1 cells, indicating that concentrations were either below the level of detection or a lack of galectin-3 release by THP-1 macrophages upon infection (Fig. 3.6 C).

Next, the surface expression of galectin-3 on differentiated THP-1 cells was examined. Galectin-3 binds to receptors present on the surface of cells, such as T cell receptor β (TCR- β) or CD98 on T cells and macrophages, respectively (Dong and Hughes

1996; Rabinovich *et al.* 2002), and some receptors belonging to the CEACAM family on neutrophils (Ohannesian *et al.* 1995). Therefore to examine the saturation of galectin-3 receptors present on the surface of THP-1 macrophages, recombinant galectin-3 (3 µg) was added to cells. No increase in the level of surface bound galectin-3 was detected following addition of exogenous protein, indicating that there were no unoccupied receptors on the surface of THP1 macrophages (Fig. 3.7). In addition, preliminary data on human primary monocytes, revealed that these cells do not express detectable amounts of galectin-3 on their surface, while incubation of the cells with exogenous recombinant galectin-3 led to detectable galectin-3 on the surface, indicating that galectin-3 is able to bind receptors on these cells (not shown).

Our data thus confirm the expression of galectin-3 on the surface of THP-1 cells and human monocytes (Novak *et al.* 2011), and suggest that galectin-3 may interact with surface ligands or receptors on phagocytic cells.

The expression of galectin-3 on the surface of phagocytes was also examined by immunofluorescence microscopy using an anti-galectin-3 antibody to detect galectin-3 on THP-1 cells or human primary monocytes infected with GFP-expressing *N. meningitidis* MC58. Cells were stained without permeabilisation to analyse surface expression. Figure 3.8 shows diffuse galectin-3 staining in both human monocytes and THP-1 cells incubated with MC58 strain for 1 hour. Galectin-3 staining on primary human monocytes was less intense than on THP-1 macrophages, in accordance with our previous flow cytometry results (not shown). Furthermore, we observed *N. meningitidis* associated with the cell surface as the extracellular non bound bacteria were removed by washing the cells, consistent with previous work (McNeil *et al.* 1994; Read *et al.* 1996; Spinosa *et al.* 2007).

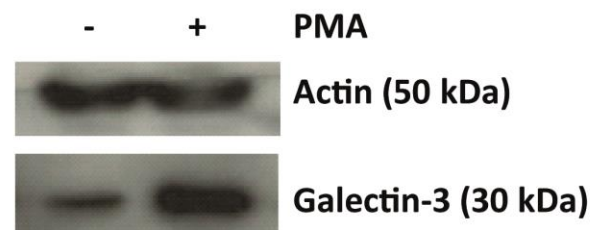
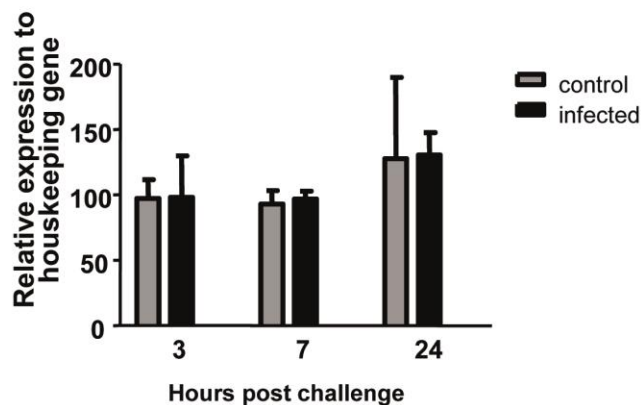
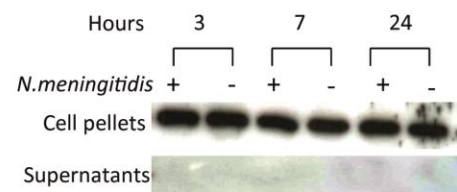
A**B****C**

Figure 3.6 Galectin-3 mRNA and protein expression in THP-1 macrophages.

(A) Galectin-3 levels in THP-1 cells detected by western blot prior to and following differentiation into macrophages by PMA. Lysates from 1×10^6 cells were subjected to SDS-PAGE and analysed using an anti-galectin-3 antibody. (B) The relative amount of galectin-3 mRNA in infected (black bars) and uninfected (grey bars) THP-1 macrophages, measured by qRT-PCR. Galectin-3 mRNA levels are comparable in infected and uninfected cells at 3, 7, 24 hours following infection with *N. meningitidis* MC58. Values were normalised to results for the β -actin gene. (C) Galectin-3 detected by western blot analysis of THP-1 cells differentiated with PMA and in cell supernatants. Lysates from 1×10^6 cells were subjected to SDS-PAGE and western blotting using an anti-galectin-3 antibody. There is no obvious difference in the amount of galectin-3 detected in cells infected with *N. meningitidis* strain MC58; no galectin-3 was detected in cell supernatants.

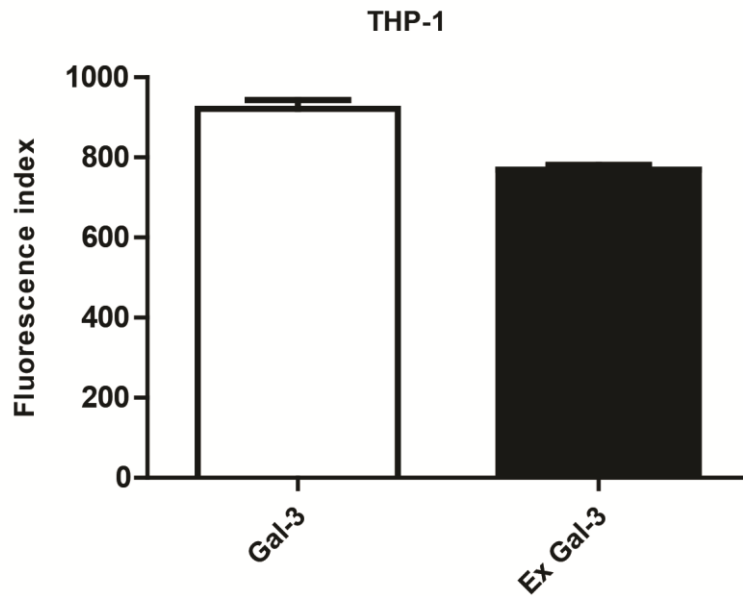


Figure 3.7 Surface expression of galectin-3 on THP-1 macrophages.

Flow cytometry analysis of galectin-3 on the surface of THP-1 macrophages before or after incubation of cells (5×10^5) with recombinant galectin-3 ($3 \mu\text{g}$). Galectin-3 is expressed on the surface of THP-1 cells and addition of exogenous galectin-3 does not alter the amount of galectin-3 detected. Experiments were performed in duplicate and error bars show the standard deviation. Data are from one representative experiment.

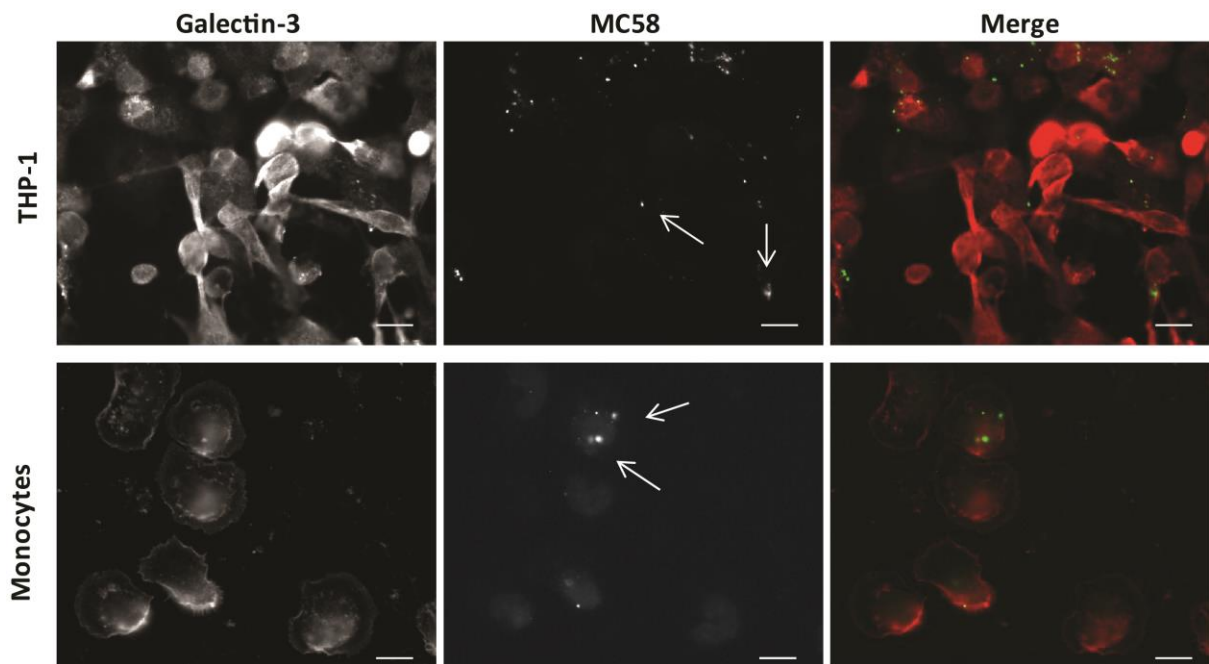


Figure 3.8 Detection of galectin-3 in THP-1 macrophages and monocytes by immunofluorescence microscopy.

Epi-fluorescence microscopy showing surface expression of galectin-3 and bacterial association with phagocytic cells. Galectin-3 (red) is expressed by THP-1 cells and human primary monocytes and is present on the cell surface. *N. meningitidis* (green, arrows) associates with the cells. Scale bars correspond to 10 μ M.

3.6 Expression of galectin-3 in epithelial cells

Galectin-3 is expressed by several human cell types other than macrophages, including fibroblasts, endothelial cells or epithelial cells (Dumic *et al.* 2006). In particular galectin-3 is present in epithelial cells from the intestine, conjunctiva, lung, kidney, breast and prostate (Lotz *et al.* 1993; Bao and Hughes 1995; Castronovo *et al.* 1996; Kasper and Hughes 1996; Pacis *et al.* 2000; Hrdlickova-Cela *et al.* 2001). Nasopharyngeal epithelial cells are among the first cells encountered by *N. meningitidis* during colonisation (Trivedi *et al.* 2011), and may represent a site of contact between bacteria and galectin-3. However, little is known about the expression of galectin-3 in the human nasopharynx (Saussez *et al.* 2008a), and there are no data regarding its modulation during infection.

We were unable to obtain tissue sections from the nasopharynx of infected individuals as it is not routinely examined in post-mortem studies. Therefore the expression of galectin-3 was examined in a human pharyngeal epithelial cell line as a model of the cells encountered by *N. meningitidis* during colonisation. Detroit 562 cells were grown in the wells of tissue culture plates with or without challenge with *N. meningitidis* MC58 at an MOI of 30. Expression of galectin-3 was not altered after 4 or 24 hours of infection (Fig. 3.9, A and B); cellular actin levels were used as a control. To further examine the expression of galectin-3 in Detroit 562 cells, we performed epi-fluorescence microscopy of uninfected and infected cells. Analysis of non-permeabilised cells demonstrated the surface expression of galectin-3 (Fig. 3.9 C). Furthermore, apparent co-localisation of bacteria and surface galectin-3 was observed by confocal microscopy, with diplococci in proximity to galectin-3, suggesting a possible interaction between *N. meningitidis* and endogenous galectin-3 at the site of bacterial attachment.

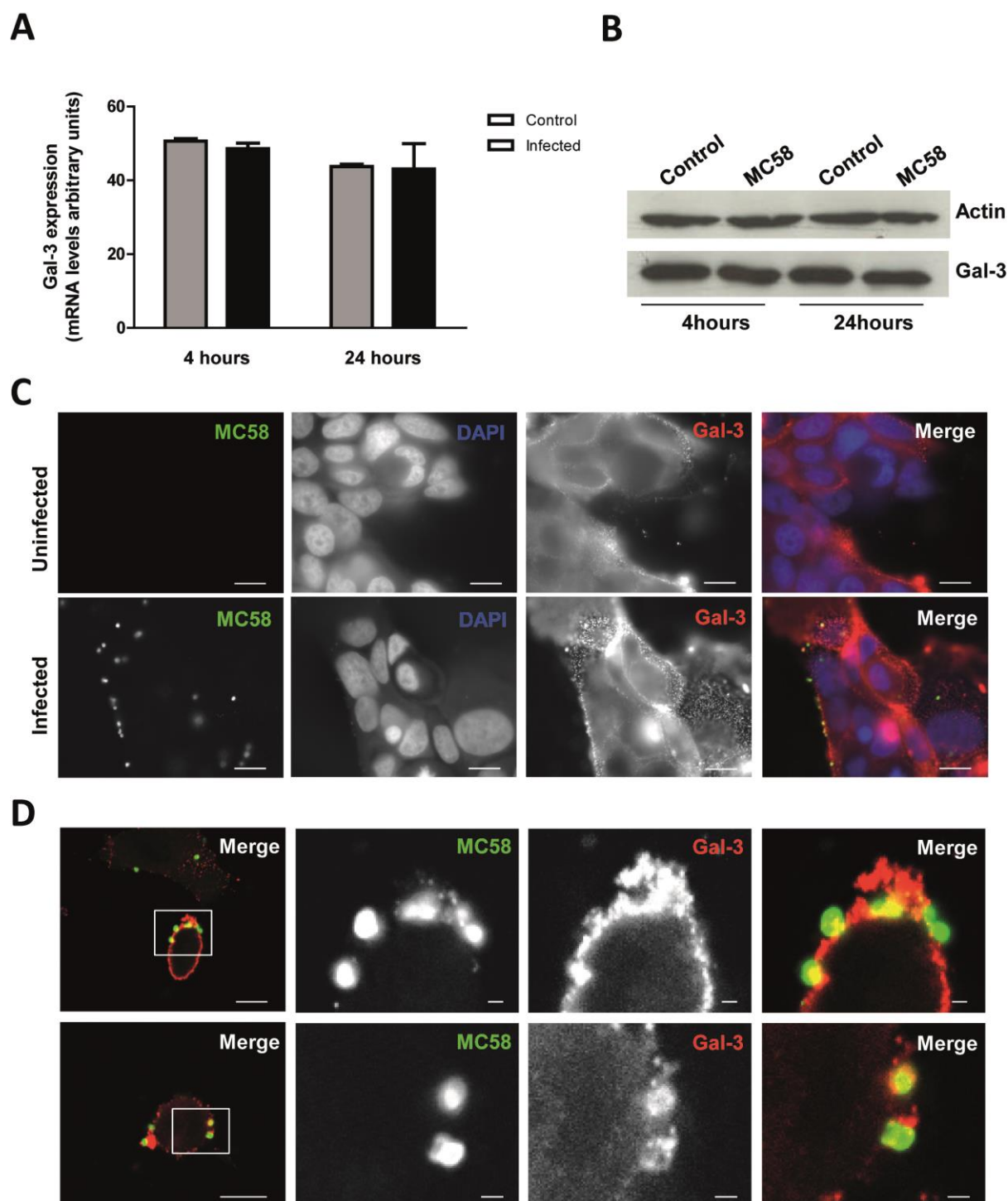


Figure 3.9 Galectin-3 expression and localisation in Detroit 562 cells infected with *N. meningitidis*.

(A) Relative amount of galectin-3 mRNA in infected (black bars) and uninfected (grey bars) Detroit 562 epithelial cells measured by qRT-PCR. Cells were infected at an MOI of 30. Values were normalised to results for expression of the β -actin housekeeping gene. (B) Western

blot analysis showing galectin-3 and β -actin in infected (MC58) and uninfected (Control) cells 4 or 24 hrs after challenge. (C) Fluorescence (C) and confocal (D) microscopy images of Detroit 562 cells stained for galectin-3 (red) and bacteria (green). Bacteria and galectin-3 were stained following 1 hour incubation with a mouse monoclonal anti-LPS antibody (α -L3,7,9) and a rabbit polyclonal anti-galectin-3 antibody, then incubated for 30 minutes with an anti-mouse Alexa Fluor 488 conjugated antibody to label bacteria and an anti-rabbit Alexa Fluor-555 conjugated antibody to label galectin-3 on the cell membranes. Accumulation of extracellular galectin-3 is observed in proximity of meningococci, suggesting co-localisation. Scale bars correspond to 10 μ M in first panel and 1 μ M in the subsequent panels.

3.7 Summary

In this chapter the presence of different galectins in tissue during meningococcal infection was analysed in a murine model of meningococcal sepsis, and the expression of galectin-3 in human tissue, in phagocytes and epithelial cells was examined. Galectin-3, but not galectin-1 and galectin-4, is present in murine tissue during meningococcal infection. This observation was confirmed by immunohistochemical analyses of tissues from patients. Of note, galectin-3 was detected both in cells and extracellularly, and there was clear co-localisation of this galectin with *N. meningitidis* in the spleen of patients with meningococcal disease. In addition, galectin-3 is expressed at both transcriptional and protein levels in the macrophage-like cell line THP-1 and the nasopharyngeal epithelial cell line Detroit 562. Furthermore, although *N. meningitidis* does not influence mRNA expression in phagocytes and epithelial cells, the presence of the protein was confirmed by microscopy, and there was some co-localisation of surface galectin-3 and *N. meningitidis* on epithelial cells.

CHAPTER 4: Binding of galectin-3 to *N. meningitidis*

In the preceeding chapter, galectin-3 was shown to be present in tissues from mice challenged with *N. meningitidis*, and patients with meningococcal disease: galectin-3 co-localised with bacterial colonies. Furthermore, galectin-3 was present in the extracellular environment in infected tissues, consistent with the hypothesis that galectin-3 interacts directly with *N. meningitidis*. Therefore, in this chapter the binding of galectin-3 to bacteria was examined. As a range of galectins are produced during inflammation (Liu and Rabinovich 2010), and there are numerous sites of inflammation during meningococcal disease, the interaction between bacteria and several galectins belonging to different families was examined.

4.1 Binding of *N. meningitidis* to galectins

Galectins can be secreted into the extracellular milieu, where they are able to bind galactoside containing molecules on the surface of cells or micro-organisms (Dumic *et al.* 2006). Initial binding experiments were performed with the serogroup B *N. meningitidis* strain MC58 (Virji *et al.* 1992a), which expresses an L3,7,9 immunotype LPS (Wakarchuk *et al.* 1996; Jennings *et al.* 1999).

The binding of four galectins belonging to the different families was examined: galectin-1 and galectin-7 (proto-type galectins), galectin-4 (tandem repeat galectin), and galectin-3 (chimera-type galectin). These galectins are present in the cytoplasm of cells, but

can be released under certain conditions, and could potentially interact with bacteria in the extracellular environment (Rabinovich and Vidal 2011).

In preliminary experiments, bacteria were fixed in paraformaldehyde (2×10^7 CFU) and incubated with galectin-3 at a range of concentrations (0.33 μ M, 1.6 μ M, 3.3 μ M). Binding was detected by FACS analysis using galectin-3 at a concentration of 3.3 μ M. Therefore this concentration was used in all experiments with this and other galectins, unless otherwise stated, as they have similar affinities for their ligands (Kaltner *et al.* 2002; Saussez *et al.* 2005; Lohr *et al.* 2007). Indeed the affinities of ligands for immobilised galectin-1, -3, -4 and -7 have K_d of between 1 and 5 μ M (Cederfur *et al.* 2008). Additionally, stoichiometry and analysis of galectin-ligand interactions indicates that a minimum concentration of 1.25 μ M is required for binding of galectin-1 to its ligands (Earl *et al.* 2010).

There was significant binding of galectin-3 to *N. meningitidis*, while none of the other galectins bound bacteria to any appreciable extent (Fig. 4.1, A and B). The lack of detectable binding of galectins other than galectin-3 underlines the specificity of binding, and directed us to characterise the interaction between *N. meningitidis* and galectin-3 in more detail.

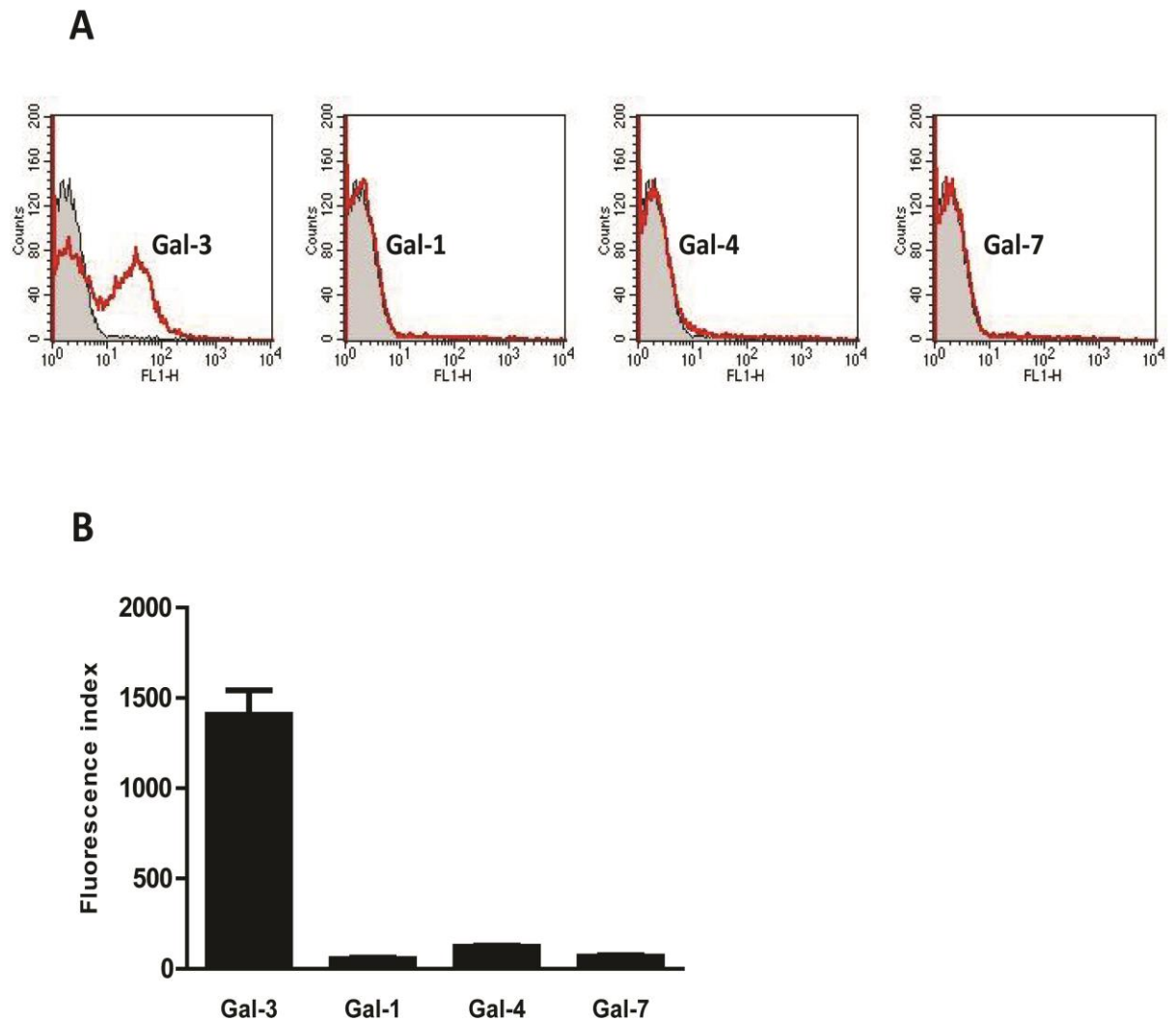


Figure 4.1 Binding of *N. meningitidis* to galectins.

(A) Representative flow cytometry traces showing binding of recombinant galectin-3 (Gal-3), galectin-1 (Gal-1), galectin-4 (Gal-4) and galectin-7 (Gal-7) to *N. meningitidis* following incubation of MC58 with galectins (3.3 μ M). Negative controls (no galectin) are shown in grey, and traces in the presence of a galectin are shown in red. (B) Quantification of flow cytometry analysis of galectin binding to *N. meningitidis*. Binding is expressed as the Fluorescence Index (% positive bacteria gated multiplied by the geometric mean fluorescence). Data are from four different experiments and error bars show the standard deviation.

4.2 Effect of lactose on galectin-3 binding to *N. meningitidis*

As mentioned in the introduction, galectin-3 interacts with various ligands via their LacNAc residues, and this interaction is mediated by the CRD of galectin-3, and can be inhibited by exogenous lactose. Galectin-3 is defined as a galactoside-binding protein and its principal ligands include lactose, N-acetyl-lactosamine and poly-lactosamine, while multivalent ligands increase the affinity of interactions (Diehl *et al.* 2010).

To examine whether the interaction between *N. meningitidis* and galectin-3 is mediated by the CRD, binding studies were performed in the presence of lactose or sucrose. Consistent with carbohydrate dependent binding of galectin-3 to *N. meningitidis*, the interaction was significantly inhibited by lactose at a concentration of 100 mM (Fig. 4.2 A, 75% reduction of galectin-3 binding, $p=0.0062$). In contrast, addition of sucrose, a disaccharide that does not inhibit CRD-mediated binding (Ochieng *et al.* 1998b) had no effect (Fig. 4.2 B). Characterisation of the lactose inhibition was confirmed by titrating concentrations of lactose and recombinant galectin-3. Results demonstrate that lactose inhibits galectin-3 binding to *N. meningitidis* in a concentration dependent manner, with 300 mM of lactose inhibiting the interaction in the presence of 3.3 μ M of galectin-3 (Fig. 4.2C, D and E). Moreover, this concentration of lactose inhibited the binding of galectin-3 to fixed bacteria (2×10^7 CFU) over a range of concentrations of recombinant galectin-3 (Fig. 4.2 D).

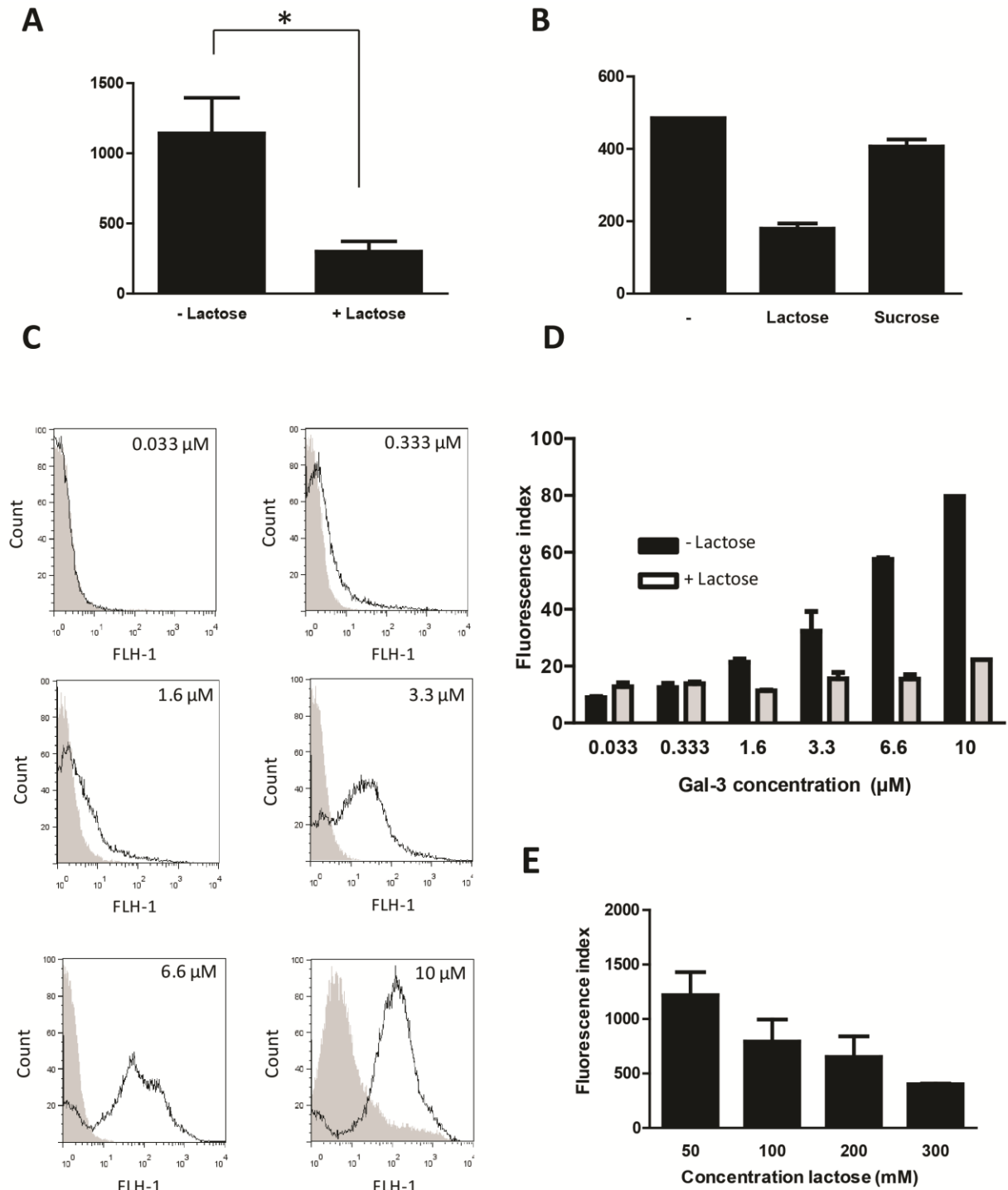


Figure 4.2 Galectin-3 binding to *N. meningitidis*.

(A) Quantification of flow cytometry analysis of galectin-3 (3.3 μM) binding to *N. meningitidis* in the absence or presence of 100 mM lactose. Lactose significantly reduces binding to *N. meningitidis* (Two-tailed, Unpaired Student's t test, * $p=0.0062$. Data are from four different experiments performed in duplicate and error bars show the standard

deviation (B) Quantification of flow cytometry analysis of galectin-3 (3.3 μ M) binding to *N. meningitidis* in the absence of disaccharides or presence of 100 mM lactose or sucrose. (C) Incubation of *N. meningitidis* with increasing concentrations of biotinylated galectin-3 shows dose dependent binding to MC58 (black traces) which is inhibited by the presence of 300 mM lactose (grey filled traces). (D) Quantification of galectin-3 binding to *N. meningitidis* in absence (black bars) or presence (grey bars) of 300 mM lactose over a range of galectin-3 concentrations (indicated). (E) Quantification of flow cytometry analysis of galectin-3 binding to *N. meningitidis* using galectin-3 at a concentration of 3.3 μ M and increasing concentration of lactose (50, 100, 200, 300 mM). Binding is expressed as the Fluorescence Index. Experiments were performed in duplicate and error bars show the standard deviation. Results were consistent on at least three occasions; data are from one representative experiment.

4.3 Binding of *N. meningitidis* galectin-3 from different species and effect of galectin-3 phosphorylation

Of all galectins, galectin-3 is the most conserved as it is found in rodents, and chickens, as well as in other species (Kaltner and Gabius 2012). As binding of galectin-3 to *N. meningitidis* involves the CRD which is highly conserved across species, binding of the meningococcus to galectin-3 from non-human sources was investigated. Murine galectin-3 shares 78% sequence identity with human galectin-3, while the CRD of chicken galectin-3 is 68% identical to the CRD of human galectin-3. Results show that chicken and mouse galectin-3 bind to *N. meningitidis* at similar levels as human galectin-3, confirming the conservation of the binding sites of galectin-3 molecules from these species (Fig. 4.3).

Galectin-3 is the only chimera type galectin, formed by a CRD connected to a N-terminal region via repeated five repeats of a six amino acid sequence rich in Gly, Tyr and Pro which is sensitive to metalloproteinases (Ochieng *et al.* 1998a; Krzeslak and Lipinska 2004) and Fig. 1.6 A). The CRD is composed of 130 amino-acids, forming a globular structure with two anti-parallel β -sheets of five and six β -strands, respectively (Fig. 1.6 B). The ND has a short sequence (12 residues) that can be phosphorylated at Ser⁵ and Ser¹¹, although the functional significance of this is not fully understood (Fig. 1.6 A). The phosphorylated form of galectin-3 is found in both the cytoplasm and nuclei of cells, while the non-phosphorylated form is present only in the cytoplasm (Mazurek *et al.* 2000). Phosphorylation reduces binding to some ligands such as asialomucin and laminin (Yoshii *et al.* 2002). As this post-translational modification may affect the binding properties of the lectin, analysis of the binding of bacteria to human phosphorylated galectin-3 was assessed. Preparation of galectin-3 phosphorylated at Ser⁵ and Ser¹¹ was performed in collaboration

with Professor Gabius using constitutively active casein kinase 1 (CK-1) and confirmed by mass spectrometry (Kubler *et al.* 2008). Flow cytometry results showed no significant alteration in the interaction of galectin-3 with the meningococcus upon phosphorylation (Fig. 4.3, $p=0.1888$) with the binding of bacteria to phosphorylated galectin-3 comparable to the binding to the non-phosphorylated protein. These data indicate that phosphorylation at Ser⁵ and Ser¹¹ does not affect the selectivity of galectin-3 for *N. meningitidis*, consistent with the observation that the phosphorylated protein can still retain its binding properties and maintain its carbohydrate-inhibitable activity (Kubler *et al.* 2008).

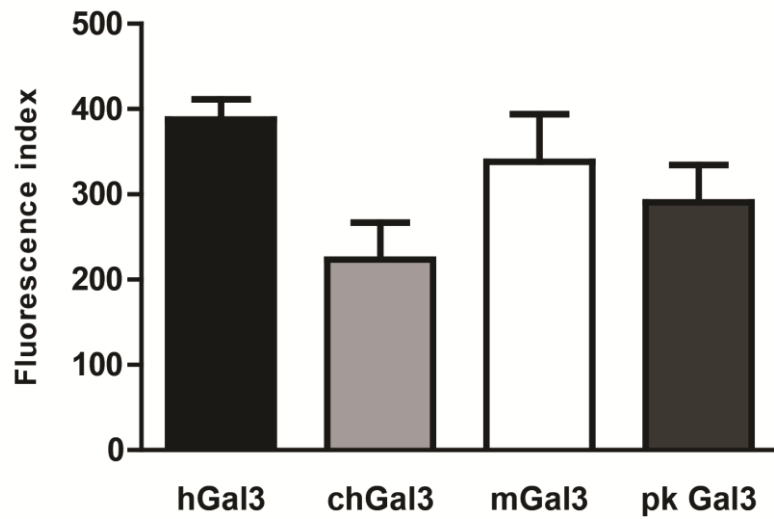


Figure 4.3 Phosphorylated and galectin-3 from several species bind to *N. meningitidis*.

Quantification of FACS analysis of binding to *N. meningitidis* using human galectin-3 (hGal3), chicken galectin-3 (chGal3), mouse galectin-3 (mGal3) and phosphorylated human galectin-3 (pk Gal3) (3.3 μ M of each protein). No significant difference was observed in binding to the different galectins.

4.4 Characterisation of the binding between *N. meningitidis* and galectin-3

4.4.1 Galectin-3 domains involved in binding

The biological activities of galectin-3 in the extracellular compartment are mainly dependent on its interaction with various ligands via CRD (Ochieng *et al.* 2004). However, the ND of galectin-3 can interact with non-carbohydrate molecules in the cytoplasm or nucleus such as Gemin-4, K-Ras or Synexin (Haudek *et al.* 2009). Furthermore the ND of galectin-3 has been suggested to interact with a region of *Salmonella* LPS that includes part of the lipid A and the inner core (Mey *et al.* 1996). Additionally, several lines of evidence indicate that galectin-3 binding to glycoproteins involves the concentration and time dependent formation of pentamers, through the association between NDs, which increases the affinity for the ligand.

Results from section 4.3 indicate that the CRD of galectin-3 binds meningococci, as lactose partially inhibits binding. However, to establish whether further domains of galectin-3 are necessary for the interaction with *N. meningitidis*, we examined the binding of truncated galectin-3, in which the N-terminal region had been removed by proteolytic treatment with collagenase D which cleaves at Met¹²⁷, as described previously ((Kopitz *et al.* 2001), Fig. 1.6 A and 4.4). This treatment leaves the CRD (residues 127-264) of the protein intact and functional (Agrwal *et al.* 1993).

As shown in Fig. 4.5, proteolytic removal of the N terminal section of galectin-3 abolished binding to wild-type strain MC58 ($p=0.0016$), indicating that the CRD alone was not sufficient to support galectin-meningococcal interactions, and that the N-terminal region of the protein is necessary for binding. A concentration of 3.3 μM for both full length

and truncated galectin-3 was used in the assay. These data are consistent with oligomerisation of the protein being necessary for galectin-3 binding to the meningococcus or with the ND itself being involved in the interaction of full length galectin-3 with *N. meningitidis*.

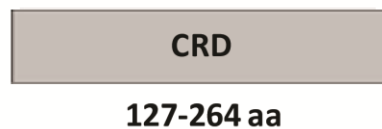
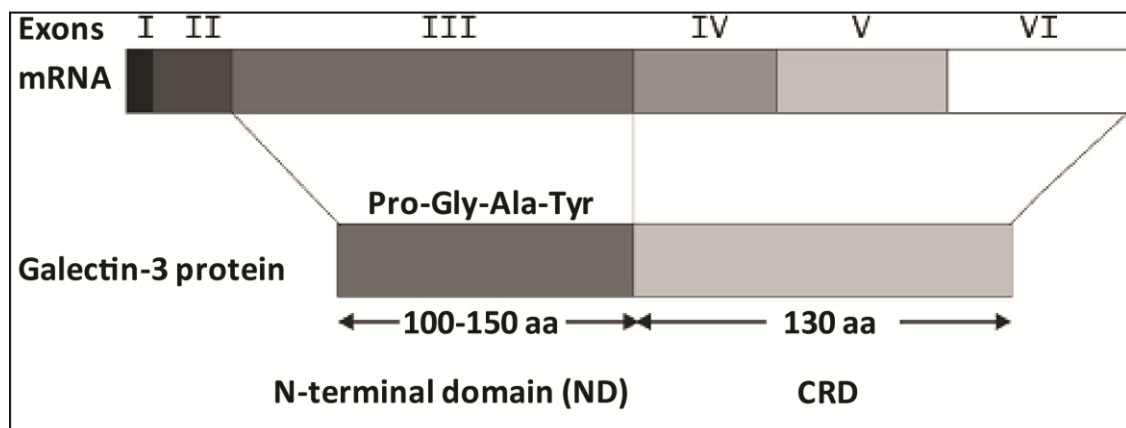


Figure 4.4 Cleavage sites in galectin-3.

Schematic representation of the mRNA and domain structure of galectin-3. The gene encoding galectin-3 is formed by six exons and five introns where the exons I and II encode the 5' untranslated region, exon III encodes the ND and exons IV, V and VI encode the CRD. The N-terminal domain of about 130 amino acids rich in Pro-Gly-Arg-Tyr residues (ND, dark grey) and carbohydrate recognition domain of about 130 amino acids (CRD, light grey) are indicated. The ND of the protein is removed by treatment with collagenase D.

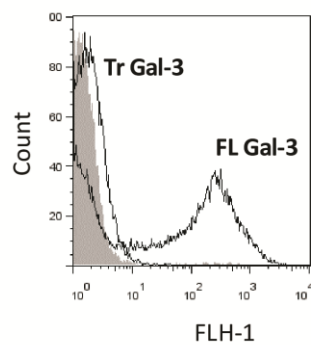
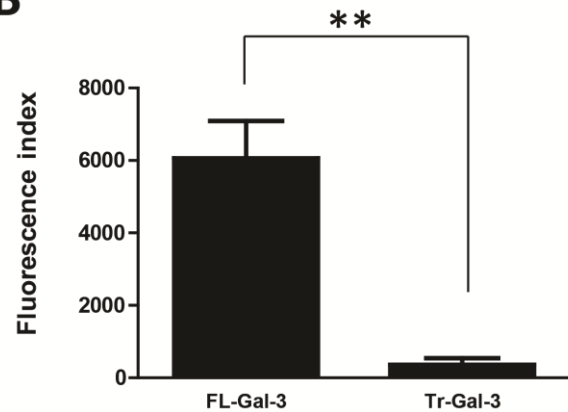
A**B**

Figure 4.5 Binding of truncated galectin-3 to *N. meningitidis*.

Representative flow cytometry traces showing binding of full length galectin-3 (3.3 μ M) to *N. meningitidis* (black trace, FL Gal-3) and galectin-3 consisting of the CRD alone (3.3 μ M, black trace, Tr Gal-3), with the negative control (no galectin-3) shown in grey. (B) Quantification of flow cytometry analysis of binding of full length galectin-3 and truncated galectin-3 to *N. meningitidis* MC58. Tr Gal-3 binding to *N. meningitidis* is significantly reduced compared to the full length protein (Unpaired Student's test, $p=0.0016$). Data are from four different experiments and error bars show the standard deviation. The binding is expressed as the Fluorescence Index.

4.4.2 Full length LPS is required for galectin-3 binding to *N. meningitidis*

In almost all Gram negative bacteria which have been reported to bind galectin-3, the target ligand is LPS (Mey *et al.* 1996; Gupta *et al.* 1997; Fermino *et al.* 2011); this includes the LPS of *N. gonorrhoeae*. Therefore it was likely that LPS is involved in the attachment of galectin-3 to the meningococcal surface.

Meningococcal LPS consists of lipid A, which anchors the molecule in the outer membrane (Fig 1.3 A) and an oligosaccharide portion. Lipid A contains five acyl chains joined to two glucosamine residues (Erridge *et al.* 2002). These are linked by two Kdoresidues to an oligosaccharide with an inner core di-heptose-N-acetylglucosamine backbone; the two Hep residues provide the point of attachment for a variety of outer core oligosaccharides (Monteiro *et al.* 2003). The outer core is formed by Gal, GlcNAc and Gal, and includes LNnT, the last two residues of which constitute LacNAc, a known galectin-3 ligand (Cederfur *et al.* 2008). The addition of HepII and GlcNAc to HepI (with or without substitutions) defines the α -chain, while the addition of Glc to the HepI and the whole outer core defines the β -chain. In addition, Neu5Ac (Fig 1.3 A) can be attached to LPS at the terminal Gal of certain LPS immunotypes (Gilbert *et al.* 1997).

Galectin-3 has affinity for Lac and LacNAc, but also for larger oligosaccharides including those containing poly-N-acetyl-lactosaminoglycan (Leffler and Barondes 1986; Sparrow *et al.* 1987). Previous work on *N. gonorrhoeae* showed that galectin-3 binds the LPS α -chain composed of Glc, Lac, LNnT, and neolactoheptaosyl (a derivative of neolactotetraosyl ceramide), the latter with higher affinity (John *et al.* 2002). This is consistent with galectin-3 preferentially binding to poly-lactosaminylated substrates that are composed of β 1-3 linked LacNAc residues (Massa *et al.* 1993).

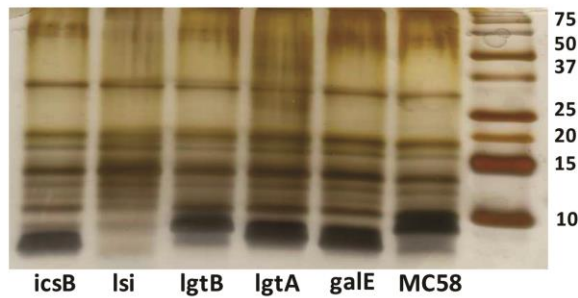
Meningococcal LPS differs from gonococcal LPS as its α -chain contains fewer saccharide residues: while LNnT is present in the LPS from both species, in meningococcal LPS this group is attached to HepI via a Glc, and in the gonococcus there are GalNAc and Gal residues in addition (Fig. 1.3, A and B) (Yamasaki *et al.* 1999).

The contribution of *N. meningitidis* LPS to galectin-3 binding was investigated using strains with defined mutations leading to truncation of the LPS molecule (provided by Dr. Derek Hood, University of Oxford); this allowed assessment of the contribution of the individual saccharide units to galectin recruitment. Galectin-3 binding was examined to the wild-type strain MC58 and isogenic strains with mutations in genes encoding glycosyl transferases responsible for the addition of carbohydrates to the LPS molecule (Fig 1.3 A). The *lgtA* and *lgtB* mutants lack enzymes responsible for the biosynthesis of LacNAc, while the *galE* mutant lacks the LNnT structure (Jennings *et al.* 1995b); the *icsB* mutant lacks the gene encoding the enzyme responsible for the addition of glucose to HepI (van der Ley *et al.* 1997) and the *lsi-1* mutant lacks the gene encoding the enzyme responsible for the addition of HepII to HepI. However, mutation of *lsi-1* also affects the α -chain of LPS; this mutant also lacks the entire oligosaccharide portion except the first heptose (Jennings *et al.* 1995b).

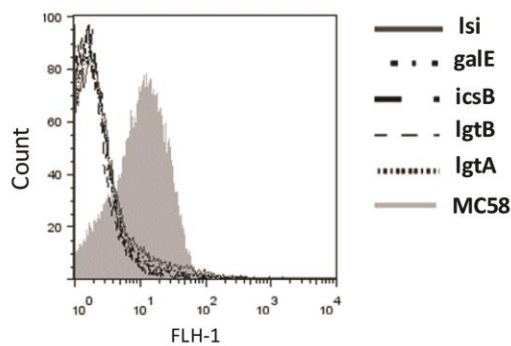
Initially, gel electrophoresis and silver staining was performed on LPS preparations from the mutants and the wild-type strains to confirm that they express truncated versions of LPS (Fig. 4.6, A). Migration of LPS was consistent with the effect of the mutations on the α - and β -chains of the molecule (Lesse *et al.* 1990). Analysis of galectin-3 binding to whole fixed *N. meningitidis* by flow cytometry demonstrated that all mutants expressing a truncated LPS molecule showed significantly reduced binding to galectin-3 compared to the wild-type strain (Fig. 4.6B and C, MC58 $\Delta galE$ $p=0.0032$, MC58 $\Delta lgtA$ $p=0.0076$, MC58 $\Delta lgtB$ $p=0.0003$, MC58 $\Delta lsi-1$ $p=0.0062$, MC58 $\Delta icsB$ $p=0.0043$), demonstrating that full

length LPS is required for galectin-3 binding to the meningococcal surface. As mutation of *lgtB* alone was sufficient to abolish galectin-3 binding, the terminal galactose and an intact LacNAc moiety are required for the interaction.

A



B



C

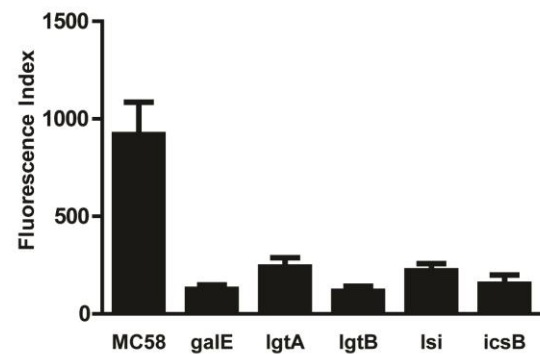


Figure 4.6 Meningococcal LPS is required for binding of galectin-3.

(A) Silver staining of LPS preparations from strains MC58 Δ icsB, MC58 Δ lsi, MC58 Δ lgtB, MC58 Δ lgtA, MC58 Δ galE and MC58. (B) Representative FACS tracing of the binding of galectin-3 (3.3 μ M) to strains of *N. meningitidis* with full length (MC58) and truncated versions of LPS (MC58 Δ galE, MC58 Δ lgtA, MC58 Δ lgtB, MC58 Δ lsi and MC58 Δ icsB, dotted black traces). (C) Quantification of binding of galectin-3 (3.3 μ M) to strains of *N. meningitidis* with full length (MC58) and truncated versions of LPS. There was a significant reduction in the binding of galectin-3 to all strains with truncated LPS (MC58 Δ galE $p=0.0032$, MC58 Δ lgtA $p=0.0076$, MC58 Δ lgtB $p=0.0003$, MC58 Δ lsi $p=0.0062$, MC58 Δ icsB $p=0.0043$). Data are from four different experiments and error bars show the standard deviation.

4.4.3 Effect of sialic acid and LPS immunotype on binding to galectin-3

N. meningitidis strain MC58 expresses L3,7,9 immunotype LPS which can be modified by the addition of an α 2,3-linked sialic acid (Mandrell *et al.* 1991); an LPS specific sialyl transferase (*Lst*) is responsible for the linkage of sialic acid to the terminal galactose of the α -chain (Mandrell *et al.* 1990). Studies with glycan microarrays have demonstrated that the presence of sialic acid on terminal residues of glycans can inhibit binding of some galectins, although this did not affect galectin-3 (Ahmad *et al.* 2004b; Stowell *et al.* 2008b). To determine whether LPS sialylation alters galectin-3 binding to *N. meningitidis*, we compared the binding to wild-type strain MC58 and an isogenic mutant lacking *Lst*. In parallel, to further understand the impact of sialic acid on galectin-3 binding to *N. meningitidis*, we also examined the effect of the presence of the polysaccharide capsule, which consists of α 2,8-linked polysialic acid. The gene *siaD* encodes the 2,8 polysialyl transferase required for capsule synthesis (Freiberger *et al.* 2007), while *siaC* is necessary for sialic acid biosynthesis (Edwards *et al.* 1994); therefore, MC58 Δ *siaC* is unable to express a capsule or sialylate its LPS without exogenous CMP-NANA. We analysed the effect of galectin-3 interaction with wild-type MC58 and isogenic mutant strains MC58 Δ *siaD*, MC58 Δ *siaC*, and MC58 Δ *lst* (Exley *et al.* 2005) and Table 2.1). While there was no significant difference in galectin-3 binding to the strains, there was a consistent trend to increased binding to MC58 Δ *siaC* (Fig. 4.7, $p=0.0671$).

Next, we chose a panel of disease causing isolates that belong to different serogroups and express different LPS immunotypes (Table 2.1), as shown by differences in the migration of LPS (Fig. 4.8 A). Analysis by flow cytometry demonstrated that, while there was some variation in the relative level of binding, strains belonging to different serogroups were able to bind galectin-3 (Fig. 4.8 B). Interestingly, all the strains which bind galectin-3

express an LPS immunotype containing LNnT and thus a terminal LacNAc. Z2491 is a serogroup A isolate which expresses an L9 immunotype LPS (Choudhury *et al.* 2008), while the serogroup B strains MC58 and H44/76 (Plested *et al.* 1999), the serogroup C strains 8013 (Geoffroy *et al.* 2003) and FAM18 (this study, data not shown) all express L3,7,9 immunotype LPS. The only strain which did not show similar binding of galectin-3 was F8238, a serogroup A isolate which expresses immunotype L10 LPS that is not thought to include LacNAc or LNnT moieties (Maslanka *et al.* 1997).

Taken together, our data demonstrate that full length galectin-3 binds to the extracellular pathogen *N. meningitidis* and LPS on the bacterial surface is required for this interaction. Along with our observation that there is co-localisation of galectin-3 and *N. meningitidis* in tissue, exogenous galectin-3 secreted by immune cells could bind to bacteria during disseminated disease. Given the known oligomerisation and cross-linking properties of galectin-3 (Ahmad *et al.* 2004a), we hypothesised that this could affect the ability of bacteria to interact with host molecules and cells.

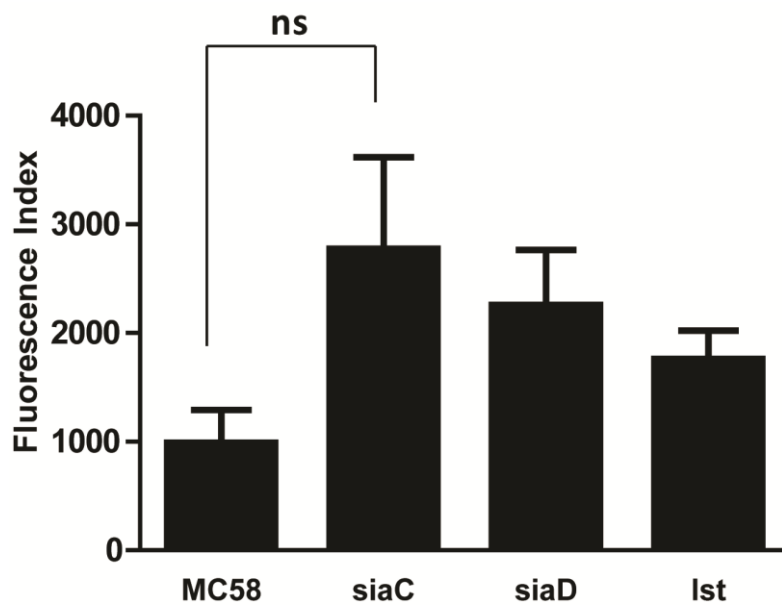


Figure 4.7 The effect of sialic acid on interaction of *N. meningitidis* with galectin-3.

Quantification of flow cytometric analysis of galectin-3 binding shows no significant difference in binding to the wild-type strain and a strain lacking LPS sialylation (MC58 Δ *st*), or capsule (MC58 Δ *siaD*). A tendency to increased galectin-3 binding was detected to the strain lacking sialic acid (MC58 Δ *siaC*, $p=0.0671$). Data are from four different experiments performed in duplicate, and error bars show the standard deviation.

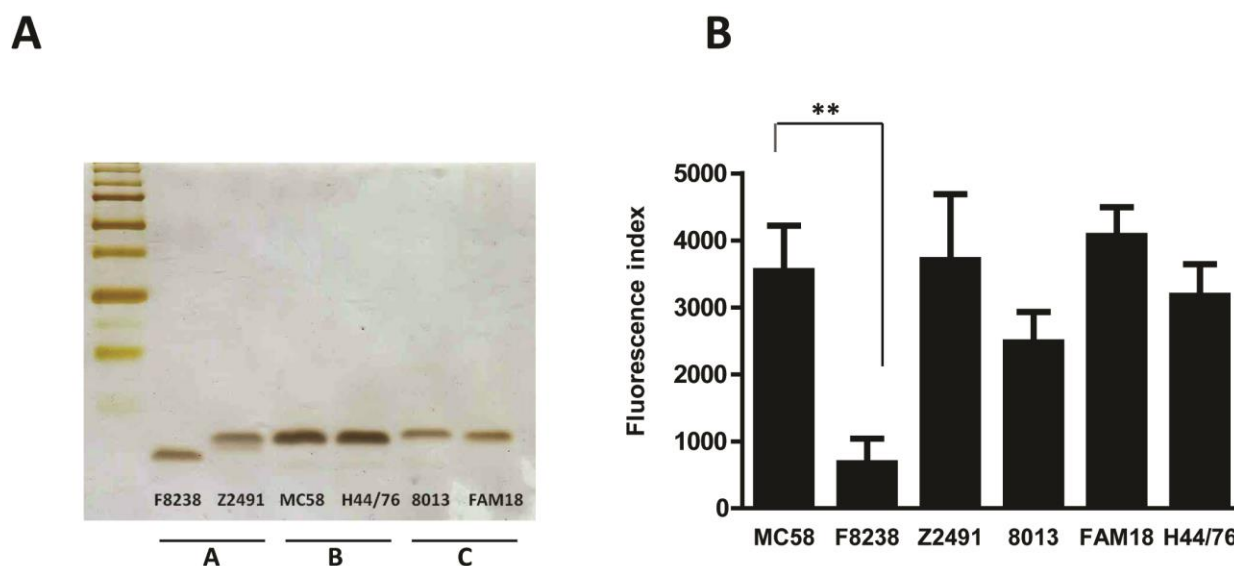


Figure 4.8 Galectin-3 binding to *N. meningitidis* is independent of serogroup.

(A) Silver staining showing LPS for *N. meningitidis* clinical isolates. Bands are the different sizes of LPS, indicating various immunotypes. (B) Binding of galectin-3 to serogroups A, B, and C *N. meningitidis*. Fixed bacteria were incubated with 3.3 μ M galectin-3 for 1 hr and binding was detected by flow cytometry. Galectin-3 binding to *N. meningitidis* strains F8238 and Z2491 (serogroup A), MC58 and H44/76 (serogroup B), 8013 and FAM18 (serogroup C) demonstrates that binding is independent of serogroup. Interaction of the strain F8238 with galectin-3 is significantly reduced (**, Unpaired Student's t-test, $p=0.0098$) compared to strain MC58. Binding is expressed as the Fluorescence Index. Data are from four independent experiments and error bars show the standard deviation.

4.5 Summary

Work in this chapter characterised the binding of the meningococcus to galectin-3 using *N. meningitidis* serogroup B MC58 and recombinant human galectins. Only the chimera-type galectin-3 was shown to bind *N. meningitidis*; therefore this interaction was analysed in detail. The interaction was dependent on meningococcal LPS (which contains the known galectin-3 ligand LacNAc) and the galectin-3 CRD, as lactose partially inhibits binding. However, almost complete loss of binding was also observed with only the CRD of galectin-3 demonstrating that, while this region is necessary for binding, it is not sufficient. This suggests that oligomerisation of galectin-3 might be important for interaction. No statistically significant effect on the interaction between galectin-3 and *N. meningitidis* was observed when sialic acid was removed from LPS. Moreover, meningococcal strains belonging to different serogroups are able to bind galectin-3, with LPS from all the strains which bound galectin-3 containing LacNAc, indicating that the interaction is dependent on the immunotype of the strain.

CHAPTER 5: Consequences of galectin-3 binding to *N. meningitidis* on interaction with cells

5.1 Influence of galectin-3 on bacteria-cell interaction

Galectin-3 has diverse functions depending on its localisation. Intracellular galectin-3 can regulate cell growth, differentiation and apoptosis, while extracellular galectin-3 has been implicated in cell adhesion and phagocytosis (Bornstein and Sage 2002; Melo *et al.* 2011). Furthermore, secreted galectin-3, which is soluble and present in serum and bronchoalveolar lavage fluid (Sano *et al.* 2000; Wanninger *et al.* 2011; Yilmaz *et al.* 2011; Taniguchi *et al.* 2012), can modulate inflammatory responses and bacterial uptake by neutrophils (Farnworth *et al.* 2008). Given the multiple regulatory effects of galectin-3 on cells and especially its influence on immune responses, this lectin is considered to be important during bacterial infection, and can have either a beneficial or detrimental effect on the infecting organism. Several functions have been attributed to galectin-3 during different infections and are mainly dependent on its extracellular location. For example, intracellular galectin-3 accumulates transiently in the vicinity of invading bacteria that lyse their vacuole and has been proposed as a marker of vacuole lysis for Gram positive and Gram negative bacteria, but no function has been defined in this context (Paz *et al.* 2010). On the other hand, cell surface galectin-3 enhances adhesion of *H. pylori* to human epithelial cells (Fowler *et al.* 2006a), while soluble galectin-3 may regulate the innate immune response against *Rhodococcus equi* by impairing IL-1 β production by macrophages (Ferraz *et al.* 2008). Moreover, galectin-3 is a negative regulator of LPS function during *Salmonella*

infection, as mice lacking galectin-3 produce excessive pro-inflammatory cytokines and nitric oxide, and are more susceptible to LPS shock (Li *et al.* 2008).

As demonstrated in the preceding chapters, galectin-3 levels increase in murine and human tissue following meningococcal infection, and *N. meningitidis* interacts with galectin-3 via its LPS. Therefore in this chapter the potential consequences of this interaction were examined.

As *N. meningitidis* replicates and disseminates in the bloodstream, where it encounters immune cells including phagocytes, we sought to examine the function of extracellular galectin-3, either in its surface-associated or soluble form, during phagocytosis. To analyse the effect of galectin-3 on cells, we used the THP-1 cell line and human primary monocytes obtained from the blood of healthy donors. Phagocytic assays were performed to evaluate the function of both endogenous and exogenous (added recombinant) galectin-3 on bacterial association with cells and non-opsonic uptake. As meningococcal disease occurs soon after exposure of non-immune individuals to *N. meningitidis*, we studied bacterial uptake in the absence of human sera and antibodies. The effect of galectin-3 was also analysed by silencing the gene encoding this lectin in THP-1 macrophages, using siRNA and evaluating the ability of these cells to interact with bacteria. The effect of soluble galectin-3 was examined by pre-incubating bacteria with recombinant galectin-3 prior to incubation with THP-1 macrophages or human primary monocytes.

5.2 Intracellular survival of *N. meningitidis* in phagocytes

An *in vitro* model, which allows the assessment of bacterial association with and internalisation into cells was initially established to analyse the interaction of *N. meningitidis* with cells.

Monocytic cell lines such as THP-1 or U-937 have been widely used to investigate cellular differentiation (Tsuchiya *et al.* 1980; Estrella *et al.* 2011). These cells express characteristic markers of immature monocytes in suspension and differentiate into adherent macrophage-like cells by treatment with compounds including PMA (Kohro *et al.* 2004). Differentiation can be monitored by changes in morphology, adherence, phagocytosis, surface markers, release of TNF- α and expression of specific genes (Maess *et al.* 2010). These parameters determine whether differentiated THP-1 cells have morphological and functional features of primary macrophages (Auwerx 1991; Schwende *et al.* 1996). Furthermore, these cells have been employed to study bacterial infection (Read *et al.* 1996; Kalmusova *et al.* 2000; Ramarao and Meyer 2001; Jack *et al.* 2005).

Here differentiated THP-1 macrophages were challenged with *N. meningitidis* in the absence of human serum. Although bactericidal activity of human complement against *N. meningitidis* can confer protection against disease (Drogari-Apiranthitou *et al.* 2002), non-opsonic uptake and intracellular killing of the bacterium by monocytes and macrophages have also been demonstrated (McNeil *et al.* 1994). In particular, phagocytosis of *N. meningitidis* in absence of complement has been attributed to the presence of phagocytic receptors on macrophages such as the SRs and siglecs, (Jones *et al.* 2003; Varin *et al.* 2010). Preliminary experiments were performed to optimise the differentiation of THP-1 into macrophages (data not shown). PMA at a concentration of 100 $\mu\text{g/ml}$ for 72 hours allowed

differentiation of 5×10^5 cells per well, which adhered to the surface of tissue culture flasks and underwent visible morphological changes. Differentiation was analysed by microscopy (to examine changes in cell shape), and by flow cytometry to examine the presence of surface receptors such as CD11b and CD18 (not shown) which are up-regulated during cell differentiation (Liu *et al.* 2008).

Initial challenge of 5×10^5 cells per well for one hour allowed *N. meningitidis* to interact with cells which were then washed to remove unbound bacteria. To determine the total number of bacteria associated with cells (both the extracellular fraction bound to cells and the intracellular fraction), THP-1 macrophages were lysed with saponin and viable bacteria recovered by plating. To determine the number of bacteria which were internalised by macrophages, gentamicin ($100 \mu\text{g/ml}$) was added for 15 minutes to cells to kill extracellular bacteria, and viable bacteria recovered (Vaudaux and Waldvogel 1979).

Using *N. meningitidis* strain MC58 at different MOIs, we were able to demonstrate that THP-1 macrophages interact with meningococci depending on the number of bacteria (Fig. 5.1 A and B); the total number of cell associated and internalised bacteria is proportional to the initial inoculum over a range of MOIs (10, 50, 100). For all subsequent experiments with THP-1 macrophages, an MOI of 30 was used. Next, the ability of THP-1 macrophages to kill *N. meningitidis* was investigated by determining the number of intracellular bacteria at different time points after challenge. Cells were subjected to initial bacterial challenge for one hour, then gentamicin was added and cells incubated at 37°C for further time periods (60 and 180 minutes, Fig. 5.2) before bacteria were recovered. To establish whether bacterial recovery was dependent on uptake by the THP-1, control cells were treated with cytochalasin D (CCD), which prevents phagocytosis by interfering with

polymerisation of actin and impairing cytoskeletal re-arrangements (Schliwa 1982; Bailly *et al.* 1991; Wakatsuki *et al.* 2001).

Bacterial association with THP-1 macrophages increased over time in presence or absence of CCD (Fig. 5.2 A). As expected, the internalisation of bacteria is significantly higher in the absence of CCD (Fig. 5.2 B). Moreover, the number of intracellular bacteria decreases over time (Fig. 5.2 B), indicating that THP-1 macrophages efficiently kill meningococci.

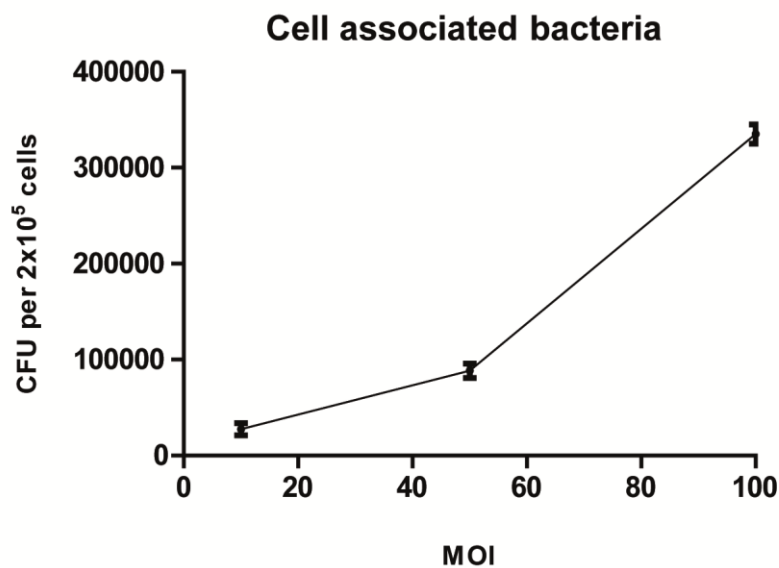
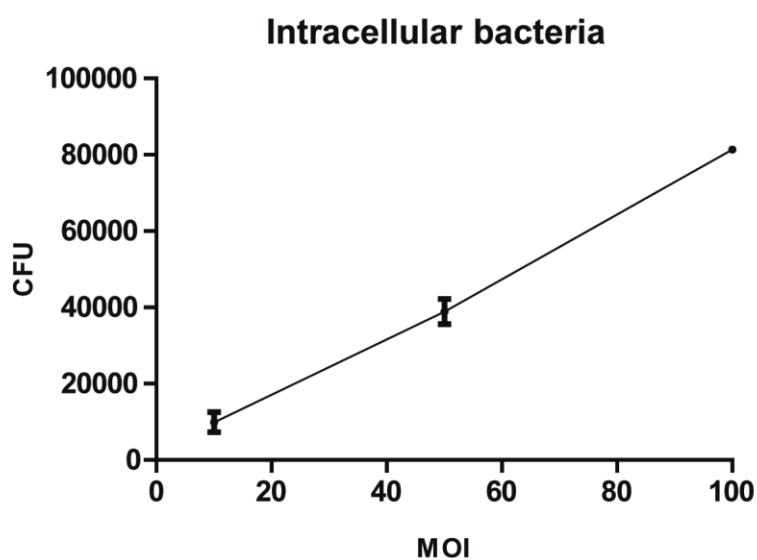
A**B**

Figure 5.1 Association to and bacterial uptake by THP-1 macrophages.

(A) Bacterial association with cells shown as the total number of recovered from 2×10^5 THP-1 macrophages 1 hour after challenge with *N. meningitidis* MC58 at increasing MOIs. (B) The number of intracellular bacteria recovered from THP-1 macrophages 1 hour after challenge with different MOIs. Data are from one representative experiment; similar results were obtained on at least three occasions. Error bars show the standard deviation.

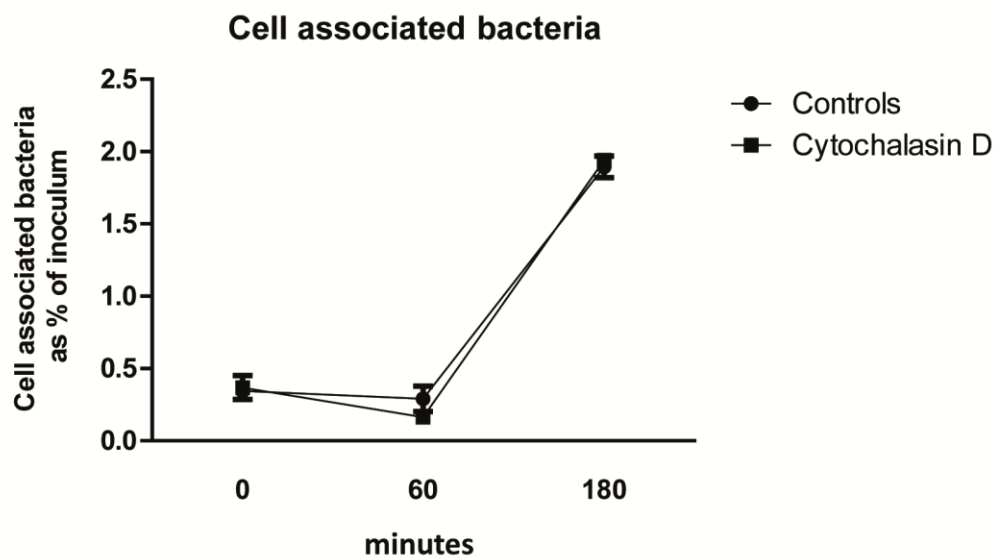
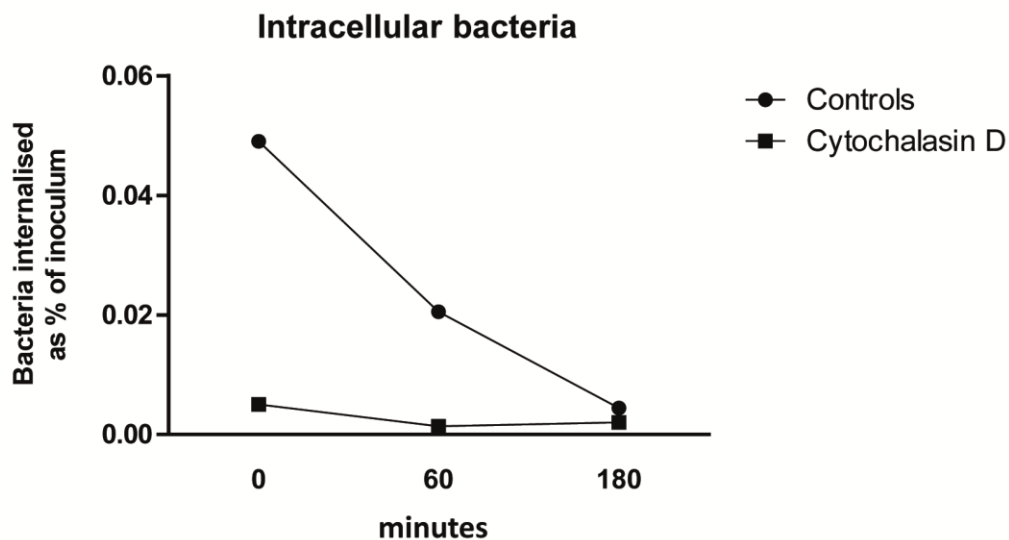
A**B**

Figure 5.2 *N. meningitidis* is internalised and killed by THP-1 macrophages.

The number of bacteria associated with (A) and internalised by (B) THP-1 macrophages shown as the percentage of bacteria in the inoculum. (A) Times are shown after an initial 1 hour incubation in presence (squares) and absence (dots) of Cytochalasin D. (B) Gentamicin was added to cells to kill extracellular bacteria and bacteria recovered immediately (0 minutes), or following incubation for a further hour (60 minutes) or three hours (180 minutes). Data are from one representative experiment. Error bars indicate the standard deviation and the experiment was repeated on at least three occasions.

5.3 Role of endogenous galectin-3 on the association with or uptake of *N. meningitidis* by phagocytes

As consistent association with and internalisation of *N. meningitidis* into THP-1 cells was observed, next we investigated the effect of galectin-3 on these processes. Given the role of endogenous galectin-3 in phagocytosis of both IgG-opsonised erythrocytes and apoptotic cells *in vitro* and *in vivo* (Sano *et al.* 2003), the influence of endogenous galectin-3 in THP-1 cells during interactions with *N. meningitidis* was evaluated.

Knock-down of galectin-3 in THP-1 macrophages was achieved through transient silencing of the galectin-3 gene by transfection with small interfering RNAs (siRNA). Gene silencing was confirmed by western blot analysis (Fig. 5.3 A) and was reproducible on at least four occasions (not shown).

The effect of galectin-3 on signaling by THP-1 cells following infection was analysed by measuring levels of TNF- α , a key pro-inflammatory cytokine (Schwende *et al.* 1996). This cytokine is markedly elevated during meningococcal disease (Deghmane *et al.* 2009). The production of TNF- α was significantly increased in cells infected with MC58 (Fig. 5.3 B); silencing of galectin-3 did not affect significantly the production of this cytokine, suggesting that THP-1 cells lacking galectin-3 are able to respond to bacteria by secreting pro-inflammatory mediators.

Additionally, bacterial association with and phagocytosis of *N. meningitidis* was examined using THP-1 macrophages transfected with galectin-3 siRNA or transfected without oligonucleotides (mock). Results show that knock-down of galectin-3 had no detectable effect on either the number of bacteria associated with cells or the number of internalised bacteria (Fig. 5.4 A and B, cell associated bacteria $p=0.4739$, internalised

bacteria $p=0.3327$). These data indicate that endogenous galectin-3 is not required for the binding and/or uptake of *N. meningitidis* by THP-1 macrophages.

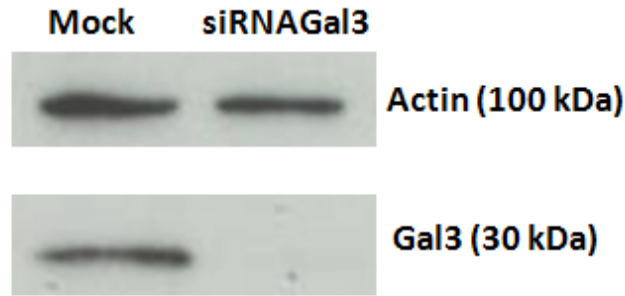
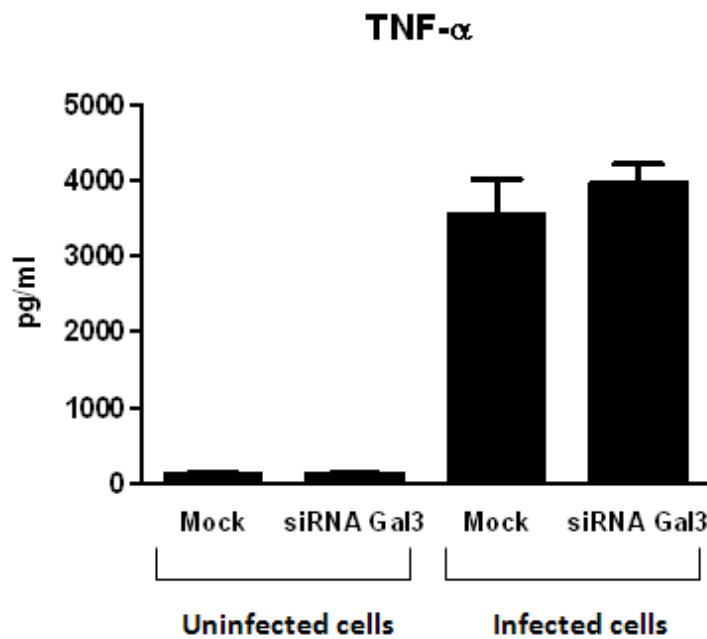
A**B**

Figure 5.3 Effect of galectin-3 silencing on THP-1 cells.

(A) Analysis of siRNA mediated knockdown of galectin-3 by western blot. Similar levels of actin (50kDa) are observed in both mock treated cells (Mock) and siRNA treated cells (siRNA Gal3), while galectin-3 (30 kDa) was detected only in mock control cells. (B) Levels of TNF- α in supernatants of wild-type (Mock) or siRNA Gal-3 cells (siRNA Gal3) uninfected or infected with *N. meningitidis* MC58 at an MOI of 30 (indicated). The levels of TNF- α increase significantly in both mock and siRNA Gal-3 infected cells (mock cells $p=0.0176$, siRNA Gal-3 cells $p=0.0039$). Data are from one representative experiment performed in duplicate and error bars show the standard deviation.

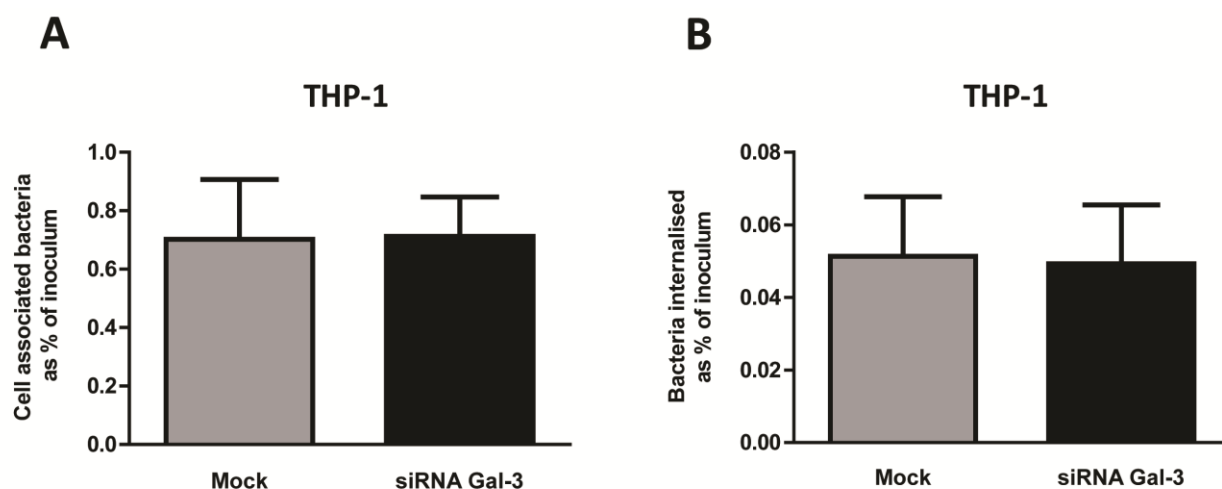


Figure 5.4 Effect of endogenous galectin-3 on the interaction of *N. meningitidis* with THP-1 macrophages.

The interaction of meningococci with THP-1 macrophages is shown as the percentage of cell associated (A) or internalised bacteria (B) relative to the initial inoculum, with control (Mock, grey bar) and galectin-3 knock-down cells (siRNA Gal3, black bar). (A) Bacterial association with THP-1 macrophages after 1 hour of infection. No significant difference in bacterial association was seen (One tailed, paired Student's t test, $p=0.4739$) (B). Bacterial internalisation by THP-1 macrophages after 1 hour of infection. There was no significant difference in bacterial internalisation in control or knock-down cells (One tailed, paired Student's t test, $p=0.3327$). Results are the mean of five different experiments and error bars show the standard deviation.

5.4 Role of exogenous galectin-3 in meningococcal infection

5.4.1 *Effect of exogenous galectin-3 on interactions of N. meningitidis with phagocytic cells*

Knock down of galectin-3 did not affect the adhesion or uptake of meningococci by THP-1 macrophages, suggesting that endogenous intracellular or cell surface galectin-3 is not required for phagocyte interactions with *N. meningitidis*. However, extracellular galectin-3 was demonstrated to associate with *N. meningitidis* by immunohistochemical analysis in Chapter Three, indicating that galectin-3 may interact with bacteria in the extracellular space.

Therefore, as extracellular galectin-3 enhances phagocytosis of *S. pneumoniae* by murine neutrophils (Farnworth *et al.* 2008), we examined whether binding of exogenous galectin-3 to *N. meningitidis* prior to encounter with phagocytes influences the interaction between meningococci and these cells.

THP-1 cells were differentiated into macrophages and challenged at an MOI of 30 for one hour with wild-type MC58 *N. meningitidis* which had been pre-incubated with 3.3 μ M galectin-3 (a concentration at which binding is detected) or PBS. The number of cell associated and internalised bacteria was determined after one hour, and calculated as percentage relative to the inoculum. However, there was considerable variation in the number of cell associated bacteria in independent experiments. Therefore, the number of cell associated bacteria following pre-incubation with galectin-3 was normalised for values obtained without additional lectin (*i.e.* bacteria pre-incubated with PBS, shown as 100% in figures). A 1.6 fold increase in association was observed when meningococci were pre-incubated with recombinant galectin-3 (Fig. 5.5 A, $p=0.0201$). In contrast, exogenous galectin-3 had no detectable effect on the internalisation of bacteria ($p=0.1499$ Fig. 5.5 B).

To investigate whether galectin-3 has a similar effect with human primary monocytes, monocytic cells were isolated from PBMCs, as adherent cells (Fuss *et al.* 2009). The purity of monocytes was examined by fluorescence microscopy for their characteristic “kidney shape” of nuclei and the presence of CD14 (de Almeida *et al.* 2000; Mayer *et al.* 2011). An MOI of 50 and 90 minutes of challenge with *N. meningitidis* were established as conditions for infection of human primary monocytes, in contrast to THP-1 macrophages which required a lower MOI and a shorter period of infection.

Similar to THP-1 macrophages, a significant increase in bacterial association to monocytes was observed after pre-incubating MC58 with galectin-3 compared to controls (1.9 fold increase, Fig. 5.6 A $p=0.0002$). The percentage of bacteria internalised by monocytes was comparable for bacteria pre-incubated with PBS or galectin-3 (Fig. 5.6 B), as observed with THP-1 macrophages. Taken together, these results demonstrate that exogenously added galectin-3 influences the association of bacteria to both macrophages and monocytes without affecting internalisation.

To establish whether expression of bacterial LPS was required for the galectin-3-mediated enhanced association of *N. meningitidis* to monocytes, binding of the MC58 Δ *lgtB* strain, which does not bind galectin-3 was examined (Fig. 5.6 C and D). First we examined whether changes in the LPS structure affect adherence of bacteria to the cell surface. As shown in Fig 5.6 C, loss of *lgtB* did not affect the association of bacteria with THP-1 macrophages. We therefore compared the influence of exogenous galectin-3 on the association of MC58 and MC58 Δ *lgtB* with macrophages. While there was a significant increase in the association of the wild-type strain to monocytes following pre-incubation with galectin-3 (Fig. 5.6 D, $p=0.0249$), exogenous galectin-3 had no effect on the adhesion of

MC58 Δ *lgtB* (Fig. 5.6 D, $p=0.2613$), indicating that full length LPS is required for the galectin-3 enhanced interaction of *N. meningitidis* with monocytes.

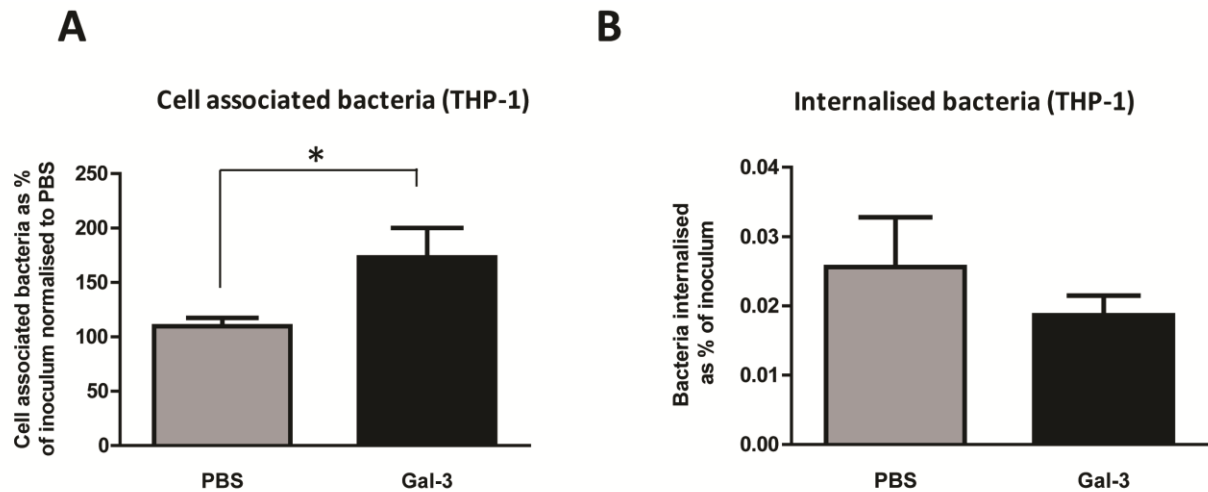


Figure 5.5 Exogenous galectin-3 promotes interaction of *N. meningitidis* with THP-1 macrophages.

(A) Association of meningococci to THP-1 macrophages analysed by bacterial recovery following infection of bacteria previously incubated with 3.3 μ M galectin-3 (black bars) or PBS (grey bars). Bacterial association is shown as relative to the number in the inocula, and normalised to results obtained with bacteria pre-incubated with PBS (shown as 100%). The association of bacteria pre-incubated with galectin-3 is significantly increased compared to bacteria pre-incubated with PBS (One-tailed paired Student's t test, $*p=0.0201$). Data are from six experiments and error bars show the standard deviation. (B) Uptake of meningococci by THP-1 macrophages shown as the percentage of internalised bacteria relative to the inoculum.

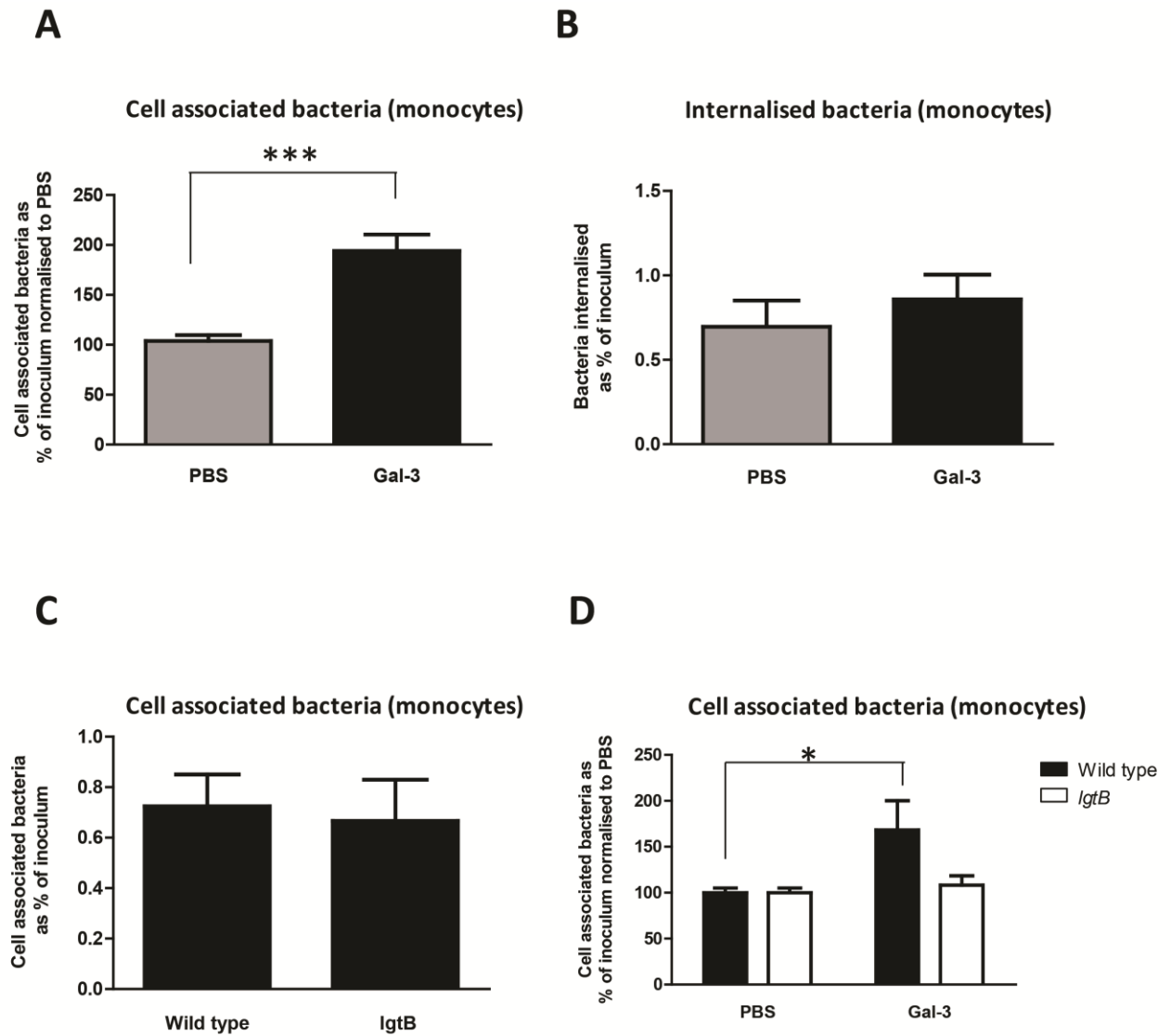


Figure 5.6 Influence of exogenous galectin-3 on the interaction of *N. meningitidis* with human primary monocytes.

(A) Association of *N. meningitidis* with primary human monocytes following pre-incubation of MC58 with galectin-3 (3.3 μ M) or PBS prior to infection for 90 mins. Bacterial association is shown as the percentage of bacteria associated to cells relative to bacteria in the inocula; results are normalised to bacteria pre-incubated with PBS (shown as 100%). Association of bacteria to cells in presence of galectin-3 is significantly higher than in absence of galectin-3 (One-tailed paired Student's *t* test, ****p*=0.0002). Data are from five different experiments performed in duplicate, and error bars show the standard deviation. (B) Percentage of internalised bacteria relative to bacteria in the inoculum at 90 minutes post-

challenge following pre-incubation with PBS (grey bar) or galectin-3 (black bar). (C) Percentage of MC58 and MC58Δ*gtB* associated with primary monocytes relative to bacteria in the inoculum. Comparable levels of MC58 and MC58Δ*gtB* are associated with cells. (D) Association of meningococci with human monocytes following pre-incubation of MC58 (black bars) or MC58Δ*gtB* (white bars) with galectin-3 or PBS. Bacterial association is shown as percentage of bacteria associated with cells relative to bacteria in the inocula; results are normalised to bacteria pre-incubated with PBS (shown as 100%). Association of MC58Δ*gtB* is unaltered in presence or absence of galectin-3 in contrast to the wild-type strain, which shows a significantly increased adhesion to monocytes when pre-incubated with galectin-3 (One-tailed paired Student's *t* test, **p*=0.0249). Data are the mean of nine different experiments and error bars show the standard deviation.

5.4.2 Exogenous galectin-3 has no effect on adherence of meningococci to epithelial cells

The finding that galectin-3 promotes the association of *N. meningitidis* with macrophages and monocytes prompted us to examine whether this phenomenon is observed with other cell types which express a different array of cell surface molecules.

In the extracellular space, galectin-3 binds to a variety of extracellular matrix components like laminin, fibronectin and collagen IV (Nangia-Makker *et al.* 2008; Melo *et al.* 2011). We used Detroit 562 cells, a human pharyngeal cell line which represents the first cellular barrier that *N. meningitidis* encounters when it colonises the human host. Detroit 562 cells have been employed to study bacterial infections (Sanchez *et al.* 2011) and express receptors involved in *N. meningitidis* adherence, such as the tetraspanins CD9, CD63 and CD151 (Green *et al.* 2011). Other important receptors for adhesion of *N. meningitidis* to epithelial cells are CEACAMs, and Detroit 562 specifically express CEACAM1 on their surface ((Ohannesian *et al.* 1995) and not shown). CEACAM-1 is also a receptor for galectin-3 on neutrophils (Feuk-Lagerstedt *et al.* 1999; Kuespert *et al.* 2011).

Therefore, to investigate whether pre-incubation of *N. meningitidis* with exogenous galectin-3 has an effect on bacterial interaction with epithelial cells, adhesion/invasion assays following procedures previously validated in the laboratory were performed. Detroit 562 cells were challenged with *N. meningitidis* pre-incubated with recombinant galectin-3 or PBS, using the procedure described for assays with phagocytes. To examine bacterial invasion, cells were treated with gentamicin to kill extracellular bacteria. In contrast to experiments with phagocytic cells, pre-incubation of bacteria with galectin-3 did not affect meningococcal association with or internalisation into Detroit 562 cells (Fig. 5.7).

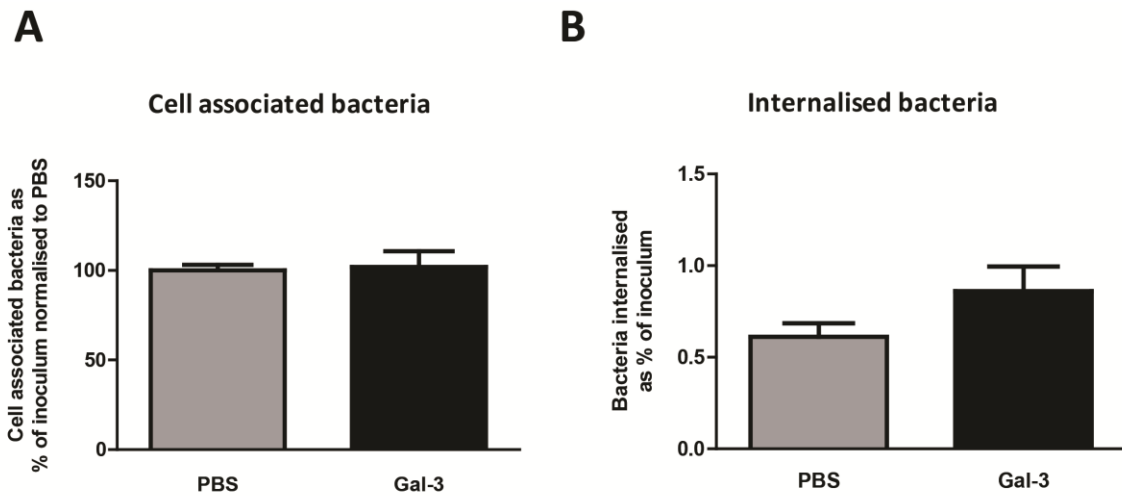


Figure 5.7 Exogenous galectin-3 does not affect the interaction of *N. meningitidis* with epithelial cells.

(A) Association of meningococci with Detroit 562 epithelial cells following pre-incubation of bacteria with 3.3 μ M galectin-3 (black bars) or PBS (grey bars) prior to infection. Bacterial association is shown as percentage of bacteria associated to cells relative to bacteria in the inocula and results are normalised to bacteria pre-incubated with PBS (shown as 100%). (B) Meningococcal internalisation by Detroit-562 cells measured as a percentage of the inoculum. Bacteria were recovered following gentamicin treatment to kill extracellular bacteria. No significant difference in the percentage of bacterial association or internalisation was observed following pre-incubation with galectin-3. Data are the mean of three different experiments, and error bars indicate the standard deviation.

5.5 Summary

In this chapter we analysed the possible functional consequences of endogenous and exogenous galectin-3 in meningococcal infection with particular interest in the interaction of the bacterium with cells.

Silencing galectin-3 in THP-1 cells demonstrated that endogenous galectin-3 does not influence the association and internalisation of bacteria into macrophages. However, extracellular galectin-3 plays an important role in the interaction with cells. While exogenous galectin-3 does not influence interactions with epithelial cells, it promotes the attachment of bacteria to the surface of monocytes and macrophages; this is dependent on galectin-3 binding to bacteria via LPS, as no enhancement of attachment was observed when the *lgtB* mutant was pre-incubated with galectin-3.

CHAPTER 6: Discussion

In this thesis, the interaction between *N. meningitidis* and galectins was studied. Galectins are host molecules formerly thought to bind only endogenous glycans and mediate developmental processes such as cell differentiation and apoptosis. More recently it has been increasingly recognised that galectins also function as molecules able to bind glycans on pathogenic micro-organisms (Vasta 2009). The importance of studying these lectins in microbial infection is supported by the observation that some galectins are up-regulated in response to infection and can promote subversion of immune responses, with important consequences on the pathogenesis of infection (Vray *et al.* 2004). Furthermore, certain galectins such as galectin-4 and galectin-8 possess bactericidal activity, and may represent crucial effectors of innate immune responses.

This work focuses on meningococcal disease, an infection caused by *N. meningitidis*, a commensal Gram negative bacterium of the human nasopharynx, but also an extracellular pathogen able to cause serious blood and CNS conditions (Gammelgaard *et al.* 2011). The outcomes of meningococcal infection may be devastating and can lead to neurological debility and death (Stephens *et al.* 2007); therefore a better understanding of the pathogenesis of this infection is urgently needed.

Meningococcal infection occurs when bacteria reach the bloodstream and the presence of bacteria in the circulation causes an intense inflammatory reaction due to the presence of high levels of circulating LPS (Andersen 1989; Brandtzaeg *et al.* 1989b).

Galectins are lectins which can be present in human fluids including serum and bronchoalveolar lavage fluids where they can bind to and interact with micro-organisms (Ochieng *et al.* 2004; Wang *et al.* 2005; Barrow *et al.* 2011). Given the importance of

inflammation in meningococcal disease and the recent focus on these proteins as mediators of inflammation and soluble PRRs, we analysed the interactions between galectins and the extracellular pathogen *N. meningitidis*.

Meningococcal disease is characterised by bacterial dissemination to organs including the brain, lung, kidney, heart, liver and spleen ((Guarner *et al.* 2004) and not shown). Here we assessed the presence of galectins in murine and human tissues by immunohistochemistry, and found that levels of galectin-3 increase in tissues from mice and patients upon infection with *N. meningitidis*.

We did not detect galectin-1 in tissues, although it shares some functions with galectin-3 in inflammatory responses (Almkvist and Karlsson 2004) and competes for the same cellular ligands (Kopitz *et al.* 2001). Lack of expression was also observed for another galectin, galectin-4, which has recently been proposed to contribute to the innate immune response to bacterial infection as it can bind the O-antigen of *E. coli* 086 resulting in bacterial lysis (Stowell *et al.* 2010). Of note, this lectin does not bind the meningococcus which does not express the O-antigen. All the reagents used to detect galectin-1 and galectin-4 have successfully been used for immunohistochemistry in previous studies (Kaltner *et al.* 2002; Saussez *et al.* 2005; Lohr *et al.* 2007), so the failure to detect these lectins is unlikely to have resulted from a technical problem. However, positive control tissue, including kidney or cells of the respiratory tract, could have been used. Given the over-expression of galectin-3 during *N. meningitidis* disease, we decided to concentrate our analysis on this chimera-type galectin.

We observed expression of galectin-3 in several tissues from mice, and its presence in the CNS (in particular the cerebellum, meninges and choroid plexus) underlines the potential relevance of this protein in meningococcal infection, as this bacterium can cause

meningitis. Little is known about galectin-3 expression during CNS infection even though it has been found in brain tumors (Park *et al.* 2008).

Galectin-3 in the CNS seemed to be restricted to cells and was not obvious in the extracellular space. The exact localisation of bacteria at this site was not assessed in our samples, but it is possible that immune cells such as recruited macrophages, neutrophils or dendritic cells, as well as the inflammatory glial cells, might be responsible for the high expression of galectin-3 in the brain during meningococcal meningitis.

Interestingly, in other tissues including the heart, liver, lung, pancreas and spleen, galectin-3 was detected in both intracellular and extracellular sites, especially in association with bacterial colonies; this suggested a possible direct interaction between the protein and bacteria.

Next we showed that human, mouse and chicken galectin-3 binds to *N. meningitidis* serogroup B strain MC58 through a CRD-LPS dependent interaction. These results are entirely consistent with the known reactivity of galectin-3 with LacNAc and LNnT (Seetharaman *et al.* 1998), components of meningococcal LPS (Plested *et al.* 1999).

The observation that binding of galectin-3 to meningococci occurs at a relatively low concentration of galectin-3 (0.33-10 μ M), while the concentration of lactose needed to inhibit the interaction is high (100 mM) is indicative of high avidity of this galectin with bacteria, likely due to pentamerisation of full length galectin (Ahmad *et al.* 2004b). Interestingly, *N. meningitidis* expresses high levels of LPS (Brandtzaeg *et al.* 2001) and this may be important in mediating reactivity to galectin-3.

Binding was not detected to the truncated form of galectin-3, which is devoid of the N-terminal section that contributes to oligomerisation. Several lines of evidence indicate that multivalent oligomerisation is required for the biological function of galectin-3 (Woo *et*

al. 1991; Lukyanov *et al.* 2005; Fermino *et al.* 2011). Indeed, lectin-monosaccharide interactions are relatively weak, with a dissociation constant of 10^{-4} M. Galectin-3 monomers are in equilibrium with higher oligomers in solution, while galectin-3 precipitates as a pentamer with multivalent oligosaccharides (Ahmad *et al.* 2004b). Therefore, initial binding of a galectin-3 monomer may be followed by recruitment of further molecules forming a complex of multivalent interactions, with the functions of galectin-3 dependent on both ligand cross-linking and self-oligomerisation. Our results support this hypothesis as the involvement of the ND indicates that binding of galectin-3 to *N. meningitidis* is achieved through multivalent interactions with both itself and bacterial LPS molecules.

Furthermore, in comparison with previous data (John *et al.* 2002), our experiments yield similar reactivity as seen for galectin-3 and *N. gonorrhoeae*; gonococcal LPS purified from 7.5×10^7 bacteria was bound by 300 µg/ml galectin-3, while we detected binding of 2×10^7 bacteria to 10 to 300 µg/ml galectin-3 (from 0.33 to 10 µM).

Of note, preliminary data (described in the Appendix chapter) suggested that galectin-3 was not the only galectin able to bind *N. meningitidis*. Our study showed that galectin-8 also binds *N. meningitidis* and indicates that the sialic acid capsule on the surface of *N. meningitidis* may mask the ligand(s) for galectin-8 (Appendix 2). Moreover, the low level of galectin-1 binding to *N. meningitidis* seems to be due to the rapid oxidation of this lectin. In fact binding of galectin-1 to *N. meningitidis* was observed only when the lectin was treated with an irreversible alkylating agent that stabilises its structure (Appendix 1). However, these data have not been confirmed and further analyses are required to assess whether galectins other than galectin-3 could influence meningococcal infection and

whether they involve mechanisms dependent on the specific protein expression, concentration or localisation.

We found that intact LPS is required for the interaction with galectin-3, as mutation of *lgtB* was sufficient to abolish binding. Although the *lsi-1* mutant (lacking the enzyme responsible for synthesis of the α -chain) showed a significant reduction in galectin-3 binding, this is likely due to the interdependence of synthesis of the α - and β -chains; there is no discernible β -chain extension in the *lsi-1* mutant (Jennings *et al.* 1995a). The presence of the inner core may also be important for binding by allowing appropriate positioning or presentation of LPS and/or by supporting multiple interactions and galectin-3 oligomerisation, as recently described for glycoprotein ligands of galectin-3 (Krzeminski *et al.* 2011). Importantly, since the homodimeric proto-type galectin-7 as well as the tandem-repeat-type galectin-4 did not recognise the meningococcus, the mere presence of the LacNAc epitope (which is bound by all three galectins with similar ΔG -values (Dam *et al.* 2002) does not predict binding, and indicates a specific interaction of galectin-3 with *N. meningitidis*.

Meningococcal strains are classified into different immunotypes based on variations in LPS composition (Plested *et al.* 1999) with differences in both inner and outer core structures (Tsai *et al.* 1987). In agreement with this, we observed a correlation between LPS immunotype and galectin-3 binding. All strains which bound galectin-3 express an LPS immunotype containing LNnT; the only strain which did not show similar binding of galectin-3 was F8238, a serogroup A isolate which expresses LPS immunotype L10 that is not thought to include the LacNAc and LNnT moieties (Maslanka *et al.* 1997). Interestingly, individual meningococcal strains have the potential to express alternative outer core structures through phase variation (Jennings *et al.* 1999; Berrington *et al.* 2002). For example, phase

variation leading to switching off expression of the *lgtA* gene results in loss of the LacNAc and LnNT structures and a switch from immunotype L3 to L8 (Jennings *et al.* 1995b). Our results using a defined LPS mutant lacking *lgtA* demonstrate that this phase variation event would abolish galectin-3 binding. Of note, L3,7,9 immunotype LPScan undergo sialylation which enhances serum resistance (Unkmeir *et al.* 2002),but further elucidation of the consequences of galectin-3 binding may reveal that the capacity to bind this lectin also confers a survival advantage to the meningococcus during infection.

Modifications of meningococcal LPS such as sialylation might affect the ability of the bacteria to bind galectin-3, although we only found a trend to increased binding to an *lst* mutant compared with the wild-type strain. The presence of terminal sialic acid on cell surface glycans can inhibit the activity of some galectins. For example, the N-terminal CRD of galectin-9 which does not recognise α 2,3-sialylated N-glycans (Solis *et al.* 2010), while α 2,3- sialylation does not affect galectin-3 recognition of glycans (Stowell *et al.* 2008a). However, removal of the both sialic acid capsule and the terminal sialylation of LPS tended to increase binding of galectin-3 possibly due to an improved accessibility of galectin-3 to LPS, although this finding was not statistically significant.

Given the ability of *N. meningitidis* to interact with galectin-3, we sought to determine the consequences of the interaction in meningococcal infection, in an attempt to assess whether it results in beneficial or detrimental effects for the pathogen.

Lectin-carbohydrate interactions are a proposed mechanism of pathogen recognition and/or adherence to cells (Sato *et al.* 2009) as described for galectin-3 and several micro-organisms (John *et al.* 2002; Fowler *et al.* 2006a; Stowell *et al.* 2010). Galectin-3 was initially identified as intracellular protein, mediating endogenous functions, but is also on the cell surface and in the extracellular space as a soluble protein (Hughes 1999). One of the

functions of galectin-3 is to promote adherence of parasites or bacteria to human cells; for example, exogenously added galectin-3 mediates *T. cruzi* adhesion to human coronary artery smooth muscle cells (Kleshchenko *et al.* 2004).

N. meningitidis is an extracellular pathogen, but a key event in meningococcal infection is the interaction with host cells (Carbonnelle *et al.* 2006). *N. meningitidis* encounters different cell types from the state of colonisation to the onset of systemic disease. These include epithelial cells in the nasopharynx, resident macrophages in the submucosa or monocytes in the bloodstream (Corbett *et al.* 2004). Our IHC data demonstrate presence of galectin-3 in both intracellular and extracellular spaces, and we investigated the role of galectin-3 in both these locations.

We found that the epithelial nasopharyngeal cells as well as THP-1 macrophages and monocytes express galectin-3. Although we did not detect changes in the expression of surface galectin-3 in both epithelial cells and phagocytes during infection *in vitro*, we analysed the consequences of the bacteria-lectin interaction on the association of bacteria with different cell types.

Galectin-3 expression has been demonstrated in human nasopharyngeal epithelial tissues (Saussez *et al.* 2008b) and is proposed to contribute to epithelial cell adhesion through interactions with extracellular matrix. Moreover, pathogens such as *H. pylori* and *P. aeruginosa* bind galectin-3 on the surface of epithelial cells, and galectin-3 has been proposed as a receptor for *N. gonorrhoeae* as it is constitutively expressed at the apical side of the non-ciliated fallopian tube cells which selectively bind gonococcus (John *et al.* 2002).

We found that the protein is detectable on the surface of Detroit 562 cells and confocal microscopy indicated that galectin-3 co-localises with meningococci. It is therefore possible that galectin-3 on the surface of these cells could contribute to meningococcal

attachment. However our experiments did not address this directly; we found that pre-incubation of bacteria with exogenous galectin-3 did not affect bacterial association to the cell surface, which may be expected if surface galectin-3 was the direct target for meningococcal LPS binding. To conclusively address the role of epithelial cell surface galectin-3 as a receptor for *N. meningitidis* more detailed studies using blocking antibodies for galectin-3 or siRNA are required.

To investigate the role of galectin-3 in meningococcal interactions with phagocytic cells, we used differentiated THP-1 cells as a source of macrophage-like cells, and human primary monocytes obtained from healthy donors. Gene silencing by siRNA was employed to gain insights into galectin-3 function in adhesion/uptake. siRNA knock down of galectin-3 in THP-1 macrophages did not alter their capacity to bind or phagocytose *N. meningitidis*, indicating that neither surface expressed nor intracellular galectin-3 is necessary for meningococcal recognition and uptake by macrophages.

Galectin-3 accumulations have been described in macrophages infected with *Shigella*, *Listeria* and *Mycobacteria* (Beatty *et al.* 2002; Paz *et al.* 2010), but no function in phagocytosis of micro-organisms by macrophages have been attributed to galectin-3 to date. In contrast, exogenous galectin-3 has been shown to have a role in the phagocytosis of *S. pneumoniae* by neutrophils (Farnworth *et al.* 2008); therefore, we were interested to examine the role of exogenously added galectin-3 on bacterial-interaction with cells.

A specific effect of recombinant exogenous galectin-3 was observed with *N. meningitidis* infection of phagocytes. Pre-incubation of *N. meningitidis* with exogenous galectin-3 led to a significant increase in *N. meningitidis* interaction with monocytes and macrophages yet did not alter the number of internalised bacteria. This effect was dependent upon expression of full length LPS molecules which allow galectin-3 binding and

was not observed with Detroit 562 cells. However, the percentage of meningococcal adhesion and bacterial uptake relative to the initial inocula was only around 1% and 0.01% respectively, so differences in the internalised population may have been difficult to detect. Of note all experiments were performed in absence of opsonising antibodies, so the extent of interaction with cells might be due to the lack of opsonisation. Previous studies also conducted in absence of opsonisation showed relatively low levels of bacterial association to phagocytes (Jack *et al.* 2005; Casey *et al.* 2008). It has been reported that 30% of meningococci adhere to THP-1 macrophages and 1-2% of meningococci are internalised by macrophages when an MOI of 200 is used for infection, with an average of 0.1 internalised bacteria per cell (Casey *et al.* 2008). In our experiments, we used an MOI of 30 to infect THP-1 macrophages and an MOI of 50 to infect human primary monocytes, and this might explain the low recovery of viable bacteria. Variability due to different technical procedures used might also influence the outcome of bacterial recovery. For this reason, the study of *N. meningitidis* phagocytosis could be undertaken using different techniques such as microscopy or FACS analysis in which fixed bacteria are used.

Macrophages produce antimicrobial peptides which are released into the extracellular space and are responsible for extracellular killing (Zaiou 2007), while intracellular killing mechanisms include production of ROS and phagolysosomal activity (Dyet and Moir 2006). Therefore, the increase in *N. meningitidis* on the surface of cells in the presence of galectin-3 might reflect increased adhesion or reduced killing; experiments with siRNA of THP-1 cells indicate that the effect of galectin-3 is on adherence to cells.

It is possible that soluble galectin-3 binds *N. meningitidis* in the extracellular environment and cross links the bacterium to the surface of cells. The increased attachment of the meningococcus to the surface of macrophages and monocytes but not to epithelial

cells indicates a specific role restricted to phagocyte interactions and may be related to different composition of receptors known to recognise *N. meningitidis*. A multimeric interaction could lead to clustering of cell surface glycans and/or lectin-mediated binding to receptors for *N. meningitidis*. Galectin-3 may act indirectly by modulating the expression of non phagocytic receptors (not present on epithelial cells) and regulate their function. A relevant example is the ability of galectin-3 to inhibit T cell activation by promoting TCR down regulation (Chen *et al.* 2009). Known receptors implicated in meningococcal interaction with phagocytes include TLRs, macrophage receptor with collagenous structure (MARCO), SRs and siglecs (Jones *et al.* 2003; Pluddemann *et al.* 2009). Galectin-3 could modulate the expression of these receptors and increase bacterial association with cells or may induce re-positioning of these receptors allowing interaction with the bacterium. The hypothesis of galectin-3 modulating siglec-5, siglec-9 and SR-A was tested in preliminary studies (not shown), but no regulation of surface expression of these receptors was observed on addition of this lectin to THP-1 cells. However, other receptors involved in binding of *N. meningitidis* on phagocytic surface could be included, and the effect of galectin-3 on the conformation or location of receptors was not analysed.

Ultimately, galectin-3 may indirectly affect adherence/uptake or interfere with TLR activation, given that LPS is a potent immune activator through TLR-4 dependent signalling (Zhang and Ghosh 2000). Previous experiments with *Salmonella* showed that galectin-3 is a negative regulator of LPS function (Li *et al.* 2008), but whether the binding to LPS is required for this regulation has not been investigated.

Alternatively, it has been shown that pre-incubation of exogenous galectin-3 with purified LPS dramatically increases LPS binding to the surface of neutrophils (Fermino *et al.* 2011). The authors propose that modification of LPS structure upon galectin binding or

oligomerisation crosslinks LPS receptors such as CD14 and TLR4 on the cells surface. Therefore we propose galectin-3 as a bacterial recognition molecule for *N. meningitidis*, but further studies are needed to fully elucidate the molecular basis of galectin-3 mediated interactions with cells and to investigate how this affects the host immune and inflammatory responses during infection.

In summary, our work demonstrates that *N. meningitidis* interacts with galectin-3, a modulator of inflammation which has roles in many physiological and pathological conditions. We propose that this interaction could occur during meningococcal infection at sites where local concentrations of galectin-3, secreted by activated immune cells, accumulate and allow binding to the surface of bacteria. A consequence of this interaction would be the increased bacterial adhesion to the surface of phagocytes, which could eventually lead to enhanced meningococcal survival, for example by preventing phagocytosis or affecting the activity of the phagocyte through altered TLR ligand presentation.

6.1 Future studies

Although confocal microscopy demonstrated the co-localisation of the meningococcus with endogenous galectin-3 on Detroit 562 epithelial cells, further studies are required to address whether this has consequences on bacterial adhesion. As discussed above, adhesion assays using blocking antibodies directed against galectin-3 or silencing galectin-3 expression in epithelial cells could be performed to directly address this.

Our data suggest that increased bacterial attachment is dependent on the presence of full LPS on *N. meningitidis*. However, the mechanism of increased bacterial attachment to phagocytic cells needs to be elucidated. The role of possible phagocyte receptors involved in binding, and understanding whether recognise bacteria or galectin-3 could provide a more complete understanding of this phenomenon. Specific experiments would include confocal microscopy to visualise the presence and location of further known receptors for *N. meningitidis* or galectin-3 receptors. However, this would not exclude the possibility that unknown receptors present on the cell surface are involved.

The investigation of the influence of galectin-3 on innate immune responses to *N. meningitidis* is also important and has not been directly addressed in our study. Data on galectin-3 as a modulator of inflammation have been previously reported, and highlighted the potential role of galectin-3 as a key component in the host defence against microbes. Most *in vivo* data suggest a pro-inflammatory role for galectin-3 during acute inflammation, whereas it is implicated in resolution of inflammation during chronic infections (Ruas *et al.* 2009). The specific functions of this lectin need to be investigated and vary enormously from one infection to another. For example, galectin-3 has been showed to be pro-inflammatory in *Candida* infections but mediates a Th2 response in *Salmonella* infection (Li *et al.* 2008). Therefore, analysis of cytokine expression in galectin-3 knock down THP-1

macrophages could provide further insights into galectin-3 modulation of immune responses to *N. meningitidis* in human cells.

In summary, more specific studies directed to analyse the role of endogenous galectin-3 during meningococcal infection would be required. Future work using galectin-3 knock out animals will be performed to examine the role of endogenous galectin-3 during meningococcal disease. This could provide important insights into understanding the effects of galectin-3 on the pathology of infection in a broader context.

6.2 Conclusions and general considerations

Many recent studies indicate that host galectins can represent novel PRRs able to target glycans on the surface of bacteria, viruses and parasites (Sato *et al.* 2009). Much is known aboutabout galectin binding specificity and affinity, but the majority of the carbohydrate ligands are yet to be identified, and studies of specific galectin-micro-organism interactions are required.

We show that *N. meningitidis* is able to interact with several galectins, and show that binding to galectin-3 enhances bacterial binding to phagocytic cells without increasing internalisation. This could enhance survival of the bacterium in the human host and represent another way for micro-organisms to disseminate, avoid intracellular killing and/or modulate host immune responses.

APPENDIX: Binding of galectin-1, -8 and -9 to *N. meningitidis*

As galectin-3 interacts with *N. meningitidis* and influences phagocyte-bacteria interactions, the binding of other galectins to the bacterium was also assessed. In chapter 4, no evidence was found for an interaction between the meningococcus and galectin-1 -4 and -7. Preliminary data (*i.e.* experiments performed on a single occasion only) were obtained for interactionsof further galectins belonging to different families and *N. meningitidis*. Specifically, binding of *N. meningitidis* MC58 strain to modified galectin-1, galectin-8 and galectin-9 was investigated. These galectins have been studied in the context of different infections and several of their roles have been identified. For example, galectin-1 interacts with influenza virus and HIV-1 (St-Pierre *et al.* 2011; Yang *et al.* 2011); galectin-8 has microbicidal activity, as it recognises and kills blood group antigen-bearing pathogens by altering membrane integrity (Stowell *et al.* 2010), while galectin-9 accelerates*Klebsiella pneumoniae* infection by reducing levels of IL-17 (Wang *et al.* 2011).

All these galectins can be released into the extracellular compartment in response to inflammation,hence galectin recognition of potential meningococcal ligands could influence the pathogenesis of disease.

Appendix 1 Binding of *N. meningitidis* to galectin-1

Galectin-1 is a member of the proto-type galectins, a homo-dimer formed by two identical CRDs linked together. Similar to other galectins, it has several biological functions, including the ability to stimulate cell growth, induce apoptosis and activation of human T cells (Sanford and Harris-Hooker 1990; Perillo *et al.* 1995; Pace *et al.* 1999; Galvan *et al.* 2000). Furthermore, galectin-1 regulates leukocyte turnover by inducing exposure of phosphatidyl serine (PS) on the surface of neutrophils (Dias-Baruffi *et al.* 2003); the full length protein is required for this function, as a mutant C2S within the dimerization interface (the linker region between two CRDs of the protein) impairs galectin-1 dimerisation and also fails to induce PS exposure (Dias-Baruffi *et al.* 2003). In addition, galectin-1 can be inactivated by oxidation which induces formation of three disulphide bonds that interfere with ligand binding (Stowell *et al.* 2009b).

Results (in chapter four) showed that galectin-1 does not bind *N. meningitidis*, suggesting that *N. meningitidis* does not express ligands for galectin-1, or that potential oxidation of this galectin prevents binding. Therefore we investigated binding of *N. meningitidis* to forms of galectin-1 that were stabilised by treatment with alkylating agents, DTT or IAA, to prevent oxidative damage (Stowell *et al.* 2009a). IAA covalently binds the thiol group of cysteine preventing the formation of disulphide bonds, while DTT is a reducing agent able to form a six-membered ring with an internal disulphide bond.

In addition, a form of galectin-1, C2S, which contains a point mutation in the dimer interface was used in binding assays. The mutant C2S contains a sulphydryl group substituted with a hydroxyl group, and this stabilises the sugar binding activity in the absence of reducing agents (Stowell *et al.* 2009a).

A concentration of 3 μM of galectin-1 was used for experiments, as the minimum concentration required for binding to its ligands is 1.25 μM (Earl *et al.* 2010). Galectins were provided by Professor Gabius and treatment of recombinant galectin-1 with DTT and IAA were performed as previously described (Stowell *et al.* 2009a). Our results show that galectin-1 binds *N. meningitidis* only when treated with DTT (Fig. A.1), but not with IAA. DTT and IAA are both alkylating agents, and should both stabilise the protein; however, only DTT is an irreversible alkylating agent as it reduces disulfide bonds (Lindstrom *et al.* 1973). Of note, the C2S mutant version of galectin-1 did not bind the meningococcus (Fig. A.1).

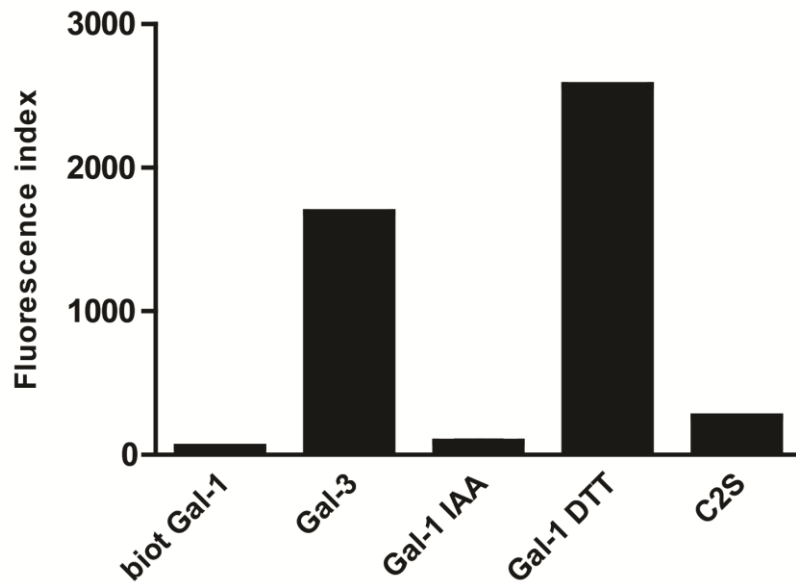


Figure A.1 Binding of recombinant human galectin-1 to *N. meningitidis*.

Quantification of flow cytometry analysis of galectin-1 (Gal-1), galectin-3 (Gal-3), IAA treated galectin-1 (IAA Gal-1), DTT treated galectin-1 (DTT Gal-1) and C2S mutant of galectin-1 (C2S Gal-1) binding to *N. meningitidis*. *N. meningitidis* binds galectin-1 only when oxidation is prevented by DTT treatment, while it does not bind galectin-1 when it is mutated in the C2S region or when treated with IAA. Galectin-3 was used as positive control.

Appendix 2 Binding of *N. meningitidis* to galectin-8

Galectin-8 belongs to the tandem-repeat galectins, and is secreted into the extracellular space where binds diverse ligands, especially glycoproteins such as integrins (Patnaik *et al.* 2006). It is involved in the regulation of cell adhesion, growth and differentiation (Bidon *et al.* 2001), and signalling of responses in leukocyte populations, although the contribution of each CRD remains unknown (Stowell *et al.* 2008b). Galectin-8 has also been studied in infections and has been recently identified as a danger receptor for *Salmonella* invasion, as it binds host glycans present on damaged *Salmonella*-containing vacuoles and restricts bacterial replication (Thurston *et al.* 2012).

Galectin-8 exists as a monomer of two CRDs that provides differential glycan binding specificity (Barondes *et al.* 1994; Hadari *et al.* 1995; Leffler *et al.* 2004). The specific ligands of galectin-8 include Neu5Ac α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc (Carlsson *et al.* 2007), where the sialic acid is attached on the galactose in a 2,3linkage (Ideo *et al.* 2003). The terminal galactose of the outer core of *N. meningitidis* LPS expressed by MC58 can be α 2,3-sialylated through the action of Lst (Gilbert *et al.* 1996). Therefore we hypothesised a possible interaction between galectin-8 and MC58 (Tsai and Civin 1991).

Binding assays were performed using 3 μ M full length human galectin-8 or its N-terminal CRD, with fixed *N. meningitidis* MC58 or strains lacking sialic acid (MC58 Δ siaC) or capsular polysaccharide (MC58 Δ siaD). Galectin-8 binding to wild-type MC58 was detected, although there was no significant difference in binding of full length and ND galectin-8 (Fig. A.2, A). Moreover, increased binding was observed with the MC58 Δ siaD mutant compared to MC58 (Fig. A.2, B), indicating that loss of the capsule might enhance the accessibility of meningococcal ligands for galectin-8. However, MC58 Δ siaC strain does not show the same

effect, suggesting that bacteria might not be able to sialylate their LPS and that α -2,3-sialylation of meningococcal LPS is important for galectin-8 recognition. The extent of sialylation of MC58 LPS was not assessed in these experiments, and binding to MC58 Δ /st was not assessed due to these constraints.

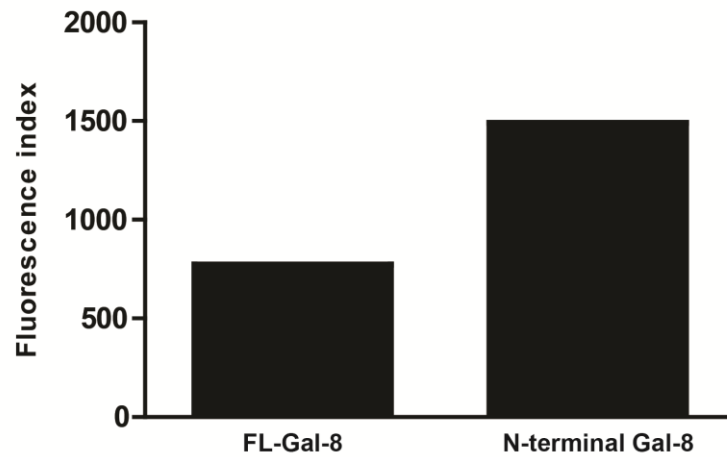
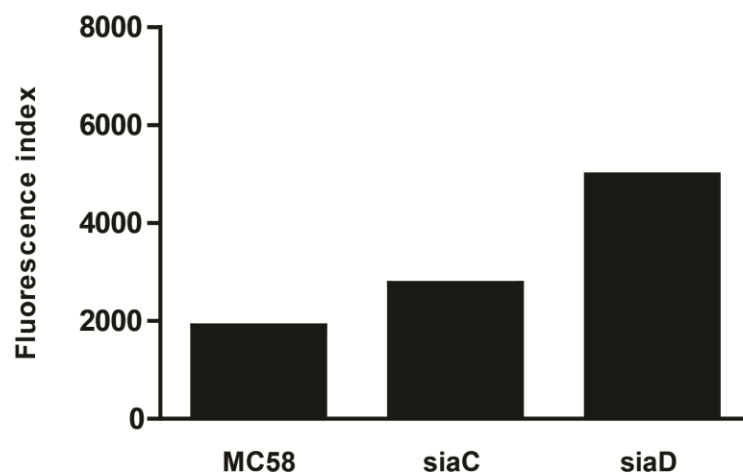
A**B**

Figure A.2 Binding of recombinant human galectin-8 to *N. meningitidis*.

(A) Flow cytometric quantification of galectin-8 binding to MC58 shows no significant difference in binding of full length galectin-8 (FL-Gal-8) and the N-terminal CRD of galectin-8 (N-terminal Gal-8). (B) Flow cytometric quantification of galectin-8 binding to *N. meningitidis* showing increased binding of MC58 Δ siaD (siaD) to galectin-8.

Appendix 3 Binding of *N. meningitidis* to galectin-9

Galectin-9 belongs to the tandem-repeat galectins and regulates many biological functions (Hirashima *et al.* 2004). It is formed by two distinct CRDs joined by a flexible peptide linker; the N-terminal CRD and the C-terminal CRD share only 39% identity, bind different sets of saccharides, and display different activities (Tureci *et al.* 1997; Li *et al.* 2011). Evidence indicates that the C-terminal CRD is more potent in activating dendritic cells and inducing cell death than the N-terminal CRD (Li *et al.* 2011).

Galectin-9 recognises T cell surface glycoprotein receptors such as Tim-3 and CD44 (Sanchez-Fueyo *et al.* 2003; Katoh *et al.* 2007; Hastings *et al.* 2009). Binding of galectin-9 to Tim-3 on activated Th1 and Th17 cells influences adaptive immunity and progression of autoimmune diseases (Li *et al.* 2011). Moreover, this lectin has been proposed to be an important regulator of infectious diseases, as it enhances survival of *K. pneumoniae* infected mice by decreasing lung bacterial clearance (Wang *et al.* 2011).

Binding assays with 3 µM recombinant full length or N-terminal CRD of galectin-9 and fixed *N. meningitidis* showed that both the full length galectin-9 and the N-terminal CRD galectin-9 bind to *N. meningitidis* (Fig. A.3), although the consequences of this interaction were not addressed in this study.

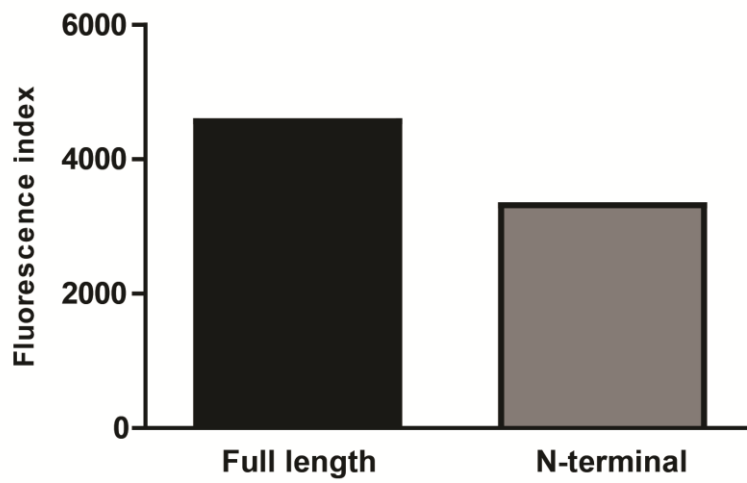


Figure A.3 Binding of recombinant human galectin-9 to *N. meningitidis*.

Quantification of flow cytometry analysis of full length galectin-9 (Full length) or N-terminal CRD of galectin-9 (N-terminal) binding following incubation of fixed MC58. *N. meningitidis* binds both full length and ND galectin-9. Binding is expressed as the fluorescence index.

Summary

The work in the Appendix describes preliminary data on the binding of meningococcus to further human galectins, examined by flow cytometry using fixed whole *N. meningitidis*, serogroup B strain MC58.

We show that *N. meningitidis* binds the form of galectin-1 stabilised with the irreversible alkylating agent DTT which does not undergo oxidation and retains its binding ability; however, galectin-1 stabilised with IAA and the mutant of galectin-1 in its linker region (C2S) do not interact with bacteria. Furthermore, galectin-8 binding to *N. meningitidis* is observed to be similar for both wild-type strains and strains lacking sialic acid on their surface, suggesting that sialic acid may not influence the interaction. In addition, binding of full length and ND galectin-9 to *N. meningitidis* was observed, indicating that ND is responsible for interaction.

In conclusion, we observe an interaction between meningococci and different galectins belonging to different galectin groups, but the experiments were not repeated and the consequences of these interactions have not been investigated.

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Declaration of originality

I certify that all the work described here is my own unless otherwise referenced.