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INNATE IMMUNE RECOGNITION OF *NEISSERIA MENINGITIDIS* at the NASOPHARYNX

Raed Saleh Albiheyri

ID 4172411

School of Life Sciences,
University of Nottingham

Supervisors

Dr. Luisa Martinez-Pomares, Dr. David Turner

and Dr. Neil Oldfield

DECLARATION

I hereby declare that all the work described in this thesis is my own unless otherwise stated and that this thesis has not been submitted, neither in whole or in part, elsewhere for another degree.

Raed Saleh Albiheyri

PhD Student

Molecular Bacteriology and Immunology Group

University of Nottingham

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ABSTRACT

Neisseria meningitidis is considered as the main suspect behind the majority of meningitis and sepsis cases globally. The major component of the outer membrane in *Neisseria*, lipooligosaccharide (LOS), is recognized by Toll-like receptor 4 (TLR4), triggering a pro-inflammatory cytokine response. Particularly, TLR4 recognizes the lipid A moiety of the LOS molecule, and the recognition is determined by the chemical composition of lipid A. The meningococcal lipid A normally has six acyl chains, the optimal number of acyl chains for TLR4 activation. Acyl chain addition is regulated by the products of two genes (*lpxL1* and *lpxL2*), which are present in a number of Gram-negative bacteria as well as *N. meningitidis*. Unlike the pathogenic B serogroup, the Y serogroup clonal complex 23 is pathogenic, in spite of being naturally *LpxL1*-deficient. We aimed to investigate how the changes in LOS structure could affect the recognition of *Neisseria* serogroups by the host, therefore, both B and Y serogroups variants have been used to infect human differentiated bronchial epithelial cells (Calu-3), cultured in an air-liquid interface. In addition, mutant serogroup B strains lacking *lpxL1* have been generated, as well as a complemented mutant strains expressing *LpxL1*. LOS analysis by silver staining suggested the presence of changes

in the LOS of the *lpxL1* mutant strains that could not be rescued by the complemented *lpxL1* gene. In addition, the expression status of selected *Neisseria* phase variant genes during the infection has been investigated but no changes were observed. Therefore the work was redirected to study bacterial behaviour during the infection of Calu-3. Cells analysis included bacterial growth, bacterial adhesion and invasion, damage to the epithelial barrier and TNF- α production. Differentiated Calu-3 cells were infected with N222.1 (Y serogroup, cc23) and two isolates, designated N459.3 and N459.6. These isolates were obtained as two colony picks from a single nasopharyngeal swab taken from the same individual as isolate N222.1 but six months later, and have identical strain designations (*i.e.* ST, PorA and FetA types) to N222.1. Furthermore, allelic variation was detected in only five genes for a whole genome comparison of N459.1 to N222.1. Both N222.1 and N459.6 increased the permeability of the epithelial barrier at 12 h post-infection and induced production of high levels of the pro-inflammatory cytokine TNF- α . In contrast, N459.3 did not affect the permeability of the epithelial cell layer or induce TNF- α production. Expression of Opa proteins, a family of *Neisseria* adhesins that interact with CEACAMs in host cells, varies among isolates and could contribute to differences in their

phenotype. These observations contrast with those obtained with control serogroup B strains (MC58 and H44/76; cc32) and another MenY isolate, N59.1 (cc174). The latter isolate, in contrast to N222.1, readily adhered/invaded the epithelial layer, did not disrupt the epithelial barrier, but promoted TNF- α production. Further work demonstrated that blocking of TNF- α production during N222.1 infection does not protect the epithelial layer from infection-induced damage. Preliminary signalling studies showed that N222.1, N459.3 and N459.6 trigger distinct signaling pathways.

The distinct patterns of bacterial behaviour exhibited by isolates N459.3 and N459.6 (with identical strain designations and contemporaneously present in the Nasopharynx of the same carrier) in our *in vitro* infection model suggest that virulence in *N. meningitidis* is intrinsically linked to subtle genomic changes. The temporal stability of the core bacterial genome and our observation that tract lengths of phase-variable loci do not alter during *in vitro* infection assays could enable us to use this and other infection models as platforms to link particular bacterial genomic traits to specific and reproducible phenotypic traits, and hence to assess the risk of closely related isolates causing invasive disease.

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LIST OF ABBREVIATIONS

A20	tumor necrosis factor inducible protein A20
ALI	air-liquid interface
AP	alternative pathway
APAF-1	apoptotic protease-activating factor 1
ATs	autotransporters
BHI	Brain Heart Infusion
CCs	clonal complexes
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CFU	colony forming units
c-Jun	transcription factor AP-1
CP	classical pathway
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	cerebrospinal fluid
DCs	dendritic cells
dH₂O	Distilled Water
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's modified Eagle's medium
DR	death receptor
dRS3	duplicated repeat sequence 3
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FAM	6-carboxyfluorescein
FBS	fetal bovine serum
fhbp	factor H binding protein
gDNA	genomic DNA

GEIs	genomic islands
GGIs	gonococcal genetic islands
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Hep	heptose
HGT	horizontal gene transfer
hpi	hours post infection
i.v.	Intravenous
IFN-γ	interferon gamma
Ig	immunoglobulin
IKAP	IKK complex-associated protein
IκB	inhibitor of NF- κ B
IKK	I κ B kinases
IL	interleukin
IL-1R1	IL-1 to its type 1 receptor
IL-1RAcP	IL-1 receptor accessory protein
IMD	invasive meningococcal disease
INFβ	interferon beta
INPCR	inverse PCR
IRAK	IL-1 receptor-associated kinase
ATM	nuclear kinase ataxia telangiectasia mutated
ITs	immunotypes
LAMP1	lysosomal-associated membrane protein 1
LB	Luria Bertani
LBP	LPS-binding protein
LGT	lateral gene transfer
LNnT	lacto- <i>N</i> -neotetraose
LOG	logarithmic
LOS	lipooligosaccharide

LP	lectin pathway
LPS	lipopolysaccharide
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MASPs	MBL-associated proteins
MBL	mannose-binding lectin
MDA	meningococcal disease-associated
MEKK1	MAP/ERK kinase kinase 1
MEM	minimal essential medium
MLST	Multilocus sequence typing
M-MLV	Moloney Murine Leukemia Virus
MOI	multiplicity of infection
MUC5AC	mucin protein
MΦ	macrophages
N	number of repeats
NANA	N-acetylneuraminic acid
NEMO	NF-κB essential modifier
NETs	Neutrophil extracellular traps
NF-κβ	Nuclear Factor-Kappa B
NHBE	Primary human bronchial epithelial cell
NIK	NF-κβ-inducing kinase
NIMES	Neisserial intergenic mosaic elements
ns	not significant
OD	optical density
OMPs	outer membrane proteins
OMVs	outer membrane vesicles
Opa	opacity protein
Opc	opacity protein 5c

ORFs	open reading frames
OS	oligosaccharide
PAIs	pathogenic islands
PAMPs	pathogen-associated molecular patterns
PARP	poly(ADP-ribose)polymerase
PBMCs	Peripheral Blood Mononuclear Cells
PBS	phosphate buffered saline
PI3K	phosphatidylinositol 3-kinase
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
PV	phase variable
qPCR	Quantitative real-time PCR
RelB	Reticuloendotheliosis oncogene cellular homolog B
RIP	receptor-interacting protein
RIPA	radio-immunoprecipitation assay
ROS	Reactive oxygen species
ROX	6-Carboxy-X-Rhodamine
RPPA	Reverse Phase Protein microarray
SD	standard deviation
SDS	sodium dodecyl sulphate
STs	sequence types
T5SS	type V secretion systems
TAK1	TGF-beta-activated kinase 1
TCC	terminal complement complex
TCR	T cell receptor
TD	translocator domain
TEER	Transepithelial electrical resistance
Tfp	type IV pili
T_h	helper T cells

TI-2	T cells independent antigens
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TPL-2	tumour progression locus 2 kinase
TRADD	TNFR-associated death domain protein
TRAF	TNFR-associated factors
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	regulatory T cells
UTRs	untranslated regions
UV	ultraviolet
WT	wild type
ZO-1	zonula occluden 1
β-actin	beta actin

CHAPTER ONE

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 *Neisseria*

The genus *Neisseria* is a member of the family *Neisseriaceae*, which also includes the genera *Moraxella*, *Kingella*, and *Acinetobacter* (Baron, 1996). The name *Neisseria* came from the name of the German bacteriologist Albert Neisser, who discovered its first member, *Neisseria gonorrhoeae*, in 1879 (Bergua *et al.*, 2010). The genus *Neisseria* includes Gram-negative aerobic and non-spore-forming diplococci that occasionally form tetrads with the adjacent sides flattened. It is a large genus that encompasses commensal bacteria that tend to colonise the mucosal surfaces of many animals. *Neisseria* is both catalase and oxidase positive, and its species can be identified by their ability to produce acid from a set of sugars. For instance, *N. gonorrhoeae* produces acid from glucose only; whereas *N. meningitidis* generates acid from both glucose and maltose (Knapp and Holmes, 1983).

1.2 *Neisseria* species

The genus *Neisseria* includes 12 species inhabiting human mucosal surfaces; most of them are non-pathogens that colonise the upper respiratory tract. Only two known species regularly cause disease in humans; *N. gonorrhoeae*, the

causative agent of gonorrhoea and *N. meningitidis*, a major cause of meningitis and septicaemia (Meningococemia) worldwide (Harrison *et al.*, 2008). Both species are intimately related to *Neisseria lactamica*, a harmless commensal bacteria present during adolescence. All three species are only found colonising the human mucosal surfaces, where the meningococcus and *N. lactamica* commensally inhabit in the Nasopharynx and the gonococcus inhabits the genitourinary tract (Maiden, 2008).

Despite the fact, that *N. meningitidis* and *N. lactamica* are intimately related, with approximately 60% genomic similarity, there are significant genomic dissimilarities between both species that affect their interaction with the host. For instance, the genes required for capsule formation are absent in *N. lactamica* while they are present in *N. meningitidis* (Bennett *et al.*, 2010). The capsule allows bacteria to escape from the antimicrobial activities of the immune system, prevents dehydration during the pre-infection phase and is considered an important virulence factor (Wong *et al.*, 2011). A major outer membrane protein, known as PorB, is present in both *N. lactamica* and *N. meningitidis*. The binding specificity of *N. lactamica* PorB to Toll-like receptor 2 (TLR2, see below) is different from that of the meningococcus (Wong *et al.*, 2011); the PorB from *N. lactamica* induces a poor proinflammatory

response and reduced TLR2 expression in human airway epithelial cells, an effect also seen with live *N. lactamica* (Liu *et al.*, 2010). Another study (Tezera *et al.*, 2011) found that *N. lactamica* induced a weak inflammatory response regarding cytokine and chemokine secretion, compared to *N. meningitidis*. In addition, they have concluded that *N. lactamica* plays a key role in suppressing pathogen-induced inflammation in the nasopharyngeal mucosa through TLR1/2 stimulation, activation of Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) and inhibition of Nuclear Factor-Kappa B (NF- $\kappa\beta$) activity.

1.3 *Neisseria meningitidis*

1.3.1 Meningococcal typing and sub-typing

Three typing methods are usually used for the study of *N. meningitidis*. The first method is immunological typing, which is based on the detection of highly immunogenic outer membrane proteins (OMPs) and the capsular polysaccharide. Secondly, electrophoretic typing using protein SDS-PAGE (PolyAcrylamide Gel Electrophoresis) is a high-resolution method compared to immunological typing, since amino acid variants that are immunologically equivalent can be separated and distinguished. Finally, sequence typing provides the highest level of resolution as it can distinguish among allele

variants invisible to immunological and electrophoretic typing. Advances in DNA sequencing technology have made typing through sequencing less costly, and sequencing has become the preferred method of typing (Wilson, 2005).

Multilocus sequence typing (MLST) compares 450– 500-bp nucleotide sequences for internal fragments of seven 'housekeeping' genes: *abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm* (Read, 2014). Distinct alleles at each of the loci are assigned numbers to produce a seven-digit allelic profile. The identified sequence types (STs) in this way can be grouped into closely related clonal complexes (CCs) (Maiden *et al.*, 1998). This system of typing was embraced, developed and popularised by the European Society for Meningococci with the support of the EU, and rapidly acquired an international credibility (Read, 2014). The further resolution has been made using whole genome sequencing, and in 2014, there are over 10,000 STs published on the Neisseria MLST website (<http://pubmlst.org/neisseria/>), which was developed by Keith Jolley in the University of Oxford (Jolley and Maiden, 2010). 41 of the 46 CCs submitted in PubMLST have been associated with invasive disease and five comprise carriage isolates (Read, 2014).

1.3.2 Epidemiology

According to the Centre for Disease Control and Prevention (CDC), meningococcal disease can be considered as an outbreak if the occurrence of the disease reaches 3 or more cases within 3 months, with an attack rate of at least 10 cases per 100,000 population; this is approximately 10 times greater than normal rates. Outbreaks may be restricted to a close population or may involve a larger community. For example, an outbreak of meningococcal disease mainly by strain W135 occurred in Mecca, Saudi Arabia, in 2000 Hajj season (Matsika-Claquin *et al.*, 2001). This outbreak was discovered when four cases caused by meningitis W135 were identified in France after returning from Hajj (two cases were fatal). Strain W135 is rare in France. In addition, an increase in the number of cases (from 96 to 129) in England and Wales has been reported due to the same reason (Hahne *et al.*, 2002).

The incidence rate of meningitis worldwide is about 500,000 cases annually, with more than 50,000 deaths. Most of these cases took place in the Sahara area in Africa, which is called "Meningitis belt" and extends from Ethiopia in the east to Senegal in the west (18 countries) and has a population of about 300 million people. In industrialised countries in the past decades, meningococcal disease has become more

infrequent, and no general outbreaks have occurred. Approximately 1-2 cases per 100,000 population have been reported, although numbers fluctuate from year to year (Maiden and Caugant, 2006).

In the UK, an increase in serogroup Y disease (from 34 cases in 2007 to 91 in 2011, 80 in 2012, 78 in 2013 and 75 in 2014) has been observed (www.gov.uk/government/publications), mainly caused by 4 clonal complexes cc23 (56% of isolates), cc174 (21%), cc167 (11%), and cc22 (8%) (Ladhani *et al.*, 2012). The median age for the cases was 60 years with 19% mortality, while the underlying percentage was 39% (Ladhani *et al.*, 2012). University students living in residential halls seem to be at high risk of serogroup Y attack, as serogroup Y meningococcal strain carriers are common among students (Bidmos *et al.*, 2011).

Incidence of meningococcal disease varies in human populations from rare to over 1000/100,000 cases/year (Stephens, 2007). Despite effective antibiotics and partially effective vaccines, meningococcal diseases still have high morbidity and mortality. Rates of meningococcal disease are highest in young children; adolescents and young adults aged 14–24 (Goldschneider *et al.*, 1969a). The frequency of meningococcal disease during the last 30 years differs from 1–

3/100,000 in most industrialized countries to 10– 25/100,000 in some third-world countries (Van Deuren *et al.*, 2000).

Although the incidence of disease caused by serogroup B is lower than serogroup A, it causes more outbreaks with larger morbidity and mortality. Several outbreaks by serogroup B have occurred in Europe (Iceland, Norway, Belgium and Spain), the USA, and South American countries (Cuba, Colombia, Brazil and Chile) (Jódar *et al.*, 2002). Since 1991, the number of serogroup B meningitis cases have increased in New Zealand with a high rate of morbidity and mortality (Dyet and Martin, 2006). In contrast, meningococcal disease due to serogroup B infection has lower disease incidences in Africa and the developing world countries (Jódar *et al.*, 2002).

Emerging in the mid-1990s, serogroup Y strains have caused increased rates of disease in parts of the US and Israel, but have not yet caused many cases in other parts of the world (Racoosin *et al.*, 1998, Stephens, 1999, Djibo *et al.*, 2003). Children and young adults have been the most susceptible groups; however serogroup Y also has a tendency to cause disease in the elderly, and to cause pneumonia more frequently than strains of other groups (Jódar *et al.*, 2002). In endemic situations, serogroup B is most common in infants while Y in elderly people (Stephens *et al.*, 2007).

1.3.3 Meningococcal vaccines

Among the six meningococcal serogroups of public health importance (A, B, C, W , Y and recently X), serogroup B is the main cause of invasive meningococcal disease in England and Wales (Gray *et al.*, 2006). In contrast to other serogroups, serogroup B has a poorly immunogenic capsular polysaccharide. The capsular polysaccharide (α 2–8) linked polysialic acid (N-acetylneuraminic acid, NANA) of B serogroup is very similar to the glycosyl residues found in adhesion molecules of the brain. This is the major cause of the delay in the development of capsule-based vaccines. In addition, the weak response to the alternative non-capsular vaccines (Feavers and Pizza, 2009), has made prevention and control of serogroup B meningococcal infection an ongoing problem (Tezera *et al.*, 2011).

In the 1960s quadrivalent vaccines against serogroups A, C, Y and W were developed by using their purified capsular oligosaccharides as antigens. More effective second generation vaccines now have been introduced, which are conjugated to carrier proteins such as CRM₁₉₇ (a non-toxic mutant of the diphtheria toxin) (Pace, 2009). Polysaccharide vaccines rely on humoral responses that are T cell-independent, which may result in short-lived immune protection. On the contrary,

conjugated vaccines generate long-term immunity against the targeted antigen via T-cell-dependent responses (Playfair and Bancroft, 2013). Conjugated vaccines have the ability to elicit immunologic memory; to reduce the nasopharyngeal carriage and establishment of population protection; and to effectively protect young children (<24 months) who may have a poor response to usual polysaccharide vaccines (Crum-Cianflone and Sullivan, 2016).

The first conjugate vaccines targeting *N. meningitidis* against serogroup C were introduced in the United Kingdom in 1999, and have shown immunogenicity and safety in all age groups (Serruto *et al.*, 2012). There was a significant reduction in carriage of serogroup C meningococci in the UK after introducing conjugate C polysaccharide vaccine, suggesting that vaccination induces adequate mucosal immunity to inhibit serogroup C carriage (Maiden and Stuart, 2002). These successful vaccines were followed by the generation of new conjugated quadrivalent meningococcal vaccines against A, C, Y and W serogroups, identified as MenACWY-CRM₁₉₇, by using their oligosaccharides and the carrier protein CRM₁₉₇ (Dull and McIntosh, 2012).

Initially, vaccines targeting serogroup B, for example MeNZB®, were developed based on the use of outer membrane vesicles

(OMVs), which are produced naturally by meningococcus and the released vesicles composed of outer membrane proteins, lipids and periplasmic components (Crum-Cianflone and Sullivan, 2016). However, the high variability of PorA protein among different MenB strains, which is the major protective antigen of the OMV-based vaccines, is a serious obstacle in the production of a globally effective anti-serogroup B vaccine (Serruto *et al.*, 2012).

The recent consecutive breakthroughs using “reverse vaccinology,” have led to successful development of serogroup B meningococcal vaccines based on conserved proteins (Caron *et al.*, 2011, Folaranmi *et al.*, 2015). These new serogroup B vaccines have many advantages, such as their broad range of coverage, which may protect against most serogroup B strains as well as some non-serogroup B strains; of note, however, these vaccines may fail to protect against some of serogroup B meningococcal strains (Crum-Cianflone and Sullivan, 2016). The recombinant protein vaccine against serogroup B MenB-4C (Bexsero) is approved in Europe, Canada, Australia and the US; and MenB-FHbp (Trumenba) is approved for use in the US. Multiple doses of current serogroup B vaccines are required, with two doses of MenB-4C given at (0 and ≥ 1 month) and three doses of MenB-FHbp at (0, 2 and 6 months); the same serogroup B vaccine should be used for

all doses (Crum-Cianflone and Sullivan, 2016). Quadrivalent can be used in conjunction with serogroup B vaccines during the same visit, but should be given at different body sites (Crum-Cianflone and Sullivan, 2016).

1.3.4 Virulence factors of *N. meningitidis*

Meningococci usually avoid the immune system of the host and establish infection through the contribution of several meningococcal surface structures. Capsular polysaccharide, outer membrane proteins and lipooligosaccharide (Rosenstein *et al.*, 2001, Stephens, 2007) are major components linked to meningococcal virulence but the complete nucleotide sequences of several pathogenic *Neisseria* strains have facilitated the identification of numerous novel virulence factors (Bentley *et al.*, 2007, Tettelin *et al.*, 2000a).

Additionally, virulence may be related to additional mechanisms including rapid doubling time and release of outer membrane blebs or vesicles (Brandtzaeg, 2006), phase and antigenic variation related to genetic switches, molecular mimicry and, less defined, the possible release of toxins (Stephens, 2007). Some of the major virulence factors will be discussed below.

1.3.4.1 Capsule

The capsule plays a vital role in invasive meningococcal disease (IMD). This has been supported by several clinical and epidemiologic studies as nearly all clinical isolates recovered from infected patients with invasive disease are encapsulated (Johswich *et al.*, 2012). The capsule of the pathogenic meningococcal serogroups differs in both chemical structure and antigenic properties (Morley and Pollard, 2001). The meningococcal capsular polysaccharides consist of sialic acid derivatives, except for serogroup A, and enhances its survival inside the host by providing the meningococcus with anti-phagocytic properties (Stephens, 2009).

The biogenesis of the meningococcal capsule is encoded by a cluster of genes known as the capsule biosynthesis locus (*cps*) (Frosch *et al.*, 1989). It consists of four distinct functional regions with one region containing an operon (*sia*) responsible for the synthesis of the sialic-acid-containing capsule, and another operon (*ctr*) involved in polysaccharide transport (Swartley *et al.*, 1996). Interestingly, capsule switching due to the genetic exchange of portions of the capsule biosynthetic operon is an important immune escape mechanism (Swartley *et al.*, 1997).

1.3.4.2 Lipooligosaccharide

Lipooligosaccharide (LOS, endotoxin) of meningococcus is structurally different from that of other gram-negative bacteria (Rosenstein *et al.*, 2001). It is composed of an oligosaccharide (OS) portion attached to a phosphorylated di-heptose (Hep) inner core linked to a lipid A moiety embedded in the outer membrane (Stephens, 2009). It is structurally related to lipopolysaccharide (LPS), but it lacks repeating polysaccharide O-antigens and is thus referred to as lipooligosaccharide (LOS). The OS portion consists of a variable sequence of sugar residues, which in meningococci and gonococci is composed of glucose, galactose, N-acetylglucosamine, and N-acetylneuraminic acid (NANA) (Kahler *et al.*, 1998). Meningococcal LOS production, like many other virulence factors, is phase variable, which allows switching between immunotypes (Berrington *et al.*, 2002).

1.3.4.3 Pili

N. meningitidis is genetically capable of producing antigenically different pili (Nassif, 1999). Class I and Class II are two major classes of meningococcal pili, both are members of the methylphenylalanine pili family, termed type IV pili, which are widely found in Gram-negative bacteria (Tzeng and

Stephens, 2000b). Type IV pili are largely homopolymers of a single 15-20 kDa pilin protein subunit (Craig and Li, 2008) termed pilin (PilE). The helical assembly of pilin leads to the formation of the fibre. In addition, several minor subunits (pilC, pilV and pilX) can be incorporated into the fibre and modulate its function. The components involved in pili assembly, retraction and specific functions are distributed around and span both bacterial membranes, from the inner side of the cytoplasmic membrane to the outer membrane. Fifteen genes are involved in pilus biogenesis, which can be divided into four genetically dissociable steps: assembly, functional maturation, emergence on the cell surface and counter-retraction (Carbonnelle *et al.*, 2005). A number of pilins are known to undergo post-translational modifications, such as glycosylation and phosphorylation, which can indirectly have an effect on cellular interactions, perhaps by affecting the agglutination of pili (Nassif *et al.*, 1997). Indeed, pili have been shown to be associated with a number of functions such as host cell adhesion, which is the first step in meningococcal host-cell interactions (Tzeng and Stephens, 2000b), and facilitation of DNA uptake during transformation, a property that contributes to meningococcal virulence by promoting genetic adaptability (Mattick, 2002). They are also involved in twitching motility, due to their ability to retract into

the bacterium from which they originate. By this process, a level of movement on cell surfaces of around 1 mm per second may be attained, and the distance between the bacterial and eukaryotic membranes may also decrease enabling the uptake of DNA and facilitating intimate cellular interactions through integral outer membrane adhesions (Winther-Larsen *et al.*, 2005). Moreover, pili of pathogenic *Neisseria* are associated with microcolony formation and autoaggregation which are required for efficient initial bacteria-cell interaction (Pelicic, 2008). As in the case of genes involved in LOS synthesis, Type IV pili are subjected to phase and antigenic variation (Nassif *et al.*, 1997).

1.3.4.4 Major outer membrane proteins

Meningococci express numerous major outer membrane proteins (OMPs), which are also related to meningococcal virulence. These proteins have been grouped into five major classes based on their apparent molecular weights on sodium dodecyl sulphate (SDS) polyacrylamide gels and peptide analysis (Tsai *et al.*, 1981). OMPs are been studied as potential vaccine candidates in *N. meningitidis* (Chiou *et al.*, 2006). *N. meningitidis* also express class 5 proteins which include opacity protein (Opa) and opacity protein 5c (Opc). A meningococcal genome has up to four or five distinct Opa

proteins sequences, the same strain can express up to four Opa, and up to two can be expressed simultaneously on the same meningococcus (Estabrook *et al.*, 2004). These proteins impart opacity to colonies and are highly immunogenic. They play an important role in adhesion and invasion of host epithelial, endothelial and phagocytic cells (De Jonge *et al.*, 2003a). Firstly, Opa proteins are integral outer membrane proteins which are synthesized as precursors containing signals for inner membrane transport (Hauck and Meyer, 2003). These proteins are eight-stranded transmembrane beta-barrel structures with four surface-exposed loops. Opa proteins also show phase and antigenic variation (Stern *et al.*, 1984). With regard to Opc proteins, they are 10-stranded β -barrell transmembrane proteins with 5 surface-exposed loops (Virji *et al.*, 1999). Opc proteins are also involved in cell interactions, adherence to eukaryotic cells and are a target of bactericidal antibodies (Tzeng and Stephens, 2000b). Moreover, Opa and Opc proteins can extend the meningococcal survival within monocytes/macrophages (McNeil *et al.*, 1994, Gammelgaard *et al.*, 2011). Subsequently, Opa and Opc proteins facilitate the entry of meningococci into the host and to the circulation (Virji *et al.*, 1996b, Lee *et al.*, 2007, Callaghan *et al.*, 2008, Hill *et al.*, 2010).

1.3.4.5 Autotransporters

Autotransporters (ATs) are produced by a large variety of Gram-negative bacteria, and many of them have virulence-related functions. ATs are classified as Type V secretion systems (T5SS) (Henderson *et al.*, 2000). Compared to the other secretion pathways reported in Gram-negative bacteria, the mechanism of AT seems to be relatively simple pathway (Henderson *et al.*, 1998, Henderson and Nataro, 2001). Although AT does not require devoted secretion machinery, it depends on general machinery required for bacterial physiology, *i.e.* the Sec system (general export pathway for periplasmic and outer-membrane proteins), and the Bam complex (assembles integral OMPs into the outer membrane) (Grijpstra *et al.*, 2013). ATs are modular proteins consisting of an N-terminal signal sequence, a translocator domain (TD) and, in between, a secreted passenger domain (van Ulsen *et al.*, 2003). The signal sequence directs the precursor over the inner membrane via the Sec system (Grijpstra *et al.*, 2013). After transport across the inner membrane, the TD is assembled via the Bam complex to form a β -barrel into the outer membrane, and the passenger is transported to the cell surface via a mechanism that is still not entirely clear (Grijpstra *et al.*, 2013).

In general, the ATs vary widely in function but often act as enzymes, adhesins or cytotoxins (Grijpstra *et al.*, 2013). Eight open reading frames (ORFs) putatively encoding ATs has been identified using a systematic search of the sequenced genomes of *N. meningitidis* strains MC58 and Z2491 (van Ulsen *et al.*, 2001). Among those ORFs is the *iga* gene, which encoding the IgA1 protease, while other ORFs are encoding proteins homologues to a variety of adhesins, a haemagglutinin and a serum-resistance protein (van Ulsen *et al.*, 2003). Meningococcal IgA protease contributes to the attachment, colonization, and intracellular survival of meningococci in the host (Gammelgaard *et al.*, 2011). That is probably due to its alteration effect on the lysosomes based on protease-mediated degradation of lysosomal-associated membrane protein 1 (LAMP1) (Lin *et al.*, 1997, Ayala *et al.*, 1998, Hill *et al.*, 2010). Another meningococcal AT is NalP, which was described by (Turner *et al.*, 2002). They demonstrated that NalP/AspA is a serine protease and that the serine residue of the active site is involved in autocatalytic processing, resulting in secretion of the passenger domain. Thus, these proteins might play important roles in the virulence of *N. meningitidis* by displaying diverse functions (see Table 1.1).

Table 1.1: Overview of the function diversity displayed by selected ATs of *N. meningitidis*

Protein	Functions	Reference
IgA protease	Protease, immunomodulation, DNA binding	(Lin <i>et al.</i> , 1997, Pohlner <i>et al.</i> , 1987a, Pohlner <i>et al.</i> , 1987b, Jose <i>et al.</i> , 2000, Lorenzen <i>et al.</i> , 1999)
Nhha	Adhesion, Macrophage apoptosis	(Scarselli <i>et al.</i> , 2006, Sjolinder <i>et al.</i> , 2012)
NaIP	Processing of ATs and OMPs	(van Ulsen <i>et al.</i> , 2003)

1.3.5 *N. meningitidis* genome characteristics

N. meningitidis has developed several strategies to evade the immune system (Pizza and Rappuoli, 2015). These strategies can be better explained by bearing in mind the nature of the Neisserial genome. The progression in the field of molecular biology and the advanced technologies have a tremendous impact on improving our understanding of the complex Neisserial machinery. The machinery that control *N. meningitidis* growth, colonization and invasion have been better understood by employing sophisticated approaches such as whole-genome sequencing and microarrays (Gasparini *et al.*, 2015). In addition, these functional genomics investigations have helped to explain the unusual Neisserial intrastrain variation and environment adaptation. Other

methods, *e.g.* genome-wide association studies, have clarified the host's susceptibility to the microbe and the pathogen-host interaction (Gasparini *et al.*, 2015). Besides the enhancement of our knowledge of the biology and pathogenesis of *N. meningitidis*, Genomics and postgenomics studies have proved to be tremendously useful in exploring candidate antigens and in developing more efficient vaccines (Del Tordello and Serruto, 2013).

N. meningitidis is a naturally competent pathogen and has a highly dynamic, plastic, and flexible genome with a size range of just above 2,000 kb (Schoen *et al.*, 2009). Unlike other microbial genomes, the Neisserial genome lacks some of the typical two-component systems and sigma factors (Power and Moxon, 2006). Neisserial pathogenicity is polygenic (Marri *et al.*, 2010), and is acquired by a range of diverse pathogenic islands (PAIs or genomic islands, GEIs); comprising gonococcal genetic islands (GGIs) (Snyder *et al.*, 2005) and meningococcal disease-associated (MDA) island (Joseph *et al.*, 2011).

Antigenic variation is one of the most effective strategies used by pathogens to evade immune recognition and also to provide functional variants that alter the interaction between virulence factors and host molecules. Basically, the Neisserial

chromosome can engage horizontal or lateral gene transfer (HGT/LGT) (mostly via transformation and, to a lesser extent, via conjugation and phage transduction) and allelic exchange/rearrangement of genes or gene portions taken up from the environment (Gasparini *et al.*, 2015). In addition, its frequent surface structural variation is caused by homologous intragenic recombination, as the Neisserial genome contains multiple copies of certain genes, *e.g.* opacity proteins and pilins (Cahoon and Seifert, 2011).

Additionally, the *N. meningitidis* harbours a number of prophages. Known as duplicated repeat sequence 3 (dRS3) is the most extensively studied sites for phage integration (Power and Moxon, 2006), which is a member of the family of Neisserial intergenic mosaic elements (NIMES). Some plasmids, for instance, pJS-A and pJS-B, have a significant role in this mechanism (van Passel *et al.*, 2006).

Another surface modulation occurs via phase variation of a huge number of genes. Phase variation is a reversible switch for the expression states of genes [expressed (ON) and unexpressed (OFF)] state or a switch between two forms of a gene product (Moxon *et al.*, 2006). The variability of the Type IV pili, the LOS antigen, and the outer membrane Opa proteins

are major advantages to *N. meningitidis* facilitating its success in pathogenesis.

Phase variation in bacteria can be mediated in three ways: (i) by invertible DNA segments, (ii) differential methylation, and (iii) changes in stretches of homopolymeric nucleotides or short tandem repeats (microsatellites) that modify the number of repeats (Rotman and Seifert, 2014). Most phase variation events in the Neisseria are mediated by increase or reduction in the number of polynucleotide repeats during replication in a process known as slipped-strand mispairing, which occurs when one repeat is paired with an adjacent repeat on the opposite strand (Levinson and Gutman, 1987).

Genomes of the pathogenic Neisseria are estimated to have more than 100 phase variable genes overall, with about 80 incidences per strain (Rotman and Seifert, 2014). Many genes identified as virulence factors are phase variable, facilitating immune evasion. The evidence for immune avoidance based on phase variation includes the *in vitro* resistance of *N. meningitidis* to bactericidal antibodies against the LgtG LOS modifier (Bayliss *et al.*, 2008), as well as the outer membrane porin PorA (Tauseef *et al.*, 2013). Nevertheless, it should be considered that not necessarily all phase-variable genes express surface antigens or are involved in pathogenesis.

Hence, both antigenic and phase variations are involved in facilitating the escape of *Neisseria* from the immune recognition (Power and Moxon, 2006, Rotman and Seifert, 2014).

1.3.6 *N. meningitidis* colonization

Normally, meningococci are carried in asymptomatic commensal situations, but in some circumstances may cause a local inflammation, invasion of mucosal surfaces with access to the bloodstream which may result in sepsis and/or meningitis. The establishment of meningococcal colonization start with adhesion to the mucosal surface, and then to epithelial cells of the Nasopharynx. Different meningococcal cell-surface and secreted virulence factors are involved in colonization, persistence, and damage within the host.

Type IV pili are involved in many stages of meningococcal infection, such as adhesion to endothelial cells, bacterial aggregation, twitching motility, bacterial migration and natural transformation (Carbonnelle *et al.*, 2006). The meningococcal membrane opacity proteins, Opa, and Opc, mediate intimate adhesion to host cells via binding to carcinoembryonic antigen cell adhesion molecule (CEACAMs) receptors and extracellular matrix components, respectively (Virji, 2000). Most *N. meningitidis* strains possess four different Opa proteins, of

which OpaA and OpaJ bind to CEACAM1, whereas OpaB and OpaD bind to CEACAM1 and CEA (de Jonge *et al.*, 2003b). However, those four Opa proteins might not be equivalent in facilitating the adhesion to host cells (Virji *et al.*, 1993, McNeil *et al.*, 1994, de Jonge *et al.*, 2003b). Opc proteins are also involved in bacteria/host cell interactions, adherence and are a target of bactericidal antibodies (Tzeng and Stephens, 2000b). As a result, Opa and Opc proteins can ease the meningococcal entry into the host and to the circulation (Virji *et al.*, 1996b, Lee *et al.*, 2007, Callaghan *et al.*, 2008, Hill *et al.*, 2010). Although the previously mentioned adhesins of meningococcus represent the most important and the most studied, additional meningococcal antigens have been suggested to have a role in adhesion.

Among these antigens, there are two meningococcal autotransporters NalP and MspA, which are phase variable outer membrane/secreted proteins (Oldfield *et al.*, 2013). Thus far, the precise biological significance of NalP and MspA remains unclear (Oldfield *et al.*, 2013). However, a role for MspA in meningococcal adhesion to host cells has been suggested, and NalP was demonstrated to alter the secretion profile of adhesion molecules App and IgA1 protease (Turner *et al.*, 2006).

NadA (*Neisseria* adhesin A) is a trimeric autotransporter which mediates binding to epithelial cells by forming stable trimers on the bacterial surface (Capecchi *et al.*, 2005), that interacts with the Heat shock protein 90 (Hsp90) (Montanari *et al.*, 2012). It might also be involved in host invasion since it can hamper the monocytes apoptotic cell death (Franzoso *et al.*, 2008). Another autotransporter might facilitate as an adhesin is NhhA (*Neisseria* hia/hsf homologue), with homology to Hia and Hsf adhesins of *Haemophilus influenzae* which binds to extracellular matrix proteins, heparan sulfate and laminin, and facilitates attachment to host epithelial cells (Scarselli *et al.*, 2006, Sjolinder *et al.*, 2008).

The presence of multiple adhesins in the meningococcus (Figure 1.1) with different targeted receptors suggests that they may interact jointly with different receptors on the same target cells for better adhesion, or may mediate Neisserial adhesion to different cell types at different stages during infection.

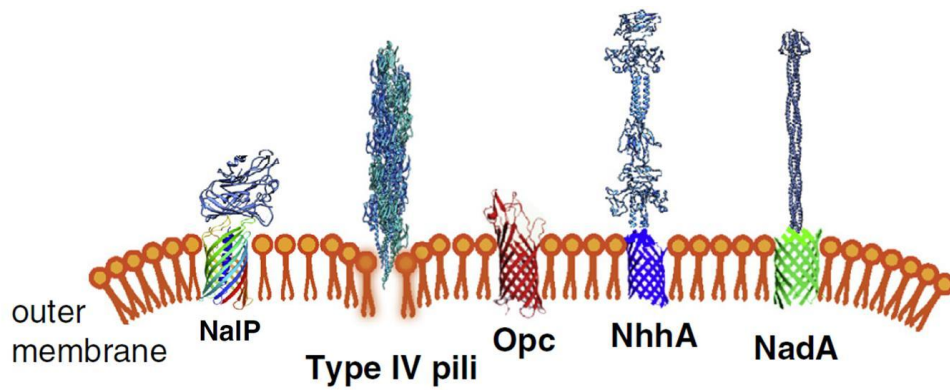


Figure 1.1: Schematic illustration of some *Neisseria meningitidis* outer membrane proteins involved in colonization.

Adapted from (Pizza and Rappuoli, 2015).

1.3.7 Pathology

Meningococcal pathogenesis is multifactorial and is determined by a number of environmental factors, host factors and different bacterial virulence factors. Environmental factors: dry and dusty weather conditions, such as in sub-Saharan Africa (meningitis belt), are more likely to affect the integrity of the mucosal surfaces in the human nasopharyngeal and promote invasion by meningococci (Gammelgaard *et al.*, 2011). Another factor is the spread of meningococci carried by smokers (Coen *et al.*, 2006), as the meningococcal carriage is frequently higher in smokers compared to non-smokers. Since, smoking disrupts the epithelial barrier of the upper respiratory tract and the mucosal immune system, and impairs the ciliary activity of epithelial cells (Gammelgaard *et al.*, 2011). Hence,

the integrity of the respiratory and nasopharyngeal epithelium is vital in the host resistance against meningococcal invasion.

Human factors: the age of host plays an important role in invasive meningococcal disease. Social behaviour and close contact between individuals, such as deep kissing and sleeping in dormitories, encourages the pathogen transmission (Gammelgaard *et al.*, 2011). In addition, a range of host genetic factors are linked to the acquisition of meningococcal disease, such as gene polymorphisms of the LPS binding protein, the LPS binding receptor, the bactericidal permeability increasing protein, mannose-binding lectin, and complement deficiencies and defects (Brandtzaeg *et al.*, 2001, Hill *et al.*, 2010, Emonts *et al.*, 2003b). Moreover, upper respiratory infections and/or chronic irritation or inflammation of the mucosa are considered as influencing factors for meningococcal invasion and colonization (Griffiths *et al.*, 2007).

Bacterial factors: *N. meningitidis* produces a number of virulence factors e.g. IgA protease, LOS and polysaccharide capsule, which are involved in immune evasion and pathology (see 1.3.4, 1.3.6 1.3.9). It involves a complex interaction between the host immune system and the organism, which

would influence the severity of disease (Mogensen *et al.*, 2006).

In the normal state, *N. meningitidis* is carried by approximately 10 % of healthy individuals without causing disease. The carriage percentage is likely to increase in closed and semi-closed communities, such as university halls and military bases. The meningococcal carriage state could last for several months or be sporadic or incidental. Nasopharyngeal colonization by *N. meningitidis* induces an antibody response, involving IgA, IgG and IgM classes, within a few weeks after acquisition of the bacterium, which might act as a vaccination (Kremastinou *et al.*, 1999). The exposure to non-pathogenic or cross-reacting species like *Neisseria lactamica* increases the level of IgG during lactation (Yazdankhah and Caugant, 2004).

Meningococci colonise upper respiratory mucosal surfaces of the nasopharyngeal cavity and are transmitted from person-to-person via respiratory secretions and less efficiently by droplets (Tezera *et al.*, 2011). Mucosal immunity can be identified by increasing concentrations of IgA in saliva. Neisserial induction of an IgA protease activity is lower in strains isolated from carriers than in invasive meningococcal strains. The mucosal immunity is not sufficient to prevent meningococcal colonisation, but it can stop the epithelial

invasion (Yazdankhah and Caugant, 2004). It has been suggested that the presence of anti-meningococcal specific IgA in the blood, which is produced post-infection, could obstruct meningococcal complement-mediated lysis by IgG or IgM and may increase the incidence of meningococcal disease (Griffiss and Goroff, 1983). This blocking effect is most likely to be directed against the capsule. Interestingly, an association between the level of specific IgA and the meningococcal epidemic has been reported (Yazdankhah and Caugant, 2004, Pollard and Frasch, 2001).

Meningococci may pass through the mucosal epithelium via phagocytic vacuoles as a result of endocytosis (Stephens *et al.*, 1983). Once the mucosal barrier is crossed, bacteria gain access to the bloodstream to cause infection and trigger the complement system, inflammatory response and the coagulation and fibrinolysis pathways (Emonts *et al.*, 2003a). Multiplication of meningococci correlates with the systemic release of inflammatory cytokines, which are key elements in the pathogenesis of meningococemia (Brandtzaeg *et al.*, 1992b).

1.3.7.1 Meningitis

Meningitis is an inflammation of the membranes that surround the brain and spinal cord, involving the arachnoid, the pia

mater, and the interposed cerebrospinal fluid (CSF). Besides other causes of meningitis (viral meningitis, tuberculous meningitis, fungal meningitis and non-infectious causes), bacterial meningitis is caused predominately by three organisms *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* (Ginsberg, 2004).

The inflammatory process extends throughout the subarachnoid space around the brain and spinal cord, and the ventricles (Mace, 2008). It is the most common clinical presentation of meningococcal disease; more than 60% of patients in industrialised countries develop meningitis without septic shock (Ovstebo *et al.*, 2004).

Meningitis presents with a variety of symptoms depending on the age of the patient. Sudden onset of fever, headache and neck stiffness are common in adult patients. Other signs and symptoms of meningitis may include nausea, vomiting, photophobia and an altered mental status (Loring, 2004). A skin rash may also develop because of inflammation and bleeding in small blood vessels throughout the body, including those under the skin (Nielsen *et al.*, 2001). However, in infants and younger children, non-specific symptoms including poor feeding, irritability, a high pitched cry and a bulging fontanel are commonly present (Nadel and Kroll, 2007).

Meningococci that are transmitted by aerosol or contact with secretions colonize upper respiratory mucosal surfaces (e.g., the Nasopharynx), may spread to adjacent mucosal surfaces (e.g., lower respiratory tract), and may invade epithelial surfaces and gain access to the bloodstream to produce systemic and focal infections. The meningococcal attachment to the epithelial cells seems to be mandatory for nasopharyngeal colonization. By employing its virulence factors and adhesins (see 1.3.4 and 1.3.6), meningococci become able to dive deeply into the mucus and attach to epithelial cells then internalise via parasite-induced endocytosis, then accumulate in phagocytic vacuoles which might be released into interepithelial spaces below epithelial cell tight junctions. Meningococci can also survive inside monocytes/macrophages, which make them are serving as bacterial carriers facilitating the meningococcal transfer into and within the circulation (Gammelgaard *et al.*, 2011). As monocytes/macrophages form over 10% of the existing cells in the nasal mucosa, the meningococci have a great chance of dissemination (McNeil *et al.*, 1994). In a different scenario, meningococci may attack epithelium damaged by smoking, co-infections or other environmental factors (Tzeng and Stephens, 2000a).

Subsequently, meningococci can enter the bloodstream and multiply and accumulate in the meninges, pericardium and large joints, causing a systemic release of inflammatory cytokines (IL-1, IL-6, TNF- α). However, it is unclear how meningococci enter the CSF. These inflammatory cytokines released in meningococcal bacteremia might enhance the permeability of the blood-brain barrier and may allow meningococcal entry into the CSF. In addition, inflammatory cytokines together with other mediators (e.g., nitric oxide), can induce leukocyte infiltration across the blood-brain barrier, which can cause breakdown of the blood brain barrier associated to edema, release of metalloproteases and induction of cellular apoptosis (Brandtzaeg *et al.*, 1992a, Tzeng and Stephens, 2000a).

1.3.7.2 Meningococcal sepsis

The series of events leading from septicemia to septic shock probably is initiated by toxins that either are liberated by the microorganisms (*Staphylococcus aureus* exotoxins) or bound to their cell walls (endotoxin of Gram-negative aerobes). The Gram-negative aerobes, such as (*Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae*), can also produce endotoxins in a free form which are also originated from their cell wall. Gram-negative endotoxin also is known as

lipopolysaccharide (LPS) and in the case of *N. meningitidis* lipooligosaccharide (LOS) (Luce, 1987). Meningococcal sepsis is the most severe form of infection caused by *N. meningitidis*. It is characterized by widespread haematogenous dissemination but is less common than meningitis. Meningococcal sepsis may be transient, chronic and fulminant. The clinical course of severe meningococemia is rapidly progressive, with the time from onset of fever until death often as short as 12 h (Kirsch *et al.*, 1996). Clinically, the patient exhibits early signs of sepsis, including tachycardia, normal systolic blood pressure with widened pulse pressure, bounding peripheral pulses, and warm extremities with instantaneous capillary refill time. Death in cases of severe meningococemia is usually resulting from the acute circulatory failure. Disseminated intravascular coagulation (DIC) is another major source of pathology in patients with meningococcal disease (Kirsch *et al.*, 1996). Most patients with meningococemia also present a petechial or purpuric rash although in the early stage a non-blanching macular rash is not uncommon.

1.3.8 Treatment regimens

Meningococcal disease is a medical emergency and potentially fatal. It requires an immediate admission to hospital, and early

clinical and laboratory diagnosis is very important (Bosis *et al.*, 2015). At presentation, it might be difficult to distinguish between meningitis due to *N. meningitidis* and other types of meningitis. In all suspected cases with clinical symptoms of IMD, lumbar puncture is required to be performed (de Gans and van de Beek, 2002). Since delayed treatment is associated with unpleasant clinical outcome, prompt antibiotic treatment is crucial when bacterial meningitis is suspected to prevent any serious neurologic morbidity and death (Bosis *et al.*, 2015).

While the etiologic investigation is established, the initial empirical antibiotic treatment should embrace an intravenous third-generation cephalosporin (*e.g.*, 100 mg/kg/day ceftriaxone administered i.v. in one daily dose or 100 mg/kg/day cefotaxime administered i.v. divided into three daily doses), as the first choice of treatment (Bosis *et al.*, 2015). If these choices of antibiotics are not accessible, intravenous penicillin should be given (Branco and Tasker, 2010, Sabatini *et al.*, 2012). Although recent studies pointed that CSF sterilization may occur within 3-4 days of treatment, a 7-10 day course of antibiotic is preferable to avoid complicated meningococcal meningitis (Branco and Tasker, 2010, Sabatini *et al.*, 2012).

Prophylaxis of meningococcal meningitis can be managed by either vaccination or antibiotics. Disease transmission between individuals can be interrupted by the use of antibiotics, which eliminate the asymptomatic nasopharyngeal carriage state. For individuals with close contacts with the index case (*i.e.*, for more than 4 hours during the 7 previous days), oral rifampicin or ciprofloxacin are the drugs of choice according to age (Sabatini *et al.*, 2012).

1.3.9 Immune recognition

1.3.9.1 Innate immune cells response

The nasopharyngeal epithelial layer in humans hosts a number of professional innate immune cells, such as dendritic cells (DCs), macrophages and neutrophils. Once meningococci have crossed the mucosal barrier, the innate immune cells will encounter the microbe directly. The innate immune system is mainly focused on sensing unique features of pathogens, the so-called pathogen-associated molecular patterns (PAMPs) a concept known as pattern recognition. The targets of pattern recognition are products characteristic of microbial metabolism or structure (Schmitt *et al.*, 2009).

DCs connect innate and adaptive immunity systems and administrate the induction and development of the immune response. Before the maturation of DCs, they sense and

phagocytise foreign invaders (pathogens). Once DCs contact with bacteria they shift into a mature state. This process is characterised by down-regulation of their phagocytic potential and pathogen recognition receptors, and up-regulation of costimulatory signals and antigen presentation receptors. Simultaneously, DCs migrate to secondary lymphoid tissues, in order to contact naive T cells and induce T cell activation. DCs can also capture pathogens in the mucosal surfaces by detaching the tight junctions between adjacent epithelial cells (Schmitt *et al.*, 2009). Similarly, macrophages (MΦ) can take up a wide range of pathogens and present them via MHC molecules to be recognised by activated CD4⁺ T lymphocytes via T cell receptor (TCR) and as such play a key role as effector cells during inflammation.

One of the early events during the *N. meningitidis* infection is the activation of neutrophils (Heyderman *et al.*, 1999, Peters *et al.*, 2003). They can be clearly found in the cerebrospinal fluid of patients suffering from meningitis caused by meningococci, as the major population of immune cells there. The migration of these cells to sites of infection is orchestrated by chemotactic factors (Mayer-Scholl *et al.*, 2004, Kuijpers and Roos, 2004). The uptake of *N. meningitidis* by neutrophils can be facilitated by neisserial outer membrane proteins such as Porins, Opa via CD66⁺ neutrophils but not by Opc, which

also binds a variety of adhesion molecules specially on epithelial cells and play a key role in meningococcal internalisation (Klein *et al.*, 1996, McNeil and Virji, 1997, Virji *et al.*, 1996a). Another recognition system is the opsonic recognition through Fc receptors and complement receptors, which might play an important role in *N. meningitidis* capture by neutrophils. In addition, the oxidative burst in neutrophils can be modulated by Neisserial porins (Bjerknes *et al.*, 1995). Likewise, some of the previously described interactions between *N. meningitidis* and macrophages and DCs might be applicable for granulocytes as well (Schmitt *et al.*, 2009).

1.3.9.2 Adaptive Immune Response against *N. meningitidis*

N. meningitidis has several surface antigens, polysaccharides are among them, which form the capsule. Unlike proteins, polysaccharides are T cells independent (TI-2) antigens and capable of direct activation of B cells, without the involvement of the MHC. However, TI-2 antigens are incapable of inducing an efficient memory B cells, and as a result, do not develop long lasting antibodies. Instead, they are able to induce the production of short-lived antibodies belong to the IgM class (Gasparini *et al.*, 2015).

The critical role of bactericidal antibodies against the exposed surface antigens of *N. meningitidis* has been highlighted by many studies. For example, a study showed that only individuals without bactericidal antibodies have contracted the clinical disease (Goldschneider *et al.*, 1969b, Gasparini *et al.*, 2015). Additionally, the therapeutic use of immune sera, which was implemented by Flexner, successfully and markedly reduced lethality (Flexner, 1913, Gasparini *et al.*, 2015). These studies showed that these antibodies have a central role in the protection from invasive disease. In addition, the opsonisation by antibodies is very important in the protection and the recovery from meningococcal disease, as it plays an important role in the activity of neutrophils, macrophages, and DCs in combating *N. meningitidis* (Gasparini *et al.*, 2015).

Neutrophil extracellular traps (NETs) are clustering of a large number of neutrophils contains microorganisms found in the cerebrospinal fluid of patients. These NETs are known by the massive release of cathepsin G. *N. meningitidis* avoids these traps by releasing OMVs, as well as by the modification of lipid A of LOS (Lappann *et al.*, 2013).

The adaptive immune response to *N. meningitidis* has been studied in carriage stage and during both the invasive disease and the convalescence period. T cells have a double-edged

response; limit the invasiveness of the pathogen by proinflammatory T cells, and restrain virulence of the pathogen by the introduction of regulatory T cells (T_{reg}), which can be responsible for restricting the effectiveness of the protective response, particularly in children (Davenport *et al.*, 2007).

From the fourth day of infection, an increased meningococcal antibody titres can be detected, while the peaking occurs toward the end of the 3rd week or the beginning of the 4th week (Gasparini *et al.*, 2015). The antibody titres correlate with the severity of the disease and the age group of patients. Generally, the T cells' count drops in the acute stage of the meningococcal disease, while that of B cells increases; toward the end of the 2nd week, IgG levels decrease and IgM levels increase (Korzhueva *et al.*, 1984, Gasparini *et al.*, 2015). Specifically, abnormalities in T cell responses can be identified during infection, for example, an increased percentage of CD25+ and HLA-DR+ T cells, an elevation in CD4+ CD45R+ (suppressor-inducer) cells, with a resulted expression of activation antigens, and a drop in the percentage of CD4+ CDw29+ (helper-inducer) cells (Raziuddin *et al.*, 1991, Gasparini *et al.*, 2015).

The correlation between helper T cells (T_h) response and age of patients can be identified during convalescence: in particular, a T_h1 response (low IL-10/IFN- γ proportion) and a remarkably proliferative T_h2 response (higher IL-10/IFN- γ proportion) can be observed in young and old patients, respectively (Pollard *et al.*, 1999, Gasparini *et al.*, 2015). In general, a significant CD4+ T central memory response, via serum bactericidal antibodies, an indicator of protective immunity, can be observed (Cruz *et al.*, 2014). Despite that, the age correlated mucosal T effector/memory cell response may also occur with excluding the bactericidal antibodies activity (Davenport *et al.*, 2003).

1.3.9.3 Interaction of *N. meningitidis* with complement components

The first discovery of complement was in the 1890s, since it was found to assist or “complement(s)” the killing of bacteria by heat-stable antibodies present in normal serum (Walport, 2001). There are more than 30 proteins involved in the complement system, which are either present in a soluble form of proteins in the blood or as membrane-associated proteins (Sarma and Ward, 2011). Complement activation leads to a cascade of sequential enzymatic reactions, *i.e.* complement activation pathways, leading to the formation of

the potent anaphylatoxins C3a and C5a. These anaphylatoxins elicit a variety of physiological responses that range from chemoattraction to apoptosis (Sarma and Ward, 2011). Initially, complement was thought to be limited to an innate immune response where a robust and rapid response is needed against pathogens. However, lately increasing evidences are showing that complement also plays an important role in the adaptive immune response involving T and B cells to help in pathogens elimination (Dunkelberger and Song, 2010, Molina et al., 1996) and in producing immunologic memory preventing from the reoccurrence of infection (Sarma and Ward, 2011). It is also believed to be involved in tissue regeneration, tumor growth (Qu et al., 2009) and in different human pathological states (Wagner and Frank, 2010).

The complement system pathways

The complement system plays a crucial role in innate immune response against the meningococcal disease. Cases with terminal complement deficiencies are more susceptible to invasive meningococcal disease (Figuerola and Densen, 1991). The complement system has a three main pathways: (i) the classical pathway (CP) in which the C1 component binds to the antigen–antibody complex; (ii) the lectin pathway (LP) through

the recognition of specific microbial patterns including saccharides and related molecules by lectin (iii) the alternative pathway (AP) which is C3-dependant (Lewis and Ram, 2014).

The CP is activated when antibody/antigen complexes are formed by the binding of IgG or IgM to foreign and non-self antigens. The C1 complex is a multimeric complex consisting of C1q, C1r and C1s molecules (Sarma and Ward, 2011). The binding of C1q to the exposed Fc portion of IgG or IgM results in the activation of C1r and C1s. Then the C1s molecule cleaves C4 and C2 forming the CP C3 convertase, C4bC2a (Sarma and Ward, 2011).

The LP is activated by either mannose-binding lectin (MBL) or Ficolin when they bind to carbohydrate moieties on surfaces of pathogens, such as parasites, yeast, bacteria and viruses. MBL and Ficolin they are both circulating in the serum in complex forms with MBL-associated proteins (MASPs) (Sarma and Ward, 2011). There are four structurally related MASPs: 1, 2, 3 and a truncated MASP2 which is known as MAP19 (Sorensen *et al.*, 2005). The binding of MASP2 to pathogens induces conformational changes resulting in its autoactivation, which cleaves C4 into C4a and C4b. The binding of C4b to the surface of the pathogens induces C2 to bind, which is also cleaved by MASP2 to form C2b and C2a. The binding of C4b

and C2a together forms the LP C3 convertase C4bC2a (Sarma and Ward, 2011). The other MASPs' exact role is not fully known; however, MASP1 can help in enhancing complement activation by cleaving C2 but not C4 (Wallis, 2007).

The AP is triggered by carbohydrates, proteins and lipids structures on the surfaces of foreign and non-self bodies (Qu *et al.*, 2009). C3 molecule is frequently cleaved at a low level ("tick over") forming C3b fraction, which binds to foreign targets such as bacteria (Sarma and Ward, 2011). Subsequently, Factor B is recruited to the bound C3b, then it is cleaved by Factor D to form the C3 convertase C3bBb (Kemper *et al.*, 2010).

The C3 convertases, C4bC2a of CP and LP and C3bBb of the AP, consequentially cleave C3 to form C3a and C3b. C3b acts as an opsin to help in phagocytosis as well as further amplifying of complement activation (Sarma and Ward, 2011). Additionally, C3b binds the C3 convertases to form a complex known as C5 convertases (C3bBbC3b and C4bC2aC3b) (Sarma and Ward, 2011). The C5 convertases cleave C5 forming C5a and C5b molecules. C5b binds C6 and C7 and then C8 and multiple molecules of C9 binding to the C5bC6C7 complex, resulting in the formation of membrane attack complex (C5b-9, MAC) also called as the terminal complement complex

(TCC) (Sarma and Ward, 2011). The MAC complexes form pores on the targeted pathogens by inserting itself into their cell membrane, leading to cell lysis (Sarma and Ward, 2011).

In addition to the three main complement pathways, complement activation products can be generated by proteases released by neutrophils and macrophages, as well as factors such as Kallikrein, plasmin and factor XIIa (Hageman factor) (Sarma and Ward, 2011). Thrombin, a member of the coagulation pathway, in turn, can generate C5a locally in C3 deficient mice which are incapable of generating C5 convertase conventionally (Huber-Lang *et al.*, 2006).

Factor H binding protein

Many proteins have been identified to play a role in complement activation. The factor H binding protein (fhbp) is the most studied among them (Pizza and Rappuoli, 2015). It interacts with an inhibitor of the alternative complement pathway, known as human Factor H (Madico *et al.*, 2006, Schneider *et al.*, 2006). The fhbp/factor H binding enhances serum resistance to the meningococcus, which allows the bacterium to grow in the blood (Pizza and Rappuoli, 2015). Interestingly, the side chains of fHbp that interact with factor H resemble the glycosaminoglycan binding region of factor H on host cells, which might allow the meningococcus to recruit

factor H by mimicking the host through fhbp (Schneider *et al.*, 2009).

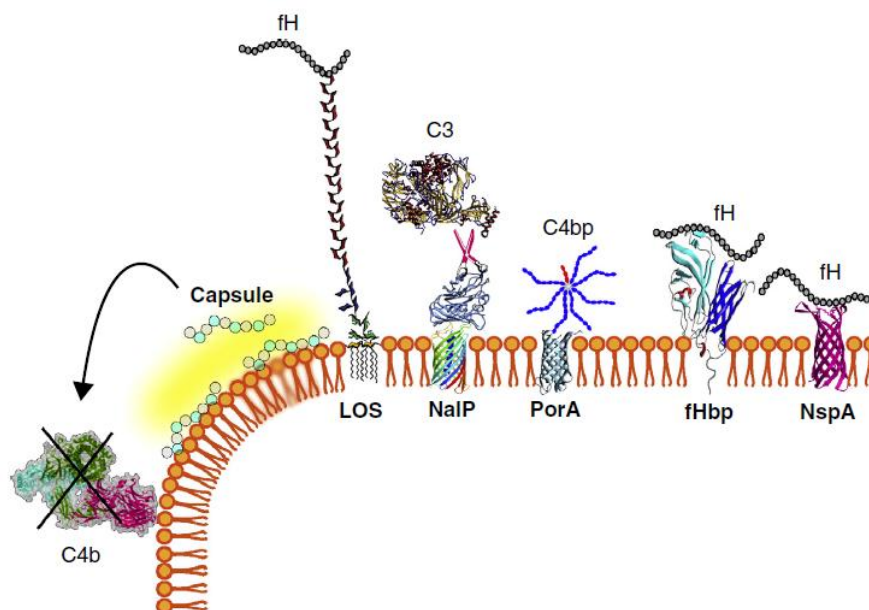
Redundant complement escape mechanisms

Recently, a number of other surface-exposed antigens were identified to inhibit the alternative complement pathway, suggesting that besides the fHbp characteristics meningococcus possesses a variety of mechanisms that enhance the evasion of human immune system. NspA (*Neisseria* surface protein A) is like fhbp, which can bind human factor H at the domains 6 and 7, and acts jointly with fHbp (Lewis *et al.*, 2010, Lewis *et al.*, 2012). PorB2 (Porin B2) shows the ability to bind human fH as well as to regulate the alternative complement pathway in baby rabbit and infant rat (Lewis *et al.*, 2014). In the event of C3 fragments became in contact with the bacterial surface, the sialylation level of LOS has an impact on the binding efficiency of domains 18–20 in factor H C-terminal to bacteria (Lewis *et al.*, 2012).

The capsular polysaccharides also can modulate multiple pathways of the complement system. Agarwal *et al.* recently demonstrated that the capsular polysaccharide of serogroups B, C, W, and Y inhibits the CP by inducing less C4b deposition, which limits the capability of antibodies to mediate bacterial killing. In this study, they showed that different strains

exhibited different susceptibility to anti-fHbp antibodies and complement, in spite of similar antibody binding to the bacterial surface, which suggests potential implications for the activity of vaccines based on fHbp antigen (Agarwal *et al.*, 2014). Also, PorA has been shown to bind a major CP inhibitor that C4b-binding protein (C4bp), in order to escape CP complement activation (Jarva *et al.*, 2005).

An additional mechanism to evade the complement system is mediated by NalP, a serine protease autotransporter (van Ulsen *et al.*, 2003), which cleaves C3 four amino acids upstream from the natural C3 cleavage site. That produces shorter C3a-like and longer C3b-like fragments liable to degradation, resulting in lower deposition of C3b on the bacterial surface which enhances the meningococcal serum resistance (Del Tordello *et al.*, 2014). These meningococcal mechanisms to evade complement may have important implications for meningococcal vaccines based on one antigen.



Schematic illustration for some of the *N. meningitidis* OMPs interacting with complement components.

Capsule (capsular polysaccharide); LOS (lipooligosaccharide); NalP (Neisseria autotransporter serine protease); porA (porin A); fhbp (factor H binding protein); NspA (Neisseria surface protein A). Adapted from (Pizza and Rappuoli, 2015).

1.3.9.4 Other strategies to evade the immune system

Although the temperature fluctuation is underscored, it plays an important role in the microbial pathogenesis, colonisation, invasion, and immune evasion. In contrast to mammals that exhibit body thermo-stabilisation, other animals' and pathogens' temperature fluctuates on a daily basis. In a recent study by Loh and collaborators, they identified the molecular bases of this temperature-dependent immune evasion strategy in *N. meningitidis* (Loh *et al.*, 2013). They have discovered

three thermo-sensor RNAs located in the 5' untranslated regions (UTRs) of genes involved in the capsule biosynthesis, fHbp expression, and LOS sialylation. Temperature elevation, as during inflammation by co-infection with other pathogens (e.g. influenza virus), "alarms" the meningococcus and activates its immune evasion mechanisms. Although this could be a possible explanation for how the meningococcus determines to transform from a commensal pathogen into a virulent one, however, the precise nature of this mechanism remains unclear (Gasparini *et al.*, 2015).

Clustered regularly interspaced short palindromic repeats- (CRISPR-) Cas9 is another molecular mechanism allows *Neisseria* to evade immune surveillance. Cas9 is an essentially two-faced mechanism, while, on one hand, is being involved in gene expression and regulation, as it limits the possibility of editing the *Neisseria* genome via HGT/LGT or the insertion of exogenous DNA fragments, and therefore it restricts the microbial variability. On the other hand, it can repress the expression of bacterial lipoprotein that facilitates evasion of TLR2 by the bacteria (Sampson and Weiss, 2014).

Another mechanism is known as the molecular decoy, in which the bacteria deceive the host immune system. For instance, the antigenic subdomain of fprB can bind antibodies, which is

not essential for functioning as autotransporter (Saleem *et al.*, 2013). However, *fprB* is subject to a high degree of antigenic variation, is a phase-variable gene, is involved in nutritional immunity, and moreover exploits a molecular decoy (Gasparini *et al.*, 2015). That makes *fprB* a useful example to show how *Neisseria* can simultaneously use the previously described immune evasion strategies.

1.3.9.5 Toll-like receptors

So far, 10 members of the human Toll-like Receptor (TLR) family have been characterized. These receptors recognise a variety of microbial products and fragments (Zhang *et al.*, 2007). For example, TLR2 recognises lipoproteins and lipoteichoic acid, TLR3 and TLR7 recognise several forms of viral RNA, TLR4 recognises LPS, flagellin is recognised by TLR5 and unmethylated CpG DNA motifs by TLR9. TLRs are type-1 transmembrane proteins containing an extracellular domain composed of a leucine-rich repeat region and an IL-1 receptor homologous cytoplasmic moiety. Nowadays, three members of TLR family have been shown to be involved in the process of *N. meningitidis* recognition: TLR2, TLR4 and TLR9 (Schmitt *et al.*, 2009).

TLR4

The main function of TLR4 is the recognition of the LPS molecule in gram-negative bacteria (Miller *et al.*, 2005). It works collaboratively with a co-receptor molecule known as MD-2, which binds to TLR4 in the Golgi apparatus (Fitzgerald *et al.*, 2004). The TLR4/MD2 complex works alongside other proteins in the process of LPS recognition. One of these proteins is an acute-phase serum protein referred to as LPS-binding protein (LBP), which is mainly produced in lung and liver (Schmitt *et al.*, 2009). LBP enhances the sensitivity of LPS binding to CD14, another LPS recognition protein (Jack *et al.*, 1997).

The lipid A moiety in the meningococcal LOS molecule is the portion recognised by TLR4/MD2, resulting in immune cell activation. However, the number of acyl chains in the lipid A is an important factor in triggering the proinflammatory response via TLR4 and the hexa-acylated lipid A is considered to contain the optimum number of acyl chains to be recognised by TLR4 (Zughaier *et al.*, 2004, Zughaier *et al.*, 2006, Zimmer *et al.*, 2007). The addition of acyl chains is regulated by two lauroyl acyltransferase genes (*lpxL1* and *lpxL2*), which are present in a number of gram-negative bacteria as well as *N. meningitidis* (van der Ley *et al.*, 2001). A natural *lpxL1* mutation has been

identified in serogroup Y *N. meningitidis* (mainly cc23 isolates), which can be considered as a method to evade immune recognition post-crossing the epithelium (Ladhani *et al.*, 2012).

The meningococcal LOS recognition by TLR4 provokes both MyD88-dependent and independent signalling pathways (Zughaier *et al.*, 2005). The MyD88-dependent pathway leads to the activation of MAPKK and IKK complex resulting in activation and nuclear translocation of AP-1 and NF- κ B, respectively (Bagchi *et al.*, 2007). The MyD88-independent pathway has TRIF as the main adaptor protein instead of MyD88 and can associate with TRAF6 to activate AP-1 and NF- κ B as well. TRIF can additionally interact with TRAF3 and the phosphatidylinositol 3-kinase (PI3K)-AKT pathway resulting in the nuclear translocation of IRF3 and IRF2, correspondingly. NF- κ B activation leads to the production of TNF α , IL-1, 6 and 8, whereas IRF3 leads to INF β and RANTES production.

TLR2

TLR2 ligands are significantly broader than those for TLR4. The reason behind this fact might be because TLR2 can recognize its ligands as a heterodimer in association with TLR1 or TLR6 (Kirschning and Schumann, 2002, Wetzler, 2003). The signalling pathway activated by TLR2; has been shown to be

dependent on two functional proteins at least, MyD88 and TIRAP, which are known to cooperate with TLR4 also (Kirschning and Schumann, 2002, Beutler, 2004). However, in the manner of a TLR1- and TLR6-independent recognition, there is recent proof that TLR2 homodimers can recognise di- and triacylated lipopeptides (Buwitt-Beckmann *et al.*, 2006). In the case of *N. meningitidis*, a previous study has shown evidence of the ability of the OMP *PorB* to activate cells by directly binding TLR2. Additionally, a complex of TLR2/TLR1 can trigger cell activation by binding *PorB* (Massari *et al.*, 2006). Meningococcal *PorB* influences the maturation of murine DCs through TLR2, resulting in an increased expression of CD86 and both classes of MHC molecules (Massari *et al.*, 2003, Singleton *et al.*, 2005).

TLR9

TLR9 is located within endosomal compartments in contrast to TLR2 and TLR4, which are basically localised at the cell surface. TLR9 functions as a recognition tool for bacterial unmethylated CpG motifs, as in the mammalian DNA CpG motifs are mostly methylated and present at low frequency (Hemmi *et al.*, 2000). Several studies have suggested that live *N. meningitidis* and its purified genomic DNA are capable of

stimulating IL-8 production and NF- κ B activation via TLR9 (Schmitt *et al.*, 2009).

1.4 Conclusion

From the previously reviewed literature, the importance of understanding the interaction of *N. meningitidis* with host cells has been highlighted. Our work is focusing on the interaction between *N. meningitidis* and host respiratory epithelium, which forms the first barrier that confronts meningococcal colonisation and invasion. We aim to study the impact of Neisserial antigenic variation on the response of epithelial cells against different meningococcal strains and variants, mainly from serogroup B and Y. The study will cover different aspects, such as cytokines response, phase variation, adhesion/invasion pattern and the damage of epithelial monolayer.

CHAPTER TWO

CHARACTERISATION OF *NEISSERIA MENINGITIDIS* LPXL1 MUTANTS

2. GENERATION AND CHARACTERISATION OF *NEISSERIA MENINGITIDIS* LPXL1 MUTANTS

2.1 Introduction

Antigenic variation is one of the most effective strategies used by *N. meningitidis* to evade immune recognition, as well as to provide functional variants that alter the interaction between virulence factors and host molecules. *N. meningitidis* undergoes antigenic variation via several strategies, such as HGT/LGT (Gasparini *et al.*, 2015), homologous intragenic recombination (Cahoon and Seifert, 2011), prophages (Power and Moxon, 2006) or phase variation (Moxon *et al.*, 2006) (see 1.3.5). The variability of LOS and other neisserial antigens is a major advantage to *N. meningitidis* facilitating its success in pathogenesis. The production of meningococcal LOS, similar to other meningococcal virulence factors, is liable to phase variation, which facilitates the immunotype switching (Figure 2.1) (Berrington *et al.*, 2002, Saunders *et al.*, 2000, Jennings *et al.*, 1999). Moreover, there are increasing evidences for immune avoidance based on phase variation includes the *in vitro* resistance of *N. meningitidis* to bactericidal antibodies against the LgtG LOS modifier (Bayliss *et al.*, 2008)

N. meningitidis LOS can be divided into 12 immunotypes (L1–L12). The immunotypes (ITs) formation is controlled by LOS glycosyltransferases, which are involved in LOS biosynthesis and encoded by three genetic loci designated *Igt* (Figure 2.1). The L8 IT has a limited lacto-*N*-neotetraose (LNnT) α -chain linked to the inner core HepI, while L3 and L7 ITs have full-length LNnT α -chain and terminal sialic acid just in L3 (Zhu *et al.*, 2001).

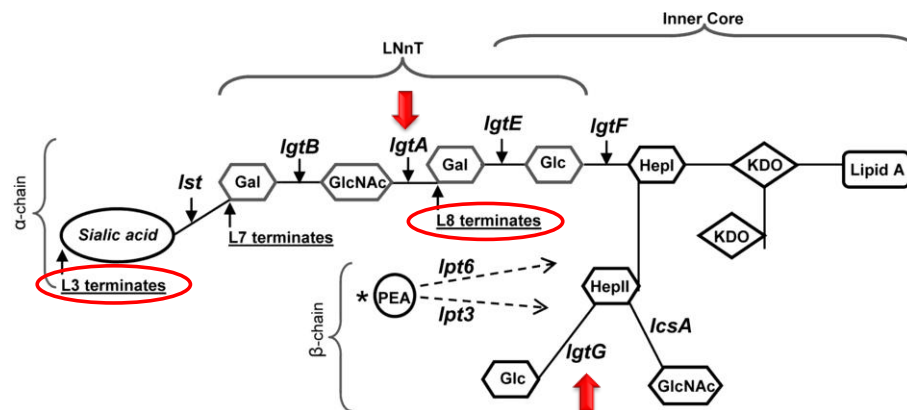


Figure 2.1: Diagram for LOS structure in MC58. MC58 can form different ITs by changing its LOS structure.

L3 IT is sialylated and has a full-length LNnT α chain. The red arrows are pointing to phase variable genes. The protein encoded by *IgtA* is responsible for truncating the LNnT α chain forming a non-sialylated L8 IT, and the product of *IgtG* is responsible for the glucose extension from the second heptose (β -chain extension). adapted (Hill *et al.*, 2010).

The lipid A moiety in the meningococcal LOS molecule is recognised by TLR4/MD-2, leading to the activation of immune

cell (see 1.3.9.5). The acylation level of lipid A plays an important role in triggering the proinflammatory response, the hexa-acylated lipid A has the optimal number for recognition (Zughaier *et al.*, 2004, Zughaier *et al.*, 2006, Zimmer *et al.*, 2007). The addition of secondary acyl chains to lipid A is mediated by proteins encoded by two genes (*lpxL1* and *lpxL2*) (van der Ley *et al.*, 2001).

Several different types of mutations (including single nucleotide deletions or insertions, or insertions of *IS1655* or *IS1301*) have been identified which inactivate *lpxL1* in certain meningococcal strains (Fransen *et al.*, 2009, Ladhani *et al.*, 2012, du Plessis *et al.*, 2014). Notably, strains from the serogroup Y associated, clonal complex 23 (cc23) harbour a single nucleotide deletion at the 5' end of *lpxL1* resulting in a lack of *LpxL1* expression (Figure 2.2). Production of a penta-acylated LOS has been suggested as a strategy to evade innate immune recognition (Ladhani *et al.*, 2012). Patients infected with *lpxL1* mutant meningococci presented significantly less frequently with rash and had higher thrombocyte counts, consistent with reduced cytokine induction and less activation of tissue factor mediated coagulopathy (Fransen *et al.*, 2009).

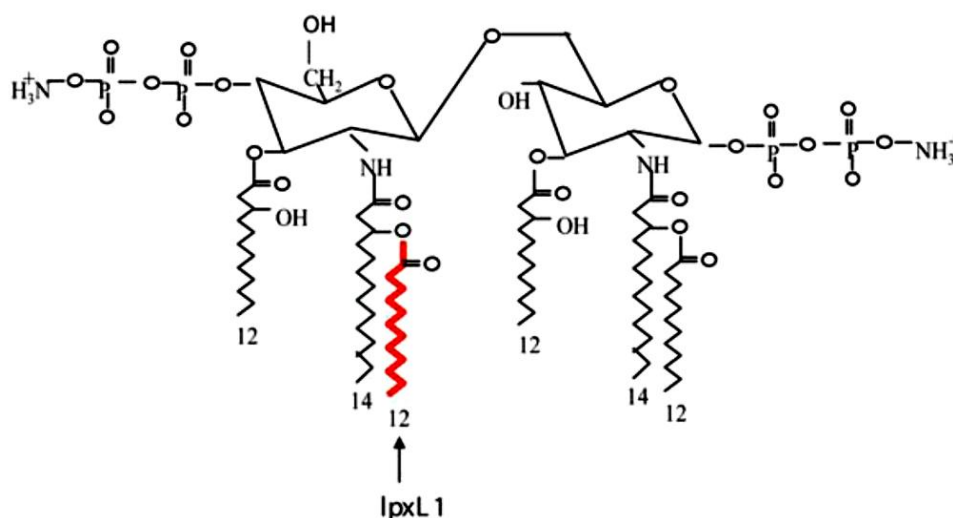


Figure 2.2: The lipid A structure of *N. meningitidis*.

Meningococcal lipid A is hexa-acylated with 12-14 carbon acyl chains. The arrow points to the acyl chain absent in strains harbouring inactivated *lpxL1* genes (Fransen *et al.*, 2009).

TLR4-mediated recognition of meningococcal LOS induces both MyD88-dependent and independent signalling pathways (Zughaier *et al.*, 2005). The MyD88-dependent pathway leads to the activation of MAPKK and IKK resulting in activation and nuclear translocation of AP-1 and NF- κ B, respectively (Bagchi *et al.*, 2007). The activation of both pathways leads to the production of several cytokines and chemokines, such as TNF- α , INF β , RANTES, and IL-1, 6 and 8.

Previously, our lab investigated the cytokines (IL-10, IL-6 and TNF- α) produced by Peripheral Blood Mononuclear Cells

(PBMCs) in response to the serogroup B strain MC58 and three different serogroup Y strains: N222 (cc23); N117 (cc167) and N52 (cc174). Only N222 harboured a mutation resulting in an inactivated *lpxL1* gene. The results showed that IL-10 production in response to N222 was significantly lower than that induced by the other strains (data not shown). In a recent study of lipid A variants isolated from patients with invasive meningococcal disease in the Netherlands revealed that *lpxL1* mutation is linked to a low IL-6 induction in human monocytic cell line Mono Mac 6 (Rodenburg *et al.*, 2012). John *et al.* found that the production of TNF- α , in Human monocyte-like cell line (THP-1) and primary human monocytes, varied by approximately 2-10-fold by differences in the state of acylation and phosphorylation of the lipid A moiety of LOS (John *et al.*, 2009).

2.2 Hypothesis

The meningococcal lipid A normally has six acyl chains, the optimal number for TLR4 activation. The addition of acyl chains in meningococci is regulated by the products of two genes (*lpxL1* and *lpxL2*). Strains from the cc23 lineage of serogroup Y meningococci are invasive, despite being naturally *lpxL1*-deficient. We hypothesised that lack of one the secondary acyl chains in lipid A would have an effect on

meningococcal recognition by immune cells and could affect the infection pattern by reducing the toxicity of the bacteria.

2.3 Aim

An MC58 derivative lacking LpxL1-production (*i.e.*, a *lpxL1*-knock-out) and a N222 derivative expressing LpxL1 (*i.e.*, a *lpxL1*-knock in) will be generated. These strains will then be used, in conjunction with wild-type controls, to infect differentiated bronchoepithelial cells (Calu-3). We aim to investigate how changes in lipid A structure can affect recognition of strains expressed hexa- or penta-acylated LOS. Bacterial growth and production of inflammatory responses will be determined. This study will help determine whether *lpxL1* mutations affect host responses.

2.4 Materials and methods

2.4.1 Bacterial strains and growth conditions

The strains used in this study are described in (Table 2.1). Meningococcal -80°C stocks were prepared in 20% glycerol/Brain Heart Infusion (BHI) broth (Oxoid). Strains were grown from -80°C stocks on Columbia Agar with chocolate Horse blood (Oxoid). Suspension cultures were grown in BHI broth. Meningococcal transformation and antibiotic selection were performed on BHI agar (Oxoid). All the meningococcal agar culture incubations were at 37°C, 5% CO₂. All the broth culture incubations were at 37°C, 200 rpm. In addition, *E. coli* JM109 chemically competent cells (Promega) were used for the propagation of plasmids. *E. coli* JM109 were cultured in Luria-Bertani (LB) broth or on LB agar (Sigma-Aldrich) and incubated at 37°C, 200 rpm for suspension cultures and static for agar plates. MC58Δ*lgtF* was used as a control for the LOS silver staining analysis, as this strain lacks the outer LOS core (LNnT α chain) due to insertional inactivation of *lgtF* (Figure 2.1).

Table 2.1: General information for the meningococcal strains used in this study

Strain	Sero-group	Clonal complex	Description	Source or reference
MC58	B	CC32	Wild type	(Tettelin <i>et al.</i> , 2000b)
H44/76	B	CC32	Wild type	(Frasch <i>et al.</i> , 1985)
N222.1	Y	cc23	Clinical carriage isolate	(Bidmos <i>et al.</i> , 2011)
MC58Δ <i>lgtF</i>	B	CC32	<i>lgtF</i> deleted and replaced with a kanamycin resistance cassette	In-house

2.4.2 Growth curves

N. meningitidis strains were cultured on Columbia Agar with chocolate Horse blood for 18 h at 37°C, 5% CO₂. Dulbecco's modified Eagle's medium-Ham's F-12 medium (DMEM F12) (Sigma) was used for the growth study, as it was the same media used to grow the bronchial epithelial cell line to be infected (Calu-3). All the optical density (OD) measurements were made using OD₆₀₀ DiluPhotometer™ (IMPLEN).

Growth curves were generated as follows: one colony from 18-hour culture plate was inoculated in 20 mL DMEM/F-12 in a 50 ml Falcon tube, and incubated at 37°C, 240 rpm, overnight. The OD₆₀₀ was measured and then the following formula was applied to calculate what volume was needed to start the experiment with.

$$\frac{\text{desired initial OD}}{\text{overnight culture OD}} \times \text{media volume} = \text{the inoculum volume needed}$$

The initial OD for this study was 0.05. The calculated volume was inoculated and the OD was measured and recorded as a confirmation for the calculation and time zero (T0) reading. Afterwards, the culture was incubated for two hours before taking the second OD reading (T2); ODs were measured hourly for 8 hours.

To equate OD to colony forming units (CFU), at each time point, duplicate serial dilutions from each culture were made in 96-well round bottom TC plates, and 50 μL of each dilution plated on a chocolate agar plate. After incubation, the number of colonies was counted, and CFU mL^{-1} calculated.

2.4.3 Cloning of *lpxL1*

MC58 genomic DNA was provided by Dr. Neil Oldfield. The *lpxL1* gene (NMB1418) was amplified using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) according to the manufacturer's instructions using oligonucleotide primers (Eurofins MWG Operon). The forward primer 1420F1 (5'-AACACATAGGCTGGTACATCGACG-3') was located 1 kb upstream of the *lpxL1* start sequence, and the reverse primer 1417R1 (5'-TTAGCAATGACCACTTCCTCGCCG-3') was located 1 kb downstream of the *lpxL1* stop sequence. Go Taq polymerase (Promega) was used to add A bases to both ends of the PCR product. The resulting PCR product (2.9 kb) was

purified using the QIAquick PCR purification kit (Qiagen) and cloned into the pGEM[®]-T easy vector (Promega) using T4 DNA Ligase (Promega) and a 3:1 insert to vector ratio overnight at 4°C. *E. coli* JM109 chemically competent cells (Promega) were transformed with the ligation mix and clones were selected by blue/white screening (Figure 2.3). Plasmids were extracted using QIAprep Miniprep Plasmid DNA purification kit (Qiagen) and analysed by restriction digestion and DNA sequencing. All gel electrophoresis was done in 0.8% agarose gels in Tris-Acetate-EDTA (TAE) buffer (40mM Tris-acetate, 1 mM EDTA, pH 8.0) at 120 V for 35 min. One correct clone was designated pRA1.

2.4.4 Construction of an MC58Δ/*lpxL1* mutant

2.4.4.1 Generation of the mutational plasmid pRA1

To generate a mutated *lpxL1* containing a kanamycin resistance cassette (*kanR*), an inverse PCR (INPCR) was performed using as template pRA1 and the primers 1418R1 and 1418F2. 1418R1 (5'-CGCGGATCCCCCGTCAGGTCGGCAATCTTGTGC-3'), is located 68bp downstream of the *lpxL1* start codon and includes a BamHI restriction site. 1418F2 (5'-CGCGGATCCGAAGACAGGGTGCGCGAACATCCG-3') is located 64 bp upstream of the *lpxL1* stop codon and also includes a

BamHI restriction site. The INPCR amplification product was BamHI-digested, self-ligated and then transformed into *E. coli* JM109 and transformants selected using ampicillin ($100 \mu\text{g mL}^{-1}$) supplemented LB agar. Plasmids were extracted using QIAprep Miniprep Plasmid DNA purification kit (Qiagen) and analysed by restriction digestion and DNA sequencing. One correct clone was designated pRA2.

The *KanR* was obtained by digesting pJMK30 with BamHI (Tunio *et al.*, 2010). The fragment was purified by gel extraction, using the QIAquick Gel Extraction Kit, then ligated to pRA2 digested overnight at 37°C with BamHI and dephosphorylated with Antarctic Phosphatase (New England Biolabs). The ligation mix was used to transform JM109 competent cells. Plasmids were extracted using QIAprep Miniprep Plasmid DNA purification kit (Qiagen) and analysed by restriction digestion and DNA sequencing. One correct clone was designated pRA3.

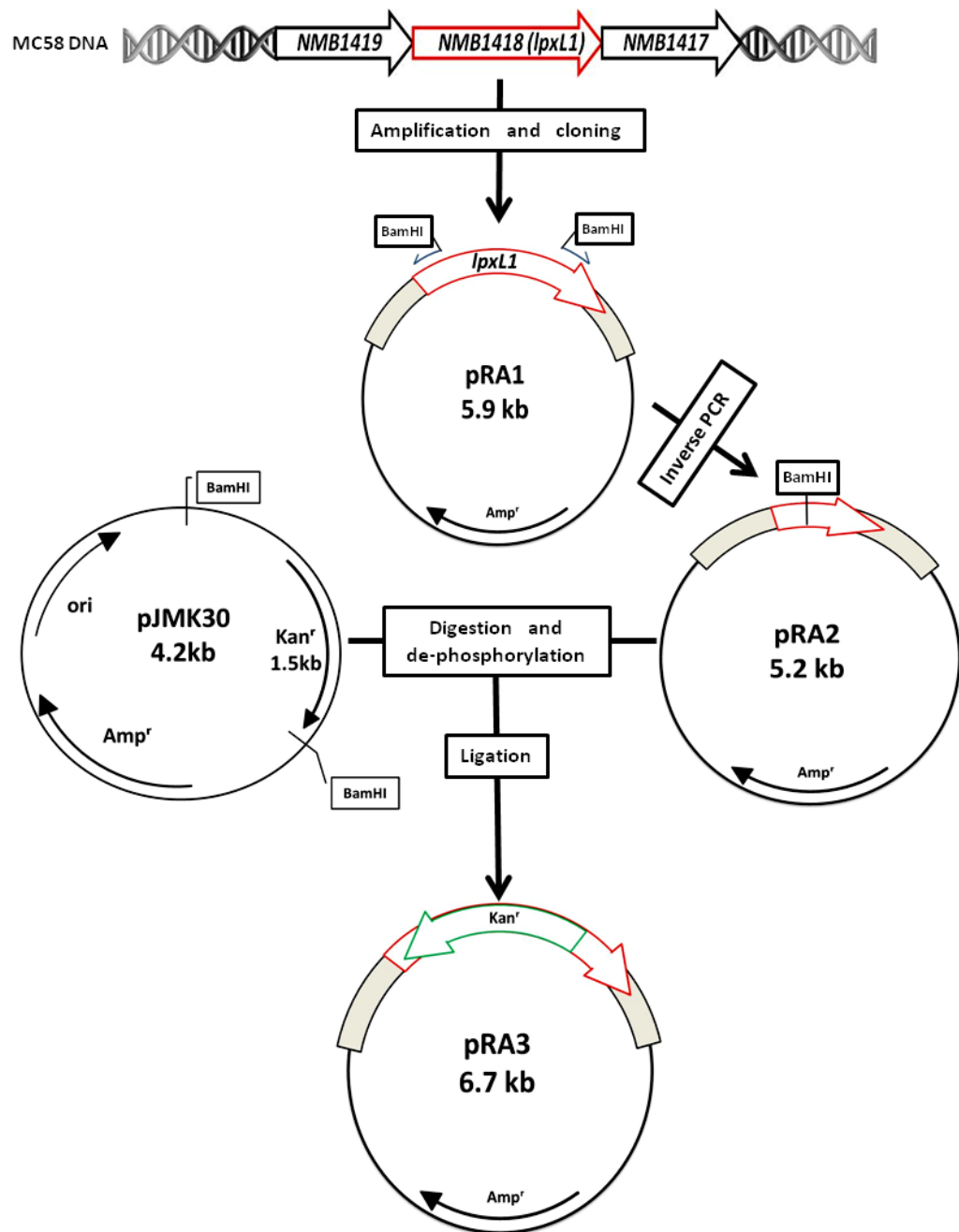


Figure 2.3: Cloning of MC58 *lpxL1* and generation of the mutational plasmid pRA3.

The diagram shows cloning of amplified MC58 *lpxL1* and flanking sequences into pGEM-T-Easy and the strategy for the generation of the deletion in *lpxL1* and replacement with *kanR*.

2.4.4.2 Natural transformation of *N. meningitidis*

Wild-type (WT) MC58 was plated out from a glycerol stock on chocolate horse blood agar and grown overnight at 37°C in the presence of 5% CO₂. Next day, BHI broth (5 mL in 15 mL Falcon tube) was inoculated with a single colony, and incubated at 37°C, 200 rpm till it reached OD₆₀₀ ≈ 0.5. A 20 µL aliquot was spotted onto a BHI agar plate and incubated for a 100 min at 37°C in 5% CO₂. A spot of pRA3 (up to 20 µL or 2 µg) was placed on the top of the bacterial growth and left in the safety cabinet till the DNA was absorbed into the bacteria. The plate was incubated for 4 h at 37°C, 5% CO₂ and the bacteria removed with a sterile plastic loop and re-suspended in 1 mL of BHI broth. A 100 µL aliquot was then spread onto a BHI plate supplemented with kanamycin (50 µg mL⁻¹), and the rest of the 1 mL bacterial suspension was spun down and the pellet re-suspended in 100 µL of BHI broth, and then spread on another supplemented BHI plate. Plates were incubated for a maximum of 2 days at 37°C in 5% CO₂. The resultant clones were picked and subcultured further onto kanamycin-supplemented BHI plate for further confirmation.

2.4.4.3 Confirmation of *lpxL1* mutation by PCR

To confirm the mutation, chromosomal DNA was purified from transformants using the QIAamp DNA Mini Kit (QIAGEN). PCR

was conducted using Taq DNA Polymerase (New England Biolabs), and using 1 µg of the purified DNA as a template in 20 µL reaction volume with combinations of the following primers: 1419F2 (5'-GTGGTGCAGATGTTGGGACTTTCG-3'); 30-KAN-CTERM (5'-GGTATGACATTGCCTTCTGCG-3'), 1420F2 (5'-CCTGCCTTGAGTTTGGCAGAATGC-3'; and 30-KAN-CTERM (5'-GGTATGACATTGCCTTCTGCG-3'.

2.4.5 MC58Δ/*px1* complementation

A plasmid named pMT2, carrying a functional copy of *lpxL1* located upstream of a erythromycin resistance cassette (*ermC*) (Figure 2.4), constructed by Dr Neil Oldfield and MSc student Michael Timpany, was used to transform MC58Δ/*pxL1* as described previously in section (2.4.4.2), but using erythromycin (5 µg mL⁻¹) as a selection antibiotic alongside with kanamycin (50 µg mL⁻¹).

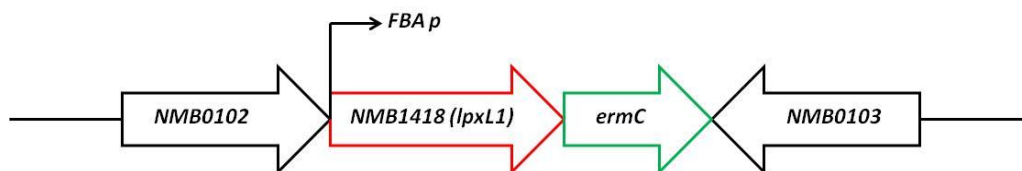


Figure 2.4: Diagram showing the site of insertion of the intact *lpxL1* gene and the selection marker within the MC58 genome between genes *NMB0102* and *NMB0103*).

To ensure high-level gene expression, the *lpxL1* ORF was placed downstream of the promoter from fructose biphosphate aldolase (FBA), a constitutively expressed enzyme from the meningococcal glycolytic pathway.

To confirm successful insertion, chromosomal DNA from transformants selected in the presence of kanamycin and erythromycin was purified using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich). Two primer pairs were used to confirm the complementation. The first set of primers, NMB102F (5'-GTTCGGCGGCTTGCGTGGTCG-3') and 1418R1 (5'-CGCGGATCCCCCGTCAGGTCGGCAATCTTGTGC-3'), were used to confirm the insertion of *lpxL1*^{Ect} and *ermC* cassette in the right position between the NMB0102 and NMB0103 genes.

2.4.6 LOS enrichment and silver staining

Meningococcal strains were grown in 5 mL of DMEM/F-12 overnight at 37°C, 200 rpm. 1 mL of exponential phase cultures (OD₆₀₀ 0.6) were spun down (6000 X *g*, 10 min). Bacterial pellets were resuspended in 200 µL of SDS-PAGE

loading buffer (SDS polyacrylamide gel electrophoresis; 100 mM Tris-Cl pH 8.0, 2% β -mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 0.2% xylene cyanol, 20% glycerol), boiled for 10 min and chilled on ice. Afterwards, proteinase K was added to a final concentration of 0.5 mg mL⁻¹ and samples were incubated at 65°C for 2 h. This procedure was based on the method from (Sprott *et al.*, 1994).

Table 2.2: Tricine SDS-PAGE gel constituents

Constituent	4% stacking gel	18% separating gel
30% Protogel acrylamide	1.5 mL	18 mL
4 x Gel Buffer (4 M Tris-HCl; 0.4% (w/v) SDS, pH 8.45)	1.86 mL	7.5 mL
Glycerol	-	3.15 mL
Distilled water	6.64 mL	1.35 mL
Ammonium persulphate (10 mg mL ⁻¹ ; Sigma)	35 μ L	120 μ L
TEMED (N, N, N', N'-tetramethyl- ethylenediamine; Sigma)	18 μ L	12 μ L

LOS preparations (30 μ L) were loaded in an 18% Tricine SDS-PAGE in a Protean II gel (Table 2.2). Remaining wells were loaded with 30 μ L SDS-PAGE loading buffer. Color Ultra Low Range Markers (Sigma-Aldrich) were run alongside the LOS preps. The gels were run for 16 hr at 15 mA per gel. To visualise LOS gels were silver stained using a method based on that of (Tsai and Frasch, 1982). First, the separating gel was fixed overnight in a solution of 40% (v/v) ethanol and 5% (v/v) glacial acetic acid. Then the gel was incubated for 5 min

in oxidative solution (0.7% w/v periodic acid, 40% (v/v) ethanol and 5% (v/v) glacial acetic acid). The gel was then washed three times in distilled water for 10 min per wash. Afterwards, the gel was transferred to a pre-staining solution (56 mL 0.1 M NaOH, 4 mL concentrated (30%) NH_4OH and 230 mL distilled water). While the gel was in the pre-staining solution and shaken gently, 10 mL of freshly made 20% (w/v) AgNO_3 were gradually added. The gel was incubated for 10 min with gentle shaking to allow staining, washed twice with distilled water (5 min each) to remove any excess stain before developing. The gel was transferred to the dark room and developed using developing solution (10 mg citric acid and 100 μL 37 % formaldehyde in 200 mL distilled water). The gel was incubated in this solution with gently shaking until the bands appeared. After the visualization of bands, the gel was rinsed several times with distilled water to halt the development.

2.4.7 Analysis of *lpxL1* transcription

Total RNA was extracted from meningococcal strains using the TRI Reagent method (Sigma-Aldrich), followed by DNase treatment with TURBO™ DNase (Ambion). cDNA was synthesised using the Power cDNA Synthesis Kit from (iNtRON). Then cDNA PCR was performed using the primers LpxL1_rtF1 (5'-GTTGGAATACGGTTTATATTGGTACGC-3') and

LpxL1_rtR1 (5'-TGCAATGTAACCGTATTGTCTGCCTCG-3'), both designed to bind to the deleted portion of *lpxL1* which was absent in the *lpxL1* mutant. The resulting PCR product had an expected size of 500 bp.

2.5 Results

2.5.1 Generation of a plasmid construct to enable mutation of MC58 *lpxL1*

Construction of an *N. meningitidis lpxL1* mutant was initiated by amplifying the *lpxL1* gene and upstream and downstream sequence from the MC58 chromosomal DNA using the 1420F1 and 1417R1 primers. The resulting 2.9 kb PCR product was successfully cloned in the pGEM-T easy vector to generate the first plasmid pRA1 with the size of 5.9 kb. Deletion of *lpxL1* was achieved by inverse PCR using pRA1 as a template and the primers 1418F2 (BamHI site) and 1418R1 (BamHI site). The reaction gave a 5.17 kb PCR product that when self-ligated generated plasmid pRA2. pRA2 was digested with BamHI and a dephosphorylation step was included in order to avoid self-ligated ligation products. The digested, dephosphorylated product was ligated to BamHI digested *KanR* cassette. JM109 cells were transformed with the ligation mix. Twelve clones were obtained and the extracted plasmids were tested by digestion with restriction enzymes (BamHI and ClaI). The digestion with ClaI confirmed the insertion of *KanR* cassette and showed its reverse orientation to the *lpxL1* gene in all clones (Figure 2.5). One of the plasmids was DNA sequenced which confirmed: (i) the presence of the deletion;

(i) the orientation of the resistance cassette; (iii) the absence of any PCR-generated errors. This clone was designated pRA3 and was subsequently used to transform MC58 WT. Therefore, the plasmid containing a mutated version of *lpxL1* in which most of the coding region is substituted by a *KanR* cassette is generated.

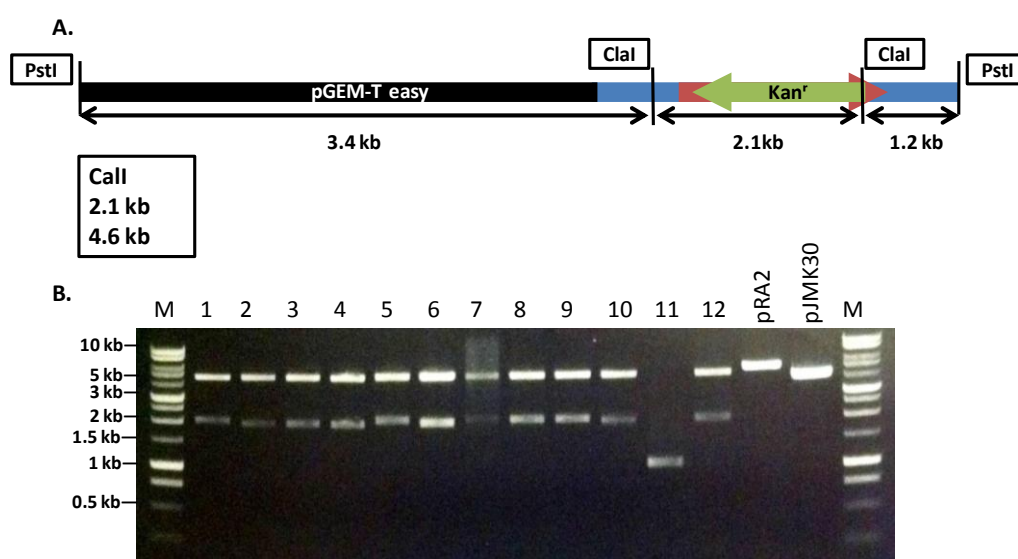


Figure 2.5: Diagram and restriction analysis of putative pRA3 clones with the enzyme ClaI.

(A) Diagram explaining the restriction sites on the pRA3 construct for the enzyme (ClaI) and the expected fragment sizes. (B) Agarose gel electrophoresis analysis shows that bands with the expected sizes were present in most clones, which confirms the *KanR* cassette insertion and its inverted orientation.

2.5.2 MC58Δ*lpxL1* generation

MC58 WT was transformed with pRA3, and initially, only one clone was confirmed to have the mutation by PCR. Therefore,

a second transformation was performed and 27 further clones were obtained. Two conformational PCR were conducted and expected product sizes from the both PCR are shown in (Table 2.3).

Table 2.3: The expected product sizes from PCRs used to confirm complementation of MC58Δ*lpxL1*

Strain	Reaction	
	1 st PCR	2 nd PCR
	NMB102F/1418R1	30-KAN-C-TERM/1420F2
MC58 WT	No product	No product
MC58Δ <i>lpxL1</i>	No product	1.2 kb
MC58Δ <i>lpxL1 lpxL1</i> ^{Ect}	0.7 kb	1.2 kb

Colony PCR analysis using the primers 1419F2 and 30-KAN-C-TERM revealed that all the clones contained the mutation as they showed the expected 500bp product (Figure 2.6 and Figure 2.7). Some of those clones were selected (B, D and E) for further examination.

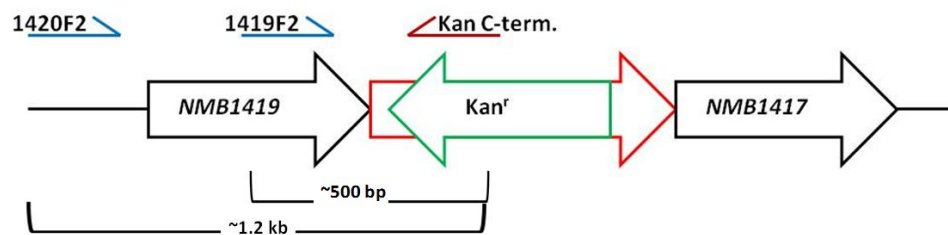


Figure 2.6: Schematic diagram showing the binding sites of the primers used to confirm the mutation in *lpxL1* (gene in red) by PCR and the resulting product sizes generated.

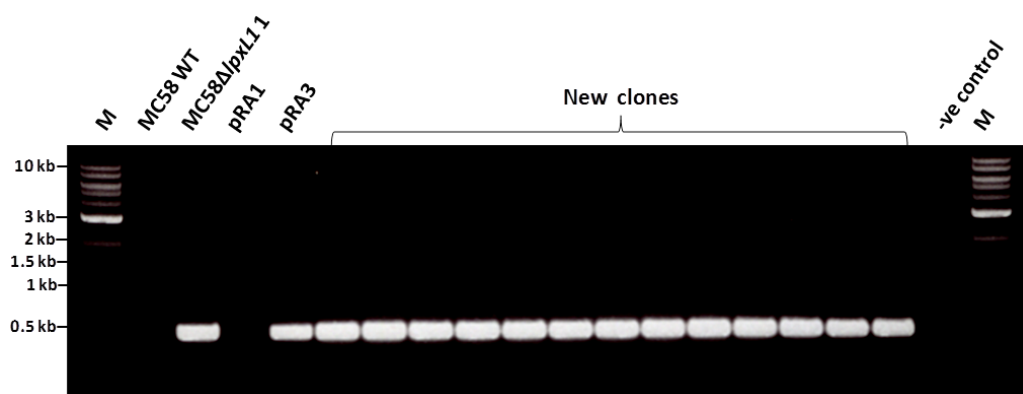


Figure 2.7: Analysis of the 1419F2/30-KAN-C-TERM PCR products by agarose gel electrophoresis.

A band with a size close to 500 bp indicated a successful transformation. This gel shows results for some clones and similar result were obtained with the rest of the clones.

The second PCR, using the primers 1420F2 and 30-KAN-C-TERM, confirmed that we had succeeded in deleting the *lpxL1* gene and replace it with *KanR* cassette (Figure 2.8 A). Furthermore, it shows that the cassette's orientation is reversed, which agrees with the pRA3 digestion analysis using *ClaI* (see section 2.5.1). The LOS extracted from selected mutated clones was then examined by SDS-PAGE and silver staining. Both D and E clones showed a size reduction in their LOS indicated by the change in their electrophoretic mobility (clone D shown in Figure 2.8 B). Whereas, the clone B failed to show a consistent LOS pattern after several silver staining attempts (data not shown).

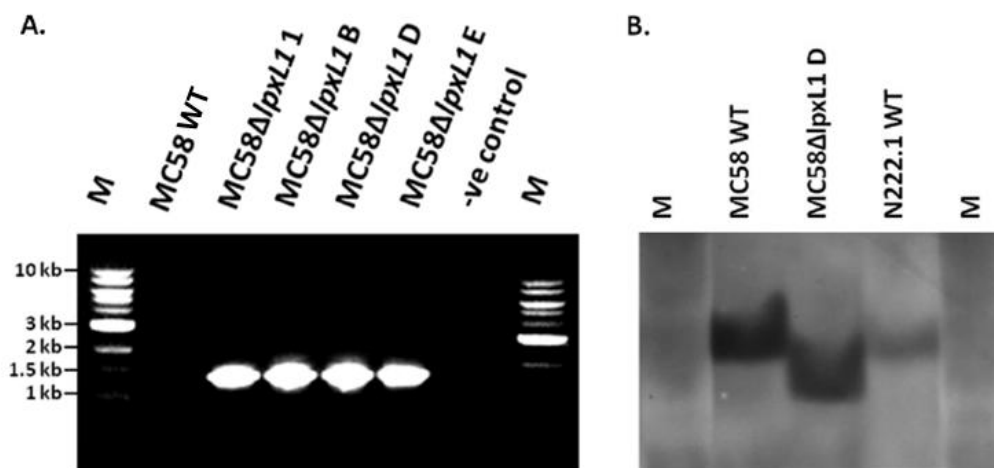


Figure 2.8: Gel electrophoresis analysis of the PCR products generated using primers 1420F2 and 30-KAN-C-TERM (A) and LOS silver staining gel analysis of MC58Δ/pxL1(B).

(A) Agarose gel electrophoresis shows bands with the expected size of 1.2 kb generated using the primers 1419F2 and 30-KAN-CTERM. (B) LOS analysis by silver staining shows a reduced size of the mutant LOS from mutant D compared to MC58 WT.

2.5.3 Growth characterisation of MC58Δ/pxL1 compared to MC58 WT

The growth characterisation for MC58Δ/pxL1 D shows that the mutation had no significant effect on bacterial growth before 5 h (Figure 2.9). This was important to establish so that any differences observed in subsequent infection assays with both strains could not be due to differential growth during the assay. All the growth curves were obtained using DMEM/F-12; the media used to grow Calu-3 cells (see 3.4.1).

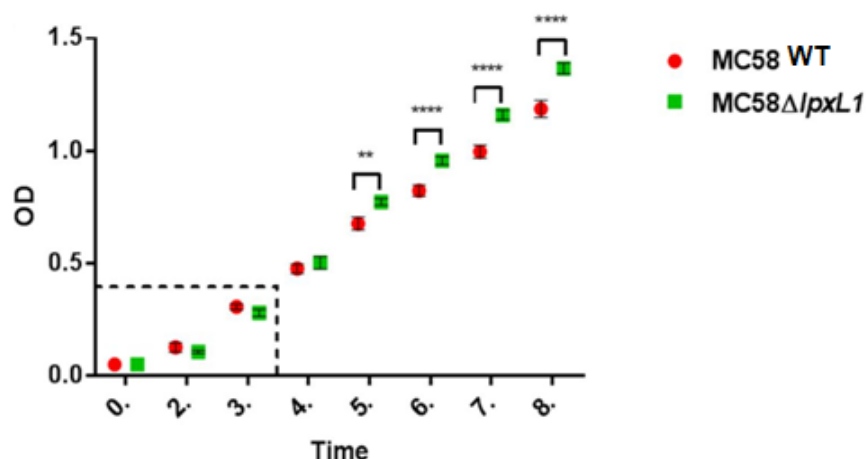


Figure 2.9: Comparison between the growth of MC58 WT and MC58Δ/*lpxL1* clone D.

The chart shows (mean±SD) of OD₆₀₀ at different time points for both strains grown in DMEM/F-12. (N=3, **P<0.01, ****P<0.001, two-way ANOVA)

2.5.4 *lpxL1* complementation in MC58Δ/*lpxL1*

Complementation of the *lpxL1* mutation was completed by an MSc student Michael Timpany. Several complemented clones were obtained by him that were confirmed by PCR (Figure 2.10 and Figure 2.11) and sequencing. Afterwards, silver staining of extracted LOS was carried out with his collaboration. Unexpectedly, all four complemented clones tested showed LOS bands with identical electrophoretic mobility to MC58Δ/*lpxL1* (Figure 2.12).

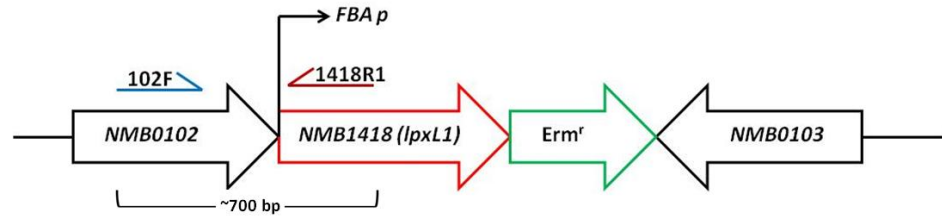


Figure 2.10: Diagram showing the binding sites of primers 102F and 1418R1 and the expected product sizes.

These primers were used to confirm the complementation of *lpxL1* gene.

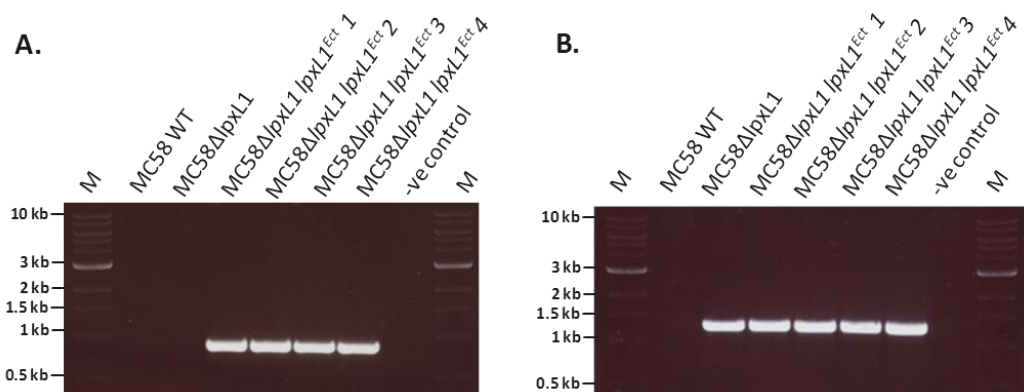


Figure 2.11: Analysis of *lpxL1* complementation by PCR.

Bands with sizes close to 700 bp (A) or 1.2 kb (B) confirmed the presence and site of insertion of the ectopic copy *lpxL1* in *MC58 ΔlpxL1 lpxL1^{Ect}*.

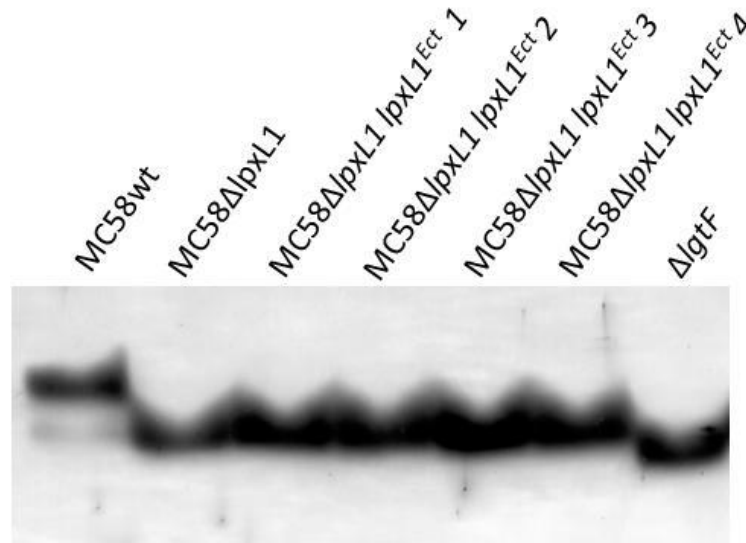


Figure 2.12: Silver staining of LOS extracted from MC58Δ*lpxL1*, MC58Δ*lpxL1 lpxL1*^{Ect} and MC58Δ*lgtF*.

LOS analysis by silver staining shows a lower molecular weight LOS in MC58Δ*lpxL1* and all MC58Δ*lpxL1 lpxL1*^{Ect} clones compared with MC58 WT. Δ*lgtF* LOS was used as a control for LOS truncation.

To confirm that the inserted ectopic copy of *lpxL1* was being transcribed, reverse transcriptase PCR was undertaken. This analysis revealed that the *lpxL1* gene was being transcribed in the WT and complemented strains, but not in the mutated strain (Figure 2.13 and Figure 2.14).

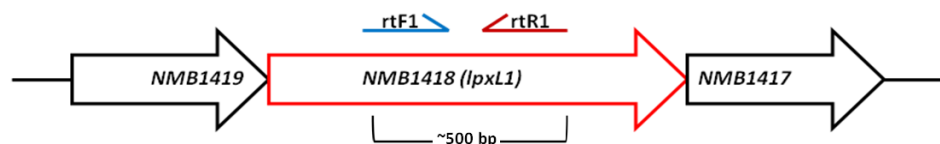


Figure 2.13: Diagram of the primer binding sites and the size of the expected RT-PCR products when using primers *lpxL1*_rtF1 and *lpxL1*_rtR1 to investigate the transcriptional status of *lpxL1*.



Figure 2.14: Analysis of *lpxL1* transcription by RT-PCR.

Bands with sizes close to 500 bp indicate *lpxL1* gene is transcribed. Genomic DNA from WT MC58 (gDNA) was used as a positive control.

These findings raised several points. Firstly, although the complementing copy of *lpxL1* was being transcribed, it remained to be determined whether the LpxL1 protein was being expressed sufficiently to restore LOS acylation to wild-type levels. This could have been addressed experimentally by immune blotting of whole cell lysates using an anti-LpxL1 specific antibody, however, none was available. A second point relates to the originally observed increased mobility of LOS following mutation of *lpxL1*. Could this have been due to phase variation of an LOS-associated gene, and not directly due to the mutation of *lpxL1*? Alternatively, was the mutation of *lpxL1* deleterious to MC58 and providing selective pressure for compensating mutations in other LOS-associated genes?

These hypotheses could each explain the lack of effect on LOS following *lpxL1* complementation.

In order to investigate further several new MC58 WT LOS preparations from independent colonies were tested using silver staining to check the consistency of LOS pattern among them (Figure 2.15). The findings showed that different MC58 WT colonies exhibited different LOS mobility patterns as judged by SDS-PAGE silver staining analysis (single or double bands). To address the compensating mutation theory, MC58 WT was transformed with genomic DNA (gDNA) from complemented strains number 3 with the intention of obtaining strains carrying both mutated and ectopic versions of *lpxL1*, yet showing the WT LOS electrophoretic mobility. 12 clones were selected in the presence of kanamycin (to select for the *lpxL1* mutation) and erythromycin (to select for the presence of the ectopic *lpxL1* expression cassette) and their LOS analysed by silver staining (Figure 2.16). These results showed that some of the clones obtained display an LOS with WT motility, whilst also exhibiting resistance to kanamycin and erythromycin simultaneously.

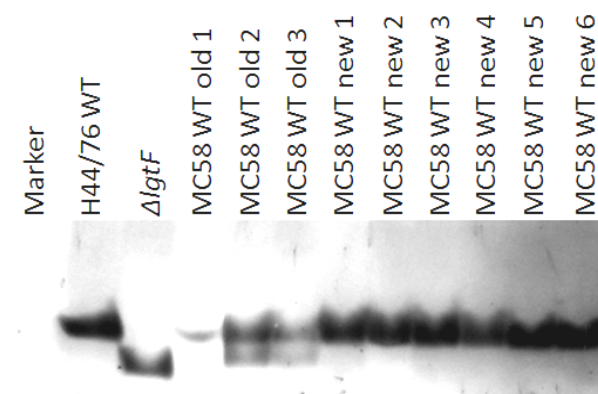


Figure 2.15: Silver staining of LOS extracted from multiple MC58 WT colonies generated at different times.

LOS analysis by silver staining shows a variation in the position of MC58 WT LOS. $\Delta lgtF$ and H44/76 LOS were used as controls.

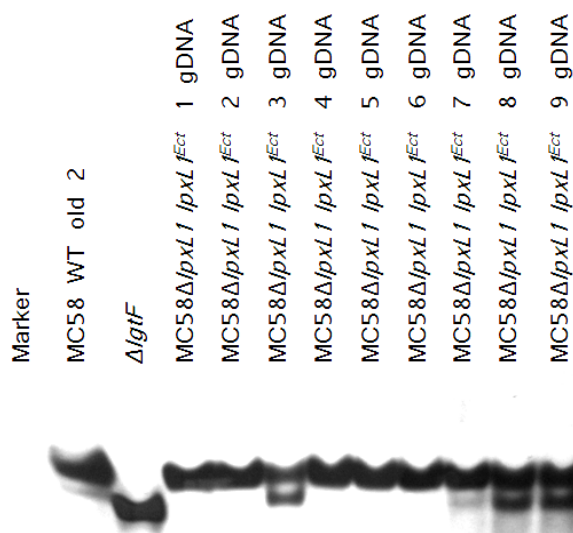


Figure 2.16: Silver staining of LOS extracted from MC58 $\Delta lpxL1$ $lpxL1^{Ect}$ clones generated by transformation with genomic DNA from strains complemented with pMT1/2 plasmids.

LOS analysis by silver staining shows a size of LOS in the complemented strains similar to that of MC58 WT, which is contrary to the complemented strains generated previously with a lower secondary LOS band. $\Delta lgtF$ LOS was used as a positive control for LOS truncation.

2.5.5 Generation of a *lpxL1* mutant and complemented mutant in *N. meningitidis* H44/76

Due to the issues apparent with our use of the MC58 strain, we introduced the same *lpxL1* mutation and complemented this mutation by using the same constructs (pRA3 and pTM2, respectively) in another serogroup B meningococcal strain H44/76. 10 colonies of H44/76 successfully transformed with pRA3 were obtained. Silver staining analysis was used to characterise those new clones. The analysis turned out that just one clone (clone No. 3) among the rest showing single banded LOS. In contrast, the complemented H44/76 Δ *lpxL1* LOS showed double bands in all the complemented clones, one matched the WT LOS position and the other corresponded to Δ *lpxL1* LOS (Figure 2.17). All the work with H44/76 was not analysed on the genomic level.

To conclude, both strains (MC58 and H44/76) are able to be *LpxL1* mutated. On the other hand, the change in the LOS seems to be not fully recovered by complementation. An attempt to generate an *lpxL1* mutation in N222.1 was not successfully accomplished, due inconsistency in the growth on BHI plates. VITOX supplement (Oxoid) was used in the media and no advantage was gained. Therefore N222.1 was excluded from this part of the study.

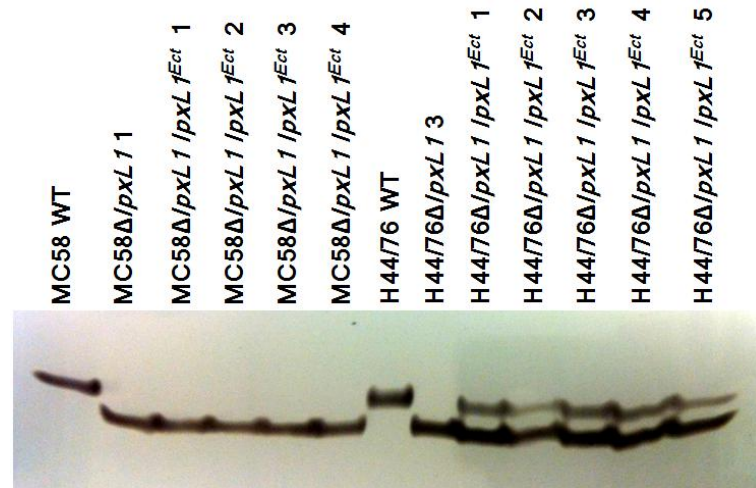


Figure 2.17: LOS silver staining analysis of Δ *pxL1* and *lpxL1*^{Ect} generated in MC58 and H44/76 backgrounds.

LOS analysis by silver staining shows a reduced size of mutated LOS compared WT in both strains. The complemented version of LOS in MC58 did not recover the WT electrophoretic mobility. The complemented version of LOS in H44/76 exhibit double bands; one matched the WT LOS position and the other the Δ *pxL1* LOS position.

2.6 Discussion

The construction of MC58 strains containing a mutated *lpxL1* gene and *lpxL1*-complemented strains appeared to have been successfully accomplished. Although all the confirmatory tests (antibiotic selection, PCR, sequencing and RT-PCR) supported the successful construct of these strains, the analysis of LOS (by SDS-PAGE and silver staining) showed that the complementation *lpxL1* did not restore the electrophoretic mobility of LOS to the MC58 WT position. Moreover, silver staining showed that different colonies of MC58 WT show variation in electrophoretic mobility of their LOS (Figure 2.15). In the case of H44/76, mutation of *lpxL1* appeared to give the same results as with MC58 (confirmed by antibiotic selection and SDS-PAGE and silver staining analysis, but not analysed by PCR). In contrast, the complementation of H44/76 resulted in clones with two LOS bands as judged by silver staining analysis; one matches the position of mutant LOS and the second band at a higher position matches the same position as the WT LOS) (Figure 2.17). The control used in the silver staining analysis ($\Delta lgtF$) proved the reliability of the SDS-PAGE analysis by showing a major difference in the electrophoretic mobility of MC58 WT LOS compared to $\Delta lgtF$, which lacks the outer core of its LOS (LNnT α chain).

The growth characterisation of MC58 Δ *lpxL1* demonstrated no significant effect of *lpxL1* mutation at the early exponential phase. This phase embraces the desired multiplicity of infection (MOI of 50 and 100) which was intended to be used in Calu-3 infection experiments. This observation matches those made in a previous study on H44/76 and H44/76 Δ *lpxL1* (Fisseha *et al.*, 2005). In another study on H44/76 (Fransen *et al.*, 2010) they showed similar growth between Δ *lpxL1* and the WT in whole blood of C57BL/6 mice. The media used in our study to grow the bacteria was DMEM/F-12, which is the same media used to grow Calu-3 cells. This was used to avoid having to grow the bacteria in BHI, and then wash and resuspend in DMEM/F-12 prior to infection, which could affect bacterial counts and viability.

N. meningitidis has several genes involved in LOS biosynthesis that are liable to phase variation (Jennings *et al.*, 1999). Among those genes are *lgtA* and *lgtG* (Figure 2.1). These genes are responsible for inducing different LOS immunotypes of MC58 and H44/76 by modifying the LNnT α chain and β chain (Jennings *et al.*, 1999, Virji *et al.*, 1995a, Saunders *et al.*, 2000). Although MC58 and H44/76 belong to the same clonal complex 32, they exhibit some differences. For example, the MC58 LOS immunotype is L8 and H44/76 is L3. The LOS of both strains is affected by phase variability; MC58

is able to switch its LOS immunotype between L8 to L3 and H44/76 can switch between L3 to L2 (Jennings *et al.*, 1999, Saunders *et al.*, 2000, van der Ley *et al.*, 2001). The size of the H44/76 genome is 2.18 Mb, with 2,480 open reading frames (ORFs), whilst the MC58 genome is 2.27 Mb with 2,465 ORFs (Piet *et al.*, 2011). A comparative study showed that H44/76 has four genes uniquely present and nine genes are uniquely present in MC58. The genome sequence of MC58 shows a ~32-kb region (NMB1124 to NMB1159) which is duplicated (NMB1162 to NMB1197), while it is only present once in H44/76 (Piet *et al.*, 2011).

As meningococcal LOS is influenced by phase variation, this might be responsible for the alterations in LOS mobility observed. Switching between the ON and OFF state of genes occurs randomly (Rotman and Seifert, 2014) and it is not clear if the difference in the LOS electrophoretic mobility was caused by the LpxL1 deficiency or by an unexpected change due to phase variation. Previous studies showed that different LOS variants (immunotypes) exhibit different LOS electrophoretic mobility (Jennings *et al.*, 1995, Jennings *et al.*, 1999).

The LOS immunotype can be concluded from the difference in the LOS electrophoretic mobility on SDS-PAGE, which can be an indicator of the difference in the molecular weight of the

LOS. In a comparative study of different mutations of MC58 expressing different LOS immunotypes, clear differences were observed in the LOS electrophoretic mobility on SDS-PAGE (Jennings *et al.*, 1995). They showed that *lgtA* gene functions as an obvious control point for switching between the L3 and L8 ITs (Figure 2.1). The homopolymeric tract of 14 Gs in the 5'-end of the *lgtA* gene grants a potential slipped-strand mispairing mechanism. That was confirmed by immune blotting and southern blotting and showed that a single G nucleotide alteration can switch the LOS immunotype between the L3 (=14 G) and L8 ($\neq$ 14 G) (Jennings *et al.*, 1995).

In addition, Vincent van Dam and Martine P. Bos stated that complementing a mutant by reintroducing an intact copy of the defective gene into it, has a risk of selecting a phase variant (van Dam and Bos, 2012). The risk comes as a consequence of picking up single colonies post transformation. The use of immunotype-specific antibodies could have helped clarify whether the different LOS bands observed were the result of phase-variable immunotype switching. For that immune blotting is a must to determine the immunotype of the MC58 WT and LpxL1 deficient in the first place. If this step confirmed that no immunotype switching occurred then the complementation method should be investigated.

On the other hand, if the immunotype switching was confirmed then a molecular approach by southern blot or Gene-scan (see 3.4.6) would clarify the expression status of *lgtA* gene. Consequently, the LOS electrophoretic mobility in SDS-PAGE would not be an adequate method to confirm the mutation and complementation. Therefore, more time-consuming and expensive analytical techniques (*e.g.*, nanoelectrospray tandem mass spectrometry) would be required to characterise and confirm the alteration of lipid A (Fransen *et al.*, 2009). If it turns out that the mutation and complementation are both a success, it would be necessary to ensure that the strains will be used for infection assays have identical immunotypes. Thus, the immunotype stability should be checked and monitored all the time.

Importantly, an *lpxL1* mutation in H44/76 has been generated in a previous study (van der Ley *et al.*, 2001), by inserting a *kanR* in the position 131bp upstream the start codon of *lpxL1* without any deletion. This mutation led to an increased LOS mobility on SDS-PAGE (van der Ley *et al.*, 2001). Similarly, Fisseha *et al* generated an *lpxL1* mutation in H44/76 by inverse PCR and generated a 282-bp internal deletion in *lpxL1* (Fisseha *et al.*, 2005). Crucially, neither study reported complementation of *lpxL1*. We undertook complementation since a complemented mutant provides confirmation that

phenotypic changes evident in the mutant are directly related to the introduced mutation.

Our findings may indicate that our mutants (which appeared to produce truncated LOS) had changes in LOS structure which were independent of the lack of LpxL1. In order to investigate that, MC58 WT was transformed by gDNA from a selected complemented strain. with the intention to see if we will be able to obtain a strain carrying both mutated and ectopic versions of *lpxL1* and showing the WT LOS electrophoretic mobility. 12 clones were selected in the presence of kanamycin (to select the mutated *lpxL1*) and erythromycin (to select the ectopic *lpxL1* expression cassette) and their LOS analysed by silver staining (Figure 2.16). Half of those clones show LOS with WT characteristics, while appears to be sensitive to both selection antibiotics. Although those clones are not investigated on the genomic levels, but this result might indicates that there are possible unintentional changes were recovered by the gDNA transformation.

Importantly, confirming the transcription of the complementing *lpxL1* gene by RT-PCR did not confirm that the transcript was successfully translated into active LpxL1. In addition, the inability to introduce a wild type *lpxL1* gene into N222.1 was another reason to redirect the project to study

other aspects of meningococcal infection. The use of N222.1 in this part of the study was crucial for investigating the role of *lpxL1* in *Neisseria* pathogenesis and the possible advantages or disadvantages might be gained by the bacteria from harbouring this type of mutation.

2.7 Summary

From the findings exhibited previously it is clear that the mutant strains are questionable due to the inability of complementation. This issue put the project in front of more encumbrances that means utilising additional investigational steps to explain our observations. Moreover, that will drive the work far away from the planned track, which is the immune response during the infection. In addition, due to limitations, we were forced to redirect the project to be able to fulfil the immunological part. Therefore, the use of bronchoepithelial cells (calu-3) as an infection model has been introduced to the project, as will be explained in the following chapters.

CHAPTER THREE

**ESTABLISHMENT AND
CHARACTERIZATION OF AN
IN VITRO INFECTION
MODEL TO STUDY THE
INTERACTION OF
NEISSERIA MENINGITIDIS
WITH DIFFERENTIATED
HUMAN BRONCHIAL
EPITHELIAL CELLS**

3. ESTABLISHMENT AND CHARACTERIZATION OF AN *IN VITRO* INFECTION MODEL TO STUDY THE INTERACTION OF *NEISSERIA MENINGITIDIS* WITH DIFFERENTIATED HUMAN BRONCHIAL EPITHELIAL CELLS

3.1 Introduction

The airway epithelium in mammals is considered to be a front line of the defence which is encountered by colonising pathogens such as bacteria, viruses, fungi and parasites. Its role is not just to provide a physical barrier against foreign bodies, its role as an immune modulator is also increasingly appreciated.

Currently, there are three types of pulmonary infection models (*in vivo* animal models, *ex vivo* organotypic cultures and *in vitro* cell culture). Due to intrinsic species differences between murine and human respiratory systems the use of mouse animal model was excluded initially from our study. Besides that, there is a rising awareness of using *in vitro* tissue culture engineering as a replacement for animal experiments in research following the principle of the 3Rs (Replacement, Reduction and Refinement) (Berube *et al.*,

2010). Cell culture models have been shown to be useful to simulate *in vivo* conditions during pulmonary infection, particularly, in elucidating the signalling mechanisms involved at the cellular level. Primary human bronchial epithelial cell (NHBE) cultures have shown the ability to simulate the physiological properties observed in the human lung (Berube *et al.*, 2010). Previous work with the culture of primary NHBE in our lab encountered significant problems/limitations. These included; the passage limitation of NHBE (no more than p4); generation of undifferentiated squamous multi-layers instead of a desired pseudostratified layer; irreproducibility and inconsistent differentiation (Sonali Singh, PhD thesis). Overall, these issues prevented the establishment of an infection model system using primary NHBE, and instead the Calu-3 cell line was utilized.

The Calu-3 cell line was derived from a 25-year-old male with a bronchial adenocarcinoma and is commercially available (Fogh *et al.*, 1977). The cells exhibit properties found in native airway epithelial cells, such as tight junctions, trans-epithelial resistance and increased short-circuit current (Isc) (Shen *et al.*, 1994, Florea *et al.*, 2003). A comparative study to evaluate the degree of differentiation in Calu-3 cells grown at the air-liquid interface (ALI) or in liquid covered

culture (LCC) revealed that the ALI culture method generated a thicker pseudostratified layer than in LCC. The formation of tight junctions, microvilli and secretory granules were also more evident in ALI culture than in LCC (Harcourt *et al.*, 2011). Recently, the ALI culture system is recognised as one of the most physiologically relevant *in vitro* models for respiratory research (Berube *et al.*, 2010). The airway bronchial epithelial Calu-3 cell cultures were firstly established in our lab by Yi-Chia Liu (PhD thesis). This demonstrated that the Calu-3 ALI culture system consistently generated differentiated epithelial cells with physiological relevant features.

Nevertheless, several other bronchial epithelial cell models are also available such as BEAS-2B and 16HBE14o-, however, no clear evidence of differentiation capacities, mucin secretion or formation of tight junctions have been reported in previous papers (Lechner and LaVeck, 1985, Cozens *et al.*, 1994, Papi and Johnston, 1999, Wan *et al.*, 2000, Berube *et al.*, 2010). A comparison of characteristics between the commonly used bronchial epithelial cells is summarised in (Table 3.1).

Table 3.1: Summary of morphological characteristics and barrier function of commonly used *in vitro* human bronchial epithelial cell models.

Cell Type	Origin	Morphology and properties	Mucus producing	Tight junction formation	Maximum TEER at ALI (Ω cm ²)
NHBE	Healthy tissue	Mixed airway cell population	YES	YES	750
Calu-3	Lung adenocarcinoma	Pseudostratified cilia-producing secretory cells	YES	YES	600
BEAS-2B	Transformed healthy tissue	Secrete cytokines and antioxidants	NO	NO	150
16HBE14o-	Transformed healthy tissue	Non-ciliated cuboidal cells	NO	YES	200

Adapted from (Sporty *et al.*, 2008, Berube *et al.*, 2010, Stewart *et al.*, 2012)

Unlike *in vivo* studies, *in vitro* models have the benefit of flexibility and controllable experimental conditions. They are also efficient to study the airway epithelial behaviour in the absence/presence of other cells such as macrophages, neutrophils, and fibroblasts, etc. (Stewart *et al.*, 2012, Zhu *et al.*, 2010, Berube *et al.*, 2010). The schematic figure of our *in vitro* airway epithelial infection model as shown in (Figure 3.1) illustrates that Calu-3 cells are seeded on top of the filter in the upper compartment (apical chamber) of the transwell and the lower compartment (basal chamber) contains the culture medium that supplies the cells with nutrients. The exposure of the cells' apical surface to air mimics the

conditions found in the human airway and drives the differentiation process towards a mucociliary phenotype. *N. meningitidis* infection was conducted by introducing the bacteria to the apical surface once the epithelium monolayer achieved polarity and integrity, in other terms, was fully differentiated. A cost-effective, reproducible and physiologically relevant human *in vitro* bronchial epithelium model for *N. meningitidis* infection was established in this chapter.

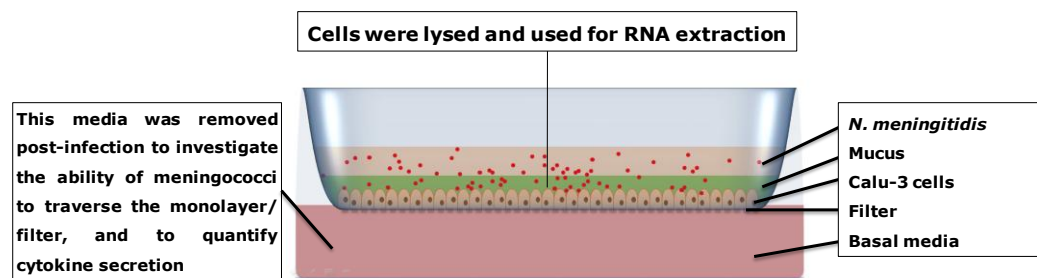


Figure 3.1: Schematic illustration of the *in vitro* respiratory epithelium model established for *N. meningitidis* infection.

Calu-3 cells grown on 0.4 μ M pore size membrane inserts at the air-liquid interface, drives the differentiation of bronchial epithelial cells into a pseudostratified mucociliary epithelium. *N. meningitidis* infection on the apical surface of the airway epithelium mimics colonisation occurring in the human respiratory system.

In the majority of published literature, Dulbecco's modified Eagle's medium (DMEM) was commonly used to grow and differentiate bronchial epithelial cells. However, as minimal essential medium (MEM) is the medium recommended by

ATCC for culturing Calu-3 cells (HTB-55), a study for evaluating the effect of media on the differentiation of Calu-3 cells has been done in our lab by PhD student Yi-Chia Liu. The study showed that Calu-3 cells grew and displayed a spindle shape in both DMEM and MEM. Calu-3 cells grew slightly faster in DMEM than in MEM and appeared more vacuolated. Both media supported the expression of Tight junction protein Zonula occluding 1 (ZO-1) by Calu-3 cells (Figure 3.2). Transepithelial electrical resistance (TEER) in Calu-3 cell cultures at ALI in DMEM was slightly higher than MEM throughout the 21 days of differentiation.

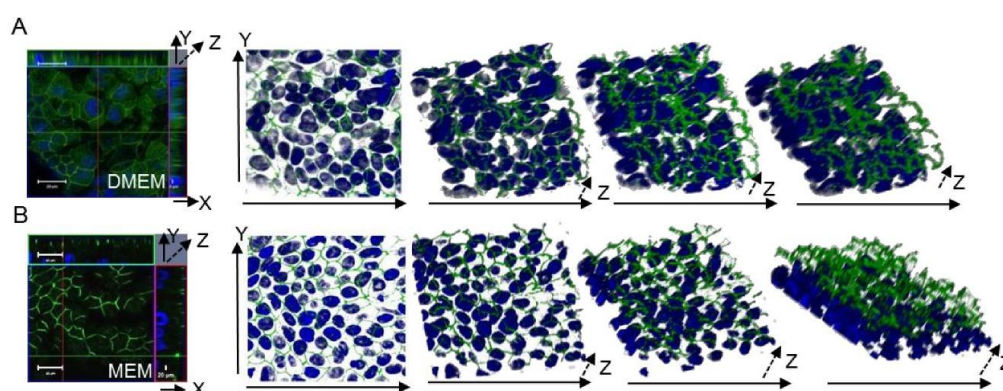


Figure 3.2: Localisation of the tight junction protein ZO-1 in Calu-3 cells cultured in ALI for 21 days.

Calu-3 cells cultured in DMEM **(A)** and MEM **(B)** expressed ZO-1. ZO-1 was enriched at the apical region of MEM cultures. ZO-1 protein was detected using mouse anti-ZO-1 antibody as described in materials and methods. The image is the representative from three independent experiments. (Yi-Chia Liu, PhD Thesis)

Calu-3 cells differentiation in DMEM was assessed using cilia associated type IV tubulin and the mucin protein, MUC5AC, the major component of airway mucus secreted exclusively by mucin-producing cells (goblet cells) (Ehre *et al.*, 2012, Hovenberg *et al.*, 1996). The morphological and topological characteristics and mucin production showed that ALI Calu-3 cultures had differentiated into a heterogeneous epithelium containing ciliated cells and goblet cells (Figure 3.3 Figure 3.4). A previous study demonstrated that Calu-3 cells grown in ALI display a columnar epithelium with a more heterogeneous apical topography and greater secretion of mucin proteins than when grown in liquid-covered culture (Grainger *et al.*, 2006). Therefore, we chose to establish ALI cultures infection model in DMEM/F-12, as an attempt to simulate the physical properties of native human airway epithelium.

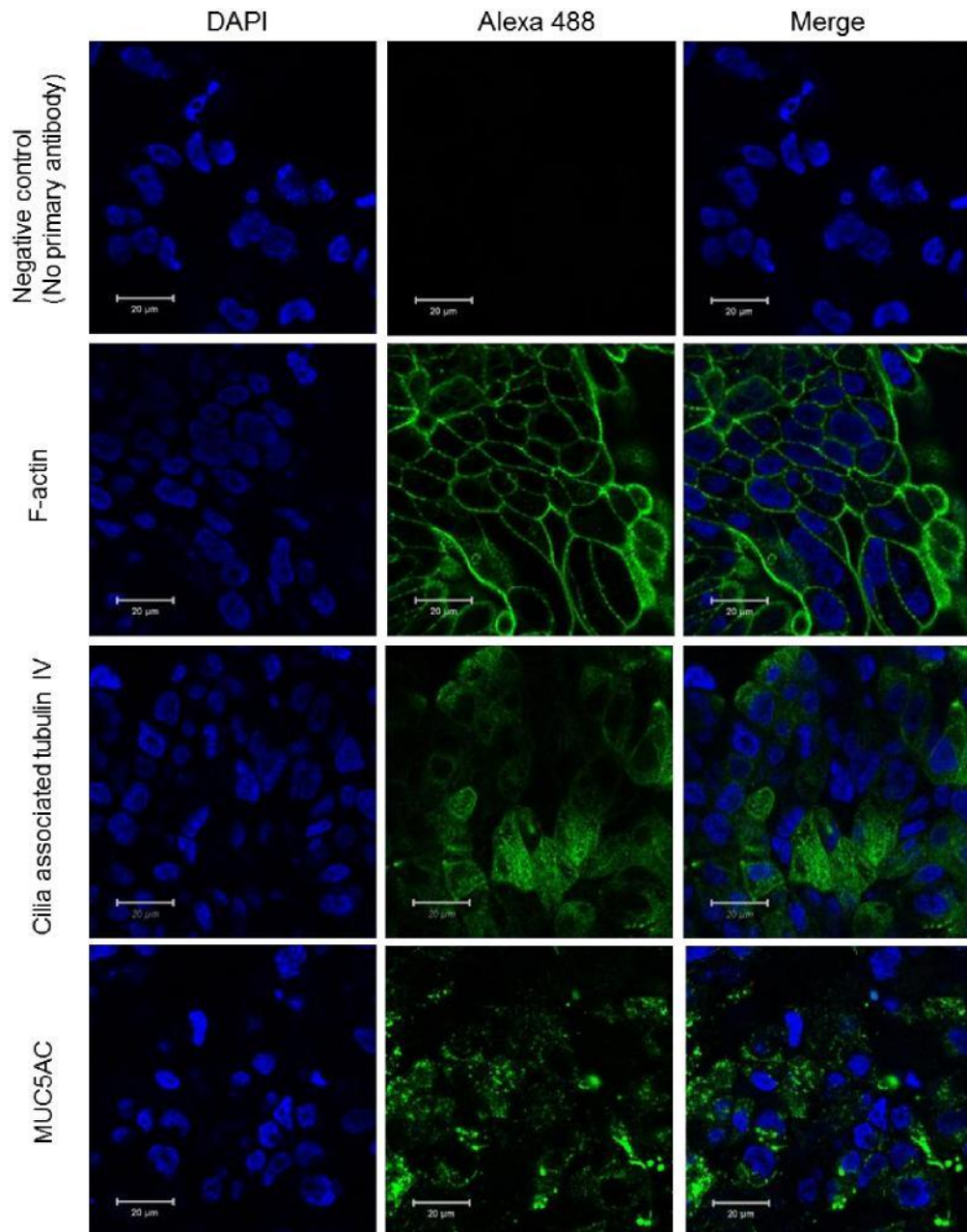


Figure 3.3: Detection of cilia and mucin expression in one-week-old Calu-3 cells cultured in DMEM at ALI.

Immunofluorescence images of cilia-associated tubulin IV and mucin MUC5AC on one-week old Calu-3 cultures. Cells were stained with anti-tubulin IV and anti-MUC5AC antibody, respectively. F-actin was detected by phalloidin conjugated with Alexa 488. Cell nuclei were stained with DAPI (blue). (Yi-Chia Liu, PhD Thesis)

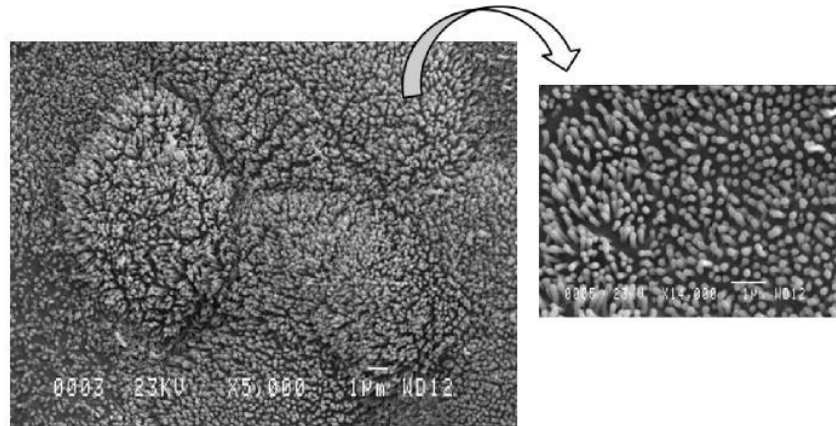


Figure 3.4: Calu-3 cells at ALI for 21 days exhibited microvilli at their apical surface.

Scanning electron microscopy images of Calu-3 cells grown at ALI. Calu-3 cells displayed short and thick microvilli at their surface. Image shown on the left is at 5,000x magnifications and individual microvilli were clearly observed when the image was zoomed-in to 14,000x magnifications (right). N=1. (Yi-Chia Liu, PhD Thesis)

3.2 Hypothesis

The first barrier encountered by air-borne pathogens is the human lung epithelium. Using differentiated human bronchial-epithelial Calu-3 cells as an *in vitro* infection model would offer an efficient and cost-effective platform to explore the early interaction between *N. meningitidis* and the host respiratory system.

3.3 Aim

Thus, the aim of the work in this chapter was to establish an airway epithelium infection model as a research

platform applicable to the study of *N. meningitidis* pathogenesis and particularly to investigate the impact of genomic variation upon infection.

3.4 Materials and methods

3.4.1 Calu-3 culture and differentiation

The handling and differentiation of Calu-3 cell culture were described previously (Grainger *et al.*, 2006). Calu-3 human bronchial epithelial cells (ATCC No. HTB-55) were obtained from the American Type Culture Collection (Rockville, MD) and used between passage 19 and 35. Newly thawed cell stocks were cultured initially in 75 cm² flasks (Corning Life Sciences) in 15 mL Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Hams (1:1) (DMEM/F-12) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), 1 x non-essential amino acid solution (Sigma), and incubated at 37°C, 5% CO₂. The medium was changed every other day, and the cells were grown to 80-90% confluency before being passaged or differentiated.

For differentiation, Calu-3 cells cultured in T-75 were washed with phosphate buffered saline (PBS) solution (Sigma) then trypsinised (0.05% trypsin, 0.02% EDTA in PBS) incubated for 10-15 min and seeded on 0.4 µm pore size, 12-well transwell polyester inserts (Corning Life Sciences). Calu-3 cells were seeded at a density of 100,000 cells per well in 500 µL of medium with 1 mL of medium in the basal chamber. After overnight culture at 37°C, 5% CO₂,

the medium in the insert was aspirated to allow the cells to differentiate at the air-liquid interface (ALI). The medium in the bottom chamber was changed every other day up to 21 days.

3.4.1.1 Transepithelial electrical resistance

The barrier function of Calu-3 cell cultures in ALI was determined by measuring transepithelial electrical resistance (TEER) using an epithelial voltohmmeter-EVOM (World Precision Instruments). Prior to the measurements, the apical surface of the Calu-3 cell culture was balanced by adding 500 μ l of culture medium for 30 min at 37°C, 5% CO₂. Effective TEER (Ω cm²) was determined by subtracting the resistance of a cell-free transwell insert. Those transwells used for TEER measurement were discarded afterward and not used for further applications.

3.4.2 Preparation of bacteria inocula for infections

The *N. meningitidis* serogroup B strain MC58 was used in this study (Tettelin *et al.*, 2000c). Growth curves were generated as described in chapter 2, to correlate the OD readings of the bacterial cultures with the colony forming unit (CFU) count. The Linear regression and square of the correlation coefficient (R^2) were generated in Prism. To calculate which exact OD₆₀₀

represents our desired inoculum (CFUs count) the following equation was applied:

$$X = \frac{Y - a}{b}$$

X is the explanatory variable (OD) **Y** is the dependent variable (CFU). While **b** is the slope of the line, and **a** is the intercept (the value of **y** when **x** (OD) = 0). Both **a** and **b** were auto-calculated by Prism.

Inoculum cultures were started with one colony from an overnight culture plate. The colony was inoculated in 20ml DMEM/F-12 in 50ml Falcon tube, and incubated at 37°C, 200 rpm, for overnight. Then a new culture was made in the next morning and grown to an OD₆₀₀ ≈ 0.45, which corresponds to ≈ 7.5 × 10⁸ CFU mL⁻¹, to achieve a multiplicity of infection (MOI) of 100. OD measurements were made using OD₆₀₀ DiluPhotometer™ (IMPLEN).

3.4.3 Infection of differentiated Calu-3

≈ 7.5 × 10⁵ differentiated Calu-3 cells were infected in duplicate with 100 µl per well of bacteria at MOI of 100; *i.e.*, 7.5 × 10⁷ CFU per well. A 1:1 dilution in pre-warmed DMEM/F-12 of the same bacterial culture was made to obtain MOI of 50; *i.e.*, 3.75 × 10⁷ CFU per well. Three samples were collected at 6 or 24 hours post-infection (hpi). First, after

aspirating the medium from the apical chamber, Calu-3 cells were lysed. The cells lysate was collected and stored at -80°C for RNA isolation (see 3.4.4). Secondly, 50 μL from the basal chamber was collected, serially diluted and plated on chocolate agar plates. Finally, the remaining basal medium was collected, centrifuged for 20 min at $6000 \times g$, and the supernatant stored at -20°C to investigate the cytokines secretion (Figure 3.1).

3.4.4 Cytokine measurement by quantitative PCR

3.4.4.1 RNA isolation

RNA was extracted from infected and uninfected Calu-3 cells using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) according to manufacturer's instruction. Briefly, cells were lysed using the lysis buffer containing β -mercaptoethanol directly in the cell culture compartment. The cell lysate was homogenised by pipetting, and homogenates transferred to a filtration column and centrifuged $16,000 \times g$ for 2 minutes, to remove cellular debris and sheared DNA. Filtrates were mixed with an equal volume of 70% ethanol and vortexed. The mixtures were transferred to the RNA Binding Column and centrifuged $16,000 \times g$ for 15 seconds. This step was followed by DNase digestion using the On-Column DNase I Digestion Set

(Sigma) according to the manufacturer's instructions. After washing, RNA was eluted and quantity/quality determined at A260/A280 using a Nanodrop 2000 spectrophotometer (Thermo Scientific).

3.4.4.2 cDNA synthesis

First strand cDNA was synthesized using random hexamers and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technologies) according to manufacturer's instructions. RNAs were mixed with random hexamers (Life Technologies) and incubated for 10 min at 70°C to eliminate any secondary structures that may have formed. The mixtures were then placed on ice for 5 min to avoid the reforming of secondary structures and master mix added. The master mix was composed of 5x first strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM MgCl₂) and 0.1 M dithiothreitol. Reverse transcriptase (1µl per reaction) was added to the complete mixture directly before starting the reaction. Reverse transcriptase negative controls were also included. Samples were incubated for 1 h at 37°C. The reaction product (cDNA) was then incubated for 10 min at 95°C to inactivate the reverse transcriptase. cDNA samples were stored at -20°C until quantitative real-time PCR analysis.

3.4.4.3 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) reaction mixtures (25 μ L per reaction) containing cDNA template ($\approx$ 2-3 μ g), 2x SYBR green qPCR master mix with 2.5 mM $MgCl_2$ (Stratagene) were used alongside with 6-Carboxy-X-Rhodamine (ROX) as a reference dye for pipetting error correction. Reactions were made using an Agilent Mx3005P qPCR system. The cycling conditions were 3 min 20 sec at 95°C and 40 cycles as follows: 95°C for 10 sec, and 59°C for 33 sec. Each sample was tested in triplicate. The efficiency of primers (

Table 3.2) was assessed by performing a standard curve with templates using 10 fold serial dilutions (from neat to 1:1000). Relative gene expression was calculated using the relative quantification ΔC_t method with a reference gene (HPRT). The method calculates the relative expression of the target gene to reference gene $[2^{(C_t(HPRT) - C_t(target))}]$, while 2 is a simplified value of the reaction efficiency. Ratio of expression was determined as follows:

Ratio of expression (fold)

$$= \frac{\text{relative expression value of the target gene in infected cells}}{\text{relative expression value of the target gene in uninfected cells}}$$

The C_t values of target and reference genes were calculated using MxPro-Mx3005P software (Stratagene).

Table 3.2: Primers used for qPCR

Primer (Accession number)	Primer Sequences	Position	Amplicon length (nucleotides)
NM_000194.2			
HPRT_f	GTAATGATCAGTCAACGGGGGAC	484-660	177
HPRT_r	CCAGCAAGCTTGCAACCTTAACCA		
NM_000758.3			
GM-CSF_f	TCTVAGAAATGTTTGACCTCC	211-231	98
GM-CSF_r	GCCCTTGAGCTTGGTGAG	291-308	
NM_000594.3			
TNFaH_f	GACAAGCCTGTAGCCCATGT	28 - 47	105
TNFaH_r	TCTCAGCTCCACGCCATT	115 - 132	

3.4.5 Adhesion and invasion assay

The adhesion and invasion assay was performed using a modified method from (Bartley *et al.*, 2013). Calu-3 cells were cultured and differentiated for 21 days in DMEM/F-12, as described in (3.4.1). Next, Calu-3 cells were infected with 100 μ L per well of MC58 to achieve MOI of 30, as described in (3.4.2). Cultures were incubated for 6 or 24 h at 37°C, 5% CO₂. At each time point, the basal medium was collected and transferred to sterile 1.5 mL tubes to investigate the bacterial traversal across the Calu-3 monolayer. Uninfected Calu-3 cell

cultures were used as a control. Afterwards, Calu-3 cells were washed twice with 450 μL per well 1x sterile pre-warmed PBS, and media and washes collected in sterile 1.5 mL tubes. Then the cells were lysed with 1 mL sterile 0.1% Saponin (Sigma) pre-warmed in PBS to investigate the bacteria associated with Calu-3 cells. Serial dilutions of each fraction were plated to assess CFU count.

To assess cellular invasion, Calu-3 cells were washed as above and incubated for 30 min with 200 μL of 200 $\mu\text{g mL}^{-1}$ gentamycin in fresh pre-warmed DMEM/F-12 medium per well. Afterwards, the Calu-3 cells were washed twice with 400 μL per well 1x sterile pre-warmed PBS, and washes were collected in sterile 1.5 mL tubes. Then the cells were lysed with Saponin as previously described. As above, serial dilutions of each fraction were plated to assess CFU count.

3.4.6 Gene-scan for phase variation study

Colonies examined in this study were obtained from the adhesion and invasion study as in (3.5.4). 10 colonies from each fraction (initial inoculum, cell-associated and internalised at 24 hpi) were collected, and each colony was suspended in 1 mL of pre-warmed sterile dH_2O . 500 μL was boiled for 10 min to be used as a DNA template for subsequent PCR. The remaining 500 μL was mixed with 2x concentrated BHI

medium containing 30% glycerol and stored at -80 °C for further investigation. All the incubations were at 37 °C in 5% CO₂.

Repeat tract analysis of PV genes was undertaken using the primers shown in (Table 3.3). Repeat tracts lengths were determined by sizing of amplification products. PCR amplification and DNA sequence analysis were performed using a modified method from (Oldfield *et al.*, 2013). Fragments were amplified from diluted DNA boilate (1:10), 1 µL per reaction, using a 6-carboxyfluorescein (FAM)-labelled primer and a non-labelled primer in a 10 µL PCR reaction. Each reaction containing 3.9 µL dH₂O, 1 µL 2mM dNTPs, 1 µL FAM-labelled forward primer (2 pM µL⁻¹), 1 µL reverse primer (2 pM µL⁻¹), 2 µL 5x colourless reaction buffer and 0.1 µL Go Taq polymerase (Promega). PCR products were A-tailed by adding a 4 µL reaction mix containing 2.76 µL dH₂O, 0.4 µL 2 mM dATP, 0.8 µL 5x colourless reaction buffer and 0.04 µL Go Taq polymerase (Promega), followed by 45 min incubation at 72°C. 0.5 µL volumes of PCR products (1:100 diluted in dH₂O) were mixed with Hi-Di Formamide and GeneScan 500 LIZ size standard (Applied Biosystems) and electrophoresed on an ABI 3130 Genetic Analyzer (Applied Biosystems). GeneScan data were analyzed using Peak Scanner v1.0 software (Applied Biosystems) using the local southern size calling method.

Table 3.3: Primers used in this study

Primer	DNA sequence
nalPF1^a	GTTGCAACAACACTTTCTGCCTGC
nalPR1^a	GCAGGTTGTCGTTGCTCATCCACG
nalPR2^a	CAGGCGCTTCCTTCCGCATATACG
mspAF1^a	TATCGGCAACAACAGGCAACAGGC
mspAR1^a	TTTTGGGCACGCGTCAGAATGCCG
mspAR2^a	GGTAGGCACCCAAATCGGCATAGC
nalPF2-FAM^b	(FAM)AAATGTGCAAAGACAGAAGCATGC
mspAF2-FAM^b	(FAM)GCAGTCCGCATGGAAGCAGACAGC
FetApro-for2^a	TTACATTTCCGACAAAACCT
FetApro-rev^a	AGAGTTCGCGCATATCGGTT
FetApro-rev-FAM^b	(FAM)AGAGTTCGCGCATATCGGTT
opc-revS^a	CTCATTAGCGGTTTGAAGCTCTTGTGCAG
Opc-for^a	GAGAATAACAATTCGTTGTA
Opc-for-FAM^b	(FAM)GAGAATAACAATTCGTTGTA
NadA-rev^a	TGGCTGTGGTCAGTACTTTGGATGG
NadA-for^a	TCGACGTCCTCGATTACGAAGGC
NadA-for-FAM^b	(FAM)TCGACGTCCTCGATTACGAAGGC
hmbR-RF4^a	GCTACTGAACACGTCGTTCC
hmbR-RF3^a	TGCCAACCTCTTTTACGAATGG
hmbR-RF3-FAM^b	(FAM)TGCCAACCTCTTTTACGAATGG
porA-p72^a	GCACGAGGTCTGCGCTTGAATTG
porA-p72-FAM^b	(FAM)GCACGAGGTCTGCGCTTGAATTG
porA-MC58-rev^a	CGCCGGCTTTGATTTCCCGTATAG

a Used for amplification and sequencing

b Used for gene-scanning with its antisense primer

3.5 Results

3.5.1 Calu-3 culture and differentiation

To determine whether differentiated Calu-3 cells in ALI culture acted as a barrier, TEER was examined. TEER in Calu-3 cell cultures at ALI in DMEM/F-12 was tracked every 7 days for 3 weeks. TEER in DMEM/F-12 culture reached $433 \pm 32 \Omega\text{-cm}^2$ at day 7 and was maintained above $1700 \Omega\text{-cm}^2$ throughout the 3 weeks (Figure 3.5). High TEER readings in DMEM/F-12 culture with a significant difference at Day 21 indicated that ALI cultures of Calu-3 cells attained barrier integrity.

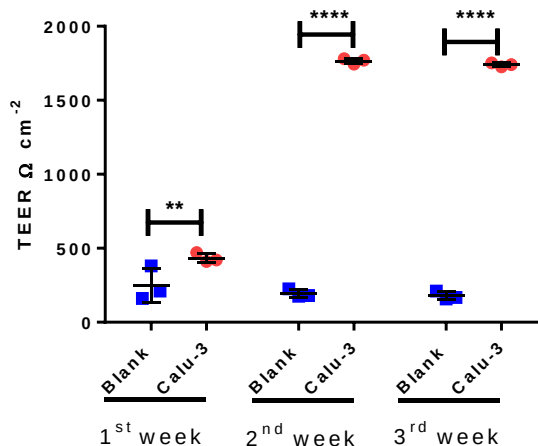


Figure 3.5: Development of transepithelial electrical resistance (TEER) in Calu-3 cultures in DMEM/F-12, (mean±SD).

At the end of week one, the confluence did not reach 100%. Then the development of tight junctions occurred, thus increasing electric resistance above $1700 \Omega\text{ cm}^{-2}$. High TEER indicates that Calu-3 cells are in a differentiated stage. (N=3, **P<0.01, ****P<0.0001, two-way ANOVA)

3.5.2 Preparation of bacteria inoculum for infections

A comparison between the growth of wild-type strain MC58 in DMEM/F-12 and BHI was performed. Choosing DMEM/F-12 to grow *N. meningitidis* was to avoid washing the bacteria prior to infection of Calu-3 cells. This washing step might reduce the number of viable bacteria and eliminate OMVs produced by *Neisseria* during growth. These OMVs could also contribute to the immune recognition of meningococcal infection. Also, the change in media composition could result in a lag in bacterial growth whilst the meningococcal metabolic machinery adjusted to suit the new media.

Figure 3.6 shows a comparison of OD₆₀₀ readings for MC58 growth in BHI and DMEM/F-12. The data indicates that DMEM/F-12 does not support MC58 growth as well as BHI; however, the differences were not statistically significant. This was in agreement with the CFU results (Figure 3.7).

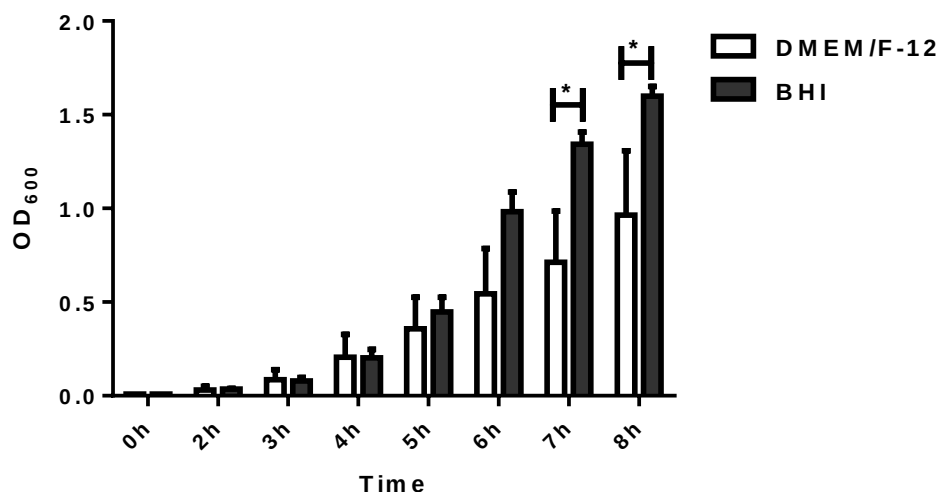


Figure 3.6: Comparison between the growth of MC58 in BHI and DMEM/F-12 determined using OD measurements.

The chart shows the differences in (mean±SD) of OD₆₀₀ readings in the both media at different time points. (N=3, *P<0.05, two-way ANOVA)

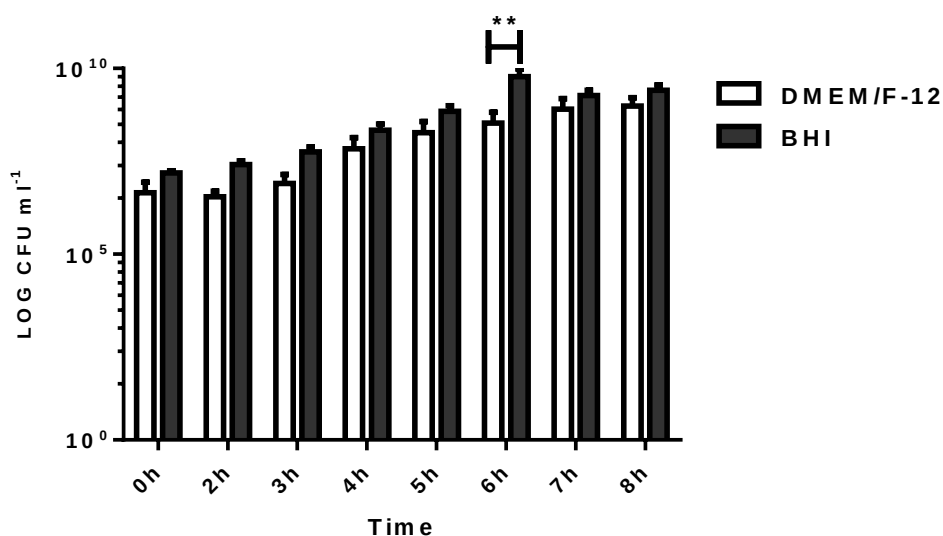


Figure 3.7: Comparison between the growth of MC58 in BHI and DMEM/F-12 determined using CFU counting.

The chart shows LOG of CFU count (mean±SD) in both media at different time points. (N=3, **P<0.01, two-way ANOVA).

In general, the data showed a strong level of correlation between CFUs and OD₆₀₀ readings in DMEM/F-12. Interestingly, our desired number of CFU (7.5×10^8 CFU/ml $\approx$

MOI of 50) always corresponded with OD₆₀₀ 0.22, which can be obtained within 3-4 hours using a starting OD₆₀₀ of 0.05. Figure 3.8 show a strong correlation by linear regression analysis, between OD₆₀₀ and CFU for MC58 strain grown in DMEM/F-12 with initial OD₆₀₀ 0.05 indicated by the value of R^2 , which is very close to 1 ($R^2 = 0.9609$).

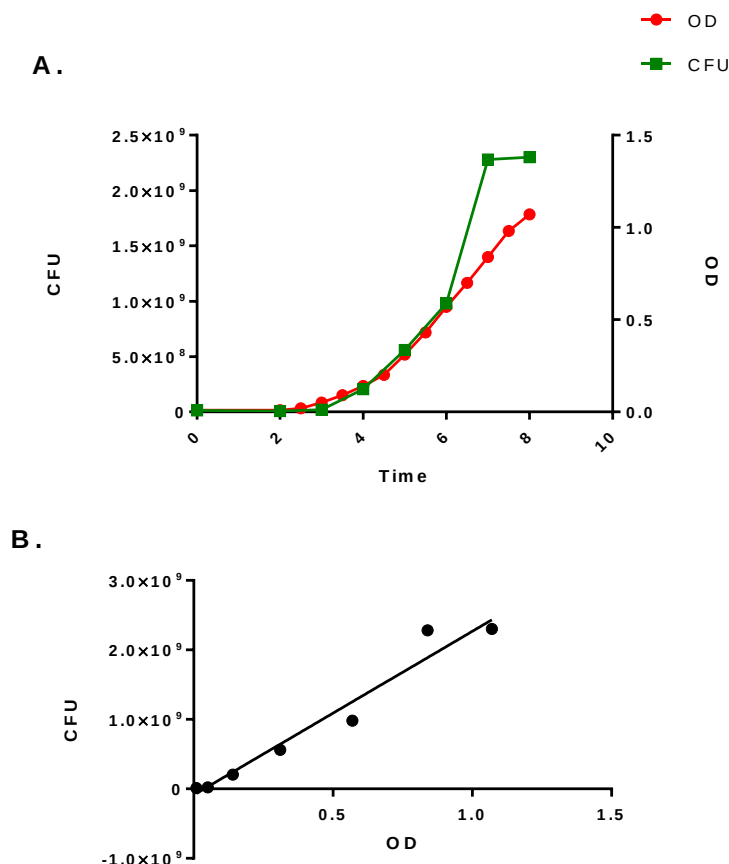


Figure 3.8: Correlation between OD and CFU for MC58 in DMEM/F-12.

(A) a stacked alignment for the mean of OD and CFU curves illustrating the strong correlation between the measurements in first 6 h of growth
 (B) the linear regression analysis shows a strong correlation between OD and CFU indicated by the R-square value ($R^2 = 0.9609$), which is very close to 1. (N=3)

3.5.3 Infection of differentiated Calu-3 and cytokines measurement

Differentiated Calu-3 monolayers, grown for 21 days on 0.4 μ M pore size transwell inserts, in ALI culture in DMEM/F-12, were infected with MC58 for 6 or 24 h at MOI of 50 and 100. The aim was to evaluate the cytokine and chemokine response of Calu-3 cells to MC58 for 6 h at MOI of 50, as well as, to assess the ability of MC58 to traverse through the monolayer/filter barrier for 6 or 24 h at MOI of 50 and 100.

The TNF- α and GM-CSF responses were determined by qPCR as fold change in comparison with uninfected Calu-3 cells. TNF- α fold change mean was $\approx 80 \pm 19$ fold increase for MOI of 50 at 6 hpi. Whereas, the mean of GM-CSF levels were increased $\approx 178 \pm 26$ fold compared to uninfected cells (Figure 3.9). These findings indicated that Calu-3 cells sense, and respond to, infection with MC58 WT. Attempts to measure levels of additional cytokines (*e.g.*, IL-6, IL-8, IL-10 and IL-1 β), to create more comprehensive secretion profiles of pro and anti-inflammatory responses against MC58 were unsuccessful due to technical difficulties.

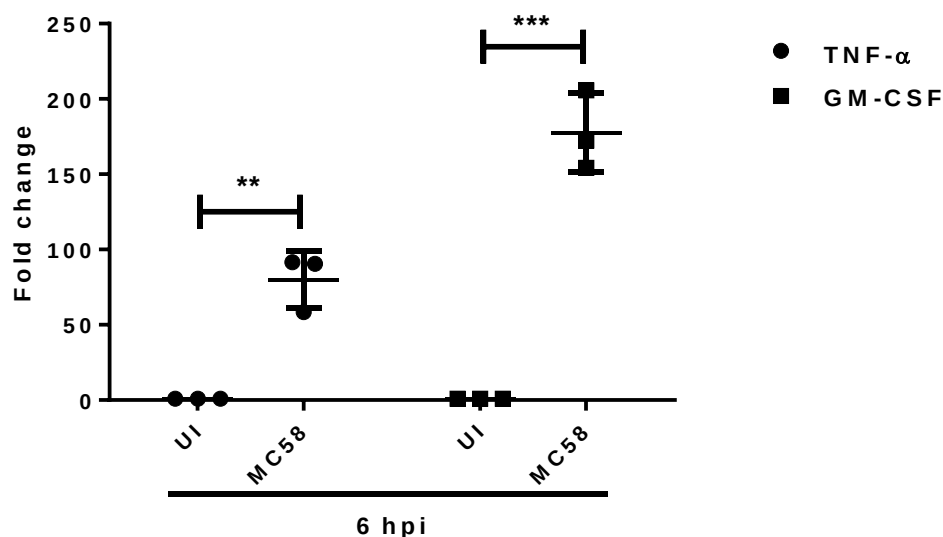


Figure 3.9: Induction of pro-inflammatory cytokines by differentiated Calu-3 cells upon MC58 infection.

Expression of TNF- α and GM-CSF was investigated in Calu-3 cells infected with MC58 at an MOI of 50 for 6 h by qPCR. The ratio of expression of TNF- α and GM-CSF induced by MC58 infection was 80 ± 19 fold and 178 ± 26 fold increase, respectively, compared to uninfected cells. Each symbol represents one experiment. Each experiment was performed in duplicate wells, and samples from each well were prepared for qPCR in triplicate. (Mean $\pm$ SD) (N=3, **P<0.01 ***P<0.001, two-way ANOVA)

The traversing ability was assessed by plating 50 μ l of basal media and 5 serial dilutions from each well on Columbia Agar with chocolate Horse blood. Next day, the plates were checked for growth to perform a CFU count. In all the conditions, MOI of 50 and 100, at 6 and 24 hpi, no bacteria were detected in the basal chamber. It worth mentioning that in one preliminary experiment bacteria were detected in the basal chamber. In this case, this was judged to have occurred due to the incomplete confluence of the Calu-3 cells. In

subsequent experiments, the confluence of the monolayers was confirmed by microscopy before infection. This observation also confirms that meningococci are capable of traversing the membrane in the absence of Calu-3 cells and illustrates the ability of an intact epithelial barrier in limiting access to deeper tissues.

3.5.4 Adhesion and invasion assay

In view of the fact that Calu-3 cells were responding to the presence of the bacteria, but no bacteria were traversing across the monolayer, we decided to study the localisation of the bacteria within the model. To do this, an adhesion and invasion study was conducted to investigate the localisation pattern of MC58 WT.

The results showed that the bacteria were replicating during the course of the infection since the total CFU count at 24 hpi was significantly higher than the total CFU count for the inoculum and at 6 hpi (Figure 3.10).

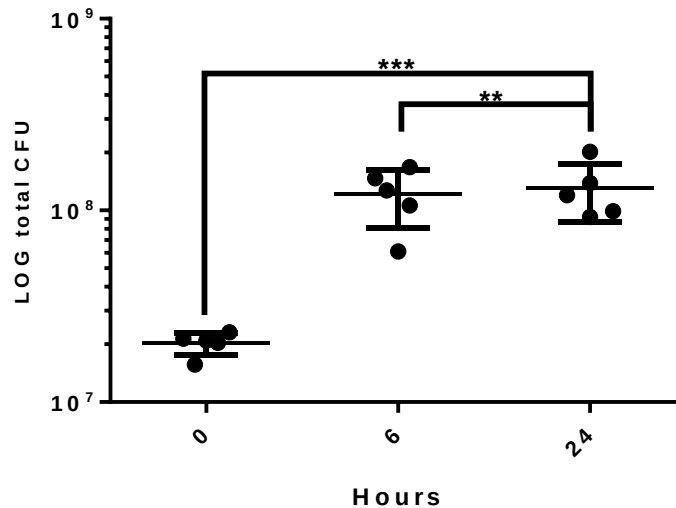


Figure 3.10: Total CFU count for inoculum and total CFU count at 6 and 24 hpi of MC58 in adhesion and invasion assay.

The chart shows in LOG scale the total CFU count at different time points: 0h (inoculum), 6 and 24 hpi. (mean±SD) (N=5, **P<0.01 ***P<0.001, one-way ANOVA).

The analysis for distribution and localisation of MC58 within the Calu-3 model is shown in Figure 3.11. The non-associated bacteria correspond to the bacterial cells recovered from the media added to those recovered by gentle washing with PBS. The data showed that the association level of the bacteria after lysing the Calu-3 cells using Saponin (*i.e.*, corresponds to adhered and internalised bacteria), dropped significantly between 6 and 24 hpi. In contrast, the numbers of invaded bacteria dramatically increased by ≈ 20 fold at 24 hpi compared to 6 hpi. The internalised bacteria were quantified by lysing the Calu-3 cells using Saponin after using gentamycin to kill non-internalised bacteria. The adhesion

level was calculated as the difference between association and invasion.

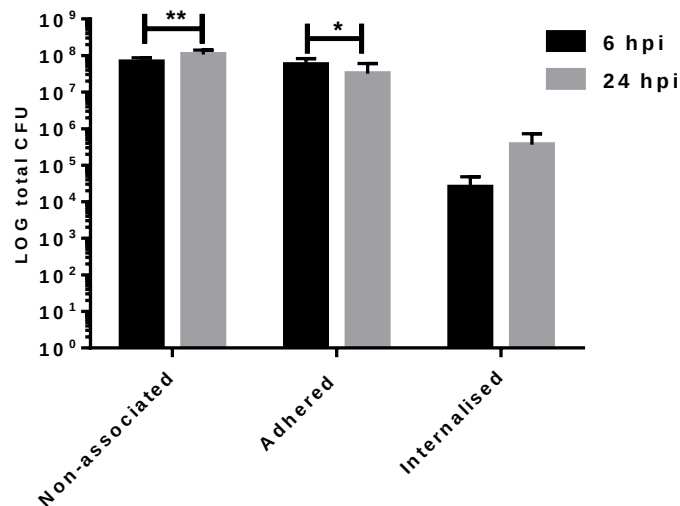


Figure 3.11: MC58 distribution during infection of Calu-3 cells at 6 and 24 hpi.

The chart shows CFU (mean±SD) for each localisation group (non-associated, adhered and internalised) at 6 and 24 hpi. (N=5, **P<0.01, *P<0.05, two-way ANOVA)

The analysis for distribution and localisation of MC58, within Calu-3 model, is presented in percentages in (Figure 3.12). The data show that the percentage of non-associated bacteria was increased almost 30% at 24 hpi in comparison with 6 hpi. The adhesion level of the bacteria to Calu-3 cells at 6 hpi (≈ 45%) dropped almost by a third at 24 hpi (≈ 16%). The percentage adhesion was calculated as the difference between association and invasion. The invasion percentage was 0.01% at 6 hpi, which was increased approximately by 20 fold at 24 hpi (0.21%).

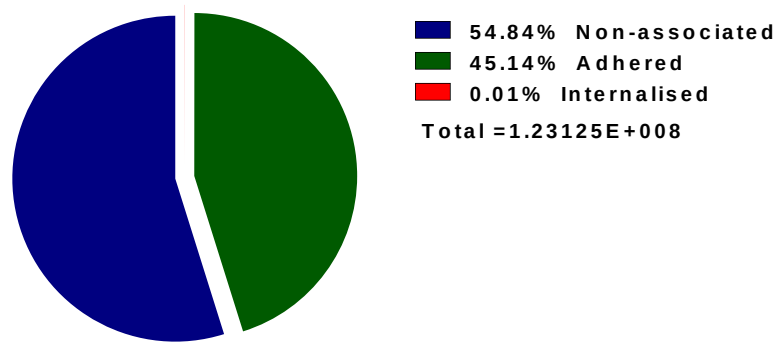
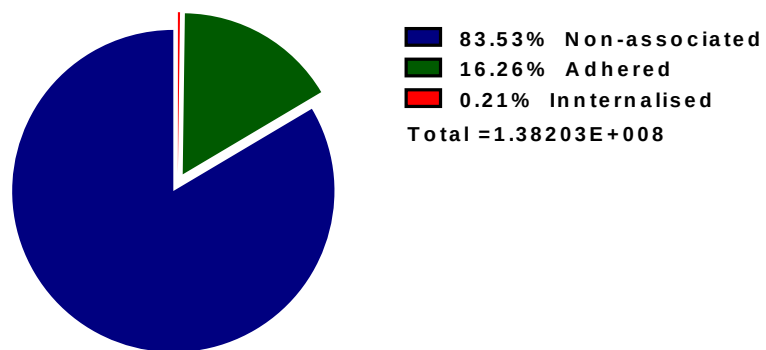
A. Percentage of MC58 association 6 hpi**B. Percentage of MC58 association 24 hpi**

Figure 3.12: Charts depict bacterial distribution within the Calu-3 model.

The pie charts show the mean of total CFU counts into percentages for non-associated bacterial cells and cell-associated (adhered and internalised) for (A) MC58 at 6 hpi, (B) MC58 at 24 hpi. No bacteria were detected in the basolateral chamber during these infections. The data represent the mean of five repeats performed independently (N=5)

3.5.5 Gene-scan for phase variation study

120 colonies were examined for ON/OFF status of seven PV genes (Table 3.4), by sizing of fluorescent PCR products. The rationale of this examination was to study the possible change in PV pattern in different localisation states of MC58 within the

Calu-3 model. The colonies were obtained from the 24 hpi time point of two independent adhesion and invasion experiments, 60 colonies each. Each experiment was carried out in duplicate transwell, and from each transwell, colonies were picked from inoculum, associated and internalised bacterial group 10 colonies each.

Table 3.4: Phase variable genes used in this study

PV gene	Function of encoded protein	Reference
<i>nalP</i>	Autotransporter protease	(Turner <i>et al.</i> , 2002)
<i>mSPA</i>	Autotransporter protease	(Turner <i>et al.</i> , 2006)
<i>hmpR</i>	Haemoglobin receptor	(Stojiljkovic <i>et al.</i> , 1995)
<i>porA</i>	Porin	(van der Ende <i>et al.</i> , 2000)
<i>nadA</i>	Adhesion/invasion	(Comanducci <i>et al.</i> , 2002)
<i>fetA</i>	Iron-regulated enterobactin receptor	(Dyer <i>et al.</i> , 1988)
<i>opc</i>	Outer membrane protein	(Olyhoek <i>et al.</i> , 1991)

Fragment size was determined by ABI 3130 Genetic Analyzer and Peak Scanner v1.0 software as described in (3.4.6). The size was recorded for the primary peak where the ratio of primary to secondary peak was >1.2 . Where the ratio was <1.2 then the sizes of both peaks were recorded and just the size of the largest peak was utilised for determining tract length. Tract lengths and expression states were acquired from (Alamro *et al.*, 2014).

The expression status of six PV genes out of seven (*nalP*, *mshA*, *hmpR*, *porA*, *fetA* and *opc*) was phase ON, while *nadA* was phase OFF (Table 3.5). This pattern of PV gene expression was uniform in all 120 colonies, suggesting a lack of selection for phase variants in MC58 after being in contact with Calu-3 cells for 24 hpi.

Table 3.5: Expression status for the phase variable genes during infection

PV genes	Tract ¹	Inoculum	Associated	Internalised
		Exp. State ² *(40) 100%	Exp. State ² *(40) 100%	Exp. State ² *(40) 100%
<i>nalP</i>	10C	1	1	1
<i>mshA</i>	9C	1	1	1
<i>hmpR</i>	9G	1	1	1
<i>porA</i>	12G	2	2	2
<i>nadA</i>	9TAAA	0	0	0
<i>fetA</i>	9C	2	2	2
<i>opc</i>	13C	2	2	2

¹Indicates that the tract length was confirmed by sequencing (Alamro *et al.*, 2014); ²Expression states were determined by Western blotting and the coding is 2 for high or ON, 1 for intermediate and 0 for OFF (Oldfield *et al.*, 2013); *Numbers between brackets resemble the number of colonies were tested, in total 120 colonies obtained from two independent experiments each was performed in duplicate

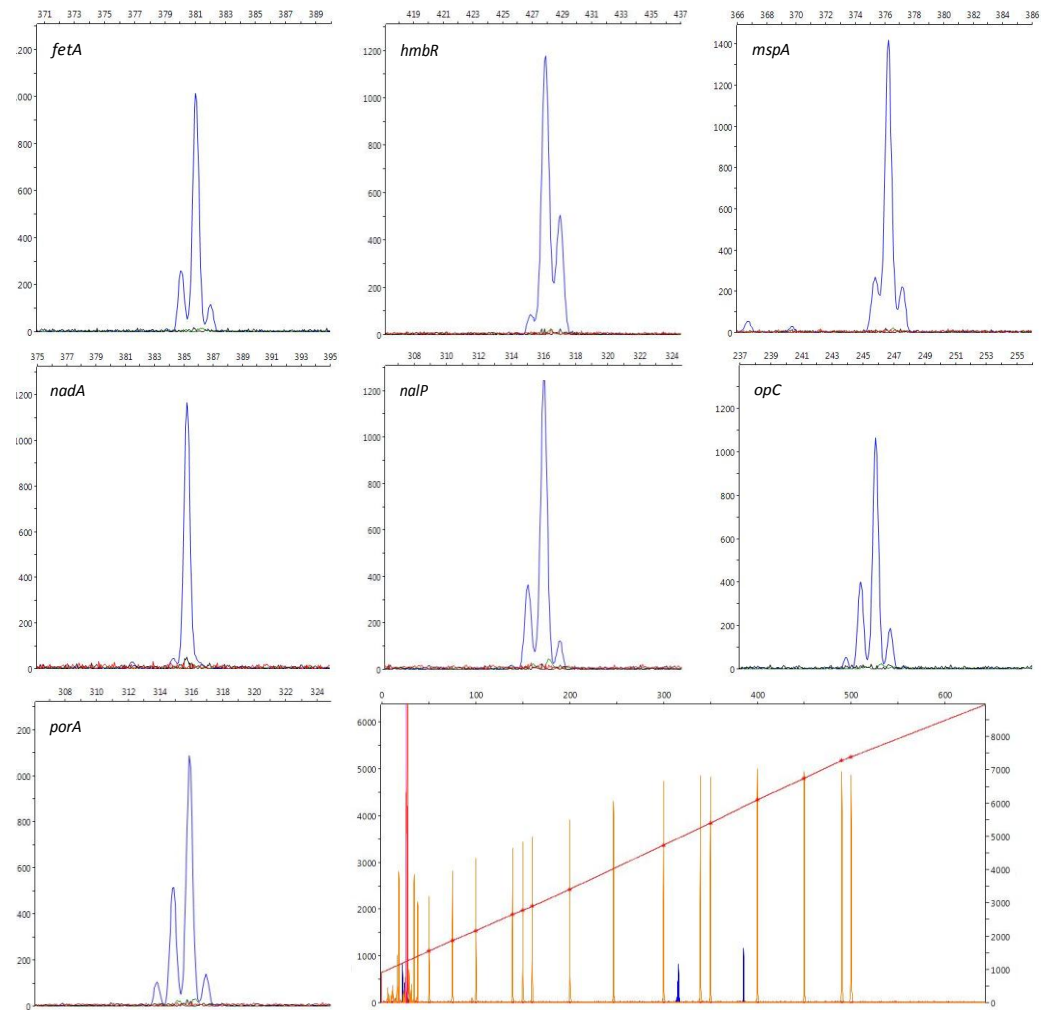


Figure 3.13: Representative plot view histograms for tested PV genes in Peak Scanner.

The data generated by ABI 3130 Genetic Analyzer was analysed in Peak Scanner v1.0 software. The figure includes peaks height in the X axis and the DNA fragment size (in base pairs) in the Y axis. In addition to a representative overlay view for fragments' peaks (blue), size standard (yellow) and the linear sizing curve (red). The change in the tract length was concluded from the fragment size.

3.6 Discussion

An *in vitro* infection model of differentiated Calu-3 cells was successfully achieved based on TEER measurements and other characteristics previously determined in our lab. The preparation of consistent inoculum of MC58 was fulfilled for MOI of 100, 50 and 30. The Calu-3 monolayer, on 0.4 µm pore size transwells, formed a barrier which could not be traversed by MC58 under the conditions tested. The Calu-3 cells responded to the challenge by elevating the expression level of TNF-α and GM-CSF. MC58 can survive within the infection model and its adhesion level was decreased at 24 hpi compared to 6 hpi. On the other hand, the invasion level was dramatically increased at 24 hpi compared to 6 hpi. MC58 phenotypic pattern ($\frac{nalP}{1} \frac{mspA}{1} \frac{hmpR}{1} \frac{porA}{2} \frac{nadA}{0} \frac{fetA}{2} \frac{opc}{2}$) was not changed after being in contact with Calu-3 cells for up to 24 hpi.

3.6.1 Calu-3 culture and differentiation

Calu-3 cells cultured at ALI have been shown to be more morphologically and functionally relevant to *in vivo* airway epithelium compared to liquid submerged culture (Grainger *et al.*, 2006). In particular, ALI cell cultures have been shown to possess a more columnar morphology and form a

thicker (20-45 μM) pseudostratified layer than submerged cultures (Grainger *et al.*, 2006).

The use of ALI Calu-3 cell culture as an infection model for *N. meningitidis* has been assessed in a previous study by (Sutherland *et al.*, 2010). Their findings suggested that the Calu-3 model could be suitable for studying the pathogenesis of infections caused by other respiratory pathogens, as well as the meningococcus. In more recent study, Barrile *et al.* (2015), compared the *N. meningitidis* infection in the Calu-3 model at different stages of cell differentiation (6, 12 and 18 days) (Barrile *et al.*, 2015). They found that the level of polarization of the Calu-3 cell monolayer plays an important role in *N. meningitidis* infection.

A previous study in our lab has established a differentiated human bronchial epithelial culture in an attempt to generate an *in vitro* model for *P. aeruginosa* respiratory infection (Yi-Chia Liu, PhD Thesis). In this study she found that the 21 days old differentiated Calu-3 cells consistently featured: (i) cilia-expression and mucus production at the apical surface visualised by histological staining, confocal and scanning electronic microscopic images; (ii) tight junctions and transepithelial resistance verified by the expression of ZO-1 tight junction protein and relatively high

TEER. The previous study of the morphology, polarity and barrier characteristics of the 21 days differentiated Calu-3 cell culture in our lab and the study of barrier functionality by TEER in this study all together are supported by the literature (Bals *et al.*, 1999, Florea *et al.*, 2001, Florea *et al.*, 2002, Barrile *et al.*, 2015, Grainger *et al.*, 2006).

Despite the fact that ALI differentiated Calu-3 cell cultures exhibited the characteristics of cultures derived from primary NHBE (Berube *et al.*, 2010), it should be noted that the Calu-3 lung adenocarcinoma cell line is hypotriploid. Thus, some properties in Calu-3 cells may not fully imitate those in the healthy native epithelium.

3.6.2 Infection of differentiated Calu-3

MC58 does not traverse the Calu-3 monolayer grown on 0.4 μm pore size transwells, which form a strong polarized epithelial barrier. The use of ALI Calu-3 cell culture as an infection model for MC58 has been assessed in previous study by Sutherland *et al.* (2010). They were able to detect bacteria traversing across the Calu-3 monolayer in small numbers (equivalent to 0.0035% of input) at 8 hpi. By 24 hpi, the number of bacteria traversing had increased by around 4 fold. They were not able to find bacteria in the basal chamber prior to 6 hpi (Sutherland *et al.*, 2010).

Importantly, although they show evidence of differentiation by confocal and electron microscopy, and TEER measurement, the protocol used in their study has major differences compared to that utilized in this study. Firstly, the Sutherland study used transwells with a pore size of 1 μm . Furthermore, they stated that less than 1 μm pore size impeded the passage of the bacteria. In fact whilst optimising our infection assay bacteria were detected traversing across 0.4 μm pores, but that was caused by an incomplete confluence of Calu-3 in some transwells. Secondly, the differentiation method they followed was by seeding (4×10^5) cells grown and differentiated for 5 days, while in this study 1×10^5 cells were seeded and differentiated for 21 days. Finally, they washed the monolayer thoroughly with media at the designated time points and then transferred into new wells. Samples were taken from the basal chambers of the new wells after 1 h and plated for the CFU count. Our method was to collect the basal media from the same well used for infection. Moreover, the monolayer washes were performed after the basal media collection to avoid damaging the monolayer and causing any artefactual effects on the CFU count.

In more recent study, Barrile *et al.* (2015), compared the ability of MC58 strain to invade and cross Calu-3 monolayer at different stages of cell differentiation (6, 12 and 18 days)

(Barrile *et al.*, 2015). They found that poorly polarized Calu-3 monolayers are more susceptible to *N. meningitidis* invasion. Calu-3 cells were grown and differentiated for 6, 12 or 18 days, and then were infected with MC58 for 8 hpi. They reported a significant reduction in the number of internalized bacteria in fully polarized cells grown for 18 days (Barrile *et al.*, 2015). They use a modified protocol from Sutherland *et al.* (2010), but they seeded (1×10^6) cells.

We can conclude from the previous comparison that the longer differentiation is crucial for better polarization, which is supported by the literature (Bals *et al.*, 1999, Florea *et al.*, 2001, Florea *et al.*, 2002, Barrile *et al.*, 2015). We believe that the level of mucus secretion plays an important role as a physical barrier during infection, which might be attenuated by short time differentiation method in other studies. This might explain the reduced ability of MC58 to traverse the Calu-3 monolayer, especially as it is a non-motile bacterium. The bacteria motility can facilitate the pathogen traverse across the mucus layer and reach the surface of epithelial cells to start the interaction with them. That is supported by the observations in the work with *P. aeruginosa* in our lab, as the bacteria were able to traverse Calu-3 monolayer, differentiated for 21 days, and damage it even at MOI of 0.5. Obviously, the motility of the bacteria is not the only

difference between *N. meningitidis* and *P. aeruginosa*, but it is one of the important virulence factors. However, these findings highlight the need for confocal and electron microscopy work to determine the mucus thickness for different differentiation protocols and to study the impact of different levels of mucus thickness on the traversing ability of the bacteria.

3.6.3 Cytokine measurement by quantitative PCR

The Calu-3 cells reacting to infection with MC58 by elevating the expression levels of TNF- α and GM-CSF. TNF- α fold change mean was $\approx 80 \pm 19$ fold increase for MOI of 50 at 6 hpi. Whereas, the mean of GM-CSF levels were increased $\approx 178 \pm 26$ fold compared to uninfected cells (Figure 3.9). TNF- α is one of the most predominant secreted cytokines and its production by epithelial cells can be stimulated by many meningococcal related factors (*e.g.*, LOS endotoxin, synthetic lipid A, nitric oxide, oxygen radicals, toxic shock toxin- 1 or by the induction of GM-CSF, TNF and other cytokines) (Wang *et al.*, 2003). GM-CSF secretion mainly requires the producer cell to be stimulated, for example by other cytokines, antigens, or inflammatory agents (*e.g.*, endotoxin). The fact that GM-CSF is produced mainly as a response to cellular activation agrees

with its role in immune and inflammatory response against *N. meningitidis*.

In a clinical study in 1995 very high values of TNF- α were found in meningococcal septic patients with fatal disease (van Deuren *et al.*, 1995). In another study, human meningioma cells were challenged with *N. meningitidis* strain MC58 (Cap⁻Pil⁺), which triggered high GM-CSF secretion (up to 500 pg mL⁻¹) when compared to uninfected cells at 24 hpi (Christodoulides *et al.*, 2002).

Inducing TNF- α production has been reported in human malignant choroids plexus papilloma cell line (HIBCPP) when challenged with MC58 encapsulated and unencapsulated (MC58siaD⁻) at MOI of 10, but higher for the unencapsulated, which induce PMNs and monocytes transmigration (Steinmann *et al.*, 2013). Cells were treated for 2 h with bacteria followed by antibiotic killing of the bacteria and further incubation up to 20 h. Another study showed that when HIBCPP were challenged with MC58 and MC58siaD⁻ at MOI of 10 for 4 h followed by antibiotic treatment and further incubation up to 24 h, both TNF- α and GM-CSF responses were detected at 24 hpi for both strains. TNF- α response to MC58siaD⁻ was detected at 4 hpi, in general, the response against MC58siaD⁻ was always higher than MC58 (Borkowski *et al.*, 2014). In

fact, the presence of the capsule functionally masks membrane proteins, such as Opa and Opc proteins, that facilitate the bacteria internalisation and the close contact to host cells (Schubert-Unkmeir *et al.*, 2010). Moreover, the capsule can inhibit cell adhesion, biofilm formation, and help in the immune evasion (Pizza and Rappuoli, 2015, Kugelberg *et al.*, 2008). Another previous study demonstrated that challenging MDM with MC58, at MOI of 30 for 20 h, induced a 4.1 fold increase in TNF- α secretion (Stevanin *et al.*, 2007).

From the previous literature, no attempts have been reported to study the cytokine responses of Calu-3 cells against MC58. However, our observations are supported by these studies on other infection models. The cytokine qPCR does not necessarily reflect the actual secreted levels as the mRNAs of TNF- α and GM-CSF, like many cytokines, display short cytoplasmic half-lives, due to repetitive 5'-AUUUA-3' motifs in their 3' untranslated regions (Enzler and Dranoff, 2003, Wang *et al.*, 2003). However, it gives a strong indication of the intracellular signalling pathways activation as a result of the Calu-3/MC58 interaction.

3.6.4 Adhesion and invasion assay

The localisation of MC58 within the Calu-3 model was studied by the adhesion and invasion assay. The results revealed that

MC58 can grow within the infection model up to 24 hpi. The association level was decreased at 24 hpi compared to 6 hpi. On the other hand, the invasion level was significantly increased at 24 hpi compared to 6 hpi. However, similar results were reported for the population of gentamicin-protected (internalised) bacteria was consistently present in infected Calu-3 cells at 8 hpi, and the number was increased by an order of magnitude by 24 hpi (Sutherland *et al.*, 2010). Their confocal microscopy work on cross-sections of the monolayer at 24 hpi revealed the presence of internalised *N. meningitidis*, including bacteria located in the basal regions of Calu-3 cells. In addition, unlike our study they detected bacteria traversing across the monolayer, but that could be due to the short time of differentiation or the pore size of basal membrane they used (1 μm). They noted that the use of a filter with pore size $<1 \mu\text{m}$ impeded the passage of bacteria, which was discussed in (3.6.2).

The internalisation of *N. meningitidis* by Human Umbilical Vein Endothelial Cells (Huvecs) was studied by Virji *et al.* in 1991. They found that the intracellular bacteria at 4 hpi, which survived the gentamicin treatment, were extremely less than the associated bacteria. A confirmation by transmission electron microscopy (TEM) demonstrated the presence of

many bacteria attached to the cell surface, while a paucity of intracellular bacteria were detected (Virji *et al.*, 1991).

In a more recent study, MC58 association was investigated in the Detroit 562 pharyngeal carcinoma epithelial cell line challenged with MOI of 100 for 6 h. They found that 1.5% of the inoculum of MC58 associated with the cells, and just 0.01% of attached MC58 invaded. In addition, they demonstrated that the removal of LOS sialylation in the absence of capsule (CAP–, NANA–) in the strain MC58 background conferred a 52-fold increase in attachment and a 70-fold increase in invasion compared to wild-type MC58 (Bartley *et al.*, 2013). In the same study, the author stated that the recovered colonies of MC58 from these assays were found to have a minor population of capsule and LOS phase variants (Bartley *et al.*, 2013).

The PV study results from two independent experiments show that MC58 expression pattern for seven selected PV genes did not change after being in contact with Calu-3 cells for up to 24

hpi; $\left(\frac{nalP}{1} \frac{mspA}{1} \frac{hmpR}{1} \frac{porA}{2} \frac{nadA}{0} \frac{fetA}{2} \frac{opc}{2} \right)$.

NadA is one of the meningococcal components that aid adhesion and invasion into epithelial cells molecules (Capecchi *et al.*, 2005). Interestingly, NadA expression state was OFF all

the time which might explain the low association percentage compared to non-association.

Several meningococcal components were described to mediate adhesion with host cells (e.g., type IV pili, Opc, Opa, NhhA, App and NadA) (Pizza and Rappuoli, 2015). Type IV pili (Tfp) has been proposed to mediate meningococcal attachment to host cells via CD46 binding (Kallstrom *et al.*, 1997). Sutherland *et al.*, have shown that MC58 Δ *pilE* (lacking Tfp) displayed a reduced association compared to MC58 WT. Another study showed that different expression pattern combinations of Pili and Opc in MC58 have diverse influences on the association, as the absence of pili decreased the association more than the absence of Opc (Virji *et al.*, 1995b).

MC58 showed the ability to change the expression state of Tfp as 2 out of 10 of the MC58 population that traversed through Calu-3 monolayer did not express Tfp, while 100% of MC58 in apical chamber expressed it (Sutherland *et al.*, 2010). Therefore, the Calu-3 monolayer model offered a good approach to investigating MC58 PV phenotypic pattern. That could explain partially not detecting any changes in the expression states of our selected PV genes, as in our assay no bacteria were traversing. On the other hand, the ON state of

the other genes could be the optimum phenotype of those genes in MC58 at this stage of infection.

3.7 Summary

The findings shown in this chapter indicate that the use of a differentiated Calu-3 model to study *N. meningitidis* interactions with the host during infection is a promising approach. Therefore, the model was then challenged with meningococcal strains from different serogroups and variants to study their different behaviour within the model and the different Calu-3 response to each one of them. This work is shown in the following chapters.

CHAPTER FOUR

**THE USE OF CALU-3 MODEL
TO COMPARE THE
INFECTION PATTERN OF
NEISSERIA MENINGITIDIS
SEROGROUPS B AND Y**

4. THE USE OF CALU-3 MODEL TO COMPARE THE INFECTION PATTERN OF *NEISSERIA MENINGITIDIS* SEROGROUPS B AND Y

4.1 Introduction

Neisseria meningitidis is considered the main cause of the majority of bacterial meningitis and sepsis cases globally (Manchanda *et al.*, 2006). Among the six meningococcal serogroups of public health importance (A, B, C, W, Y and recently X), serogroup B is the main cause of invasive meningococcal disease in England and Wales (Gray *et al.*, 2006). Carriage serogroup Y isolates are frequently found but are considered to be less invasive than other serogroups, such as A, B, and C (Stephens *et al.*, 2007). In the UK, an increase in serogroup Y disease (from 34 cases in 2007 to 91 in 2011, 80 in 2012, 78 in 2013 and 75 in 2014) has been observed (www.gov.uk/government/publications), mainly caused by 4 clonal complexes cc23 (56% of isolates), cc174 (21%), cc167 (11%), and cc22 (8%) (Ladhani *et al.*, 2012). The median age for the cases was 60 years with 19% mortality, while the underlying percentage was 39% (Ladhani *et al.*, 2012). In endemic situations, serogroup B is most common in infants while Y in elderly people (Stephens *et al.*, 2007).

Ladhani *et al.* Found that *lpxL1* mutations were present in 97% of invasive cc23 serogroup Y isolates. The high frequency of *lpxL1* mutations among invasive disease and carriage cc23 isolates suggests that *lpxL1* mutations might have occurred within the host to avoid recognition by the immune system (Ladhani *et al.*, 2012, Fransen *et al.*, 2009). University students living in residential halls seemed to be at high risk of serogroup Y meningococcal attack, as its carriage is common among students (Bidmos *et al.*, 2011).

The association between different clonal complexes and specific clinical features in different age groups is of interest. It has been reported that infection with cc23 strains with the *lpxL1* mutation were more likely to cause meningitis, mainly in young adults, and less likely to cause activation of the coagulation system in meningococcal septicaemia (rash, septic shock, or thrombocytopenia); In contrast cc174 is more associated with non-meningitis symptoms, particularly pneumonia, in older age groups (>65 years) (Ladhani *et al.*, 2012, Fransen *et al.*, 2009).

A previous study in our lab compared cytokine production in response to serogroup B strain MC58 and serogroup Y strain N222.1, using peripheral blood mononuclear cells (PBMCs). The results showed no significant differences in production of

TNF- α and IL-6 in response to N222.1 compared to MC58. In the case of the anti-inflammatory cytokine IL-10, significantly lower production was observed in the case of N222.1. This study also focused on the genetic differences between MC58 and N222.1 and uncovered an *lpxL1* mutation in N222.1 consisting of an adenosine deletion near the start codon site of *lpxL1*, MC58 (5' ATGAAATTT 3') and N222 (5' ATG_AATTT 3'). These results suggest that the level of acylation in LOS might play a role in manipulating the immune response by altering the balance between pro- and anti-inflammatory cytokines induction. This is supported by John *et al.* who showed that TNF- α production by human monocytic THP-1 cells and primary human monocytes is subject to variation by approximately 2-10 fold, caused by differences in the state of acylation and phosphorylation of the lipid A moiety of LOS (John *et al.*, 2009).

4.2 Hypothesis

We hypothesise that the Calu-3 model can be a useful tool to study responses against different meningococcal strains, as well as studying the different patterns of meningococcal infections in the respiratory tract, especially at the very early stage.

4.3 Aim

We aim to investigate the different innate immune responses in Calu-3 cells in response to different meningococcal strains of serogroups B and Y. To achieve that infection assays were performed to investigate:

- a. Bacterial adhesion and invasion.
- b. The integrity of epithelial monolayer post-infection.
- c. Cytokine responses.

4.4 Materials and methods

4.4.1 Calu-3 culture and differentiation

See (3.4.1).

4.4.2 Preparation of bacteria inoculum for infections

The strains used in this study are described in (Table 4.1). The inoculum was prepared using a method modified from (Bartley *et al.*, 2013). Bacteria were grown from -80°C stocks on Columbia Agar with chocolate horse blood and incubated at 37°C in 5% CO₂. For inoculum preparation, bacteria were harvested with an L-shaped sterile plastic spreader from an 18h agar plate by adding 2 mL of pre-warmed DMEM/F-12. Then 1 mL of the bacterial suspension was collected and normalised with pre-warmed DMEM/F-12 to an OD₆₀₀ of 0.24. Calu-3 cells were infected with 100 µL per well of the normalised inoculum. This standard was applied to all experiments to achieve MOI of 30. For each experiment, a viable count of bacteria was performed to confirm the actual MOI used as an inoculum.

Table 4.1: General information about the strains used in this study

Strain	Sero-group	Clonal complex	Description	Source or references
MC58	B	cc32	Wild type	(Tettelin <i>et al.</i> , 2000c)
H44/76	B	cc32	Wild type	(Frasch <i>et al.</i> , 1985)
N222.1	Y	cc23	Clinical carriage isolate	(Bidmos <i>et al.</i> , 2011)
N459.3	Y	cc23	Clinical carriage isolate	(Bidmos <i>et al.</i> , 2011)
N459.6	Y	cc23	Clinical carriage isolate	(Bidmos <i>et al.</i> , 2011)
N59.1	Y	cc174	Clinical carriage isolate	(Bidmos <i>et al.</i> , 2011)

4.4.3 Adhesion and invasion assay

See (3.4.5).

4.4.4 Cytokines measurement by Enzyme-Linked Immunosorbent Assay (ELISA)

4.4.4.1 TNF- α

Cytokines were measured using the DuoSet ELISA systems from R&D Systems. Mouse Anti-Human TNF- α Capture Antibody was diluted to a working concentration of 4.0 $\mu\text{g mL}^{-1}$ in PBS without carrier protein. MaxiSorp 96-well surface microplates, from Thermo Scientific, were coated immediately with 100 μL per well of the diluted capture antibody overnight at room temperature. Next morning each well was aspirated and washed three times with 400 μL per well wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4). After the last wash, the remaining wash buffer in the plate was removed by inverting the plate and blotting it against clean paper towels. Plates

were blocked by adding 300 μL per well of reagent diluent (1% BSA in PBS, pH 7.2-7.4, 0.2 μm filtered). This was followed by 1h incubation at room temperature, and then aspiration/wash step was repeated as above. 100 μL of basal media samples or standards in reagent diluent were added to each well. Afterwards, plates were covered with parafilm and incubated for 2 hours at room temperature followed by aspiration/wash as above. Biotinylated goat anti-Human TNF- α antibody diluted to 500 ng mL^{-1} in reagent diluent was added (100 μL per well). Plates were covered with a new parafilm strip and incubate for 2 h at room temperature, then aspiration/wash as above. 100 μL of streptavidin-HRP diluted 1 in 200 in reagent diluent was added to each well. Plates were then covered and incubated for 20 min in the dark at room temperature and washed as above. Then substrate solution [100 μL of a 1:1 mixture of H_2O_2 and tetramethylbenzidine (R&D Systems)] was added to each well. Then plates were incubated for 20 min in the dark at room temperature. The reaction was stopped by adding 50 μL per well of 2M H_2SO_4 . Optical density was determined using FinstrumentsTM microplate reader set to two wavelengths, 450 nm and 550 nm. Readings at 550 nm were subtracted from the readings at 450 nm. This subtraction was to correct the optical imperfections in the plate.

4.4.4.2 IL-8

As described previously in TNF- α ELISA, except that the reagent diluent was comprised 0.1% BSA, 0.05% Tween 20 in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl); pH 7.2-7.4, 0.2 μ m filtered. In addition, the antibodies used were mouse anti-human IL-8 and biotinylated goat anti-human IL-8 (DuoSet, R&D Systems).

4.4.5 Epithelial monolayer permeability assay

Calu-3 cells were cultured and differentiated for 21 days as described in (3.4.1) and infected as described in (3.4.2 and 4.4.2). At 12 hpi the basal media was collected from each well and transferred to sterile 1.5 mL tubes and stored at -80 °C for cytokine measurement. Uninfected Calu-3 cells were used as controls. Calu-3 cells were then washed twice with 450 μ l per well X1 sterile PBS, and then the inserts were transferred to a fresh 12-well plate.

Soon after, filter sterile 0.5 mL Hanks' balanced salt solution (HBSS) from (Sigma), containing 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Sigma) and 1 mg mL⁻¹ of fluorescein isothiocyanate (FITC)-Dextran 10 kD (Sigma), was added to the top chamber, and 1.5 mL of HBSS, 25 mM HEPES into the bottom chamber. 100 μ l samples were collected from the basolateral chamber every 30 min for 3 h

and placed into 96-well polystyrene black plates with a flat bottom (Costar, Sigma-Aldrich) in duplicates. A serial dilution of FITC-Dextran (10 kD) in HBSS, 25 mM HEPES in duplicate was used as a standard in the same plate. All the incubations were at 37 °C in 5% CO₂.

FITC-Dextran was detected using a FLUOstar omega multi-detection microplate reader (BMG LABTECH) with excitation and emission wavelengths of 485 nm and 520 nm, respectively.

4.4.6 Histological haematoxylin and eosin staining

Uninfected and infected Calu-3 monolayers at 6 hpi on transwell inserts were fixed overnight with 4% paraformaldehyde (16% para-formaldehyde, EM grade; Electron Microscopy Sciences) in PBS at 4°C. Following the fixation, the filters were cut out, halved, and each half was paraffin wax embedded using a Leica TP1020 automated tissue processor. Cross-sections with a 5 µm thickness of wax-embedded filters were obtained using a Leica RM2145 microtome. The cross-sections were air-dried after being mounted on glass slides, rehydrated using decreasing concentrations of ethanol and stained with haematoxylin and eosin (Raymond A Lamb Laboratory Supplies). After staining, sections were dehydrated by passing them through increasing

concentrations of ethanol, dried, and mounted using DPX mounting medium (Raymond A Lamb Laboratory Supplies). Mounted sections were viewed using a Zeiss Axiovert 25 microscope and images were captured with a detachable Canon PowerShot camera, and images were processed by ImageJ software version 1.47.

4.5 Results

4.5.1 Comparison between MC58 and N222.1

4.5.1.1 Adhesion and invasion pattern and cytokines profile

Differentiated Calu-3 cells were cultured for 21 days in 0.4 μ M pore size transwell in ALI, were infected in parallel with MC58 or N222.1 for 6 and 24 h at MOI of 30. The rationale of this experiment was to evaluate the possible different pattern of adhesion and invasion between the two strains as well as the cytokine production of Calu-3 cells after infection.

The first observation was that the Calu-3 layer was totally disrupted by 24 hpi when infected with N222.1 this was not observed in the case of MC58. During the delicate PBS wash Calu-3 cells came off the filter completely after infection with N222.1. Both strains were able to significantly grow within the Calu-3 model for up to 24 hpi as shown in Figure 4.1 and Figure 4.2. However, N222.1 seemed to take a slightly longer time to grow compared to MC58.

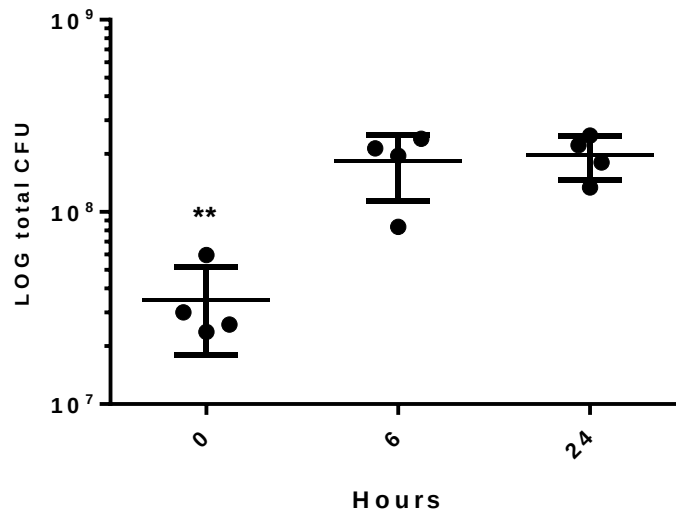


Figure 4.1: Total CFU count for inoculum and total CFU count at 6 and 24 hpi of MC58 in adhesion and invasion assay.

The chart shows in LOG scale the total CFU count at different time points: 0h (inoculum), 6 and 24 hpi. (mean±SD) (N=4, **P<0.01, one-way ANOVA)

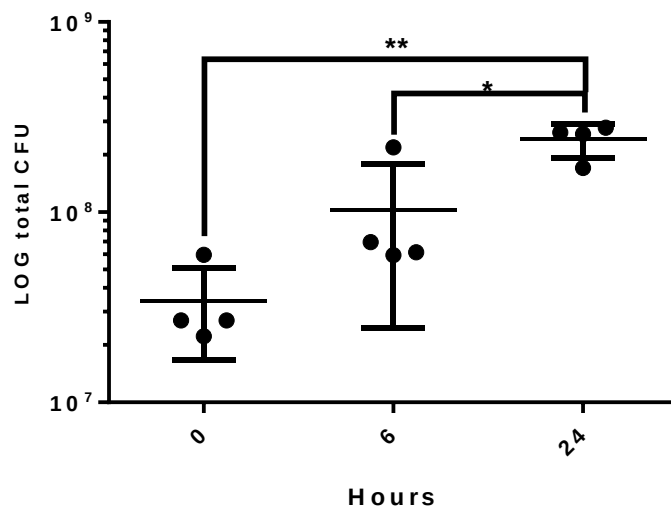


Figure 4.2: Total CFU count for inoculum and total CFU count at 6 and 24 hpi of N222.1 in adhesion and invasion assay.

The chart shows in LOG the total CFU count at different time points: inoculum (0h), 6 and 24 hpi. (mean±SD) (N=4, **P<0.01, *P<0.05, one-way ANOVA)

The growth and distribution of MC58 within the Calu-3 model (Figure 4.3), were identical to those found in the previous chapter (3.5.4), in spite of the fact that the inoculum preparation was different. Due to the total destruction of N222.1 infected Calu-3 monolayer at 24 hpi, the determination of the bacterial localisation at 24 hpi was not feasible (Figure 4.4). The comparison of the bacteria localisation between both strains at 6 hpi is presented in (Figure 4.5), which shows that N222.1 association to cells in general was not significantly lower than in the case of MC58.

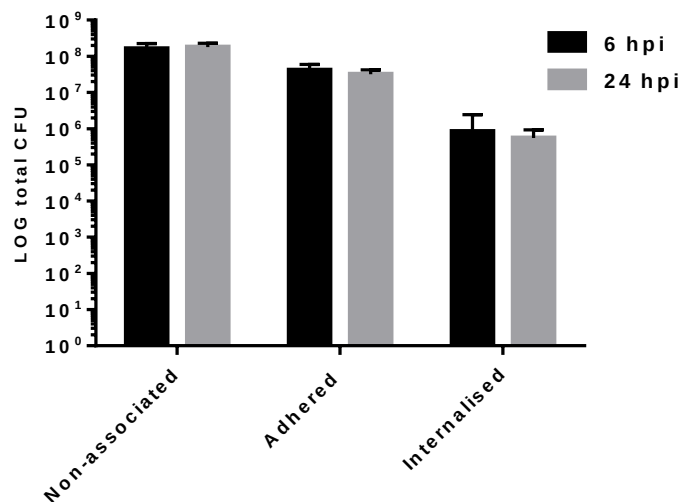


Figure 4.3: MC58 distribution during infection of Calu-3 cells at 6 and 24 hpi.

The chart shows CFU (mean $\pm$ SD) for each localisation group (non-associated, adhered and internalised) at 6 and 24 hpi. (N=4, P=0.1, two-way ANOVA)

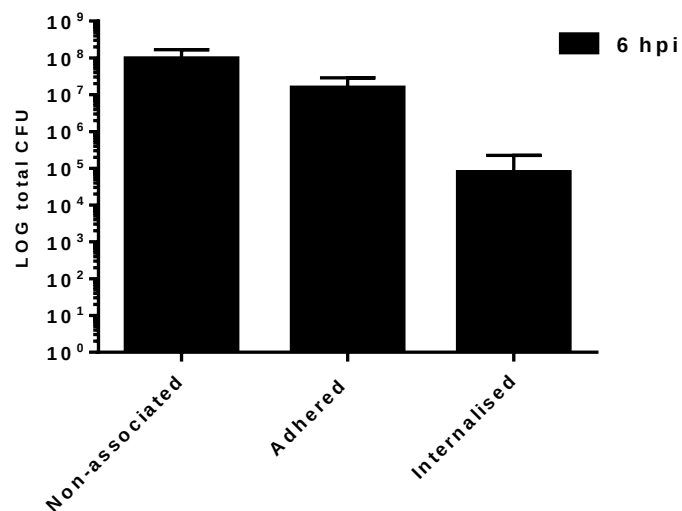


Figure 4.4: N222.1 distribution during infection of Calu-3 cells at 6 hpi.

The chart shows CFU (mean±SD) for each localisation group (non-associated, adhered and internalised) at 6 hpi. (N=4)

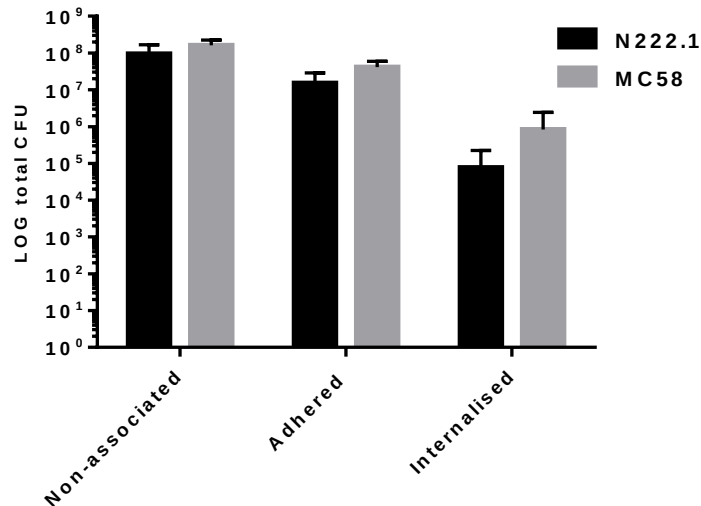
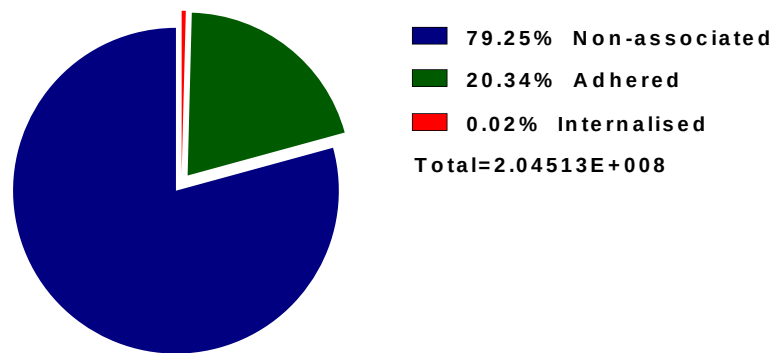
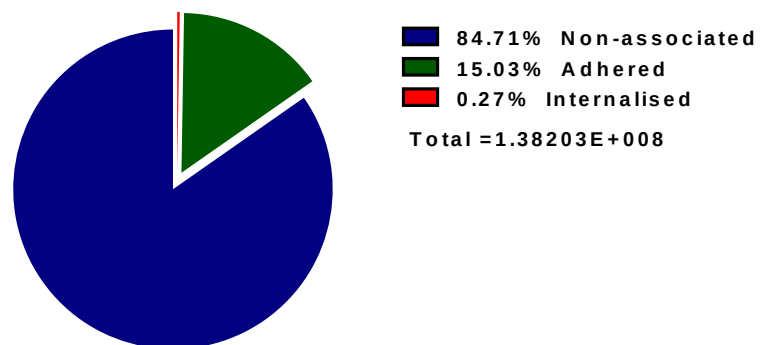
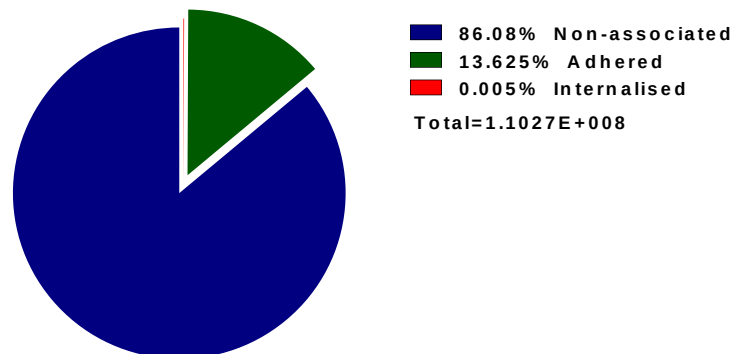


Figure 4.5: Comparison between N222.1 and MC58 localisation at 6 hpi within Calu-3 model.

The chart shows CFU (mean±SD) for each localisation group (non-associated, adhered and internalised) at 6 hpi. (N=3, P=0.1, two-way ANOVA)

The analysis of the distribution of MC58 and N222.1 within the Calu-3 model was represented as percentages as shown in (Figure 4.6). The data show that MC58 was exhibiting an identical pattern to that observed in chapter 3. By comparing the pattern of bacteria distribution and localisation of MC58 and N222.1 at 6 hpi, it is clear that the total CFU count for N222.1 (1.1×10^8) was almost one fold less than for MC58 (2.0×10^8). In general, the association percentage for N222.1 was almost 7% lower than for MC58, which means that MC58 associated slightly better to Calu-3 cells at 6 hpi. The percentage of N222.1 adhesion to Calu-3 cells at 6 hpi was 13.6%, while it was 22.3% for MC58. That means that N222.1 adhered one-third less than MC58 at 6 hpi. The adhesion percentage was calculated as the difference between association and invasion. The invasion percentage of N222.1 at 6 hpi was 0.005%, which was one-quarter of the MC58 invasion percentage (0.02%).

A. Percentage of MC58 association 6 hpi**B. Percentage of MC58 association 24 hpi****C. Percentage of N222.1 association 6 hpi****Figure 4.6: Charts depicting bacterial distribution within the Calu-3 model.**

The pie charts show the mean of total CFU counts into percentages for non-associated bacterial cells and cell-associated (adhered and internalised) for (A) MC58 at 6 hpi, (B) MC58 at 24 and (C) N222.1 6 hpi. No bacteria were detected in the basolateral chamber during these infections. The data represent the mean of four repeats performed independently (N=4).

The traversing ability was assessed by plating 50 μ l of basal media and 5 serial dilutions from each well on Columbia Agar with chocolate Horse blood. Next day, the plates were checked for growth to perform CFU count. For all the conditions (MC58 and N222.1, at 6 and 24 hpi) no bacteria were detected in the basal chamber even though the N222.1 infected monolayer was destroyed by 24 hpi.

TNF- α and IL-8 secretion by infected Calu-3 cells were quantified by ELISA as (pg mL^{-1}) and compared (mean $\pm$ SD) with uninfected Calu-3 cells at 6 and 24 hpi. TNF- α secretion levels were elevated significantly by both strains at 24 hpi compared to uninfected cells (Figure 4.7). However, N222.1 triggered a significantly higher TNF- α response ($104.8\pm15.9 \text{ pg mL}^{-1}$) compared to MC58 ($14.2\pm9.7 \text{ pg mL}^{-1}$). On the other hand, IL-8 secretion was not significantly changed by the infection with both strains at both time points; it was approximately $\pm 3000 \text{ pg mL}^{-1}$. These findings indicated that Calu-3 cells might be sensing the differences between both strains and responding differentially against each.

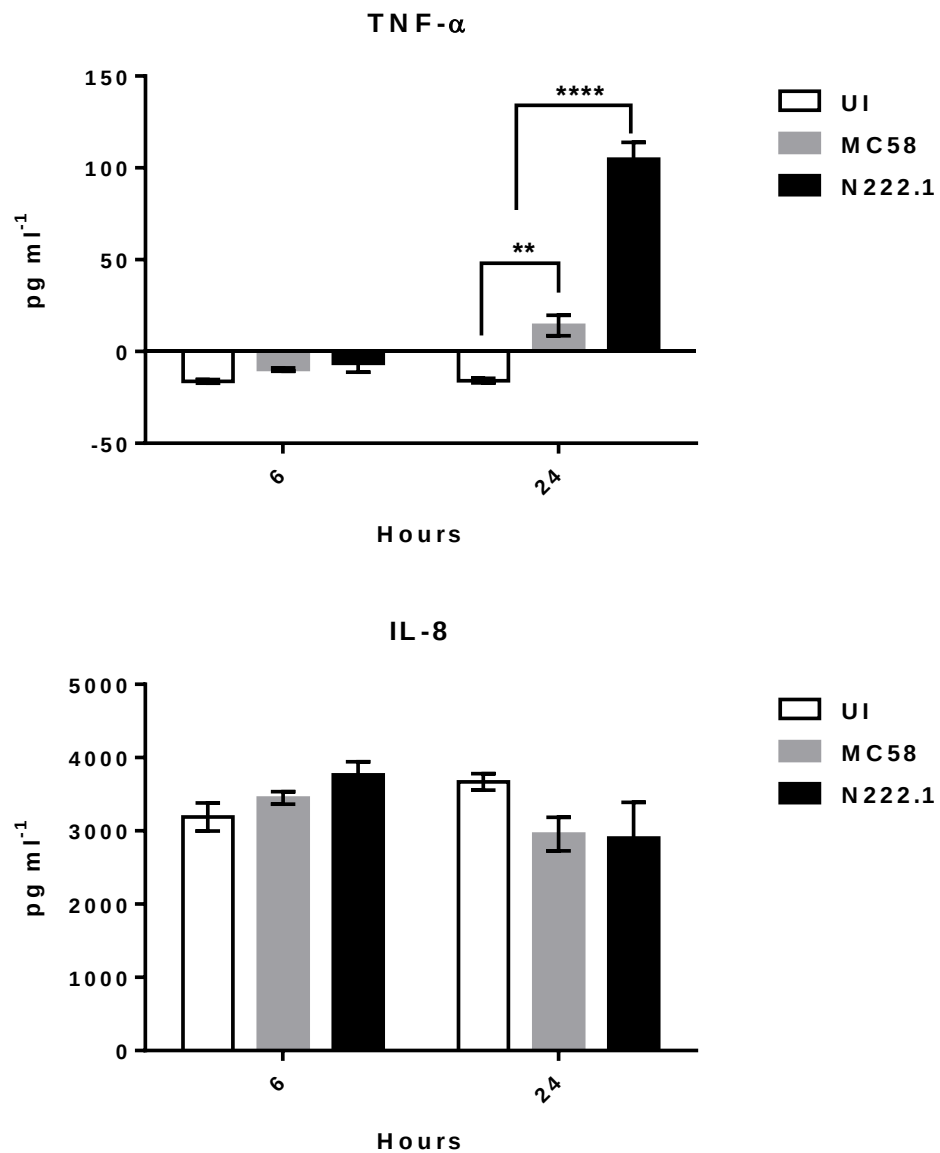


Figure 4.7: Cytokines responses of Calu-3 cells infected with MC58 or N222.1 up to 6 and 24 hpi.

The charts show (mean $\pm$ SD) of TNF- α and IL-8 production by Calu-3 cells in response to MC58 and N222.1 infections at MOI of 30 at 6 and 24 hpi. MC58 triggers a significant TNF- α response compared to uninfected cells at 24 hpi. N222.1 triggers a significantly higher TNF- α response compared to MC58 at 24 hpi. IL-8 does not show any significant differences between all the conditions at any of the time points. Uninfected Calu-3 cells were used as a control. (N=3, **P<0.005, ****P<0.0001, two-way ANOVA).

These findings encouraged us to study the integrity of the Calu-3 layer post-infection to better characterise the observed destruction of the cells by N222.1 at 24 hpi. Therefore, a permeability assay was applied using the same Calu-3 model and meningococcal strains.

4.5.1.2 Epithelial monolayer integrity and cytokine production after infection with MC58 and N222.1

Differentiated Calu-3 cells in ALI were challenged with MC58 or N222.1 at MOI of 30 for 12 hpi. The level of integrity was determined using a Dextran permeability assay. For this assay, FITC-Dextran (10,000-MW) was added to the apical side of Calu-3 cells after washes. The fluorescence in the basolateral chamber was monitored every 30 min over the next 3 h post FITC-Dextran addition. The rationale for choosing 12 hpi was because we assumed that the monolayer will not be totally damaged at that time point and can survive the washes steps before starting the assay, while we can still be able to detect the difference between both strains.

The assay revealed that the permeability of the epithelial layer was significantly increased by N222.1 infection in general, while MC58 appeared to enhance the epithelial barrier (Figure 4.8). The difference in the integrity was concluded from the level of the fluorescence unit passage into the basal chamber.

The difference between strains was clear in the first 30 min after the FITC-Dextran addition, but after 2 h the difference started to be significant in the case of N222.1 infection.

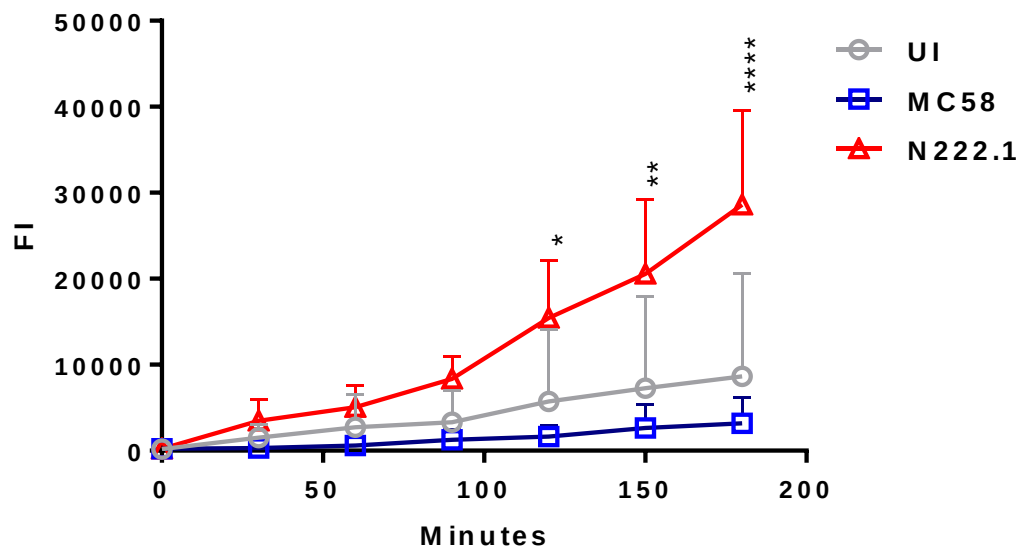


Figure 4.8: Comparison between the permeability of uninfected Calu-3 monolayer and Calu-3 cells challenged with MC58 or N222.1 up to 12 hpi.

FITC-Dextran (10,000-MW) was added to the apical side of Calu-3 monolayers infected with MC58 or N222.1 at an MOI of 30 for 12 h. Fluorescence in the basal chamber was monitored every 30 min over the next 3 h post FITC-Dextran addition. The chart shows the monolayer permeability (mean $\pm$ SD) which significantly increased after challenging the cells with N222.1 compared to uninfected Calu-3 cells as a control, while MC58 insignificantly decreased the permeability of the monolayer compared to control cells. (N=4, *P<0.05, **P<0.005, ****P<0.0001, two-way ANOVA)

Before the addition of FITC-Dextran, the basal media was collected for TNF- α measurement and replaced with the transfer media (see 4.4.5). The data in Figure 4.9 reveal that

the level of TNF- α secretion (mean $\pm$ SD) triggered by MC58 infection was 23.3 ± 2.9 pg mL $^{-1}$ at 12 hpi, which was not significantly higher than uninfected cells. N222.1 infected cells secreted significantly higher levels of TNF- α compared with both uninfected cells and infected with MC58. The level of TNF- α was 347.6 ± 105 pg mL $^{-1}$ in the case of N222.1, which was more than 3-fold higher than its level at 24 hpi (see 4.5.1). This could be explained by the extensive monolayer damage at 24 hpi.

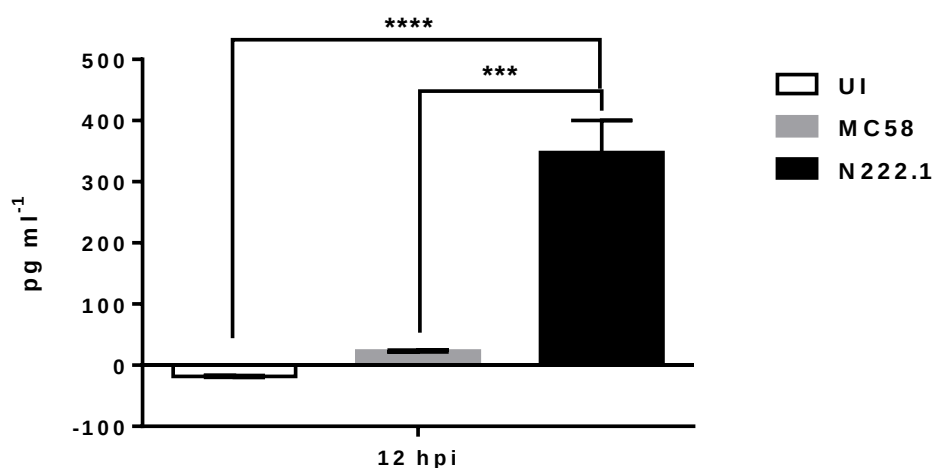


Figure 4.9: TNF- α production by Calu-3 cells infected with MC58 or N222.1 at 12 hpi.

TNF- α secretion (mean $\pm$ SD) for Calu-3 cells uninfected or infected with MC58 or N222.1 at MOI of 30 for 12 hpi, N222.1 triggers a significantly higher TNF- α response compared to MC58 and uninfected cells at 12 hpi. (N=4, ***P=0.0001, ****P<0.0001, one-way ANOVA)

To examine the level of the damage caused by N222.1 a histological analysis was done using samples from uninfected

Calu-3 and Calu-3 cells infected with MC58 or N222.1 at MOI of 30 for 6 hpi. Haematoxylin and eosin staining was performed on paraffin-embedded sections. We decided to use cells challenged for 6 hpi to ensure that the monolayer can survive the aggressive tissue processing treatments. The cross sections of uninfected Calu-3 displayed a columnar appearance, while the MC58 infected cells showed a tightly compressed monolayer (Figure 4.10). On the other hand, N222.1 infected Calu-3 monolayer displayed a poor cellular integrity, as it came off the filter and was difficult to be located near the filter in many sections. This experiment was performed one time only.

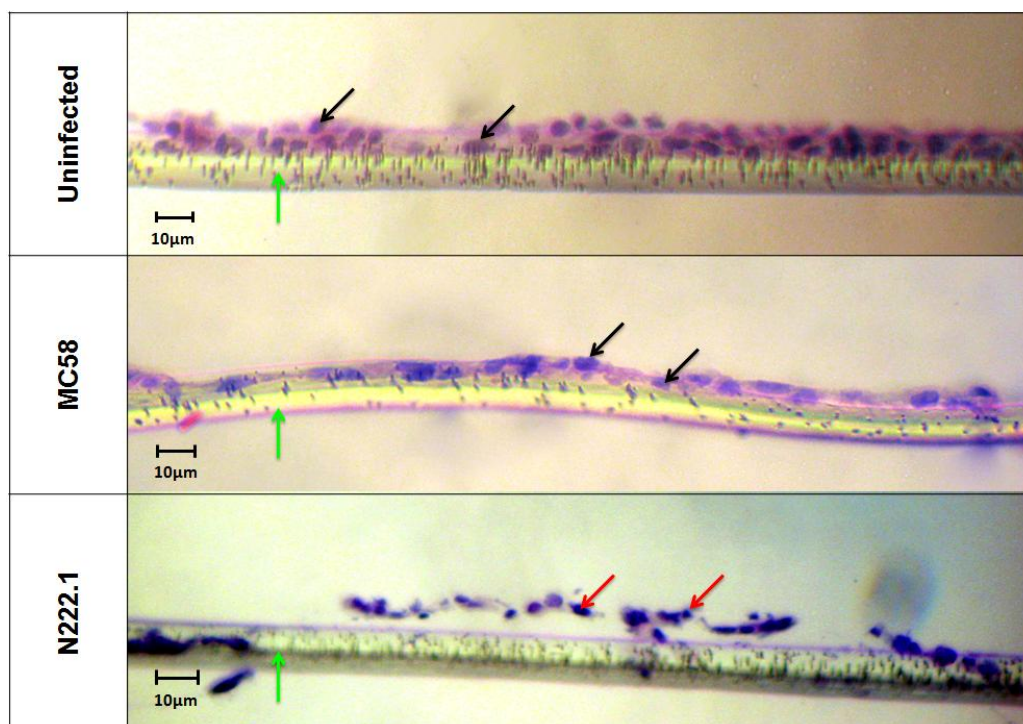


Figure 4.10: Histological staining of uninfected differentiated Calu-3 cells and Calu-3 cells infected with MC58 or N222.1 for 6 h.

Haematoxylin and eosin stained paraffin-embedded sections of uninfected Calu-3 and infected with MC58 or N222.1 at MOI of 30 for 6 hpi. Uninfected Calu-3 displayed a columnar appearance, while the MC58 infected cells showed a tightly compressed monolayer. On the other hand, N222.1 infected cultures displayed a poor cellular integrity. Black arrows point at nuclei, red arrows point at detached cells and green arrows point at 0.4 μm filters.

Altogether, the data revealed that the Calu-3 monolayer is responding differentially against different strains, additionally, it suggested that the monolayer damage and TNF- α response do not correlate to the level of bacteria association with Calu-3 cells. Thus, it was decided to introduce more different meningococcal strains to the study and investigate their

pattern of infection in the Calu-3 model to better understand the factors behind these different outcomes.

4.5.2 Comparison between H44/76 and N59.1

4.5.2.1 Adhesion and invasion pattern

Differentiated Calu-3 cultured in ALI, were infected in parallel with serogroup B H44/76 (cc32) or serogroup Y N59.1 (cc174) for 6 and 24 h at MOI of 30. The rationale of this experiment was to evaluate if there is a correlation between serogroups differences and the pattern of adhesion and invasion, as well as, cytokine responses after infection of Calu-3 cells.

Unlike in the case of N222.1, the integrity of the Calu-3 monolayer was not disrupted by N59.1 or H44/76 infection. N59.1 was able to significantly grow after infection of Calu-3 cells for up to 24 hpi as shown in Figure 4.11 similarly to N222.1. However, unlike MC58, H44/76 showed a significant increase in CFU count at 6 hpi followed by a significant reduction at 24 hpi (Figure 4.12).

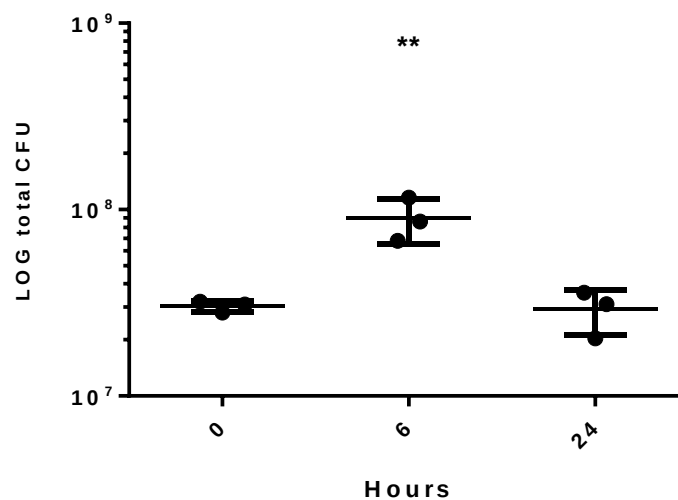


Figure 4.11: Total CFU count for inoculum and total CFU count at 6 and 24 hpi of H44/76 in adhesion and invasion assay.

The chart shows in LOG scale the total CFU count at different time points: 0h (inoculum), 6 and 24 hpi. (mean±SD) (N=4, **P<0.01, one-way ANOVA)

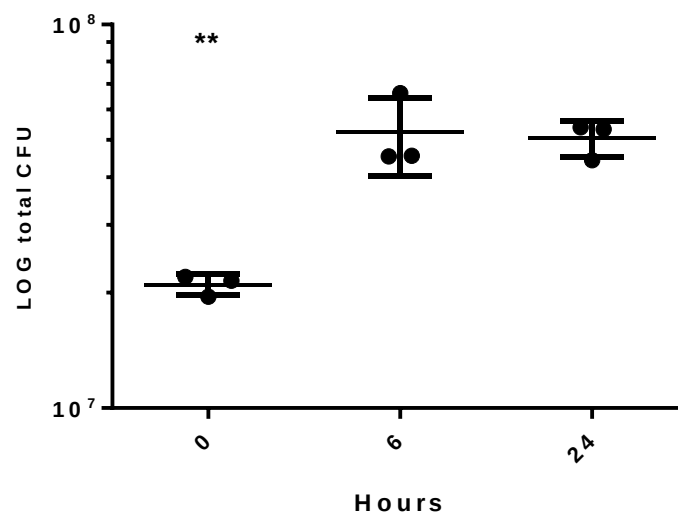


Figure 4.12: Total CFU count for inoculum and total CFU count at 6 and 24 hpi of N59.1 in adhesion and invasion assay.

The chart shows in LOG scale the total CFU count at different time points: 0h (inoculum), 6 and 24 hpi. (mean±SD) (N=4, **P<0.01, one-way ANOVA)

The findings of the analysis for the H44/76 distribution and localisation within the Calu-3 model (Figure 4.13), were dissimilar to the findings for MC58 infection (see 4.5.1). The level of H44/76 association was significantly lower. Interestingly, N59.1 localisation analysis revealed that N59.1 association to cells, in general, was the highest among all the strains tested (Figure 4.14).

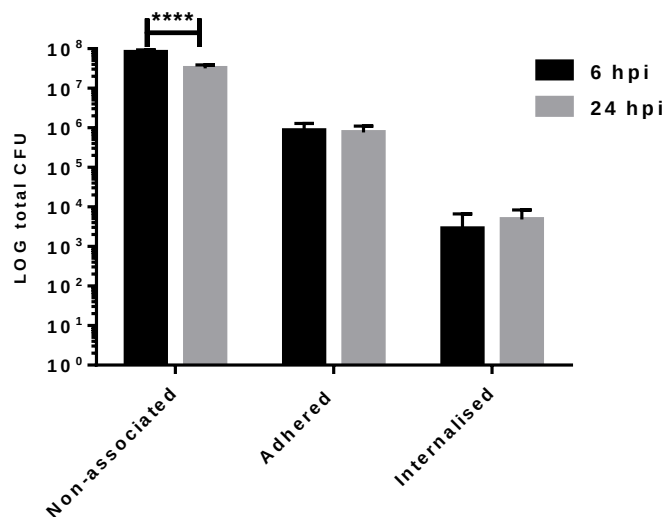


Figure 4.13: H44/76 distribution during infection of Calu-3 cells at 6 and 24 hpi.

The chart shows CFU (mean±SD) for each localisation group (non-associated, adhered and internalised) at 6 and 24 hpi. (N=3, ****P<0.0001, two-way ANOVA)

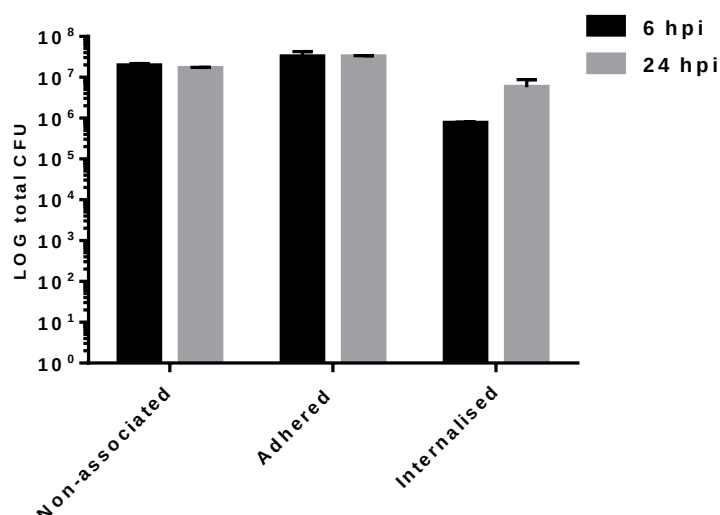


Figure 4.14: N59.1 distribution during infection of Calu-3 cells at 6 and 24 hpi.

The chart shows CFU (mean±SD) for each localisation group (non-associated, adhered and internalised) at 6 and 24 hpi. (N=4, P=0.1, two-way ANOVA)

The analysis for the distribution and localisation of H44/76 and N59.1 within Calu-3 model was presented in percentages as shown in (Figure 4.15). The data show that H44/76 exhibited the lowest level of association among all tested strains, while N59.1 had the highest (see also 4.5.1). By comparing the pattern of bacteria distribution of H44/76 and N59.1 at 6 hpi, it is clear that the total CFU count for N59.1 (5.1×10^7) was almost one-third less than for H44/76 (8.1×10^7). In general, the association level of N59.1 was almost 60 fold higher than for H44/76 at 6 hpi. The N59.1 adhesion level to Calu-3 cells at 6 hpi was 61.26%, while it was just 1.05% for H44/76. The invasion percentage of N59.1 at 6 hpi was 1.47%, which was

73 fold higher than for MC58 (0.02%) (see 4.5.1). On the other hand, H44/76 invasion percentage at 6 hpi was minimal ($\approx 0.003\%$).

At 24 hpi, the association (adhesion plus invasion) was enhanced in general for both strains. The h44/76 association was increased by more than two-fold (from $\approx 1.05\%$ to $\approx 2.34\%$), while N59.1 association increased by 7% (from $\approx 62.7\%$ to $\approx 69.6\%$). In fact, the N59.1 association increase appears to be a result of a 7-fold increase in the invasion percentage (from $\approx 1.5\%$ to $\approx 10.6\%$), while the adhesion percentage remained in the same position at 59-61%. In the case of H44/76, the increase in the association level at 24 hpi resulted from a two-fold increase in adhesion (from $\approx 1.05\%$ to $\approx 2.33\%$), and a 3-fold increase in invasion (from $\approx 0.003\%$ to $\approx 0.01\%$).

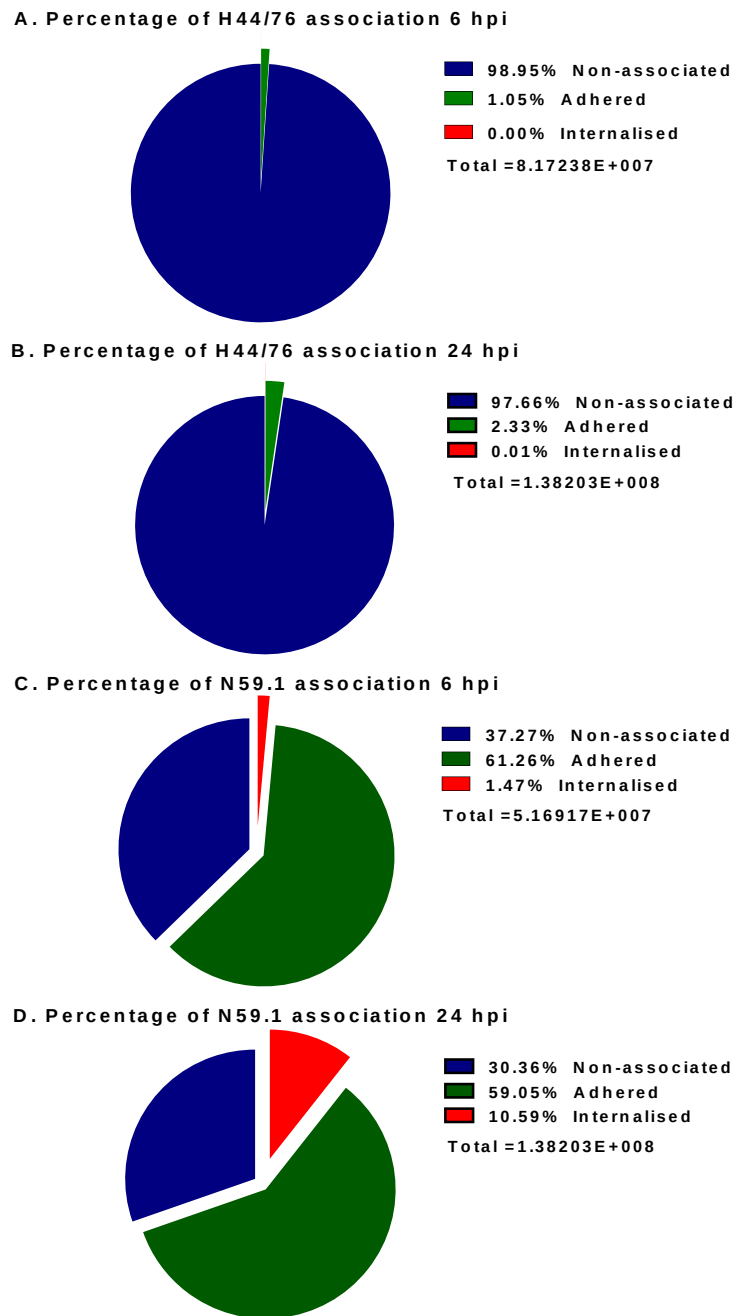


Figure 4.15: Charts depicting bacterial distribution within the Calu-3 model.

The pie chart breaking down the mean of total CFU counts into percentages for non-associated bacterial cells and associated (adhered and internalised) for (A) H44/76 at 6 hpi, (B) H44/76 at 24, (C) N59.1 6 hpi, (D) and N59.1 24 hpi. No bacteria were detected in the basolateral chamber during these infections. The data represent the mean of three repeats performed independently (N=3).

These findings suggest that the association level does not correlate with serogroup. Therefore, it was decided to compare the integrity of the monolayer post-infection with MC58, H44/76, N222.1 or N59.1 in parallel to determine if this phenotype was linked to serogroup using the permeability assay as in 4.5.1.

4.5.3 Comparison of epithelial monolayer integrity post-infection with B and Y serogroups variants

Differentiated Calu-3 cells in ALI were challenged with MC58 or N222.1 at MOI of 30 for 12 hpi. The level of integrity was determined using a dextran permeability assay. The difference in the integrity was deduced from the difference between the rates of fluorescence unit passage into the basal chamber. The assay showed that the permeability was significantly increased by N222.1, as expected, while the other strains (MC58, H44/76 and N59.1) did not impact the permeability of the monolayer (Figure 4.16). The difference was clear in the first 30 min after the FITC-Dextran addition, but after 2 h the difference started to be significant in the case of N222.1 infection. Interestingly, both MC58 and H44/76 induce a non-significant but clear reduction of the fluorescence unit passage into the basal chamber, which means that the monolayer integrity might be enhanced in some way.

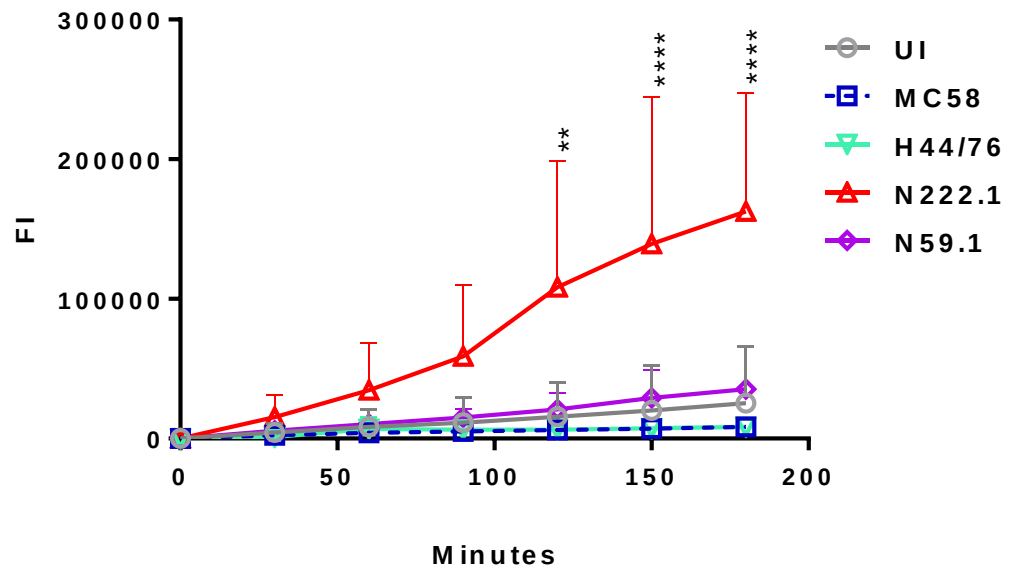


Figure 4.16: Impacts of different serogroup B and Y strains on the permeability of Calu-3 monolayer at 12 hpi.

FITC-Dextran (10,000-MW) was added to the apical side of Calu-3 monolayers previously challenged with MC58, H44/76, N222.1 or N59.1 at an MOI of 30 for 12 h, and the fluorescence in the basal chamber was monitored every 30 min over the next 3 h post FITC-Dextran addition. The chart shows that monolayer permeability (mean $\pm$ SD) significantly increased after challenging the cells with N222.1 compared to uninfected Calu-3 cells as a control, while no impact of N59.1 infection on Calu-3 permeability was observed. MC58 and H44/76 appeared to decrease the permeability of the monolayer compared to control cells (N=3, **P<0.005, ****P<0.0001, two-way ANOVA).

This result indicates that the damaging effect could be unique for N222.1 among the tested strains; therefore, we introduced two N222.1 variants, N459.3 and N459.6 (cc23), to test their behaviour on the Calu-3 model. These variants are very similar to N222.1 strain and were isolated from the same carrier, but differ in their PV pattern for some PV genes (Table

4.2). Interestingly, the impact of those variants on the barrier integrity was completely different. N459.6 infection increased the permeability significantly, although slightly less than N222.1, whereas N459.3 significantly enhanced the barrier function compared to uninfected cells, MC58 was used as a control and in this set of experiments did not affect the epithelial barrier (Figure 4.17).

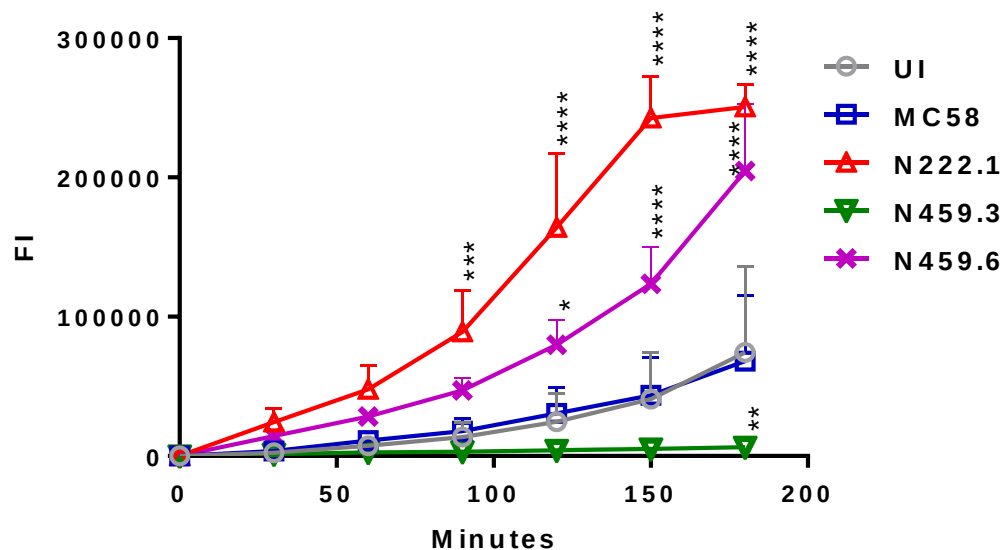


Figure 4.17: Impacts of N222.1 variants on the permeability of Calu-3 monolayer at 12 hpi.

FITC-dextran (10,000-MW) was added to the apical side of Calu-3 monolayers previously challenged with N222.1 N459.3 or N459.6 at an MOI of 30 for 12 h, and the fluorescence in the basal chamber was monitored every 30 min over the next 3 h post FITC-dextran addition. The chart shows that the monolayer permeability (mean±SD) significantly increased after challenging the cells with N222.1 and N459.6 compared to uninfected Calu-3 cells as a control, while N59.1 significantly decreased the permeability of the monolayer compared to control cells. No impact of MC58 infection on Calu-3 permeability was

observed. (N=3, *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001, two-way ANOVA).

TNF- α secretion by Calu-3 cells infected with each strain or variant was quantified by ELISA and compared with uninfected control cultures at 12 hpi. Samples obtained from the last two permeability assays were quantified in parallel. The data in Figure 4.18 show the TNF- α secretion profile triggered by each strain or variant infection. N222.1 and N59.1 induced the highest amount of TNF- α among all meningococcal isolates at 918 and 817 pg mL⁻¹, respectively. N459.6 came in the second with 416 pg mL⁻¹, which was significantly lower than N222.1 and N59.1 but significantly higher than uninfected cells. TNF- α secretion in response to MC58 infection was significantly higher than for uninfected cells (at 179 pg mL⁻¹) while TNF- α secretion after the infection with H44/76 and N459.3 infections was insignificant.

In the case of N222.1 and N459.6 infections the increased secreted level of TNF- α seemed to correlate with the level of permeability increase, but this was not the case for N59.1 infection. Moreover, the low TNF- α secretion seemed to correlate with the enhancement of the barrier function for Calu-3 monolayer. These observations will be investigated further in the next chapter of this thesis.

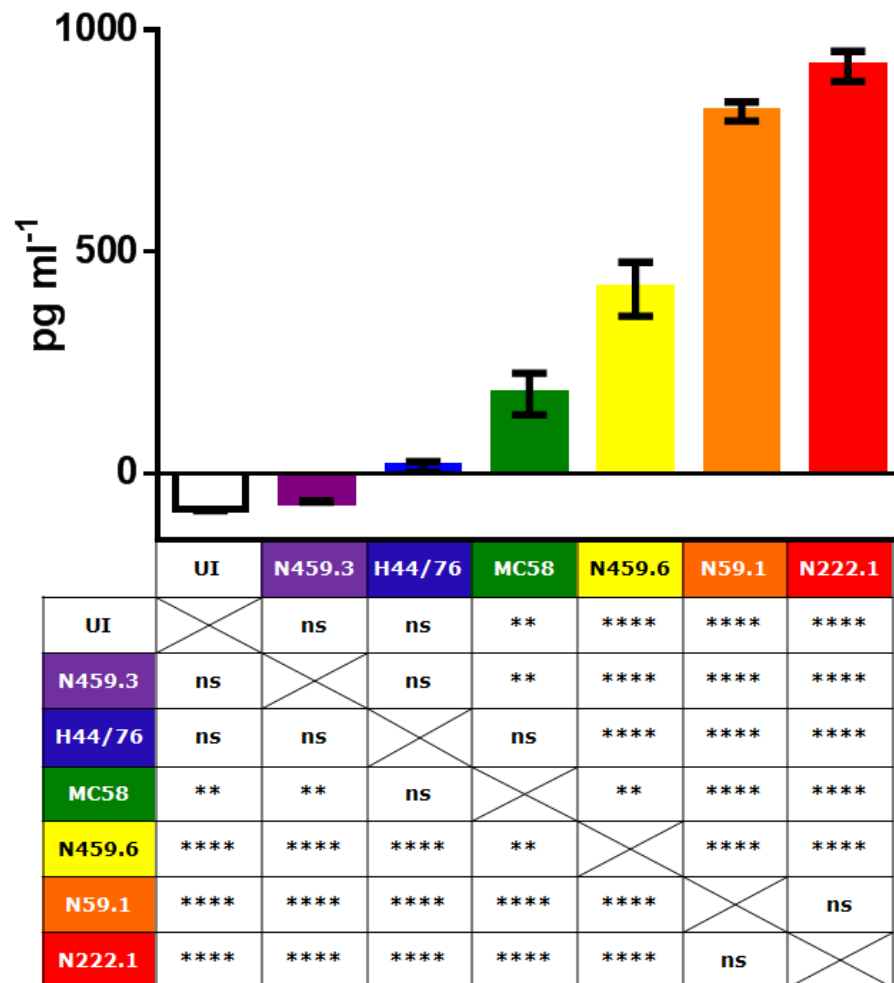


Figure 4.18: Profile of TNF- α production in response to different meningococcal strains and variants at 12 hpi.

TNF- α secretion (mean $\pm$ SD) from the permeability assay for uninfected and infected Calu-3 epithelial cells with MC58, H44/76, N222.1, N459.3, N459.6 or N59.1 at MOI of 30 for 12 hpi, N222.1, N459.6 and N59.1 significantly trigger the highest TNF- α response among all strains and variants. MC58 comes in the second place, while H44/76 and N459.3 were not triggering a significant TNF- α response. (N=4, ns P=0.1, **P<0.005, ****P<0.0001, one-way ANOVA)

Overall, the infection assays revealed that the Calu-3 infection model responded differentially against different meningococcal strains, as summarised in (Table 4.2). The sequences analysis

for 1,689 genes of *N. meningitidis* for 92 isolates, including isolates used in this study, showed the level of genetic differences between those isolates (Figure 4.19). Although, N222.1 and its variants (N459.3 and N459.6) are closely related in term of serotyping and genosubtyping, they exhibit significant differences in their infection patterns.

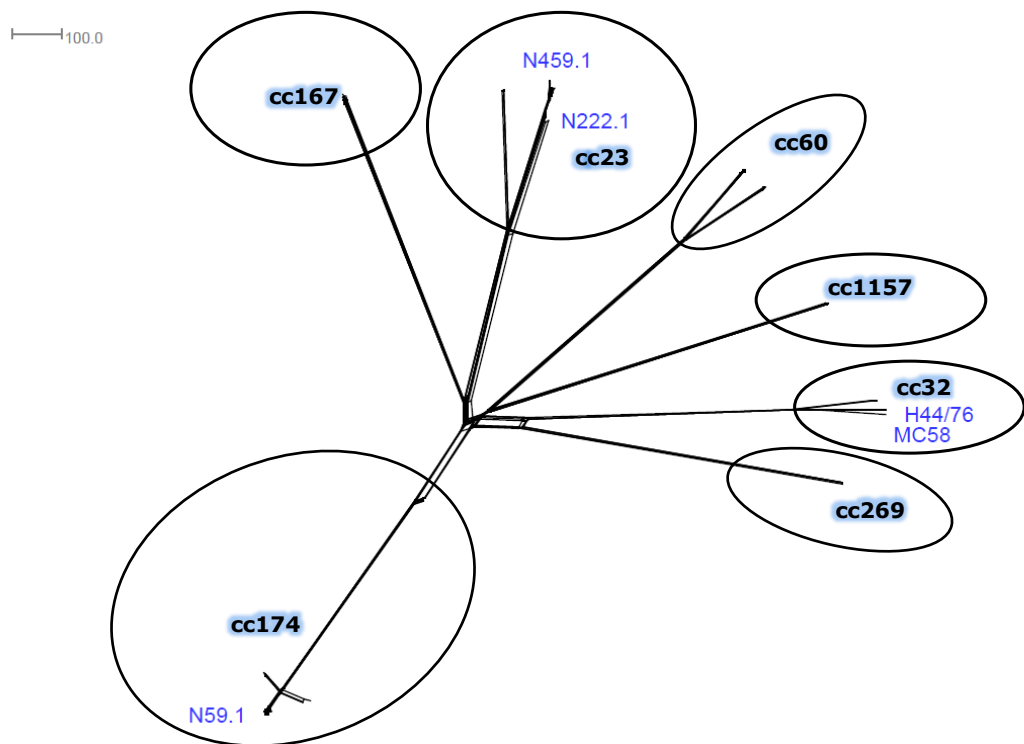


Figure 4.19: NeighborNet graph comparison for different clonal complexes of *Neisseria meningitidis*.

Sequences were analysed using BIGSdb Genome Comparator tool for 1,689 genes utilizing the publically available *N. meningitidis* pubMLST database for 92 isolates, and the graph was modified using SplitsTree software (version 4.14.2). Apart from MC58 and H44/76 the rest of the 92 isolates, which include the serogroup Y isolates used in this study, are from a carriage study on university students living in residential

halls (Bidmos *et al.*, 2011). The graph illustrates the genetic differences between the strains used in this study.

Table 4.2: Outcomes of infection of differentiated bronchial epithelial cells with different B and Y serogroup strains and their correlation with predicted expression of PV proteins.

		MC58	H44/76	N222.1 ^a	N459.3 ^a	N459.6 ^a	N59.1 ^a
	NalP	ON	ND	OFF	OFF	OFF	ON
	MspA	ON	ND	OFF	OFF	OFF	OFF
	HmbR	ON	ND	X	X	X	X
	PorA	ON	ND	ON	ON	ON	ON
	NadA	OFF	X	X	X	X	ON
	FetA	ON	ND	ON	ON	OFF	ON
	Opc	ON	ND	ON	OFF	OFF	OFF
	HpuA	X	X	ON	ON	ON	ON
Opa ^b	A	OFF	ND	OFF	ON	OFF	ON
	B	ON	ND	ON	OFF	OFF	OFF
	D	OFF	ND	OFF	OFF	OFF	OFF
	J	OFF	ND	OFF	ON	ON	OFF
Monolayer's permeability		-	-	+++	-	++	+
TNF-α production		+	+	+++	-	++	+++
Adhesion/Invasion		++	+	+	NT	NT	+++
Media colour change to yellow (acidic)	12 hpi	+	+	++	+	++	++
	24 hpi	+	+	+++	NT	NT	+++

ND (Not determined) the gene existed according to the genome analysis by BIGSdb Genome Comparator tool but not tested for PV state

X (Not existed) the genome of the strain lacks the encoding gene for this protein according to the genome analysis by BIGSdb Genome Comparator tool

NT (Not tested) no infection assay for this strain at 24 hpi

a PV data adopted from (Alamro *et al.*, 2014)

b Opa data was generated by a collaboration with Dr Christopher Bayliss, Leicester

4.6 Discussion

The adhesion and invasion assay revealed that the Calu-3 infection model responded differentially against different meningococcal strains. Strains N459.3 and H44/76 did not induce a significant TNF- α response compared to MC58, N459.6, N59.1 and N222.1. Furthermore, the disrupting effect on the barrier function of the Calu-3 monolayer was a unique feature for N459.6 and N222.1 infections. In general, the different responses against the different strains were expected, as some of those strains are different in term of serotyping and genosubtyping (Figure 4.19). However, the enormous difference in the infection patterns of N222.1 and its variants (N459.3 and N459.6) was surprising. It is interesting that N59.1 strain did not impact on the permeability of the Calu-3 monolayer, while it induced a high TNF- α response.

4.6.1 Adhesion and invasion characteristics

Different clonal complexes have different pattern of cell-surface and secreted virulence factors, which influence colonization, persistence, and damage within the host. Among these virulence factors, there are two meningococcal autotransporters NalP and MspA, which are phase variable outer membrane/secreted proteins (Oldfield *et al.*, 2013). In general, the autotransporters vary widely in function but often

act as enzymes, adhesins or cytotoxins (Grijpstra *et al.*, 2013). This far, NaIP and MspA precise biological significance remain unclear (Oldfield *et al.*, 2013). However, a role for MspA in meningococcal adhesion to host cells has been suggested, and NaIP was demonstrated to alter the secretion profile of adhesion molecules App and IgA1 protease (Turner *et al.*, 2006). Interestingly, NaIP and MspA are OFF in N222.1 and ON in MC58 (Table 4.2), which might explain the lower association of N222.1 with Calu-3 cells compared to MC58. Additionally, the members of the cc23 clonal complex, *i.e.*, N222.1, lack the gene that encodes NadA protein, another important adhesion molecule (Alamro *et al.*, 2014).

N59.1 has the highest adhesion level among all the tested strains. That might be a result of expressing the NadA adhesion molecule, which the gene encoding NadA is OFF in MC58 and is missing in the rest of the strains (Table 4.2). Beside that N59.1 has the gene encoding NaIP in the ON state, which might result in enhancing the adhesion by having multiple means to adhere to host cells. Currently, it is well known that *N. meningitidis* use a variety of adhesion strategies that interact cooperatively with different receptors on the same target cells, or may act at different stages during infection, mediating bacterial adhesion to different cell types at different sites (Liu *et al.*, 2015, Pizza and Rappuoli, 2015).

The meningococcal membrane opacity proteins, Opa, and Opc, mediate intimate adhesion to host cells via binding to carcinoembryonic antigen cell adhesion molecule (CEACAMs) receptors and extracellular matrix components, respectively (Virji, 2000). Most *N. meningitidis* strains possess four different Opa proteins, of which OpaA and OpaJ bind to CEACAM1, whereas OpaB and OpaD bind to CEACAM1 and CEA (de Jonge *et al.*, 2003b). However, those four Opa proteins might not be equivalent in facilitating the adhesion to host cells (Virji *et al.*, 1993, McNeil *et al.*, 1994, de Jonge *et al.*, 2003b). The Opa expression profile varied among the strains used in our study (Table 4.2). It was difficult to correlate a certain cell association pattern to specific Opa expression pattern. This particular point needs more investigation as Opa proteins are one of the major players in meningococcal association with host cells.

Our results suggest that different combination of adhesion molecules might play a possible role in enhancing or reducing the association of bacteria with host cells. Nevertheless, all the indicators according to our results point at NadA as the factor responsible for the high association level of N59.1, as it is the only strain expressing it. Although NadA, NalP, MspA, Opc, and Opa represent important adhesion molecules of the meningococcus, additional antigens have been described as

having a role in adhesion, such as Type IV Pili, NhhA and App (Pizza and Rappuoli, 2015). Altogether, the results of our study and the literature highlight the importance of studying the role of those antigens in cell association extensively by introducing different strains with different expression pattern of adhesion molecules to the Calu-3 model.

4.6.2 Cytotoxicity and permeability

The minor enhancement of permeability when the Calu-3 monolayer was infected with MC58, H44/76 or N459.3 has been reported in another study on MC58 (Sutherland *et al.*, 2010). Their results showed that even when MC58 traversed across the Calu-3 monolayer, the integrity of the monolayer was preserved in terms of permeability and distribution of tight junctions. This agreed with a more recent study showing that the MC58 infection does not significantly change the distribution of ZO-1, which indicates no disruption of the tight junction integrity (Barrile *et al.*, 2015). The same study reported also that the localisation of E-cadherin and integrin- β 1 was significantly modified in Calu-3 cells infected with MC58. E-cadherin and integrin- β 1 play a well-known role in the maintenance of polarized cell architecture; the authors observed a significant change in height and polarity of the Calu-3 monolayer during infection (Barrile *et al.*, 2015). This

agreed with our observation in the histology part of this study, which revealed a change in the polarity and height of infected cells with MC58 compared to uninfected cells. These observations indicate that the meningococcus does not need to either alter the integrity of the epithelial monolayer as an infection strategy nor traverse across the epithelial barrier by a paracellular route (Sutherland *et al.*, 2010, Pujol *et al.*, 1997). It is suggested that meningococci traverses the monolayer barrier using an intracellular route by manipulating the monolayer function and polarity (Barrile *et al.*, 2015).

The disruption of monolayer integrity by N222.1 and N459.6 was a feature unique to these two strains infection among all tested. Interestingly, this kind of infection strategy has been reported in another *Neisseriaceae* family member, *N. gonorrhoeae* (also known as gonococcus) (Rodriguez-Tirado *et al.*, 2012, Edwards *et al.*, 2013). Rodriguez-Tirado *et al.* have noticed that gonococcal infection to polarised endometrial adenocarcinoma epithelial cells (Ishikawa cells) induced disruption of the monolayer's junction complexes first with detachment of E-cadherin and β -catenin from the adherens junction complex, then followed by a reorganization and redistribution of the actin cytoskeleton and fibronectin within the extracellular matrix (Rodriguez-Tirado *et al.*, 2012). Edwards *et al.* demonstrated the capability of the gonococcus

to weaken the apical junction and polarity of another type of polarised human endometrial adenocarcinoma epithelial cells (HEC-1-B) by activating epidermal growth factor receptor (EGFR), which facilitates gonococcal transmigration across the epithelium (Edwards *et al.*, 2013). An earlier study on the mechanisms of attachment and invasion involved in the pathogenesis of *N. meningitidis* has shown that different strains of meningococcus are capable of disrupting the tight junction integrity of HecIB and move between two epithelial cells (Birkness *et al.*, 1995). However, we have not established that N222.1 and N459.6 are using this mechanism as a pathogenesis strategy as further analysis using microscopy work is required.

4.6.3 TNF- α production

According to our observations, we believe that the characteristics of N222.1 and N459.6 infections were different than the other tested strains. In the infections of those two strains, it is unclear whether the high levels of TNF- α caused the monolayer damage as an apoptotic effect, or the cell damage led to an elevation of TNF- α production as an inflammatory response to the damage-associated molecular pattern. TNF- α elicits a wide range of cellular effects, from proliferation and viability to inflammation and apoptosis

(Roulston *et al.*, 1998). Of interest, TNF- α receptors (TNF-R) can determine the effect of TNF- α , at least in neuronal tissues, where TNF-R1 activation leads to tissue destruction, whereas TNF-R2 has a protective effect (Wajant *et al.*, 2003). Two different studies on Calu-3 cells have reported that incubation of Calu-3 cells with TNF- α together with IFN- γ , and IL-1 β mixture, induced epithelial cell injury including tight junction disassembly and alteration of epithelial barrier permeability (Han *et al.*, 2004, Petecchia *et al.*, 2012).

A natural *lpxL1* mutation has been identified in the Y serogroup of *N. meningitidis* (mainly cc23 isolates), which can be considered as a strategy to evade immune recognition (Ladhani *et al.*, 2012). The *lpxL1* mutation has been studied in the H44/76 background, and the lack of LpxL1 caused a reduction in cytokine (*e.g.*, TNF- α , IL-6 and IL-10) production *in vivo* in a mouse model (Fransen *et al.*, 2010). In a comparative study of wild-type strains and *lpxL1* mutants looking to pro-inflammatory cytokine induction in peripheral blood mononuclear cells (PBMCs) the same reduction effect of the mutants was observed (Fransen *et al.*, 2009).

A previous study in our lab compared cytokine production in response to MC58 and N222.1 using PBMCs (unpublished). The results showed no significant differences in production levels of

TNF- α against N222.1 compared to MC58. In the case of the anti-inflammatory cytokine, IL-10 production significant lower induction by N222.1 was observed. Obviously, all these studies contradict our study in terms of TNF- α production, but that might be explained by the different models used in these studies, and the cell type is an important factor in determining the type of response elicited in response to the pathogen. Part of this previous study in our lab focused on the genetic differences between MC58 and N222.1 which uncovered an *lpxL1* mutation in N222.1, which is an adenosine deletion near the start codon site of *lpxL1*; MC58 (5' ATGAAATTT 3') and N222.1 (5' ATG_AATTT 3'). That suggests that the level of acylation in LOS might play a role in manipulating the immune response by altering the balance between induction of pro- and anti-inflammatory cytokines. This is supported by John *et al.* results which showed that TNF- α production by human monocytic THP-1 cells and primary human monocytes is subject to variation by approximately 2-10-fold, caused by differences in the state of acylation and phosphorylation of the lipid A moiety of LOS (John *et al.*, 2009). There is a lack of information in the literature reporting the response of epithelial cells to this mutant, which suggests that this particular aspect needs further investigations.

However, in the case of N59.1 infection, we demonstrated that the high levels of TNF- α did not correlate with changes in permeability. Although this might suggest that the high level of TNF- α is not linked to the permeability disruption, it is well known that TNF- α production by epithelial cells can be stimulated by many meningococcal factors (e.g., LOS endotoxin, synthetic lipid A, nitric oxide, oxygen radicals, toxic shock toxin- 1 or by the induction of GM-CSF, TNF- α and other cytokines) (Wang *et al.*, 2003). Therefore, the high levels of TNF- α are unnecessary to be triggered by the same factor as in N222.1 and N459.6, which means the whole infection scenario might be different in each strain. Indeed the different pattern of antigenic structures present in each strain may play an important role in this variation in responses. Thus, the impact of the high levels of TNF- α on permeability post infection will be investigated in the next chapter, as well as, the signalling pathways triggered post-infection (5.5.2)

4.7 Summary

It is fascinating how little variations between meningococcal strains have a huge impact on the infection pattern and the host cells response. However, these findings highlight the fact that the Calu-3 model is a useful *in vitro* platform to study the meningococcal early stages of infection and to evaluate the

impact of genetic variation on pathogenesis. Consequently, it would be desirable to invest more effort in this infection model as an important investigational tool in the area of *N. meningitidis* pathogenesis.

CHAPTER FIVE

**THE ROLE OF TNF-ALPHA IN
APOPTOTIC CELL DEATH
AND NF- κ B-INDUCED CELL
SURVIVAL**

5. THE ROLE OF TNF- α IN APOPTOTIC CELL DEATH AND NF- κ B-INDUCED CELL SURVIVAL

5.1 Introduction

The airway epithelium is among the first host line of defence and barriers participates in inflammation. The classical examination of epithelial responses to injury and inflammation highlighted the capability of these cells to act in response to environmental insults by secreting water and mucus into the airways in addition to secreting mediators (*e.g.*, cytokines and chemokines) locally and systemically (White, 2011). The outcomes of the epithelial layer response to physical injury normally lead to a series of processes including phagocytic clearance of damaged cells and material, restoration of barrier function and epithelial cells proliferation and differentiation (White and Leff, 1998).

Increasing evidence suggests that in order to achieve protection of the airway mucosa and repair in response to sustained damage programmed cell death, *i.e.*, apoptosis, needs to be regulated (White, 2011). The regulation of apoptosis is a tightly controlled process of non-necrotic cell

death and is a critical event for the homeostasis of normal tissues.

5.1.1 Mechanisms of apoptosis

Apoptotic cell death is orchestrated by highly conserved genes in eukaryotes, that permits the clearance of unwanted or damaged cells from tissue without releasing cellular contents to avoid damaging the surrounding cells or eliciting an inflammatory response (Vaux and Strasser, 1996, Strasser *et al.*, 2000). Although it is tightly and carefully regulated in normal situations, however, it might be improperly activated or inhibited in diverse pathological circumstances (Zhivotovsky and Orrenius, 2010). Unlike necrosis, the main characteristics of apoptosis include cell shrinkage, nuclear condensation, DNA fragmentation, cytoskeleton fragmentation, and eventual formation of secreted or internalised apoptotic bodies (Hotchkiss *et al.*, 2009).

Apoptosis can be initiated by several signals including death receptors, cytokines, heat and oxidative stress, and caspases (White, 2011). Two different pathways exist for caspase activation, type I (extrinsic pathway) and type II (intrinsic pathway).

5.1.1.1 Extrinsic pathway

This pathway is termed extrinsic because it is activated by extracellular signals and the ligation of cell surface death receptors (Schultz and Harrington, 2003, Strasser *et al.*, 2000, Muzio *et al.*, 1996, Medema *et al.*, 1998), members of the TNF superfamily, such as CD95 (Hotchkiss *et al.*, 2009, Hamann *et al.*, 1998), TNFR1, DR3, DR4, DR5, DR6, EDAR, and p75NTR (Weinlich *et al.*, 2010). The well-described ligands for those receptors are CD95L (FasL), TNF and the TNF-related apoptosis-inducing ligand (TRAIL).

The death receptor ligand FasL is probably the best characterized. Originally it was considered mostly a mediator of cell death but it can also serve as an effector molecule to promote cell survival (White, 2011). Initially described as a transmembrane molecule, FasL now is recognized to be cleaved from the cell surface by metalloproteases to form a soluble variant (Tanaka *et al.*, 1996, Matsuno *et al.*, 2001); The soluble FasL binds its receptor with equivalent efficiency as the transmembrane form (White, 2011).

TNF- α has complex functions in airway epithelial cells and may play a dual role as anti- and pro-apoptotic agent depending on the context. It is considered as a pro-inflammatory cytokine

that induces a number of effects on airway epithelium, such as expression of the adhesion molecule ICAM-1 (Tosi *et al.*, 1992, Kim *et al.*, 2008) as well as IL-6 and IL-8 production (Becker *et al.*, 1993, Levine *et al.*, 1993). TNF- α binds two different receptors TNFR1 and TNFR2. Both receptors can interact with a series of adaptor proteins, the TNFR-associated factors (TRAF), whereas only TNFR1, is responsible for the cytotoxic effects of TNF- α , including apoptosis (White, 2011). TRAF2 but not TRAF1 can initiate NF- κ B signaling (Hsu *et al.*, 1995) resulting in IL-8 secretion (Roger *et al.*, 2004, Becker *et al.*, 1993). The interruption of NF- κ B signaling can promote TNF- α -induced apoptosis. Equally, NF- κ B activation can inhibit TNF- α -induced apoptosis (Liu *et al.*, 2002). Hence, TNF- α -induced cell death and inflammation are balanced in a way that can be controlled by several pathways.

Another member of the TNF superfamily is TRAIL, which is a type II membrane protein that can induce apoptosis in a variety of cells (White, 2011). TRAIL binding to the death receptors DR4, DR5, and/or DcR2 may also activate the transcription factor NF- κ B, in some cell types, resulting in the transcription of genes that actually inhibit cell death signaling pathways and induce inflammation (Jeremias and Debatin, 1998, Schneider *et al.*, 1997).

5.1.1.2 Intrinsic pathway

The intrinsic pathway of apoptosis activation is not receptor-dependant but stressors-dependant. These stressors include oxidative stress, DNA damage, and starvation (Hotchkiss *et al.*, 2009, Erlacher *et al.*, 2005). This pathway is characterised by increased mitochondrial permeability and release of cytochrome c. Cytochrome c binds APAF-1 to activate procaspase-9 through cleavage (Medema *et al.*, 1998, Erlacher *et al.*, 2005, Strasser, 2005). The mitochondria thus may serve an activator of complex internal signals indicating cellular damage. This pathway activation leads to the cleavage and activation of caspases such as caspase-3 directing the cell towards an apoptotic death (Hotchkiss *et al.*, 2009).

In this chapter, we intended to explore the correlation between TNF- α expression and the hyperpermeability response of the Calu-3 monolayer, and tried to locate the position of the high TNF- α levels among the chain of infection events; whether it acted as a mediator or an outcome of the cell damage. We utilised the Reverse Phase Protein microarray (RPPA) technology to explore NF- κ B signaling post-infection, in order to know the possible key factors responsible for the

observed variations in Calu-3 responses against different meningococcal isolates.

5.1.1.3 Pathways of NF- κ B activation

In unstimulated cells, inactive form of NF- κ B exists in the cytoplasm comprising a transcriptionally active dimer bound to an inhibitor protein, I κ B. Currently, the known subunit members of the NF- κ B family in mammals are p50, p65 (RelA), c-Rel, p52, and RelB, while also multiple mammalian forms of I κ B also has been identified, namely I κ B α , β , γ (p105), δ (p100) and ϵ , and Bcl-3 (Baeuerle and Baltimore, 1996). I κ B kinases, such as IKK α and IKK β , have been shown to be activated by important inducers of NF- κ B such as IL-1 and TNF (Bowie and O'Neill, 2000). This is a crucial step in NF- κ B activation pathways by these cytokines (Regnier *et al.*, 1997, Mercurio *et al.*, 1997, Woronicz *et al.*, 1997, DiDonato *et al.*, 1997, Zandi *et al.*, 1997). The IKKs are members in a family of larger multi-protein complexes called the IKK signalosome, that contains IKAP (IKK complex-associated protein) and NF- κ B essential modifier (NEMO, also called IKK γ), which are also known to be important in NF- κ B activation pathways (Yamaoka *et al.*, 1998, Cohen *et al.*, 1998, Rothwarf *et al.*, 1998). I κ B α can be phosphorylated on two serine residues S32 and S36, by both IKK α and IKK β ,

while in I κ B β S19 and S23 (Regnier *et al.*, 1997, Mercurio *et al.*, 1997, Woronicz *et al.*, 1997, DiDonato *et al.*, 1997, Zandi *et al.*, 1997). Many upstream activators and regulators of IKKs activity have been identified, such as NIK, MEKK1 , TAK1 , protein kinase C ζ , MEKK2 and 3, and S6 kinase (Bowie and O'Neill, 2000).

The NF- κ B subunit p50 is translated as a precursor, p105, which can form a complex with p65 and inactivate it. TNF has been shown to induce p105 phosphorylation by the kinase TPL-2 (tumour progression locus 2 kinase) (Bowie and O'Neill, 2000). Therefore, it is likely that multiple pathways can regulate the activation of NF- κ B, most of which via I κ B phosphorylation by the IKK-containing signalosome.

In many cell types, NF- κ B can be activated in response to a wide range of stimuli and conditions, including viral and bacterial products, T- and B-cell mitogens, inflammatory cytokines such as IL-1 and TNF, intracellular stresses such as endoplasmic reticulum protein overload, and extracellular stresses as diverse as UV light, H₂O₂, cigarette smoke, and asbestos fibres (Bowie and O'Neill, 2000).

The NF- κ B activation pathways involving IL-1 and TNF, have been well described from receptor to nucleus (Baeuerle, 1998). Binding of TNF to the TNFR1 aggregates three TNFR1

subunits together, which induces the recruitment of TNFR-associated death domain protein (TRADD) with the death domains in the cytosolic region of TNFR1s. TRADD then, by its turn, recruits TRAF2 and receptor-interacting protein (RIP), which are involved in NF- κ B activation. Consequently, TRAF2 can interact with NIK and activate it, resulting in IKKs activation. Alternatively, the binding of IL-1 to its type 1 receptor (IL-1R1) recruits the IL-1 receptor accessory protein (IL-1RAcP), leading to IL-1 receptor-associated kinase (IRAK) activation via the adaptor molecule MyD88. As a result, IRAK activates TRAF6 which then can associate with TAK1 (Ninomiya-Tsuji *et al.*, 1999), resulting in the activation of NIK (Figure 5.1).

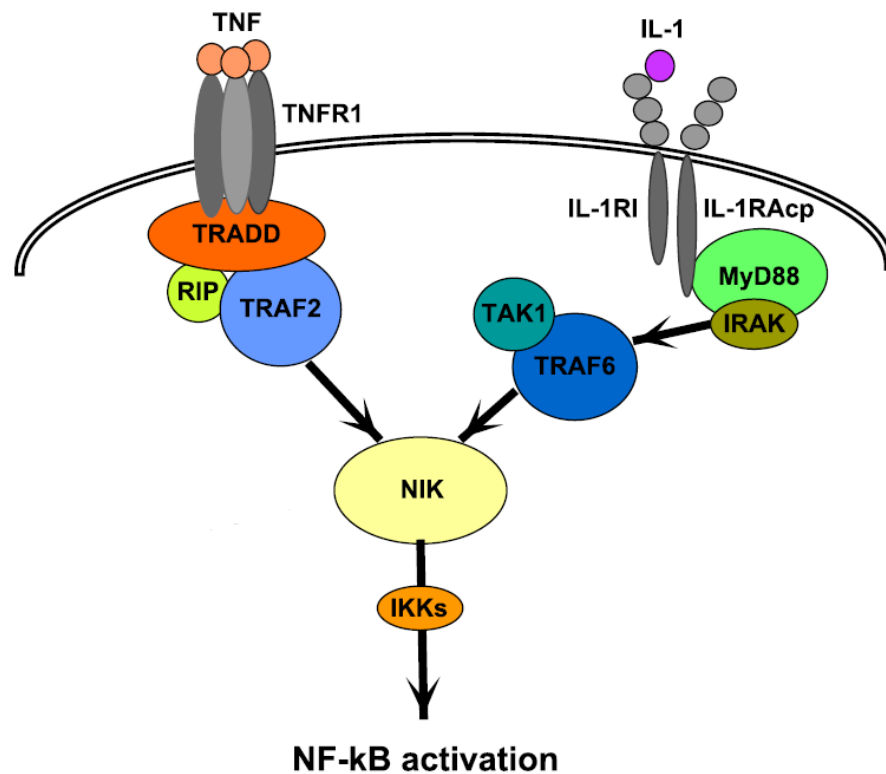


Figure 5.1: Pro-inflammatory signal pathways for NF- κ B activation.

TNF binds to TNF receptor type 1 molecules (TNFR1), resulting in the recruitment of TNFR-associated death domain protein (TRADD), which recruits TNFR-associated factor 2 (TRAF2) and receptor-interacting protein (RIP), which are involved in NF- κ B activation. TRAF2 activates NIK, which can lead to IKK activation. On the other hand, IL-1 binds to its type 1 receptor (IL-1RI), resulting in the recruitment of IL-1R-accessory protein (IL-1RAcP), and leading to the activation of the IL-1R-associated kinase (IRAK) via the adaptor molecule MyD88. IRAK then can activate TRAF6, which can associate with TAK1, resulting in NIK activation. This diagram was drawn in PathVisio 3.2.1 software utilising the information obtained from (Bowie and O'Neill, 2000).

5.2 Hypothesis

We hypothesise that the high levels of TNF- α produced by the Calu-3 model might be the reason behind the increased permeability of the epithelial layer post-infection with some isolates. We also hypothesised that there will be differences in signaling pathways triggered by different isolates.

5.3 Aim

We aim to investigate the possible difference in monolayer permeability in the presence and absence of TNF- α neutralisation during N222.1 or N59.1 infections. In addition, we aim to study the activation of the NF- κ B pathway using Reverse Phase Protein microarray in response to serogroup Y variants, *i.e.*, N222.1, N459.3 and N459.6.

5.4 Materials and methods

5.4.1 Calu-3 culture and differentiation

See 3.4.1).

5.4.2 Bacterial strains and preparation of inoculum

See 4.4.2).

5.4.3 Analysis of epithelial monolayer permeability in the presence of neutralising antibodies against TNF- α

Calu-3 cells were cultured and differentiated for 21 days as described in chapter 3. Before initiating the infection the monolayers were washed three times with pre-warmed DMEM/F-12. TNF- α was neutralised by adding goat anti-human TNF- α (anti-hTNF- α) IgG antibodies (R&D Systems) to the apical and basal chambers at a final concentration of 1 $\mu\text{g mL}^{-1}$. Cells were incubated 1 hour prior to infection at 37°C and 5% CO₂. Normal goat IgG (R&D systems) was used as a control at the same concentration. Cells were then infected with N222.1 or N59.1 for permeability assay as described in (4.4.5).

5.4.4 TNF- α measurement by ELISA

See 4.4.4.1).

5.4.5 Reverse Phase Protein microarray

Differentiated Calu-3 cells were infected with N222.1, N459.3 or N459.6 isolates for 30 minutes or 2 hours at MOI of 30. At each time point the infected cells were washed three times with ice-cold PBS followed by the addition of 100 μ L of lysis buffer [radio-immunoprecipitation assay (RIPA) buffer containing phosphatase/protease inhibitor cocktail (Thermo Fisher Scientific), and 5 μ L of benzonase (Sigma)], on ice for 10 minutes with continuous mixing. Cell lysates were collected by pipetting up and down vigorously, placed in 1.5 mL tubes, and centrifuged for 10 minutes at 16000 X *g* and 4°C to remove any cell debris. Supernatants were stored at -80°C until use.

Reverse Phase Protein microarray (RPPA) was performed within the Post-Genomic Technologies Facility, Immunology, A Floor, West Block, Queens Medical Centre (QMC) (PGT facility, QMC) by Dr. Ola Negm using proprietary methods developed there (Negm *et al.*, 2014). All primary antibodies used in this section were purchased from Cell Signaling Technology, Inc., USA.

A brief description of the method is as follow:

The total protein lysate was mixed with 4X SDS sample buffer in a ratio of 3:1 (40 μ L of protein lysates with 13.3 μ L of sample buffer). Then the mixture was boiled for 5 minutes at 95°C. The samples were loaded onto a 384-well microtitre plate (Genetix, New Milton), and each sample was serially diluted three times in 1X SDS sample buffer. Each dilution was spotted twice onto 16-pad nitrocellulose-coated glass slides (Grace Bio-Labs). The slide printing was performed by a microarrayer instrument (MicroGrid II 600, Biorobotics Inc.). The printed slides were dried and stored at -80°C.

The slide was blocked overnight at 4°C with shaking using I-Block (Tropix) (0.1 g I-Block dissolved in 50 mL PBS with 25 μ L Tween-20, heated in a microwave for 30 seconds, then mixed on a magnetic stirrer until the solution became clear, next filter sterilized with a 0.2 μ m filter). Next day the blocking buffer was discarded and the slide was washed three times with TBS-Tween 0.01% on a shaker. The slide was then incubated overnight at 4°C with shaking with primary antibodies for each targeted signaling proteins and the β -actin antibody as a protein loading control (Table 5.1). The majority of the antibodies were diluted 1:100 in an antibody diluent (Dako UK, Ely, UK).

Next day the slides were washed three times with TBS-Tween 0.01%, and then incubated for 30 minutes at room temperature in the dark while being shaken continuously with 680 CW anti-mouse antibodies to detect β -actin (red) and 800 CW anti-rabbit antibodies (green) to detect the targeted proteins. Then the slide was washed three times with TBS-Tween 0.01% followed with a final wash with ultra-pure water.

Afterward, the slides were dried and scanned using an NIR Odyssey scanner (LI-COR, Biosciences) at 700 nm (red) and 800 nm (green) channels with a resolution of 21 μ m. The scanned images were analysed using Axon Genepix Pro-6 Microarray Image Analysis software. After spot analysis, the generated data file was analysed using RPPanalyzer statistical software available free online at <http://cran.r-project.org/> (Mannsperger *et al.*, 2010, von der Heyde *et al.*, 2014). The generated data Excel format was normalised to β -actin for graphical illustration and analysis using MultiExperiment Viewer (MeV) version 4.9.0 and PRISM.

5.5 Results

5.5.1 Epithelial monolayer permeability post-infection in the presence of anti-TNF- α neutralising antibodies

Differentiated Calu-3 cells were treated with anti-TNF- α IgG antibodies or normal goat IgG as a control 1 hour prior infection with N222.1 or N59.1 to study the role of TNF- α in altering the barrier function in the Calu-3 model. The results show a minor enhancement in the barrier function for both isolates in the presence of both antibodies (Figure 5.2). In general, both strains preserved the same pattern of permeability changes found in previous infections. Uninfected cells were treated with same IgGs as a control. Interestingly, the same effect of the antibodies on permeability was observed.

To evaluate the efficiency of the anti-TNF- α treatment the basal media was collected before the FITC-Dextran addition for TNF- α measurement by ELISA. The results show that the neutralisation was effective, and TNF- α levels were lower than in the uninfected cells (Figure 5.3). These results indicate that TNF- α is not a key factor in the enhanced permeability observed in the case of N222.1 infections. Thus, we decided to study some of the signaling pathways regulating the cellular

survival and death in an attempt to understand which ones might be responsible for the change in barrier function.

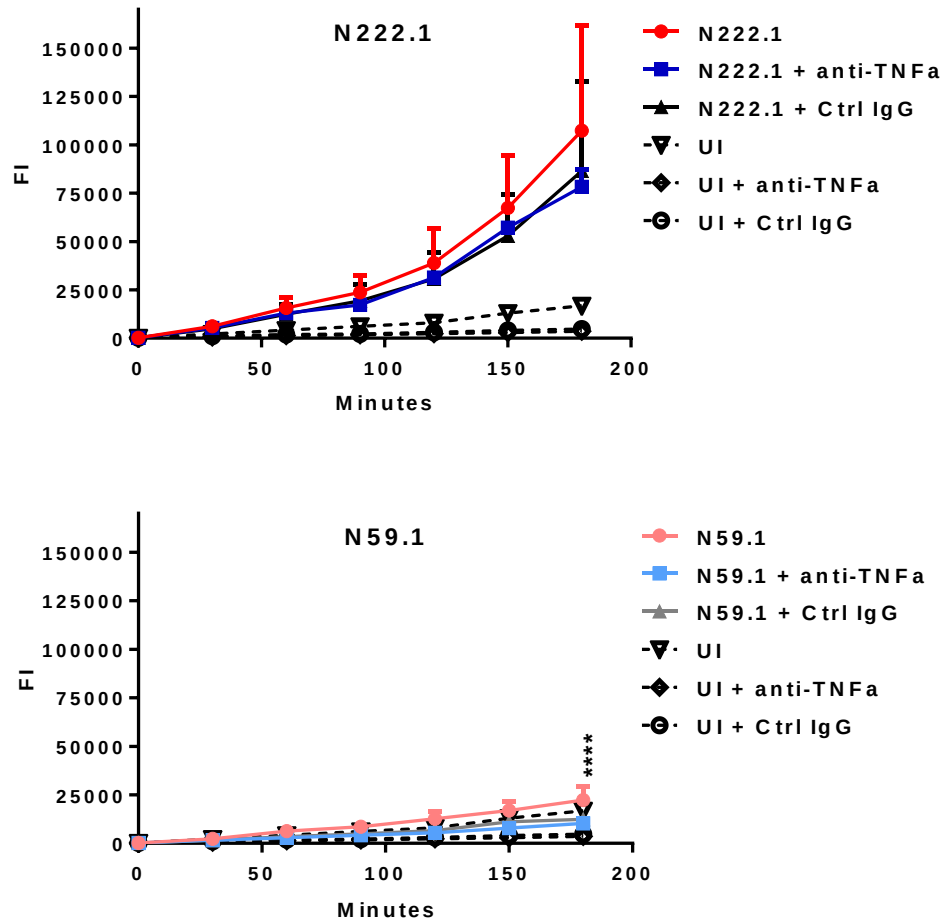


Figure 5.2: The impact of TNF- α neutralisation on the permeability of infected Calu-3 monolayer.

Differentiated Calu-3 monolayers were treated with anti-TNF- α IgG or control IgG pre-infection with N222.1 or N59.1 at an MOI of 30 for 12 hours, and the fluorescence in the basal chamber was monitored every 30 minutes over the next 3 hours post FITC-Dextran addition. The charts show the level of fluorescence (mean $\pm$ SD) which was not significantly affected by TNF- α . The same observation was found during N59.1 infection. (N=3, ****P<0.0001, two-way ANOVA)

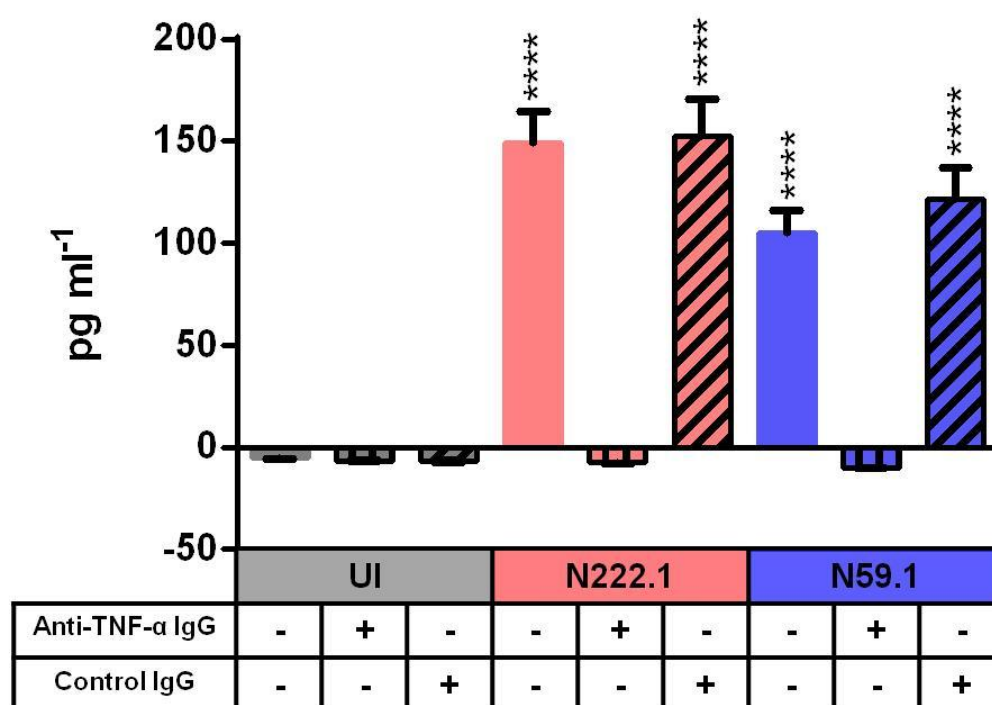


Figure 5.3: Validation of TNF- α neutralisation in Calu-3 cells infected with N222.1 or N59.1 up to 12 hpi in the presence of anti-TNF- α antibodies.

The chart shows (mean $\pm$ SD) of TNF- α production in response to N222.1 and N59.1 infections at MOI of 30 at 12 hpi, with/without TNF- α neutralisation. Both strains trigger a significant TNF- α response compared to uninfected cells, which was neutralised completely by using anti-TNF- α IgG. The control IgG did not impact the TNF- α level after treatment. (N=3, ****P<0.0001, one-way ANOVA).

5.5.2 NF- κ B signaling pathway study after infection with N222.1, N459.3 and N459.6

NF- κ B is involved in the regulation of multiple cellular processes including cell survival, cell proliferation, immunity, inflammation, and apoptosis. It is one of the most important regulators of innate/adaptive immunity, inflammatory responses, and developmental processes (Bonizzi and Karin, 2004, Ghosh *et al.*, 1998). To investigate its role in our infection assays, differentiated Calu-3 cells were infected with N222.1, N459.3 or N459.6 isolates for 30 minutes or 2 hours at MOI of 30. Cell lysates were collected and analysed by RPPA as described before (see 5.4.5). The protein signals investigated in this study (Table 5.1) covered some of the best-studied pathways leading to NF- κ B activation (Figure 5.4) Besides levels of three important proteins involved in cell's polarisation and architecture, mucus (mucin), type IV tubulin and zonula occluden 1 (ZO-1) were investigated.

Table 5.1: List of the signaling proteins used in this study

Signaling protein	Description
β -actin	Cytoskeletal protein (housekeeping)
TRAF3	TNF receptor-associated factor 3
NF- κ B p65 (p65)	Nuclear factor-kappa B p65
P- NF- κ B p65 (p65)	Phosphorylated nuclear factor-kappa B p65
NIK	NF- κ B-inducing kinase
RelB	Reticuloendotheliosis oncogene cellular homolog B
I κ B α	Inhibitor of NF- κ B alpha
P-I κ B α	Phosphorylated inhibitor of NF- κ B alpha
NF- κ B p100/p52	Nuclear factor-kappa B
P-NF- κ B p100	Phosphorylated nuclear factor-kappa B
A20	tumor necrosis factor-inducible protein A20
TRAF2	TNF receptor-associated factor 2
TRAF6	TNF receptor-associated factor 6
P-PARP	Phosphorylated poly(ADP-ribose)polymerase
cleaved caspase 9	Cleaved cysteine proteases with aspartate specificity 9
PI3K 85	Phosphoinositide 3 kinase 85
P-PI3K 85	Phosphorylated phosphoinositide 3 kinase 85
P-AKT S	Phosphorylated AKT8 virus oncogene cellular homolog Serine
P-AKT T	Phosphorylated AKT8 virus oncogene cellular homolog Threonine
p38 MAPK	p38 mitogen-activated protein kinase
P-p38MAPK	Phosphorylated p38 mitogen-activated protein kinase
ERK1/2	Extracellular signal-regulated kinase 1/2
P-ERK1/2	Phosphorylated extracellular signal-regulated kinase 1/2
c-Jun	transcription factor AP-1
P-c-JUN	Phosphorylated transcription factor AP-1

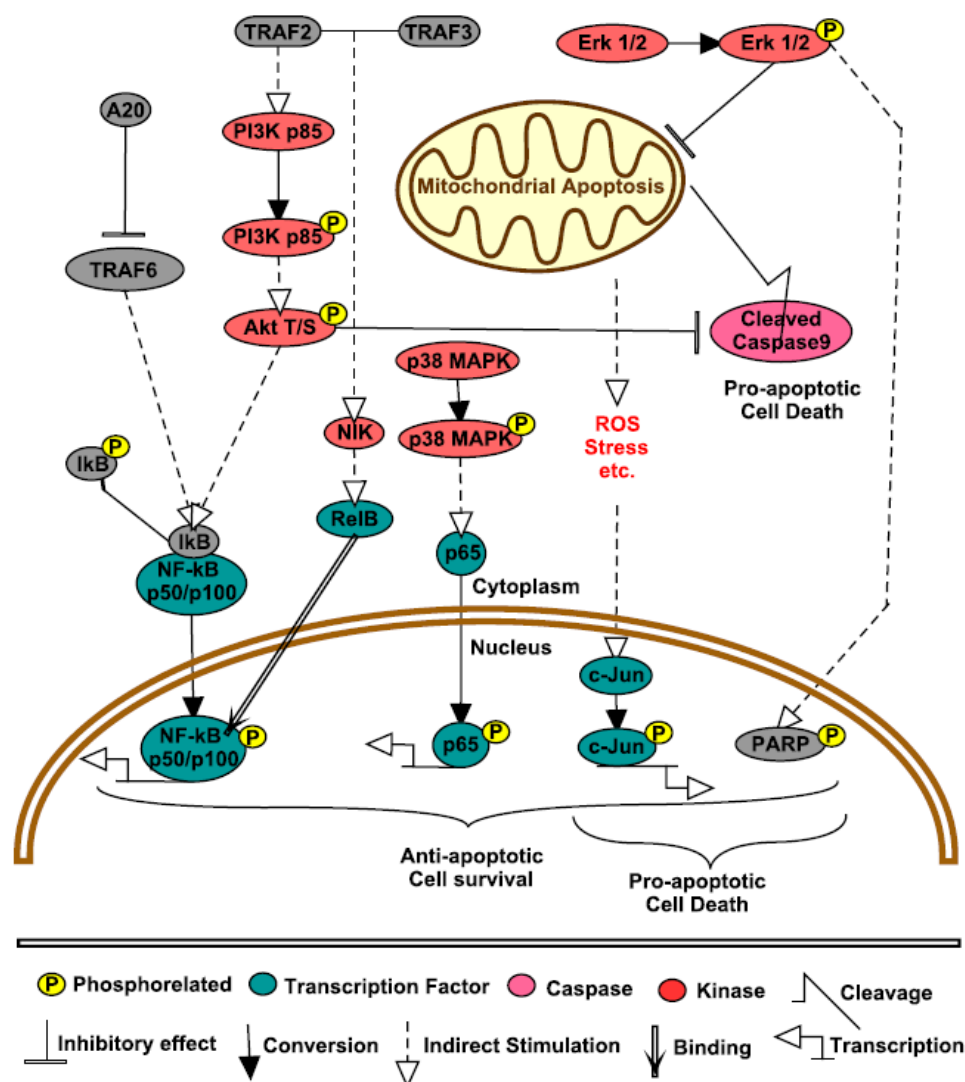


Figure 5.4: Diagram simplifying the proposed role of the investigated signals in cells' death and survival pathways.

NF-κB can be activated via binding of several ligands to their receptors (e.g., TNFR and TLR). C-Jun activation occurs through JNK, which can be activated by a variety of factors (e.g., environmental stresses and inflammatory cytokines). The phosphorylation of PARP can be mediated via phosphorylation of the Erk1/2 pathway, which is required for maximal PARP activation for DNA repair after damage. This diagram was drawn in PathVisio 3.2.1 software utilising the information obtained from <http://www.cellsignallingbiology.org/csb> (Berridge, 2014), and <http://www.cellsignal.com/> © 2016 Cell Signaling Technology, Inc.

At 30 min after N222.1 infection, slightly higher levels were observed for most signals except TRAF2 and P-c-Jun, and the highest levels were detected for the TRAF3, P-p38 MAPK and P-PARP, compared to other infected and uninfected cells, (Figure 5.5 and Figure 5.6). P-PARP and P-c-Jun can play a dual role as an anti or pro-apoptotic signal. N222.1 at 30 min developed slightly higher levels of type IV tubulin and ZO-1 compared to uninfected cells, and lower level of mucin (Figure 5.5).

At 2 hpi N222.1 infection more or less preserved the same signals level in general, except for P-p38 MAPK and Erk1/2, which were clearly reduced compared with their levels at 30 min (Figure 5.5 and Figure 5.6). At the same time, the A20 signal was higher than its level at 30m, while the P-c-Jun level was elevated to a higher position compared to uninfected cells. Interestingly, N222.1 infection developed the lowest levels of mucin and type IV tubulin compared to the uninfected cells and the infected with the other strains (Figure 5.5).

N459.3 infection at 30 min has not developed major changes in the signals level compared to uninfected cells, except a slight increase in the levels of NIK, P-NF- κ B p100, P-PARP and P-p38 MAPK; and reduction in the P-c-Jun level compared to uninfected cells. (Figure 5.5 and Figure 5.6). A minor

reduction in the mucin and type IV tubulin levels was observed at 30 min (Figure 5.5).

At 2 hpi the signals level for cells infected with N459.3 were elevated dramatically. The levels of all signals, in general, were higher than in uninfected cells and the infected with N222.1 (Figure 5.5 and Figure 5.6). The highest signals in the case of N459.3 infection were NIK and P-Erk1/2 compared to other infected and uninfected cells. The mucin level was reduced compared to uninfected cells, while the type IV tubulin and ZO-1 levels were lifted up faintly (Figure 5.5).

In general, N459.6 infection at 30 min more or less induced the same activation levels of all signals compared with N459.3 (Figure 5.5 and Figure 5.6). A little reduction in the mucin and type IV tubulin levels, compared to uninfected cells, was noticed at 30 min (Figure 5.5).

By 2 hpi N459.6 infection induced high levels of all signals in general, with a low level of P-Erk1/2 and the lowest P-p65 levels compared with all the conditions (Figure 5.5 and Figure 5.6). However, it induced the highest levels of pro-apoptotic signals I κ B α , A20 and cleaved caspase 9 compared to all other conditions. A major increase in type IV tubulin and ZO-1 levels and a reduced level of mucin was observed at 2 hpi (Figure 5.5).

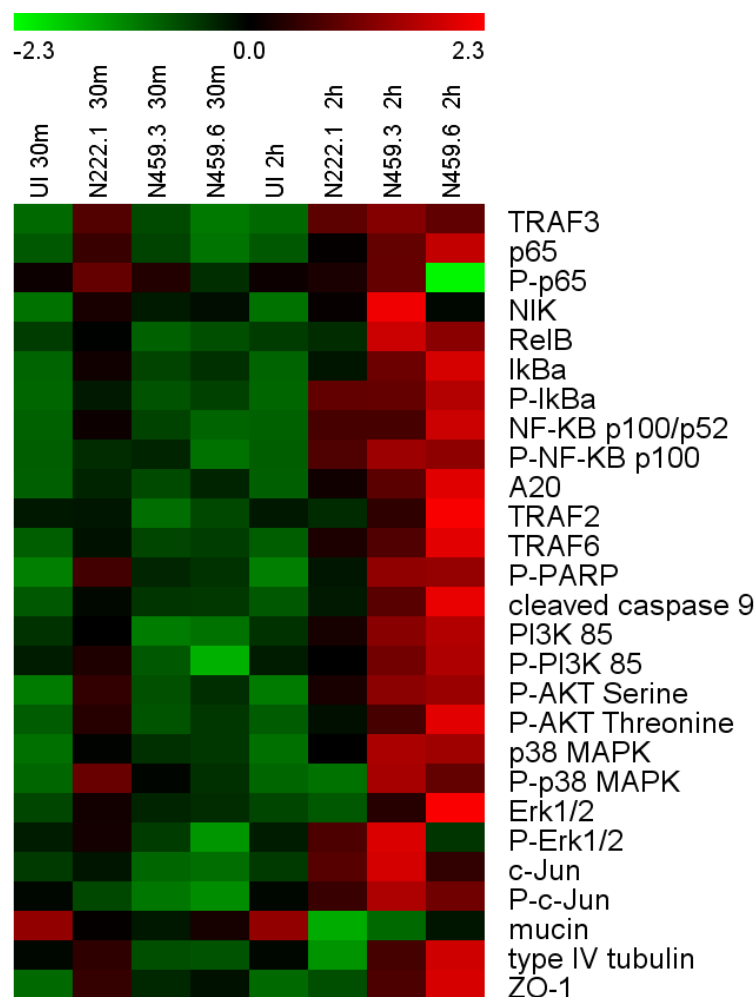


Figure 5.5: Time-course analysis of the anti-apoptotic signals activation in infected and uninfected Calu-3.

The heat map representing the activated signals using RPPA to compare Calu-3 cells infected with N222.1, N459.3 or N459.6 at MOI of 30 for 30 minutes or 2 hours post-infection. Each column represents a cell lysate sample, while each row represents a tested signaling molecule. Red and green indicate the higher and lower levels, respectively (log2 scale of normalized signal fluorescence). The data shows that the response against N222.1 infection, in general, was developed faster than its variants (N459.3 and N459.6). N459.3 and N459.6 infections were similar in the delayed response against them with a high level of activation at 2 h. All fluorescent signals were normalized to β -actin. The data represent one experiment in duplicate.

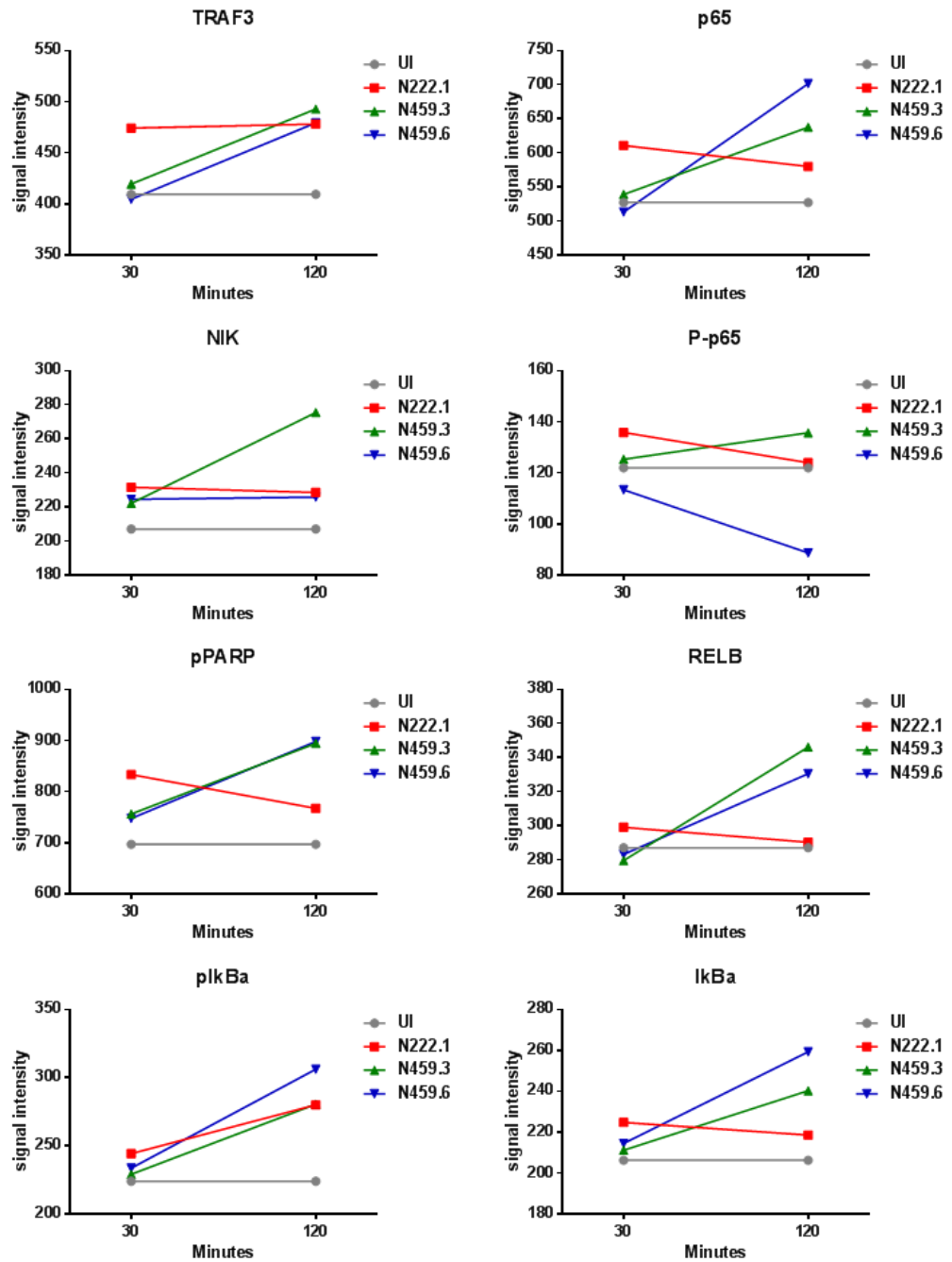
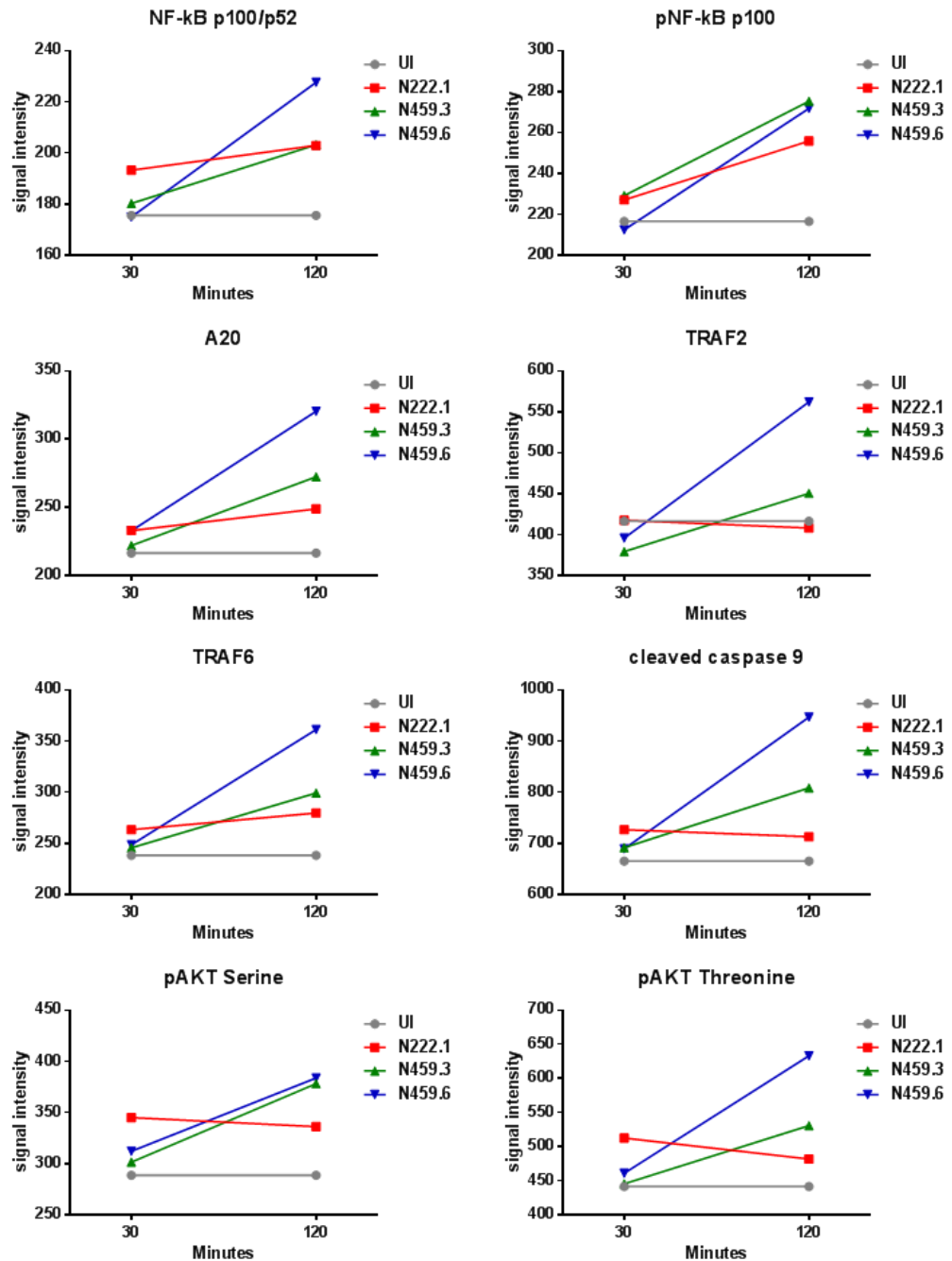


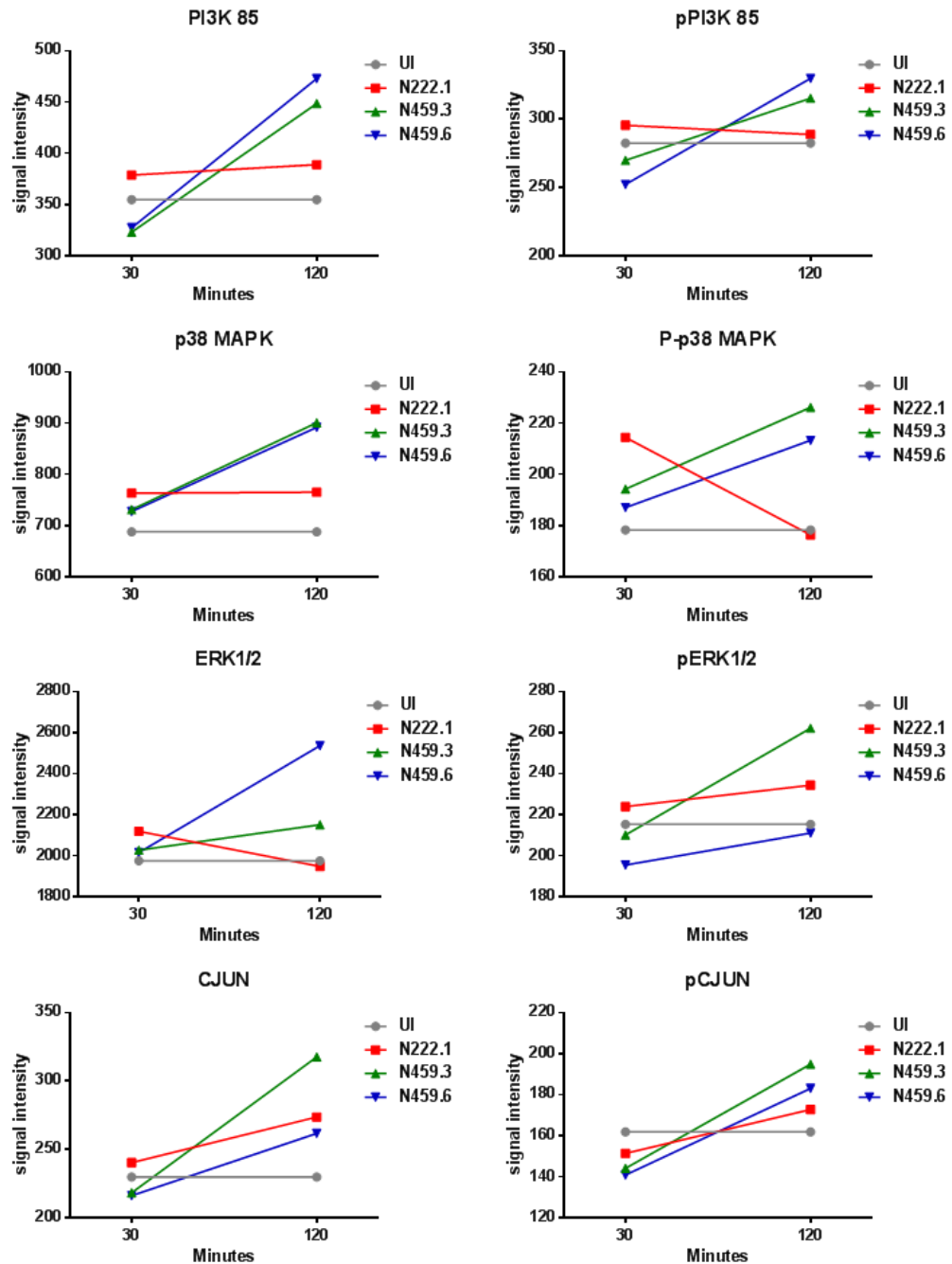
Figure 5.6: Time-course activation of all signals in infected and uninfected Calu-3.

The charts are showing comparisons between uninfected and infected Calu-3 with N222.1, N459.3 or N459.6 at 30 minutes and 2 hours post-infection. The data represent one experiment in duplicate.



Continued from figure 5.6: Time-course activation of all signals in infected and uninfected Calu-3.

The charts are showing comparisons between uninfected and infected Calu-3 with N222.1, N459.3 or N459.6 at 30 minutes and 2 hours post-infection. The data represent one experiment in duplicate.



Continued from figure 5.6: Time-course activation of all signals in infected and uninfected Calu-3.

The charts are showing comparisons between uninfected and infected Calu-3 with N222.1, N459.3 or N459.6 at 30 minutes and 2 hours post-infection. The data represent one experiment in duplicate.

Taken all together, the response against N222.1 infection developed faster than its variants and the early major increase in P-PARP level indicates that possible DNA damage has occurred. We are suggesting that the anti-apoptotic signaling might not have developed fast enough to cope with the amount of stress and damage, especially with the high level of I κ B α inhibitor compared with the other conditions. However, N459.3 and N459.6 infections have a low level of activation for all signals in general, which was followed with a remarkable increase at 2 hpi. This increase in the case of N459.6 was anti-apoptotic combined with lowest levels of P-p65 and P-Errk1/2 compared to other infections.

5.6 Discussion

The neutralisation of TNF- α has no significant enhancement of the barrier function of the monolayer post-infection with N222.1. The response against N222.1 infection developed faster than its variants with possible occurrence of DNA damage. The anti-apoptotic signaling might not have developed rapidly enough to cope with the amount of stress and damage caused by N222.1 infection. N459.3 infection was the fastest in developing the major key factor signals in cell survival response. The rapid anti-apoptotic response might turn the scale in the favour of cell survival against cell death. The delayed development of the key factor signals in the anti-apoptotic response, *i.e.* P-Erk1/2 and P-p65, by N459.6 combined with high levels of pro-apoptotic signals might explain the observation of the moderate level of barrier dysfunction and TNF- α production by N459.6 infection compared to N222.1 infection.

According to our results, it is clear that TNF- α is not a key factor in the observed alteration of the monolayer's permeability in the case of N222.1 infections. However, it is well known that TNF- α production by epithelial cells can be stimulated by many meningococcal related factors (*e.g.*, LOS endotoxin, synthetic lipid A, nitric oxide, oxygen radicals, toxic

shock toxin- 1 or by the induction of GM-CSF, TNF- α and other cytokines) (Wang *et al.*, 2003). In a study on HeLa-M cells, a link between the DNA damage and the TNF- α production has been described (Picco and Pages, 2013). Thus, it is possible that the extensive level of TNF- α in our study can be a response to cell damage rather than being the inducer to the damage. However, apoptosis can be induced by a variety of factors (*e.g.*, growth factors deprivation, TNF, TGF- β , cytotoxic chemicals and toxins) (McConkey *et al.*, 1996). The apoptosis-induced nuclear DNA fragmentation has been reported in many organisms including mammalian cells (Abastado, 1996). Therefore, the observed scenario of infection can not be framed and limited to one prospect; the window of possibilities is very wide.

Deghmane *et al.* have shown a strong correlation of cytopathic effect to Human endometrial epithelial cell line (Hec-1B) with meningococcal isolates pathological type, either invasive or carriage. Infected cells with invasive isolates presented features of apoptosis induced partially by LOS, the major bacterial endotoxin, and involved TNF- α signaling through its receptor TNFR1. In contrast, carriage isolates interfered with TNF- α -induced apoptotic signaling by increased extracellular shedding of TNFR1 resulting in the attenuation of the biological activity of TNF- α (Deghmane *et al.*, 2009). However, although

all the strains we used (N222.1, N459.3 and N459.6) are carriage isolates from the same case, and belong to the same clonal complex and sequence typing, we observed a distinct pattern of infection and response.

In another study by Deghmane *et al.*, they show that only carriage isolates were able to induce persistent NF- κ B activation in infected Hec-1B cells. ST-11 invasive isolates initially triggered a strong NF- κ B activity which was inhibited at 9 hpi and was associated with sustained JNK activation, elevated levels of membrane TNFR1, and apoptosis. At the opposite side, carriage isolates infection lead to prolonged activation of NF- κ B associated with a temporary activation of JNK, increased level of soluble TNFR1 and apoptosis inhibition (Deghmane *et al.*, 2011). Although they suggest that invasive and carriage isolates are both able to trigger the TLR4/MyD88 pathway via an LOS-dependant pathway. It is noteworthy, that our carriage strains (N222.1, N459.3 and N459.6) have an *lpxL1* mutation, which results in under acylation of lipid A endotoxin that impacts the TLR4 binding and activation (Ladhani *et al.*, 2012, Bidmos *et al.*, 2011).

The nuclear kinase ataxia telangiectasia mutated (ATM) orchestrates a range of events, such as facilitating the recognition and repair of DNA damage, the engagement of

cell-cycle checkpoints, and the activation of pro-survival and stress-response pathways (Biton and Ashkenazi, 2011). ATM can also activate cytokine secretion and programmed cell death depending on the degree and duration of DNA damage (Fumagalli and d'Adda di Fagagna, 2009, Harper and Elledge, 2007, O'Driscoll and Jeggo, 2006, Rashi-Elkeles *et al.*, 2006, Kastan and Bartek, 2004). The NF- κ B pathway can be activated by ATM, which results in various biological functions, including inhibition of apoptosis and activation of cell survival. The mechanism of NF- κ B activation by ATM shares some features with the stimulation of NF- κ B by TNF- α (Biton and Ashkenazi, 2011).

However, in the situation of excessive DNA damage, ATM utilizes the NF- κ B essential modifier (NEMO) and RIP1 kinase via autocrine TNF- α signaling to switch on cytokine production and caspase activation (Picco and Pages, 2013). Among the initial and important events in DNA damage recognition and signaling is the formation of poly (ADP-ribose) (PAR) by PARP-1, which has been demonstrated to play a fundamental role in the mechanism leading to nuclear post-translational modifications of NEMO (Stilman *et al.*, 2009). According to our results, N222.1 infection at 30 min developed faster and higher increase in P-PARP level compared to its variants, indicating that possible DNA damage has occurred. However,

the activation of NF- κ B was higher in the case of N459.3, which indicates a faster recovery from the suggested DNA damage. Noteworthy, that besides DNA damage, the activation of NF- κ B can be induced by a range of pathways, such as, cellular stress, TLRs, IL-1R, TNFRs, growth factors and hormones.

The immediate response to DNA damage is a rapid cell cycle arrest mainly based on the activation of p53, and the capability of repairing the damage determines the speed of recovery and return to the normal cell cycle (Belyaev, 2010). In the case of irreparable or incorrectly resolved damage, the cell stops proliferating and directed towards apoptosis or aging (Lukas *et al.*, 2011, Rodier and Campisi, 2011). NF- κ B activity is usually described as a pro-survival factor, However, Biton and Ashkenazi showed that the activation of NF- κ B by genotoxic agents actually switches between a pro-survival and an apoptotic cellular response to DNA damage (Biton and Ashkenazi, 2011). In their model, mild DNA damage could develop an early-phase NF- κ B response leading to cell survival during the time required to repair the injuries. Alternatively, in the case of extensive DNA damage, the initial NF- κ B response would be maintained and boosted by a TNF- α /TNFR-1 autocrine loop, which is suggested to be the reason behind the sustained activation of JNK3.

Interestingly, N459.3 infection was the faster in developing NF- κ B activation a major key factor in cell survival response, which was followed later with a further major increase in survival signals compared with N459.6 infection.

A broad range of events occurring downstream of JNK activation, ranging from cytoskeleton remodeling to the regulation of cell proliferation or apoptosis, have been described (Picco and Pages, 2013). The JNK activation leads to the increase in c-Jun phosphorylation and transcriptional activity (Dunn *et al.*, 2002). It was believed and documented that c-Jun has a positive role in neuronal cell death in hippocampal neurons exposed to kainite (Le-Niculescu *et al.*, 1999, Watson *et al.*, 1998), in contrast, an increasing evidence of the c-Jun involvement in the protection of cells from stress-induced apoptosis has been reported (Wisdom *et al.*, 1999, Eferl *et al.*, 1999). However, it has been reviewed that JNK activation is well recognized to suppress TNF- α -stimulated apoptosis (Varfolomeev and Ashkenazi, 2004, White, 2011).

To conclude, it is clear that TNF- α is not the key factor in the alteration of the monolayer's permeability in the case of N222.1 infections. That is supported with the outcome of the N59.1 infection, as it developed a similar level of TNF- α

without the permeability alteration. It is possible that N222.1 induced DNA damage, while the anti-apoptotic signaling might not have developed fast enough to cope with the amount of stress and damage, especially with the high level of I κ B α inhibitor. The delayed development of the key factor signals in the survival response by N459.6, followed with an increase for some of them, was combined with high levels of cell death signals, which might explain the observation of its moderate level of causing barrier dysfunction and TNF- α production compared with N222.1.

Although the production of Reactive oxygen species (ROS) as players of oxidative stress was not investigated, the observation of critical change in the media pH was always present in N222.1, N459.6 and N59.1 infections (see chapter 4). That was always harmonised with the high levels of TNF- α production. ROS such as superoxide and hydrogen peroxide are important components of several cellular pathways (Dan Dunn *et al.*, 2015). In associated pathways with immunity, ROS can play a role as anti-microbial effector molecules and as signaling molecules that regulate processes such as NF- κ B activation and the production of DNA-based neutrophil extracellular traps (NETs) (Nauseef, 2014, Morgan and Liu, 2011, Fuchs *et al.*, 2007). In addition, within and without the context of immunity, ROS can be involved in pathways that

regulate autophagy (Lam *et al.*, 2010, Lee *et al.*, 2012). However, several studies indicate that ROS production in the mitochondria, and once thought to be redundant to the cell metabolism, is regulated by immune signaling pathways to be involved in immune responses (West *et al.*, 2011, Bulua *et al.*, 2011). Thus, the aspect of the position of mitochondrial-induced apoptosis from our observations needs to be clarified. We believe that including more signals and strains in this investigational approach will be beneficial for understanding these variations in the infection pattern of meningococcal. Moreover, eliminating, blocking or inhibiting some of the key factors in cell signaling can be a useful approach to achieve a better understanding of the events of meningococcal infection. All the cell signalling data in this study are indicators, not certain notions and need further investigation and validation.

Although the RPPA assay was not repeated for a statistically considered number of repeats, it still indicates that the cell signalling investigation could be helpful and informative tool in the field of meningococcal variation study. The RPPA can detect the changes in cell response in the signalling level at very early stages of infection. Thus, instead of conducting long infection assays to detect the differences in the response, RPPA can cut down the time to minutes or few hours. In addition, the rapidly growing library of specific antibodies

targeting cell signaling molecules allows precise and accurate information about the cellular response against each strain or variants. On top of that, it can reduce the cost by analysing multiple samples for multiple signaling molecules at the same time with a small amount of protein (1 nL of a 2 ng/nL solution).

On the other hand, it might be financially inefficient in the case of small experiments, as the cost of printing the slide and the amount of antibodies and reagents should be spread on the samples number per slide, fewer samples more cost per sample and more samples less cost per sample. Moreover, analysing and visualising the data is quite complicated and not a straight forward process, it needs as well high computing skills.

5.7 Summary

It seems to be that the permeability modification in the case of N222.1 infections is not influenced by the high production of TNF- α . There is a blurred image, need to be clarified, of the associated events with meningococcal infection. Utilising the cell signaling approach in studying the meningococcal variation, can be a valuable addition.

CHAPTER SIX

GENERAL DISCUSSION

6. GENERAL DISCUSSION

6.1 The generation of *lpxL1* mutation

In this study, we confirmed that the construction of MC58 strains with mutated *lpxL1* gene and *lpxL1*-complemented was a success. However, the complementation did not lead to the restoration of electrophoretic mobility of LOS to the WT position. This could be due to the fact that *N. meningitidis* has several genes involved in LOS biosynthesis that are liable to phase variation (Jennings *et al.*, 1999). These genes are responsible for inducing different LOS immunotypes of MC58 and H44/76 by modifying the LNT α chain and β chain (Jennings *et al.*, 1999, Virji *et al.*, 1995a, Saunders *et al.*, 2000, van der Ley *et al.*, 2001). Switching between the ON and OFF state of genes occurs randomly (Rotman and Seifert, 2014), therefore, the observed change in the LOS electrophoretic mobility could be caused by the either *LpxL1* deficiency or an unexpected change due to phase variation. Previous studies showed that different LOS immunotypes exhibit different LOS electrophoretic mobility (Jennings *et al.*, 1995, Jennings *et al.*, 1999).

Vincent van Dam and Martine P. Bos stated that complementing a mutant by reintroducing an intact copy of the defective gene into it, has a risk of selecting a phase

variant (van Dam and Bos, 2012). The risk comes as a consequence of picking up single colonies post transformation. However, we showed that different colonies of MC58 WT show inconsistency in electrophoretic mobility of their LOS. The use of immunotype-specific antibodies could have helped clarify whether the different LOS bands observed were the result of phase-variable immunotype switching. Alternatively, a genetic approach using sequencing of *lgtA* and *lgtG* would have clarified the expression status of these genes.

Previous studies in H44/76 background generated *lpxL1* mutation, however, none has performed *lpxL1* complementation (van der Ley *et al.*, 2001, Fisseha *et al.*, 2005). Since a complemented mutant provides confirmation that phenotypic changes evident in the mutant are directly related to the introduced mutation, the complementation was a must. In order to explain the apparent differences in LOS mobility and structure, more time-consuming and expensive analytical techniques (*e.g.*, nanoelectrospray tandem mass spectrometry) would have been beneficial.

However, *lpxL1* mutation and complementation were generated to test the response against the resultant strains by Calu-3 infection model. Due to the uncertainty of complementation, it was decided to start the infection assays

with different WT isolates and study the possible different responses against them.

6.2 Establishment of Calu-3 infection model

An *in vitro* infection model of differentiated Calu-3 cells growing in ALI was successfully established in our lab. A previous study in our lab has found that the 21 days old differentiated Calu-3 cells featured a barrier characteristics (Yi-Chia Liu, PhD Thesis), this is further supported by the literature (Bals *et al.*, 1999, Florea *et al.*, 2001, Florea *et al.*, 2002, Barrile *et al.*, 2015, Grainger *et al.*, 2006). Calu-3 cells cultured at ALI have been described to be morphologically and functionally relevant to *in vivo* airway epithelium compared to liquid submerged culture (Grainger *et al.*, 2006). Sutherland *et al.* suggested that the Calu-3 model could be suitable for studying the pathogenesis of infections caused by other respiratory pathogens, as well as the meningococcus (Sutherland *et al.*, 2010). In more recent study the level of polarization of the Calu-3 cell monolayer was shown to play an important role in *N. meningitidis* infection, where the longer differentiation resulted in a better polarisation and barrier function (Barrile *et al.*, 2015).

Despite the fact that ALI differentiated Calu-3 cell cultures display the characteristics of cultures derived from primary

NHBE (Berube *et al.*, 2010), it should be noted that the Calu-3 model does not fully imitate those in the healthy native epithelium.

6.3 Infection of differentiated Calu-3

The Calu-3 monolayer formed a barrier which could not be traversed by MC58 under the conditions tested. The infection of Calu-3 cells with MC58 has shown promising consistent result regarding cytokines expression, the growth of MC58 within the infection model, MC58 adhesion and invasion pattern and MC58 phenotypic stability. Sutherland *et al.*, in 2010, reported the ability of MC58 to traverse across the Calu-3 monolayer in small numbers (Sutherland *et al.*, 2010). However, their method of differentiation and infection was different from our method.

In a more recent study, comparing the ability of MC58 strain to invade and cross Calu-3 monolayer at different stages of cell differentiation, they have found that poorly polarized Calu-3 monolayers are more susceptible to *N. meningitidis* invasion (Barrile *et al.*, 2015). Therefore, there is an agreement that longer differentiation is crucial for better polarization (Bals *et al.*, 1999, Florea *et al.*, 2001, Florea *et al.*, 2002, Barrile *et al.*, 2015).

6.4 Adhesion and invasion assay

The study of adhesion and invasion revealed that different isolates displayed different association pattern even for those closely related isolates. These differences might be resulted by their different pattern of cell-surface and secreted virulence factors, which influence colonization, persistence, and damage within the host. Among these virulence factors, there are two meningococcal autotransporters NalP and MspA, which are phase variable outer membrane/secreted proteins (Oldfield *et al.*, 2013). Interestingly, NalP and MspA are OFF in N222.1 and ON in MC58, which might explain the lower association of N222.1 with Calu-3 cells compared to MC58. Additionally, the members of the cc23 clonal complex, *i.e.*, N222.1, lack the gene that encodes NadA protein, another important adhesion molecule (Alamro *et al.*, 2014). Results of this study pointed at NadA as the factor responsible for the high association level of N59.1, as it is the only strain expressing it. Furthermore, it had the highest adhesion level among all tested isolates. In addition, it expresses NalP, another adhesion molecules, leading to the overall enhancement of the adhesion to host cells.

Apart from the autotransporters, the meningococcal membrane opacity proteins, Opa, and Opc, mediate intimate

adhesion to host cells via binding to CEACAMs receptors and extracellular matrix components, respectively (Virji, 2000). There are different Opa proteins (de Jonge *et al.*, 2003b) with variable levels of adhesion to host cells (Virji *et al.*, 1993, McNeil *et al.*, 1994, de Jonge *et al.*, 2003b). The Opa expression profile varied among the isolates used in our study. It was difficult to correlate a certain cell association pattern to specific Opa expression pattern. This particular point needs more investigation as Opa proteins are one of the major players in meningococcal association with host cells.

Besides those important meningococcal adhesion molecules mentioned above, additional antigens have been described as having a role in adhesion such as Type IV Pili, NhhA and App (Pizza and Rappuoli, 2015). Altogether, the results of our study and the literature highlight the importance of studying the role of those antigens in cell association extensively by introducing different isolates with different expression pattern of adhesion molecules to the Calu-3 model.

6.5 Cytotoxicity and permeability

In our study, the observation of minor enhancement of permeability when the Calu-3 monolayer was infected with MC58, H44/76 or N459.3 has been reported in another study on MC58 (Sutherland *et al.*, 2010). This also agreed with a

more recent study showing that the MC58 infection does not significantly disrupt the tight junction integrity, while the cells height and polarity have significantly changed during infection in an agreement with our histological observation (Barrile *et al.*, 2015). This indicates that meningococcus does not need to either alter the integrity of the epithelial monolayer as an infection strategy or traverse across the epithelial barrier by a paracellular route (Sutherland *et al.*, 2010, Pujol *et al.*, 1997). Instead, the meningococcus traverses the monolayer barrier using an intracellular route by manipulating the monolayer function and polarity (Barrile *et al.*, 2015).

However, the disruption of monolayer integrity by N222.1 and N459.6 has been reported as an *in vitro* infection strategy in *N. gonorrhoeae* in other studies (Rodriguez-Tirado *et al.*, 2012, Edwards *et al.*, 2013). However, it is not clear that N222.1 and N459.6 are using this mechanism or not, as a pathogenesis strategy as further analysis using microscopy techniques is required.

6.6 Cytokines response by Calu-3 cells

The Calu-3 cells react to infections with different meningococcal isolates differentially, especially in the case of TNF- α production. TNF- α is one of the most predominant secreted cytokines and its production by epithelial cells can be

stimulated by many meningococcal related factors (*e.g.*, LOS endotoxin, synthetic lipid A, nitric oxide, oxygen radicals, toxic shock toxin- 1 or by the induction of GM-CSF, TNF and other cytokines) (Wang *et al.*, 2003).

In our study Calu-3 cells reacted to MC58 infection by elevating the expression levels of TNF- α moderately, unlike H44/76, which triggered very low levels of TNF- α . The serogroup Y isolates triggered a spectrum of TNF- α response from very low, N459.3, to very high, N222.1. However, N459.3 and N459.6 are the closest to each other phenotypically among the other isolates, and N222.1 shares the same clonal typing with them cc23, while N59.1 is not.

Many studies highlighted the significance of TNF- α production in meningococcal infection clinically (van Deuren *et al.*, 1995), *in vivo* (Fransen *et al.*, 2010) and *in vitro* (Fransen *et al.*, 2009, Steinmann *et al.*, 2013, Borkowski *et al.*, 2014). Various factors can play an important role in the differences of cytokine production, such as lipid A acylation level (Fransen *et al.*, 2009, Fransen *et al.*, 2010) and capsule (Steinmann *et al.*, 2013, Borkowski *et al.*, 2014).

The *lpxL1* gene is naturally mutated mainly in cc23 isolates of Y serogroup of *N. meningitidis* (Ladhani *et al.*, 2012). However, results of these studies contradict our study in terms

of TNF- α production, but that might be explained by the different models used in these studies, and the cell type is an important factor in determining the type of response elicited in response to the pathogen. There is a lack of information in the literature reporting the response of epithelial cells to this mutant, which suggests that this particular aspect needs further investigations.

The presence of the capsule functionally masks OMPs, such as Opa and Opc proteins, which facilitate the bacteria internalisation and the close contact to host cells (Schubert-Unkmeir *et al.*, 2010). Moreover, the capsule can inhibit cell adhesion, biofilm formation, and help in the immune evasion (Pizza and Rappuoli, 2015, Kugelberg *et al.*, 2008). Therefore, the investigation of the capsule state among the isolates used in our study would be beneficial.

TNF- α elicits a wide range of cellular effects, from proliferation and viability to inflammation and apoptosis (Roulston *et al.*, 1998). Unlike N222.1 and N459.6, the infection case of N59.1 showed that the high levels of TNF- α did not correlate with changes in permeability. Furthermore, the neutralisation of TNF- α has no significant enhancement of the barrier function of the monolayer post-infection with N222.1. Indeed the different pattern of antigenic structures present in each strain

may play an important role in these variable responses, which could mean that the whole infection scenario of each strain might be different.

6.7 The NF- κ B pathway

Although our results suggested that TNF- α is not involved in the observed alteration of the monolayer's permeability, a link between the DNA damage and the TNF- α production has been described before (Picco and Pages, 2013). Thus, it is possible that the extensive level of TNF- α in our study can be a response to the cell damage rather than being the inducer to the damage. However, apoptosis can be induced by a variety of factors such as toxins (McConkey *et al.*, 1996). The apoptosis-induced nuclear DNA fragmentation has been reported in many organisms including mammalian cells (Abastado, 1996). Therefore, the observed infection scenario can not be framed and limited to one prospect as the window of possibilities is very wide.

The activation of NF- κ B pathway results in various biological functions, including inhibition of apoptosis and activation of cell survival. However, in the situation of excessive DNA damage NF- κ B can be activated via autocrine TNF- α signaling to switch on cytokine production and caspase activation (Picco and Pages, 2013). In our study, N222.1 infection at 30 min

developed a fast and high increase in P-PARP level, which might indicate a possible occurrence of DNA damage, while the anti-apoptotic signaling might not be developed fast enough, especially with the high level of I κ B α inhibitor. However, the high level of NF- κ B activation might indicate a faster recovery of the suggested DNA damage in the case of N459.3. Noteworthy, that besides DNA damage, the activation of NF- κ B can be induced by a range of pathways, such as, cellular stress, TLRs, IL-1R, TNFRs, growth factors and hormones.

The partially delayed development of the key factor signals in the survival response by N459.6 was combined with high levels of cell death signals, which might explain the moderate level of causing barrier dysfunction and TNF- α production. However, all these are indicators, not certain notions, and this assay needs more repeats with further investigation and validation.

Although the role of ROS and oxidative stress were not investigated, the observation of critical change in the media pH was always present in N222.1, N459.6 and N59.1 infections. ROS is considered as anti-microbial effector molecules and can play a role as signaling molecules that regulate NF- κ B activation and the production of DNA-based NETs (Nauseef, 2014, Morgan and Liu, 2011, Fuchs *et al.*,

2007). Certainly, including more signals and strains in this investigational approach would be beneficial for understanding these variations of meningococcal infection pattern.

6.8 Summary

From the findings exhibited previously it is clear the use of a differentiated Calu-3 model to study *N. meningitidis* interactions with the host during infection is a promising approach. Since it was fascinating how little phenotypic variations between meningococcal isolates have a huge impact on the infection pattern and the host cells response. Although TNF- α is an important key player in the event of the inflammatory response, it seems not to be the direct stimulus for the observed permeability modification. The image of the associated events and signaling pathways with meningococcal infection needs to be clarified, utilising the cell signaling approach in studying the meningococcal variation, in which can be a valuable addition to this approach. Altogether, these observations are stressing on the fact that the Calu-3 model is a useful *in vitro* platform to study the meningococcal early stages of infection and to evaluate the impact of genetic variation on pathogenesis. Consequently, it would be desirable to invest more effort in this infection model as an important investigational tool in the area of *N. meningitidis* pathogenesis.

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APPENDIX

Appendix 1: Total CFU count for inoculum and total CFU count at 6 and 24 hpi of adhesion and invasion assays

Figure No.	Strain	hpi	CFU mean	SD	N
Figure 3.10	MC58	0	2.03E+07	2.76E+06	5
		6	1.22E+08	4.11E+07	5
		24	1.31E+08	4.40E+07	5
Figure 4.1	MC58	0	3.49E+07	1.67E+07	4
		6	1.83E+08	6.90E+07	4
		24	1.97E+08	5.07E+07	4
Figure 4.2	N222.1	0	3.40E+07	1.73E+07	4
		6	1.02E+08	7.79E+07	4
		24	2.43E+08	4.84E+07	4
Figure 4.11	H44/76	0	3.03E+07	2.08E+06	3
		6	9.00E+07	2.43E+07	3
		24	2.91E+07	7.89E+06	3
Figure 4.12	N59.1	0	2.10E+07	1.32E+06	3
		6	5.24E+07	1.21E+07	3
		24	5.05E+07	5.41E+06	3

Appendix 2: Count for the bacterial distribution during infection of Calu-3 cells at 6 and 24 hpi.

Figure No.	Strain	Distribution	6 hpi			24 hpi		
			CFU mean	SD	N	CFU mean	SD	N
Figure 3.11	MC58	Non-associated	6.65E+07	1.98E+07	5	1.06E+08	3.41E+07	5
		Adhered	5.66E+07	2.62E+07	5	3.17E+07	2.91E+07	5
		Internalised	2.51E+04	2.32E+04	5	3.63E+05	3.68E+05	5
Figure 4.3	MC58	Non-associated	1.62E+08	6.10E+07	4	1.81E+08	4.69E+07	4
		Adhered	4.16E+07	1.78E+07	4	3.21E+07	1.02E+07	4
		Internalised	8.38E+05	1.59E+06	4	5.50E+05	3.82E+05	4
Figure 4.4	N222.1	Non-associated	9.49E+07	7.21E+07	4	-	-	-
		Adhered	1.53E+07	1.33E+07	4	-	-	-
		Internalised	7.74E+04	1.48E+05	4	-	-	-
Figure 4.13	H44/76	Non-associated	8.09E+07	1.22E+07	3	3.19E+07	7.00E+06	3
		Adhered	8.54E+05	4.43E+05	3	7.61E+05	3.49E+05	3
		Internalised	2.83E+03	3.83E+03	3	4.83E+03	3.57E+03	3
Figure 4.14	N59.1	Non-associated	1.93E+07	2.31E+06	3	1.64E+07	1.01E+06	3
		Adhered	3.17E+07	1.11E+07	3	3.20E+07	1.89E+06	3
		Internalised	7.58E+05	3.82E+04	3	5.73E+06	3.10E+06	3

Appendix 3: Readings for the cytokine responses of Calu-3 cells infected with MC58 or N222.1.

Figure No.	cytokine	hpi	UI			MC58			N222.1		
			pg/ml mean	SD	N	pg/ml mean	SD	N	pg/ml mean	SD	N
Figure 4.7	IL-8	6	3189.72	331.08	3	3449.44	147.03	3	3764.44	313.90	3
		24	3668.33	194.41	3	2956.11	395.57	3	2901.39	846.45	3
	TNF- α	6	-16.27	1.66	3	-9.82	1.63	3	-6.41	8.39	3
		24	-15.84	2.10	3	14.22	9.68	3	104.79	15.86	3
Figure 4.9	TNF- α	12	-18.35	2.93	4	23.32	2.93	4	347.63	104.97	4

Appendix 4: Readings for the TNF- α production in response to different meningococcal strains and variants at 12 hpi.

Figure No.	Strain	TNF- α at 12 hpi		
		pg/ml mean	SD	N
Figure 4.18	UI	-81.90	3.12	3
	N459.3	-62.90	2.29	3
	H44/76	15.27	19.57	3
	MC58	179.10	81.27	3
	N459.6	415.60	105.05	3
	N59.1	815.77	37.54	3
	N222.1	917.60	58.33	3

Appendix 5: Readings for the validation of TNF- α neutralisation in Calu-3 cells infected with N222.1 or N59.1 up to 12 hpi in the presence of anti-TNF- α antibodies.

Figure No.	Treatment	N222.1			N59.1		
		pg/ml mean	SD	N	pg/ml mean	SD	N
Figure 5.3	No treatment	148.99	27.03	3	104.83	19.39	3
	Anti TNF- α	-7.48	0.30	3	-9.92	0.78	3
	Ctrl IgG	152.57	31.41	3	121.69	26.98	3

Appendix 6: Readings for the comparison between the permeability of uninfected Calu-3 monolayer and Calu-3 cells challenged with MC58 or N222.1 up to 12 hpi.

Figure No.	Minutes	UI			MC58			N222.1		
		FI mean	SD	N	FI mean	SD	N	FI mean	SD	N
Figure 4.8	0	206.50	36.74	4	224.25	55.07	4	211.75	32.16	4
	30	1541.00	1629.67	4	321.50	127.99	4	3468.25	2479.22	4
	60	2733.00	3825.42	4	597.25	391.39	4	5076.75	2535.58	4
	90	3310.75	3763.76	4	1266.00	1152.14	4	8346.50	2607.96	4
	120	5738.50	8450.01	4	1661.00	1353.06	4	15454.50	6679.17	4
	150	7279.50	10725.19	4	2632.25	2738.68	4	20565.75	8660.02	4
	180	8650.50	12030.91	4	3205.00	3071.51	4	28583.75	10997.71	4

Appendix 7: Readings for the impacts of different serogroup B and Y strains on the permeability of Calu-3 monolayer at 12 hpi.

Figure No.	Minutes	UI			MC58			H44/76			N222.1			N59.1		
		FI mean	SD	N	FI mean	SD	N	FI mean	SD	N	FI mean	SD	N	FI mean	SD	N
Figure 4.16	0	223.67	126.59	3	225.67	124.43	3	223.67	125.32	3	219.00	131.67	3	205.67	118.32	3
	30	4308.00	6531.26	3	2449.33	1107.55	3	1193.33	382.12	3	15296.33	15843.90	3	5684.67	1744.23	3
	60	8261.33	12767.88	3	4043.33	1786.83	3	6934.00	8263.69	3	34620.00	33597.84	3	10336.00	3127.92	3
	90	11399.67	17937.48	3	5113.67	2369.77	3	6348.67	6054.46	3	58869.00	50801.90	3	15178.00	6317.89	3
	120	15559.67	24796.37	3	6186.33	2923.52	3	6367.00	4844.66	3	108288.00	90486.27	3	20762.33	11838.82	3
	150	20044.67	32091.07	3	7145.67	3558.01	3	7313.67	4976.96	3	139384.70	105359.70	3	29048.33	20131.71	3
	180	25483.33	40739.82	3	8447.67	4994.72	3	8274.67	4880.03	3	162704.70	84880.06	3	35470.33	31111.41	3

Appendix 8: Readings for the impacts of N222.1 variants on the permeability of Calu-3 monolayer at 12 hpi.

Figure No.	Minutes	UI			MC58			N222.1			N459.3			N459.6		
		FI mean	SD	N	FI mean	SD	N	FI mean	SD	N	FI mean	SD	N	FI mean	SD	N
Figure 4.17	0	72.33	8.39	3	70.67	12.42	3	73.67	7.64	3	71.33	8.39	3	79.00	13.00	3
	30	2465.00	1733.53	3	3555.33	893.38	3	24476.00	9975.49	3	1485.33	54.79	3	14263.67	1375.17	3
	60	7495.00	5872.68	3	11066.67	4468.29	3	47954.33	17188.31	3	2504.67	595.33	3	28366.00	4483.55	3
	90	13517.67	10815.92	3	17968.33	8719.85	3	89013.34	30092.12	3	3212.33	761.25	3	47122.67	9324.59	3
	120	24570.00	20022.51	3	30605.00	18206.51	3	164098.30	52748.18	3	4142.67	1053.97	3	79919.34	17968.95	3
	150	40886.33	33820.41	3	43703.33	27377.95	3	242537.30	30040.35	3	5247.33	1158.38	3	123612.00	26414.16	3
	180	74331.66	62125.77	3	68147.66	46906.26	3	250730.30	16055.53	3	6319.67	1310.20	3	204517.70	48049.86	3

Appendix 9: Readings for the impact of TNF- α neutralisation on the permeability of infected Calu-3 monolayer.

Figure No.	Strain	Minutes	No treatment			Anti TNF- α			Ctrl IgG		
			FI mean	SD	N	FI mean	SD	N	FI mean	SD	N
Figure 5.2	N222.1	0	148.00	1.00	3	174.33	18.50	3	168.33	6.51	3
		30	6137.33	2459.50	3	5252.00	1082.00	3	4912.00	1804.00	3
		60	15604.33	5262.50	3	12934.00	523.00	3	12379.00	5160.00	3
		90	23685.00	8613.00	3	17152.00	196.00	3	19282.33	8293.50	3
		120	38820.33	17887.50	3	31423.33	28.50	3	30642.00	13628.00	3
		150	67388.34	27025.50	3	57195.00	3132.00	3	52948.00	21320.00	3
		180	107332.30	54352.50	3	78211.34	8961.50	3	86419.00	46582.00	3
	N59.1	0	152.33	2.52	3	200.00	1.00	3	176.00	6.00	3
		30	2297.67	403.05	3	1647.67	100.03	3	1656.67	127.51	3
		60	6251.33	170.00	3	2915.33	143.00	3	3480.67	22.81	3
		90	8557.33	1102.50	3	4123.67	567.03	3	4894.67	134.69	3
		120	12679.00	3679.50	3	5420.67	87.00	3	6641.33	381.07	3
		150	16931.00	4825.00	3	7860.33	1160.01	3	11152.00	82.50	3
		180	22348.00	6894.07	3	10239.00	2016.53	3	12348.00	502.24	3