

The modulatory effects of commensal neisseriae on upper respiratory tract infections

Keith Page

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Department of Applied Sciences, Centre for Research in Biosciences,
University of the West of England, Bristol, UK

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Abstract

The human nasopharynx is a reservoir of both commensal and pathogenic bacteria that can be easily transmitted from one individual to another. It has long been hypothesised that host commensal flora give protection from carriage of pathogens and invasive disease. The commensal *Neisseria lactamica* has previously been associated with protection against the closely related human pathogen *Neisseria meningitidis*, which is thought to be due to the acquisition of cross-reactive immunity to *N. meningitidis*. The objective of this study was to identify the extent of protection by *N. lactamica* in the absence of host immune cells, using an *in vitro* model of the human nasopharyngeal epithelium with the Detroit 562 (D562) cell line.

N. lactamica has been demonstrated to attenuate the induction of innate inflammatory cytokines and chemokines from D562 cells challenged with *N. meningitidis*. For the first time in this study, *N. lactamica* was found to attenuate the induction of IL6, IL8 and TNF α from D562 cells challenged with the unrelated Gram-positive human pathogen *Streptococcus pneumoniae*. Attenuation by *N. lactamica* did not extend to suppression of MAPK pathways when stimulated with chemical agonists, but was able to suppress inflammation induced through the intracellular PAMP receptor TLR3, which is not involved in meningococcal or pneumococcal inflammation. This suggests a global mechanism of attenuation in host cells by *N. lactamica*.

N. lactamica was further demonstrated to reduce association with and invasion of D562 epithelial cells by *N. meningitidis* serogroup B (MenB) by up to 60% and 90%, respectively. This suppression was dependent on live *N. lactamica* and did not require invasion of host cells by the commensal, suggesting an active mechanism employed by *N. lactamica*. The occasional human commensal coloniser *Neisseria polysaccharea* was found to reduce adhesion and invasion of MenB to a similar degree, however the related commensal *Neisseria cinerea* was not. The reduction in MenB association with host cells protected host cells from MenB-induced apoptosis, which was mediated by activation of caspase 3.

This study demonstrates that commensal *Neisseria* spp. *N. lactamica* and *N. polysaccharea* protect the host at the nasopharyngeal epithelium from experimental colonisation and invasive disease by MenB. Additionally, commensal neisseriae protect against inflammation and cell death induced by the unrelated pathogen *S. pneumoniae*. Therefore, commensal neisseriae warrant further study to evaluate their effectiveness for use as probiotics to protect against bacterial pathogens responsible for meningitis.

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List of Abbreviations

Abbreviation	Name
ANI	Anisomycin
AP1	Activator protein 1
BSA	Bovine serum albumin
Ca	Calcium
CAMP	Commensal-associated molecular pattern
CARD	Caspase activation and recruitment domain
CEACAM	Carcinoembryonic antigen cell adhesion molecule
CFU	Colony forming units
Chop	Phosphorylcholine
chx	Cyclohexamide
DAMP	Danger-associated molecular pattern
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbant assay
EMEM	Eagle's minimum essential medium
ERK ^{MAPK}	Extracellular signal-regulated kinase
ET	Electrophoresis type
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
Gent	Gentamicin
Hi	<i>Haemophilus influenzae</i>
Hia	<i>Haemophilus influenzae</i> type a
Hib	<i>Haemophilus influenzae</i> type b
HRP	Horseradish-peroxidase

Ig	Immunoglobulin
IgA	Immunoglobulin alpha
IgG	Immunoglobulin gamma
IgM	Immunoglobulin mu
IL10	Interleukin 10
IL1 β	Interleukin 1 β
IL6	Interleukin 6
IL8	Interleukin 8
JNK ^{MAPK}	c-Jun NH(2)-terminal kinase
LPS	Lipopolysaccharide
M199	Medium 199
MAPK	Mitogen-activated protein kinase
MenA	<i>Neisseria meningitidis</i> serogroup A
MenB	<i>Neisseria meningitidis</i> serogroup B
MenC	<i>Neisseria meningitidis</i> serogroup C
MFI	Median/ Mean fluorescent intensity
Mg	Magnesium
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence type
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MyD88	Myeloid differentiation primary response gene (88)
NadA	<i>Neisseria</i> adhesin A
NALT	Nasopharyngeal associated lymphoid tissue
Ncin	<i>Neisseria cinerea</i>
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nlac	<i>Neisseria lactamica</i>
NLRP3	Nucleotide-binding oligomerization domain-like receptor (NLR) pyrin domain-containing 3
Nmen	<i>Neisseria meningitidis</i>

NOD	Nucleotide-binding oligomerization domain
Npoly	<i>Neisseria polysaccharea</i>
OMP	Outer membrane protein
Opa / Opc	Opacity-associated protein
PAMP	Pathogen-associated molecular pattern
PARP	Poly-(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBS-B	Phosphate buffered saline containing magnesium and calcium
PCR	Polymerase chain reaction
PCV13	13-valent pneumococcal vaccine
PCV23	23-valent pneumococcal vaccine
PCV7	7-valent pneumococcal vaccine
Pen/Strep	Penicillin/ streptomycin
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
Poly(I:C)	Polyinosinic:polycytidylic acid
PorB	Porin B
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
PS	Polysaccharide
RLU	Relative light units
RNA	Ribonucleic acid
Sa	<i>Staphylococcus aureus</i>
SD	Standard deviation
SEM	Standard error of the mean
slgA	Secretory immunoglobulin alpha
Sp	<i>Streptococcus pneumoniae</i>
ST	Sequence type

STRP	Staurosporine
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TNF α	Tumour necrosis factor alpha
TRAM	Toll-like receptor 4 adaptor protein
TRIF	TIR-domain-containing adapter-inducing interferon- β
VRSA	Vancomycin resistant <i>Staphylococcus aureus</i>
x-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Chapter 1

General Introduction

1.1 Background

From the moment we are born we are being colonised with a plethora of micro-organisms that when combined make up our microbiome or microbiota (Human Microbiome Project Consortium, 2012). It has been estimated that the human body is populated by approximately 10 times the number of bacterial cells than eukaryotic cells with the majority of bacteria found in the gastrointestinal (GI) tract (Savage, 1977). However, more recent studies have found that approximately equal proportions of bacteria exist on the skin (21%) and in the oral cavity (26%) as are found in the GI tract (29%), with the airways (14%) being the next most colonised (Peterson *et al.*, 2009). The microbiome during the first year or so of life has been found to be unstable and uneven, with a greater diversity in the gut, but with a more even distribution of microbial mass appearing through development (Capone *et al.*, 2011). These are predominantly commensal organisms which compete for dominance within specialised human sites resulting in similarities in microbial flora in neighbouring sites and more diversity in genera between sites. For example, predominantly *Bacterioides* are found in the gut and *Streptococcus* within the oral cavity (Morgan, Segata & Huttenhower, 2013). In health, the interaction between microbes and host cells is balanced and relatively stable, described as being in a state of homeostasis. This homeostasis between host and microbiota can become disrupted causing the induction of disease, particularly through the use of oral broad spectrum antibiotics (Willing, Russell & Finlay, 2011). This is significant as disruption of the nasal microbiota may affect the host immune response, which has been shown to be modified by communication between innate host cells of the nasal mucosa and the host immune cells (Yeh *et al.*, 2013).

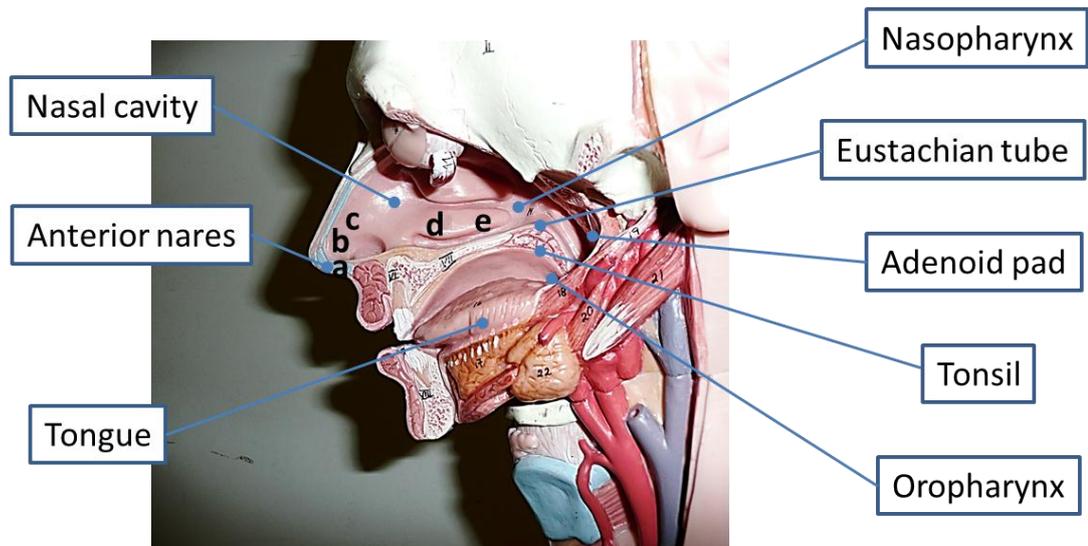


Figure 1.1 Lateral wall of human oronasal cavity.

Specific sites within the nasal cavity comprise of mucosa with epithelial types; a) skin at the nostril, b) squamous epithelium without microvilli, c) transitional epithelium with short microvilli, d) pseudostratified columnar epithelium with few ciliated cells, and e) pseudostratified columnar epithelium with many ciliated cells (Sahin-Yilmaz & Naclerio, 2011).

Earlier studies on the human microbiota used culture-dependent techniques to identify micro-organisms that could be isolated (Cartwright *et al.*, 1987). However, this could lead to both misidentification of species and false negatives due to the difficulty in culturing certain organisms. More recently in addition to culture-dependent techniques, culture-independent molecular techniques have been used to identifying organisms from samples (Hugenholtz, Goebel & Pace, 1998). This has been through the sequencing of specific genes, in particular the 16S ribosomal RNA gene (Patel, 2001; Peterson *et al.*, 2009), or most recently metagenomic studies involving sequencing of many genes from the original sample taken (Bogaert *et al.*, 2011; Belda-Ferre *et al.*, 2012).

Of particular interest to this study is the microbiota of the upper respiratory tract. Many species of bacteria have tropism for the human oronasal cavity, an ecological niche that is inhabited within the first few months of infancy (Leiberman *et al.*, 1999). *Streptococcus*, *Neisseria* and *Haemophilus* spp. are most predominant in the oro- and naso-pharynx in adults (Belda-Ferre *et al.*, 2012), whereas *Staphylococcus* spp. resides at the anterior nares (Figure 1.1) and the nasopharynx (Human Microbiome Project Consortium, 2012). In children under the age of 5 years *Moraxella* and *Haemophilus* spp are most abundant in the oro- and naso-pharynx, with *Streptococcus* and *Neisseria* spp being less abundant than in adults (Bogaert *et al.*, 2011).

Within these genus of bacteria, are the pathogenic species *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*, which are found as part of the microbiota of healthy carriers (asymptomatic) as well as carriers showing symptoms (symptomatic) of invasive disease (Cartwright *et al.*, 1987; Bogaert *et al.*, 2004). Once having colonised, any one of these species may migrate to, and cause localised inflammation in, the lung (pneumonia) or via the Eustachian tube (Figure 1.1) the middle ear (otitis media). Otitis media is predominantly caused by *S. pneumoniae* and *H. influenzae* (Revai, Mamidi & Chonmaitree, 2008). Additionally, the bacteria may move through the nasopharyngeal mucosa to cause invasive disease by entering the blood stream (bacteraemia), which may lead to systemic inflammation (sepsis). Once in the blood it may be able to invade the cerebral spinal fluid (CSF) and from there invade and cause inflammation in meningeal cells of the brain (meningitis). *S. pneumoniae*, *N. meningitidis* and *H. influenzae* have historically been the main causes of bacterial meningitis (Thigpen *et al.*, 2011).

1.2 Nasopharyngeal pathogens

1.2.1 *N. meningitidis*

The genus *Neisseria* is comprised mainly of Gram-negative diplococci and contains two species of human pathogens. *N. gonorrhoeae* is primarily found in the urogenital tract of humans and is the causative agent of the localised infection gonorrhoea. *N. meningitidis* (Nm; meningococcus) colonises the human upper respiratory tract, particularly the nasopharynx, and has been found to asymptotically colonise the oronasopharynx of approximately 10% of the population (Cartwright *et al.*, 1987), with the greatest carriage of between 25-40% occurring in older children and adults aged 15 to 25 years (Holten, Bratlid & Bøvre, 1978; Cartwright *et al.*, 1987; Christensen *et al.*, 2010). However, due to variations in sampling techniques this figure may be underestimated (Sim *et al.*, 2000). There is evidence that invasive meningococcal disease may occur approximately seven weeks post-colonisation in susceptible individuals (Neal *et al.*, 1999; Ala'Aldeen *et al.*, 2000), and that genetic factors in the host can increase susceptibility to invasive disease (Wright, Hibberd & Levin, 2009). Frequenting communal gatherings, cigarette smoking, intimate kissing and alcohol consumption can also influence acquisition of Nm (Imrey *et al.*, 1995, 1996; MacLennan *et al.*, 2006).

Nm is classified into 13 different serogroups (A, B, C, D, 29E, H, I, K, L, Y, W-135, X and Z) based on the immunological recognition of antigenic variations in the polysaccharide (PS) capsule that surrounds the bacterium. However, this classification has very recently been revised to exclude serogroup D, leaving 12 serogroups, with serogroups 29E and W-135 being renamed simply as E and W respectively (Harrison *et al.*, 2013). Of these, 6 serogroups (A, B, C, W, X, and Y) are associated with invasive disease (Rosenstein *et al.*, 2001). Further classification into serotypes is based on antibody recognition of the outer membrane protein (OMP) porin B (PorB), subserotypes by the OMP PorA and immunotypes by lipopolysaccharides (LPS) expressed by each strain or isolate (Frasch, Zollinger &

Poolman, 1985). In addition to encapsulated Nmen there are nongroupable strains that do not possess the *siaD* gene for expression of a PS capsule, which are only associated with carriage and not invasive disease (Claus *et al.*, 2002). For example the antigenic type designated for Nmen serogroup B (MenB) strain MC58 is B:15:P1.7,16:L3,8 detailing the serogroup: serotype: subserotype: immunotype of the strain (Tzeng & Stephens, 2000).

The presence of a PS capsule is associated with intracellular survival and therefore virulence (Spinosa *et al.*, 2007) although the expression of capsule is known to be switched on and off during carriage (Ala'Aldeen *et al.*, 2000). Single strains can acquire and switch capsular serogroups through genetic exchange (Lancellotti *et al.*, 2006; Beddek *et al.*, 2009) and lose the ability to synthesise capsule through genetic mutation (Weber *et al.*, 2006). More recently PCR techniques have been used to classify *Neisseria* spp. Multilocus enzyme electrophoresis (MLEE) that produces electrophoresis types (ETs) based on the mobility of enzymes through starch gels (Selander *et al.*, 1986), or multilocus sequence typing (MLST) of fragments of 7 neisserial housekeeping genes has been used to classify *Neisseria* spp. into sequence types (STs). Similar lineages are further classified into clonal complexes, each of which can include multiple serogroups (Maiden *et al.*, 1998, 2013). Through these techniques hypervirulent or hyperinvasive lineages were identified that were responsible for the majority of invasive disease, which in Europe were the ST-41-44, ST-11, ST-32, ST-8, and ST-269 complexes (Brehony, Jolley & Maiden, 2007).

The introduction of PS-conjugate vaccines to the major disease-causing serogroups, namely A, C, Y and W, has resulted in a large decline in these serogroups in the countries using vaccinations. For instance, ST-11 is predominantly associated with serogroup C Nmen (MenC), and an epidemic in the UK in the 1990s prompted the construction of a conjugate MenC vaccine (Ramsay *et al.*, 1997). After its introduction in the UK for those under the age of 19 years there was a 75% reduction in MenC carriage (Maiden *et al.*, 2008). In contrast, the capsular polysaccharide of MenB closely resembles polysialic acid in human neural cells,

and so concerns of inappropriate immune response to host antigens has restricted the use of MenB PS in vaccinations (Finne *et al.*, 1987). Despite medical interventions including antibiotic treatments, meningococcal disease still has a fatality rate of 10% (Pollard *et al.*, 2007) and since the introduction of the MenC conjugate vaccine in the UK, MenB has become the most prevalent serogroup associated with invasive disease (Maiden *et al.*, 2008).

MenB is also the serogroup most associated with epidemics worldwide and is the predominant serogroup for invasive meningococcal disease in Europe, North America and Australasia. Invasive disease from MenC is more prevalent in South America, and MenA accounts for over 90% of invasive meningococcal disease in Africa (Racloz & Luiz, 2010; Halperin *et al.*, 2012). The incidence of invasive meningococcal disease is approximately 1 case in 100,000 of the population worldwide, though 10-1000 times higher in areas of Africa known as the African meningitis belt (Halperin *et al.*, 2012). Due to the reluctance to use vaccines derived against MenB PS, many protein based vaccines have been developed (Sadarangani & Pollard, 2010). For example, a MenB outbreak in Cuba in the 1980s drove the development of a MenC polysaccharide-MenB outer membrane vesicle (OMV) based vaccine that resulted in broad coverage of sequence types and reduction in carriage of MenB in the population (Climent *et al.*, 2010). Similarly in the early 1990s in New Zealand there was an epidemic of ST-41/44 MenB that resulted in the development of a successful vaccine derived from the outer membrane proteins (OMPs) of that strain (Martin *et al.*, 1998; Oster *et al.*, 2007). However, due to the large genetic variation found particularly in the circulating MenB population there is consequently a difficulty in developing a successful broad range protein based vaccine (Racloz & Luiz, 2010).

MenB accounts for over 85% of meningococcal invasive disease in the UK (Ladhani *et al.*, 2012) and 88% in Europe (Halperin *et al.*, 2012). Recently, a vaccine (4CMenB) derived from highly conserved meningococcal surface antigens has been developed, comprising

recombinant forms of; factor H binding protein (fHbp), neisserial adhesin A (NadA), *Neisseria* heparin binding antigen (NHBA) and OMVs derived from the New Zealand MenB outbreak strain. The vaccine has shown strong immunological response in trials in adults (Toneatto *et al.*, 2011), adolescents (Santolaya *et al.*, 2012) and infants (Vesikari *et al.*, 2013) and has recently been approved for use in Europe.

1.2.2 *H. influenzae*

H. influenzae is a small Gram-negative bacterium, which is an obligate human coloniser. The unencapsulated form is termed non-typeable (NTHi) and is most commonly associated with chronic and acute otitis media in infants and sinusitis in adults (Brunton, 2006). The more virulent encapsulated forms, are categorised into six serotypes (a-f), which as is the case with the meningococcus are based on the antigenic properties of their capsular polysaccharides (PS) (Pittman, 1931). Most invasive *H. influenzae* strains are serotype b (Hib), which accounts for 8-13 million serious illnesses worldwide and 370,000 deaths in children less than 59 months (Watt *et al.*, 2009). After the introduction of Hib polysaccharide conjugate vaccines in the UK in 1992 there was a dramatic decline in the incidence of invasive Hib disease (Hargreaves *et al.*, 1996; Moxon *et al.*, 1999), which has been mirrored in other countries introducing Hib vaccines (Adegbola *et al.*, 1999).

The PS-conjugate Hib vaccine is considered to be highly successful and has achieved an approximately 90% reduction in cases of Hib infection in the UK (Ladhani *et al.*, 2009). However, since the introduction of the Hib conjugated vaccine in Brazil there has been a substantial shift to *H. Influenzae* type a (Hia) invasive disease (Ribeiro *et al.*, 2003, 2007). Unfortunately the Hib conjugate vaccine has failed in small numbers of vaccinated groups (McVernon, 2003; Ladhani *et al.*, 2010) and is occasionally unable to prevent invasive disease in premature babies, the elderly and those with immunodeficiency disorders (Foster *et al.*, 2009; Ladhani *et al.*, 2010). There has been shown to be a positive correlation

for co-colonisation by *H. influenzae* and *S. pneumoniae* in the nasopharynx demonstrated in rats (Margolis, Yates & Levin, 2010) and healthy infants (Chien *et al.*, 2012), whereas a negative correlation has been shown for co-colonisation by *H. influenzae* during *S. pneumoniae* infections (Pettigrew *et al.*, 2008). This could suggest colonisation by *H. influenzae* can protect against *S. pneumoniae* invasive disease which may involve an attenuation of inflammation at the mucosa during co-colonisation (Lijek & Weiser, 2012) and/ or recruitment of *S. pneumoniae* targeting neutrophils in the presence of *H. influenzae* (Lysenko *et al.*, 2005; Margolis, Yates & Levin, 2010).

1.2.3 *S. pneumoniae*

S. pneumoniae (pneumococcus) is a Gram-positive diplococcus that has host species and tissue tropism for the oronasopharynx of humans and other large mammals, with 91 different serotypes identified by antibody recognition of its polysaccharide capsule (Park *et al.*, 2007). *S. pneumoniae* is found in the nasopharynx of healthy individuals colonising around 70% of children (Auranen *et al.*, 2010), but is associated with disease in those under 5 years, peaking at around 12 months, (Lipsitch *et al.*, 2005). It is also common in the elderly (>60 years of age) and in immune compromised individuals and it is a leading cause of morbidity and mortality worldwide (Schranz, 2009). It has been estimated that over 800,000 infants under 5 years die from pneumococcal disease globally each year (Watt *et al.*, 2009).

Natural immunity against *S. pneumoniae* is believed to be directed against the major immunogenic cell-surface proteins such as pneumococcal surface protein A (PspA) (McCool *et al.*, 2002) and appears to be serotype and possibly strain specific. Naturally acquired IgG antibodies against *S. pneumoniae* proteins increase in age with infants, but do not protect against colonisation (Prevaes *et al.*, 2012). Maternal anti-pneumococcal protein IgG levels have also been found to not protect against colonisation of the infant, and may even

facilitate colonisation (Lebon *et al.*, 2011). Prior to the introduction of vaccination, pneumococcal infections primarily occurred in those aged under 2 and over 65 years of age, though the incidence of pneumococcal meningitis was most prevalent in those under 2 years old (Sleeman *et al.*, 2001). However, since the introduction of 7-valent pneumococcal vaccine (PCV7) in the United States in 2002 and the United Kingdom in 2006 as well as many other countries, there has been a decline in invasive pneumococcal disease (Schranz, 2009). Despite this, *S. pneumoniae* accounts for 9% of all deaths in the developing world (Myers & Gervais, 2007) and 11% of deaths in children worldwide (Watt *et al.*, 2009). Furthermore, there has been a global development of antibiotic resistance within pneumococcal isolates especially within virulence associated serotypes (Dowson *et al.*, 1989; Hsieh *et al.*, 2006; Ding *et al.*, 2009).

It has long been known that some pneumococcal isolates have natural competence, enabling transfer of genetic material facilitating capsular switching where one isolate acquires a new capsule type from another (Griffith, 1928). Serotype replacement has been observed in *S. pneumoniae* with reduction in the levels of vaccine type (VT) serotypes isolated, but an increase in disease from non-vaccine type (NVT) serotypes. Since the introduction of PCV7, serotype replacement from VT to NVT has been seen in carriage isolates from infants in the United States, (Huang *et al.*, 2005), Canada (Kellner *et al.*, 2008), and Europe (Brugger *et al.*, 2010). Whereas cases of invasive pneumococcal disease (IPD) have decreased in infants less than 5 years of age, incidences stayed largely unchanged in the elderly, even since the introduction of the 23-valent pneumococcal vaccine (PCV23) in Europe (Tyrrell *et al.*, 2009). There is a greater incidence of mixed isolates of *S. pneumoniae* in the young than in the elderly (Brugger *et al.*, 2010) suggesting a reservoir for *S. pneumoniae* in this age group. A more recent study demonstrated an overall reduction in *S. pneumoniae* carriage and invasive disease in both infants and the elderly despite a partial serotype replacement in the UK since the introduction of the PCV7 vaccine for those under 2 years of age in 2006 (Miller *et al.*, 2011) suggesting successful herd immunity.

Little change in overall pneumococcal carriage has been seen in infants in France since the introduction of PCV7 (Cohen *et al.*, 2012a), though an overall reduction has been seen since the introduction of PCV13 (Cohen *et al.*, 2012b). There has been an increase in NVT serotypes seen in cases of invasive pneumococcal disease (IPD) in Europe (Aguiar *et al.*, 2010; Miller *et al.*, 2011; Spijkerman *et al.*, 2012), Canada (Tyrrell *et al.*, 2009) and Asia (Ho *et al.*, 2011).

It has however been observed that the introduction of the PCV7 vaccine has had some unforeseen effects on the nasopharyngeal microbiota. Studies in the United States have demonstrated that post-PCV7, in cases of acute otitis media in infants there is a reduced carriage of VT isolates, and an increase in NVT isolates, with additionally an increase in non-typeable *H. influenzae* carriage in the nasopharynx and middle ear (Block *et al.*, 2004; Casey, Adlowitz & Pichichero, 2010). The nasopharyngeal colonisation of both infants and their parents shows an increase in both *H. influenzae* and *S. aureus* following the implementation of the PCV7 vaccine in the Netherlands (Spijkerman *et al.*, 2012). It has been suggested that colonisation by *S. pneumoniae* may give cross-reactive immune protection against *S. aureus* acquisition as shown in mice (Lijek *et al.*, 2012). *S. pneumoniae* may also inhibit the growth of *S. aureus* by production of hydrogen peroxide (Regev-Yochay *et al.*, 2006), though the protective effect of hydrogen peroxide production in an *in vivo* animal model contradicts this (Margolis, 2009). With the observations that *H. influenzae* may protect against invasive disease by *S. pneumoniae* (section 1.2.2) the polymicrobial interactions of these bacteria during commensal and pathogenic states may warrant further investigation.

1.2.4 *S. aureus*

S. aureus is a Gram-positive coccus identified as both coagulase and catalase positive, and capable of growth on high salt agar. It can also be sub-typed by plasmid restriction

endonuclease and immunotyping out of 11 recognised polysaccharide capsules (Karakawa *et al.*, 1985; Sompolinsky *et al.*, 1985). *S. aureus* was first discovered to be encapsulated in 1931 with strains M and Smith (Gilbert, 1931). These were later recognised as having serotype 1 and 2 capsule respectively, with the other serotypes often referred to as being microcapsular having only a thin capsule (Sompolinsky *et al.*, 1985). Serotypes 5 and 8 account for 16-26% and 55-63%, respectively, of isolates recovered from humans with 60-90% of invasive isolates expressing capsule (Sompolinsky *et al.*, 1985; Hochkeppel *et al.*, 1987; Sutter *et al.*, 2011). All invasive isolates in one study possessed either the *cap5* or *cap8* gene required for capsule production, but interestingly 82% of antibiotic resistant isolates did not express detectable levels of either capsule (Sutter *et al.*, 2011).

S. aureus colonises a range of animal hosts including humans, where it has tropism for the anterior nares (Figure 1.1) and skin with between 20 and 40% of people being nasal carriers (van Belkum *et al.*, 2009; Sakwinska *et al.*, 2010; Conlan, Kong & Segre, 2012). A more recent study reported only 5% of 11 month old infants were carriers but found similar levels (20%) to those previously reported in adults (Spijkerman *et al.*, 2012). Individuals are considered to be either carriers or non-carriers of *S. aureus* in the nasopharynx (van Belkum *et al.*, 2009).

Only a small number of cases of bacteraemia or meningitis in infants are caused by *S. aureus* (4%), but there is approximately 25% incidence of morbidity (Shane *et al.*, 2012). It has been demonstrated that *S. aureus* carriage is a risk factor for post-surgical infections (Lidwell *et al.*, 1966; Pignatari *et al.*, 1990) and is becoming difficult to control since the emergence of antibiotic resistant strains such as methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA) (Sievert *et al.*, 2008).

Since the introduction of Hib, pneumococcal and meningococcal vaccination programmes, *S. aureus* carriage has increased (Bogaert, De Groot & Hermans, 2004; Spijkerman *et al.*, 2012), which has primarily been associated with a decrease in pneumococcal carriage

(Regev-Yochay *et al.*, 2004; Spijkerman *et al.*, 2012). An increase in carriage would suggest that there may be an increase of invasive disease. Since the introduction of PCV7 in the US, *S. aureus* infections have increased by more than 60% and hospitalisations caused by MRSA have increased by more than 50% (Klein, Smith & Laxminarayan, 2007).

Following this increase in *S. aureus* infections since the 1960s, particularly in neonates, it has been suggested that *S. aureus* be the next target for vaccinations (Lee *et al.*, 2010). A serotype 5 and 8 conjugate vaccine (StaphVAX) was able to raise IgG antibody response to type 5 and 8 capsular polysaccharide, but did not affect nasopharyngeal carriage of capsule positive *S. aureus* in a phase I clinical trial (Creech *et al.*, 2010). It has been observed that naturally acquired IgG antibodies against *S. aureus* proteins does not protect against colonisation and antibody titre reduces with increased age in infants (Prevaes *et al.*, 2012). Currently there are no viable vaccine candidates available (Huda *et al.*, 2011). The polymicrobial interactions of these and other common inhabitants of the human nasopharynx have been cited as important in the maintenance of carriage without invasive disease, but such interactions are not thoroughly understood (Blaser & Falkow, 2009).

1.3 Host-pathogen interactions

1.3.1 Host immunity to bacterial capsular polysaccharide

In the absence of damage, the skin provides an effective protection against invasion by microbes in healthy individuals. The mucosa of the gastrointestinal and respiratory tracts however, require more direct contact with nutrients and gases and subsequently are more exposed to direct contact with microbes than other sites and therefore possible invasion. To compensate for this man has evolved other mechanisms of protection at the mucosa to provide a barrier to infection. Innate mechanisms of protection include the epithelial barrier itself as well as secretion from the epithelial barrier of a heavily glycosylated family

of proteins called mucins that help to make up the gel-like substance mucous. Mucous along with serous fluid makes up saliva. Saliva also contains secreted anti-microbial compounds such as lysozyme (Feiner, Meyer & Steinberg, 1946), defensins (Zasloff, 1992), and exogenous vesicles called exosomes (Kesimer *et al.*, 2009), amongst others.

In addition to the innate immune mechanisms, B cells that differentiate to mature plasma cells are generated in response to predominantly exogenous antigens such as pathogen associated molecular patterns (PAMPs) to produce immunoglobulin (Ig) molecules that recognise these antigens and facilitate a targeted response by immune cells. The primary classes of Ig molecules at the mucosa are IgA, and to a far lesser extent IgG and IgM (Brandtzaeg, Fjellanger & Gjeruldsen, 1970; Nagura *et al.*, 1979), whereas IgG is most predominant in the blood (Sirisinha & Charupatana, 1970). IgG is a monomeric molecule, as is IgA, though IgA is found primarily as a dimeric molecule at the mucosa (secretory IgA; sIgA), where two IgA molecules are connected by a j-chain (Wilson & Williams, 1969). Both are produced against specific antigens whereas IgM is a pentameric molecule with greater avidity to antigens, but less specificity than either IgA or IgG.

Due to it being monomeric and therefore having lower mass, IgG can diffuse through the mucosal epithelium (Natvig *et al.*, 1997). In contrast secretory IgA and IgM are actively transported through the epithelium by secretory epithelial cells, explaining the higher quantities of these molecules in the saliva (Brandtzaeg, Fjellanger & Gjeruldsen, 1970; Bouladoux *et al.*, 2012). IgG is produced by B cells largely found systemically in the blood or spleen (Sirisinha & Charupatana, 1970), whereas IgA is predominantly generated locally by B cells in the nasopharyngeal associated lymphoid tissue (NALT; Ostergaard, 1977), being the tonsils and adenoids (Figure 1.1). Due to their localities induction of IgA may have greater protection against colonisation compared to induction of IgG, which may have greater protection against systemic infection.

Bacteria have evolved many mechanisms and structures to adapt to the environment that they occupy. It has been argued that many of these adaptations are to resist attack by bacteriophage (Sompolinsky *et al.*, 1985; Labrie, Samson & Moineau, 2010) such as phase variation of surface proteins (Alexander, Richardson & Stojiljkovic, 2004; Zaleski, Wojciechowski & Piekarowicz, 2005) and restriction systems to protect against the uptake of exogenous DNA (Vovis & Lacks, 1977). The bacterial polysaccharide (PS) capsule may also have evolved to confer resistance to bacteriophage, as has been demonstrated in *Escherichia coli* (Scholl, Adhya & Merrill, 2005) in addition to offering protection from desiccation after entering host cells (Crook *et al.*, 1989; Spinosa *et al.*, 2007).

The presence of the PS capsule does not confer an advantage during mucosal association or invasion (Read *et al.*, 1992) and expression is even down-regulated during mucosal colonisation (Deghmane *et al.*, 2002), but it confers bacterial survival during invasive disease and systemic dissemination by shrouding otherwise exposed surface proteins and resisting cellular digestion (Crook *et al.*, 1989; Virji *et al.*, 1992b; Spinosa *et al.*, 2007). It has even been postulated that *N. meningitidis* may utilise its ability to withstand phagocytic digestion by hijacking neutrophils to migrate to the cerebrospinal fluid (Criss & Seifert, 2012).

The PS capsules of *H. influenzae*, *S. pneumoniae* and *N. meningitidis* despite having varied molecular structures are alone poorly immunogenic in infants and do not induce generation of a significant antibody response (Monto, Brandt & Artenstein, 1973; MacDonald *et al.*, 1998). The former may be due to the B cell subsets that generate anti-polysaccharide antibodies not being fully developed in neonates and young infants (Mosier, Mond & Goldings, 1977). The latter may be due to PS inducing only a T cell-independent type 2 (TI-2) activation of B cells that results in the mitogenic and rapid induction of cross-reactive IgM (Kehrl & Fauci, 1983), but less induction of the more specific IgG (Barrett & Ayoub, 1986). It has been recently suggested that neutrophils may also play a role in TI-2 activation

of B cells and class switching of IgM producing B cells to IgG producing cells (Puga *et al.*, 2012).

As a consequence, many PS vaccines incorporate an immunogenic carrier protein, such as tetanus toxoid, diphtheria toxoid, CRM197 mutant diphtheria toxoid or the outer membrane protein complex of *N. meningitidis* (Nmen OMP) conjugated with the capsular PS. Such conjugate vaccines have been developed for several serogroups of *N. meningitidis*, several serotypes of *S. pneumoniae* and Hib, and induce a T cell-dependent immune response that primes the immune memory to future infections by the induction of IgG antibodies from memory B cells (Kurikka, 1996; MacDonald *et al.*, 1998; MacLennan *et al.*, 2000). However, it has been observed that the generated memory B cells are dispersed systemically and only transiently found at the mucosa (Clarke *et al.*, 2012) suggesting greater protection against invasion than colonisation. Older children and adults with immune compromising conditions can still be at risk of invasive disease from these pathogens, as are the elderly, due to a waning ability to produce long term antibody responses (Baxendale *et al.*, 2010b, 2010a) and an increase in regulatory cytokine IL10 producing activated B cells (Bancos & Phipps, 2010).

1.3.2 Toll-like receptors

The first line of defence from invasive microorganisms is not the long-term memory of acquired immunity, but the recognition of specific bacterial molecules by the innate immune system (Kawai & Akira, 2010). The epithelial mucosal barriers of the upper respiratory and gastrointestinal systems are the first point of contact in the innate immune system for mucosal colonisers. All cells in the human body possess pattern-recognition receptors (PRRs) that have evolved to specifically recognise conserved components of micro-organisms. These bacterial ligands are known as pathogen-associated molecular patterns (PAMPs) or commensal-associated molecular patterns (CAMPs). Examples of these

bacterial ligands include lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, and lipoteichoic acid (LTA) found in the cell wall of Gram-positive bacteria (Takeuchi & Akira, 2001; Kawai & Akira, 2010).

The primary bacterial receptors of most host cells are Toll-like receptors (TLRs), of which 10 functional forms have been found in humans (Kawai and Akira, 2010). Mice have 12 functional TLRs, TLRs 1-13 where TLR10 is non-functional, and have been used as models to identify which bacterial ligand is associated with each host cell TLR and determine the subsequent immune reaction (Kawai & Akira, 2010).

TLRs are transmembrane receptor molecules found at the host cell surface as well as within intracellular compartments such as endosomes and lysosomes (Kawai & Akira, 2010). TLR activation leads to homo or hetero dimerization of the receptor and downstream activation of intracellular signalling molecules, such as, MyD88, TRIF, and TRAM, as well as activation of the MAPK pathways (Peroval *et al.*, 2013). Such signalling leads to activation and/or suppression of transcription factors such as IRF3 and NFκB that control the expression of genes for inflammatory cytokines/chemokines and many other host cell responses (Figure 1.2).

Using over-expression of specific TLR's in a cell line, cell membrane expressed TLR4 has been shown to recognise LPS from Gram-negative bacteria such as MenB and Hib (Mogensen *et al.*, 2006a), and in some studies the active pneumolysin from Gram-positive *S. pneumoniae* (Beisswenger, Lysenko & Weiser, 2009). The TLR4 homodimer associates with the co-receptor CD14, and the accessory molecules LPS-binding protein (LBP) and MD-2 for LPS-induced signalling (Elson *et al.*, 2007). LPS-induced activation of airway epithelial cells is dependent on CD14 which is mostly found soluble within the serum (Schulz *et al.*, 2002).

The cell membrane expressed TLR2 forms heterodimers with either TLR1 or TLR6 before interaction with ligands (Triantafilou *et al.*, 2006). It has been shown to recognise PAMPs from all four pathogens evaluated in this study (Mogensen *et al.*, 2006b; Schmalzer *et al.*, 2009) and is the primary PRR for *S. pneumoniae*-stimulated cytokine responses (Beisswenger, Lysenko & Weiser, 2009). However, it is important to note that Gram-positive bacteria also signal through the 23S ribosomal RNA (rRNA) receptor TLR13 in mice models (Oldenburg *et al.*, 2012; Hidmark, von Saint Paul & Dalpke, 2012). Following interaction with ligands, TLR2 may further associate with co-receptors CD14 or CD36 which enhance the ligand-induced inflammatory response (Triantafilou *et al.*, 2006). Once activated, TLR2 receptors co-localise forming clusters which then internalise in endosomal vesicles (Triantafilou *et al.*, 2006).

In contrast, TLR3 forms a homodimer and is expressed on endosomes within the host cell cytoplasm that recognises double stranded ribonucleic acid (dsRNA) to induce inflammation (Alexopoulou *et al.*, 2001). Exposing mice to viral or synthetic dsRNA induced host cell death in intestinal epithelial cells (Zhou *et al.*, 2007). Apoptosis has been shown to be induced by RNA released from neighbouring necrotic host cells interacting with TLR3 (Lai *et al.*, 2010). When induced via TLR3 activation, apoptosis is dependent on signalling through TRIF, NF κ B, the extrinsic initiator caspase 8 and the effector caspase 3 (Salaun *et al.*, 2006). As well as inducing apoptosis, a TLR3 agonist induced the secretion of proinflammatory cytokines IL6 and IL1 α , and the chemokine IL8 in nasopharyngeal epithelial cells (Matijevic, Marjanovic & Pavelic, 2009). IL6 induced by a TLR3 agonist can be inhibited by activation of TIRAP (also called MAL, Figure 1.2) (Kenny *et al.*, 2009), which may explain the inhibition of TLR3 induced inflammation by *Staphylococcus epidermidis* LTA interaction with TLR2 (Lai *et al.*, 2010).

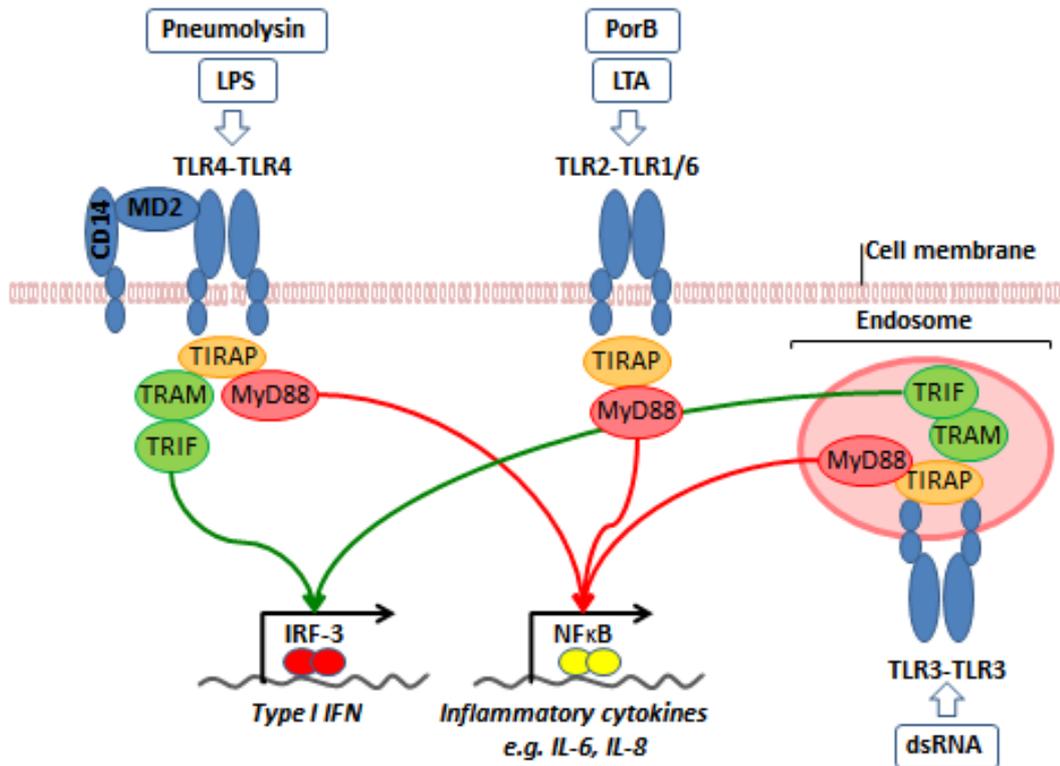


Figure 1.2 Toll-like receptor (TLR) signalling and targeted inflammatory response

The TLR4 homodimer primarily recognises LPS from the outer cell membrane of Gram-negative bacteria, but has also been shown to recognise secreted pneumolysin from *S. pneumoniae*. TLR2/6 and TLR2/1 heterodimers recognise the cell wall component LTA from Gram-positive bacteria, as well as other ligands such as the membrane protein porin B (PorB) of pathogenic and commensal *Neisseria* (Massari *et al.*, 2006; Toussi *et al.*, 2012). The encircled molecules are TLR-associated intracellular signalling molecules that ultimately lead to inflammatory cytokine induction by transcription factors IRF3 and NFκB. TLR signalling can be MyD88-dependent or independent depending on the receptor and agonist (Kawai & Akira, 2010).

1.3.3 Mitogen-activated protein kinases

The addition of phosphate groups to cell signalling proteins (phosphorylation) is a mechanism of ‘switching on’ cell signalling pathways by protein kinases for recognition by phospho-binding proteins (Jin & Pawson, 2012). Within the host, mitogen-activated protein kinases (MAPKs) are a family of such serine/threonine-specific phosphorylating proteins involved in intracellular signalling from multiple extracellular signals. These include

cytokines, hormones, growth factors and bacterial ligands, which participate in cell functions such as induction (Johansen *et al.*, 2006; Lee *et al.*, 2011) and inhibition (Chen *et al.*, 2003; Yang *et al.*, 2011) of inflammation, apoptosis and internalisation of bacteria (Griffiss *et al.*, 1999; Shinzawa *et al.*, 2009). MAPKs are sub-divided into three main families named after the final kinase in the signalling pathway; extracellular signal-regulated kinases (ERK^{MAPK}) 1 and 2, c-Jun N-terminal kinases (JNK^{MAPK}) 1, 2 and 3, and p38 proteins (p38^{MAPK}) α , β , γ and δ (Cano *et al.*, 1995; Gupta *et al.*, 1996; Davis, 2000).

N. meningitidis was found to activate p38^{MAPK} in endothelial cells independent of surface expression of the bacterial opacity protein Opc. Expression of Opc activated JNK^{MAPK} which was found to be important in invasion of endothelial cells by *N. meningitidis* and was inhibited by blocking the host cell-cell adhesion, integrin receptor on endothelial cells (Sokolova *et al.*, 2004). Neisserial PorB has been demonstrated to signal through TLR2/1 and subsequently induce inflammation via ERK^{MAPK} (Toussi *et al.*, 2012). Pneumolysin from *S. pneumoniae* was shown to induce inflammation through ERK^{MAPK}, JNK^{MAPK} and p38^{MAPK} in a TLR4-dependent manner in nasopharyngeal epithelial cells (Dogan *et al.*, 2011), and *H. influenzae* induces over-production of mucins in epithelial cells by up-regulating p38^{MAPK} (Komatsu *et al.*, 2008).

1.4 Peroxisome proliferator activated receptors

Peroxisome proliferator activated receptors (PPARs) belong to the superfamily of nuclear receptors (NRs) involved in the transcriptional regulation of metabolism, growth, differentiation, inflammation and apoptosis in many different cell types. PPARs largely only found in the nucleus, bound to nuclear receptor response elements in the promoter regions of their target genes (Guan *et al.*, 2005). The PPARs are maintained in a repressed state by nuclear receptor co-repressors until ligand binding, when the co-repressor protein complex is disassociated and co-activators are recruited (Guan *et al.*, 2005).

There are three different subtypes of PPARs in humans, namely PPAR α , PPAR β/δ and PPAR γ . Each PPAR forms an obligate heterodimer with the retinoid-X-receptor (RXR) that allows cytoplasmic to nuclear shuttling, and each PPAR can interact with different ligands and target genes containing peroxisome proliferator response elements (PPREs) producing different biological responses (Braissant & Wahli, 1998).

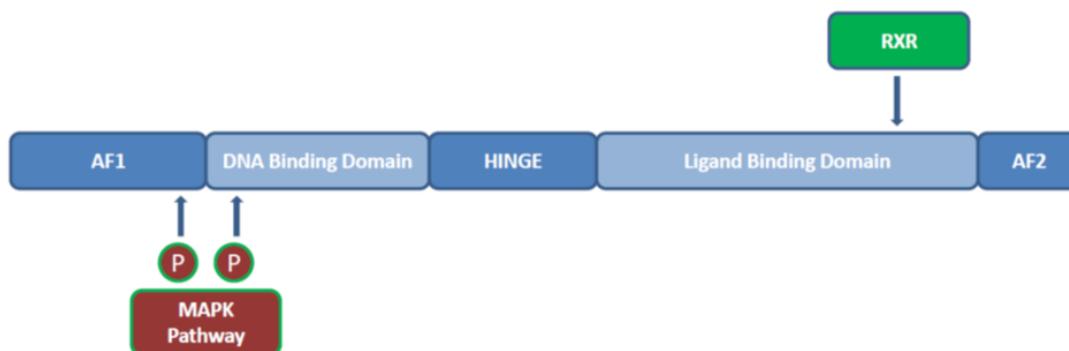


Figure 1.3 Structure of PPAR γ and its functional domains

PPARs consist of an N-terminal region with a transactivation domain, activation function 1 (AF1), with a highly conserved DNA-binding domain (DBD) of two zinc fingers at this N-terminal region. A short hinge region connects this region to a ligand-binding domain (LBD) at the C-terminal region, with an activation function 2 (AF2) domain at the C-terminus of the LBD. While the AF1 functions in a ligand-independent fashion the AF2 domain is generally ligand-dependent (Desvergne & Wahli, 1999).

PPAR γ is expressed in a wide range of cell types including colon (Fajas *et al.*, 1997; Patel *et al.*, 2005), lung (Dubuquoy *et al.*, 2003) and nasal (Ogasawara *et al.*, 2010) epithelial cells. PPAR γ agonists are known to reduce inflammation in monocytes and macrophages by suppressing the production of inflammatory cytokines (Ricote *et al.*, 1998; Jiang, Ting & Seed, 1998). PPAR γ can be activated by both agonistic ligand binding and phosphorylation (Figure 1.3), which increases ligand-independent transcriptional activity (Diradourian, Girard & Pégrier, 2005). As well as its role as a transcription factor, ligand-dependent activation of PPAR γ can also act as a transcriptional repressor, particularly of inflammatory

gene expression of IL-6 and TNF α in monocytes (Jiang, Ting & Seed, 1998), by antagonising pro-inflammatory transcription factors such as AP-1, STAT and NF κ B (Ricote *et al.*, 1998).

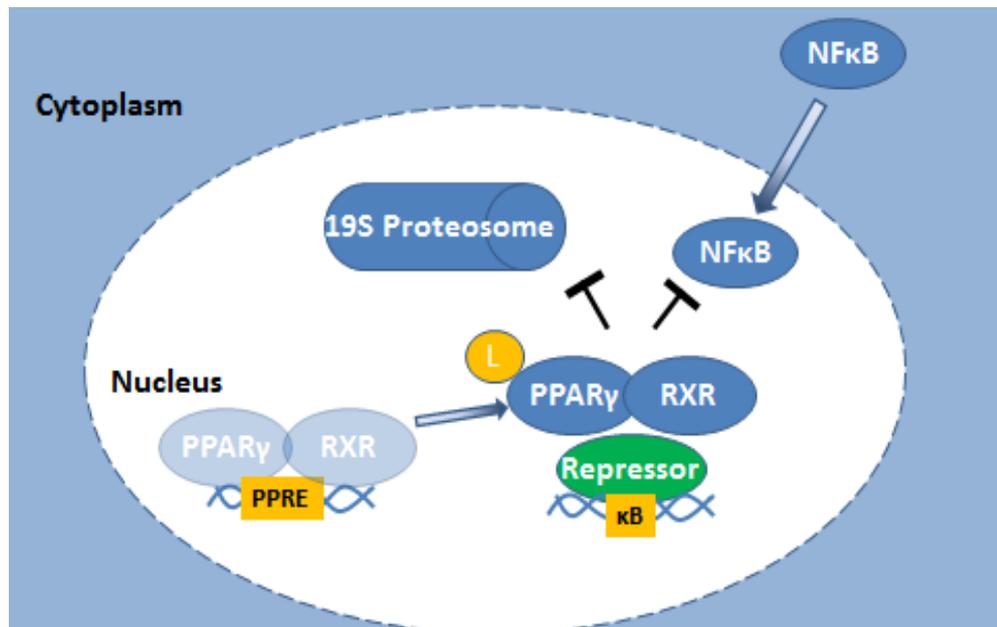


Figure 1.4 **Repression of NF κ B-dependent gene transcription by PPAR γ**

On ligand (L) activation, the ligand-dependent sumoylation of PPAR γ leads to PPAR γ binding to DNA-bound repressor complexes of PPAR γ -independent genes, lacking PPRE sequences. This prevents 19S proteasome degradation of the repressor complexes associated with these genes and continued repression of gene transcription in the presence of activated inflammatory transcription factors such as NF κ B (Pascual *et al.*, 2005).

PPAR γ can also act as a transcription repressor of genes with non-PPRE binding elements (Figure 1.4). PPAR γ is a phosphoprotein that can be phosphorylated by activators of the MAPK pathway, such as ERK^{MAPK} and JNK^{MAPK} (Hu *et al.*, 1996; Camp, Tafuri & Leff, 1999; Adams *et al.*, 1997). This modification reduces the ligand-dependent transcriptional activity of PPAR γ (Adams *et al.*, 1997). In colon epithelial cells a PPAR γ agonist induced transient phosphorylation of PPAR γ through the MAPK pathway. Following this PPAR γ reduced PPRE-dependent transcriptional activity, whilst increasing PPAR γ -NF κ B interaction thus decreasing transcriptional activity at κ B transcription sites (Chen *et al.*, 2003). The effects of PPAR γ on related pathways are also coming to light. Using PPAR γ agonists increases

phosphorylation of protein kinase C (PKC) in human nasal epithelial cells (HNECs), and the expression of tight junction associated molecules. This was then prevented by both PPAR γ and PKC inhibitors (Ogasawara *et al.*, 2010).

1.4.1 PPAR γ and bacterial interactions

It has been demonstrated that colon epithelial cells of patients with ulcerative colitis have impaired expression of PPAR γ (Dubuquoy *et al.*, 2003). Inducing PPAR γ was shown to be dependent on TLR4 signalling by bacterial LPS and partially dependent on I κ B kinase- β (IKK β) (Dubuquoy *et al.*, 2003). IKK β phosphorylates the inhibitory I κ B to release and activate NF κ B, suggesting that colonic flora may utilise PPAR γ to inhibit activation of NF κ B.

PPAR γ also protects mice against experimental colitis and inhibits the induction of IL6, TNF α and IL1 β expression in colon epithelial cells (Adachi *et al.*, 2006). Inducing PPAR γ activity reduces both inflammation and apoptosis induced by *Helicobacter pylori* LPS in the gastric mucosa of rats by inhibition of genes transactivated by NF κ B (Slomiany & Slomiany, 2002). The suppression of NF κ B by direct association with PPAR γ has also been demonstrated in colon epithelial cells exposed to *Bacteriodes thetaiotaomicron*, a prevalent anaerobe of the human intestine (Kelly *et al.*, 2004). *N. lactamica* has been shown to suppress inflammation induced by *N. meningitidis*, PAM3Cys (TLR2 agonist) and inflammatory cytokine IL1 β , utilising PPAR γ as shown by a PPAR γ antagonist (Tezera *et al.*, 2011). This illustrates that some commensal bacteria may utilise PPAR γ to inhibit the host inflammatory response induced by pro-inflammatory stimuli.

1.4.2 Other host-microbe interactions

In addition to evolved mechanisms of host cells to recognise and initiate responses to non-host molecular patterns, bacteria have evolved multiple mechanisms of interacting with

host cells. Neisseriae possess type IV pili that are also found on a variety of both Gram-positive and Gram-negative bacteria (Pelicic, 2008). Type IV pili are bacterial transmembrane structures that in neisseriae are predominantly comprised of pilin (or PilE) subunits, that form long filaments that bundle in threes to form spiralling tri-helical fibres (Hélaine *et al.*, 2005; Craig *et al.*, 2006). Pilin fibres associate with host epithelial and endothelial cells (Virji *et al.*, 1992a), as do pilin associated PilC proteins (Kirchner & Meyer, 2005).

Bacterial pili are multifunctional structures that allow twitching motility (Merz, So & Sheetz, 2000), walking and crawling on solid surfaces (Gibiansky *et al.*, 2010), uptake of exogenous DNA (Long *et al.*, 2003) and bacterial aggregation (Hélaine *et al.*, 2005). They form interactions with host cells such as exerting retractile force to maintain association (Mikaty *et al.*, 2009; Biais *et al.*, 2010) and signalling through host receptors using retractile tension (Brissac *et al.*, 2012).

Neisserial type IV pili have been demonstrated to enhance meningococcal interaction with the mucosal epithelium, especially at sites of mucosal damage that can be induced by the pathogen (Virji *et al.*, 1992a; Rayner *et al.*, 1995). The pili of pathogenic neisseriae are required for cortical plaque formation that facilitates invasion (Merz, Enns & So, 1999; Morand *et al.*, 2009) and may interact with the host receptor CD46 (Källström *et al.*, 1997, 1998; Sjölander & Jonsson, 2007). However, another unidentified receptor has been suggested (Kirchner & Meyer, 2005), which may be the host integrin receptors which were found to co-immunoprecipitate with *N. gonorrhoeae* pili (Edwards & Apicella, 2005).

Phosphorylcholine (ChoP or PCho) is a component of platelet-activating factor (PAF) which is the natural ligand for the receptor of PAF (rPAF) in humans. ChoP was first found to be incorporated into the cell-wall teichoic acid and lipoteichoic acid (LTA) of *S. pneumoniae* (Mosser & Tomasz, 1970). It has since been found to be incorporated in the lipopolysaccharide (LPS) of other human nasopharyngeal colonisers *H. influenzae* (Kolberg,

Høiby & Jantzen, 1997) and commensal *Neisseria* including *N. lactamica*, and as a modification to pili on pathogenic *Neisseria* (Serino & Virji, 2000). The incorporation of ChoP has been shown to enhance adhesion and invasion of *H. influenzae* and *N. lactamica* to airway epithelial cells, but increases complement and C-reactive protein mediated killing in the serum, suggesting an adaptation for typically commensal colonisers (Weiser *et al.*, 1998; Serino & Virji, 2002).

Carcinoembryonic antigen-related cell adhesion molecules (CEACAM) are host receptors, expressed on a wide range of epithelial cells, that are utilised by many Gram-negative nasopharyngeal colonising bacteria for association with the host (Voges *et al.*, 2010). In particular *Neisseria* spp. expressing the opacity protein Opa such as *N. meningitidis* (Virji *et al.*, 1996) and *N. lactamica* (Toleman, Aho & Virji, 2001) or *H. influenzae* expressing the P5 protein (Hill *et al.*, 2001) have a greater association with CEACAM. Exposure to the proinflammatory cytokine interferon gamma (IFN γ) can increase NF κ B activity, which in turn, upregulates CEACAM1 expression and *N. meningitidis* adhesion and invasion of epithelial cells (Griffiths *et al.*, 2007). However, the purified Opa from *N. meningitidis* was found to inhibit inflammation induced through TLR2 when expressed by *E. coli* (Slevogt *et al.*, 2008). Recently it has been suggested that *N. meningitidis* and therefore possibly other *Neisseria* spp. possess another means of interacting with CEACAM1 other than Opa (Kuespert, Roth & Hauck, 2011).

The neisserial opacity protein Opc allows association of pathogenic neisseriae to primarily endothelial cells, but also epithelial cells in a serum-dependent manner, allowing interaction with host integrins using host serum proteins vitronectin and fibronectin (Virji *et al.*, 1995b; Unkmeir *et al.*, 2002). Additionally the neisserial minor adhesin NadA also adheres to host β 1 integrins (Nägele *et al.*, 2011). *S. aureus* is also known to utilise host fibronectin when expressing fibronectin-binding protein (FnBP) to adhere and invade host cells via α 5 β 1 integrin (Agerer *et al.*, 2003), and *S. pneumoniae* is known to utilise

vitronectin to engage $\alpha 5\beta 3$ integrin of epithelial and endothelial cells for the same purpose (Bergmann *et al.*, 2009). Internalisation of bacteria by this mechanism is dependent on integrin-linked kinase (ILK) activation (Bergmann *et al.*, 2009), which is also upregulated in endothelial cells when challenged with Nmen (Schubert-Unkmeir *et al.*, 2007) and promotes an inflammatory response (Assi *et al.*, 2011).

1.5 Pathogen-induced host-cell death

Pathogenic bacteria have been shown to have greater association with damaged sites within the epithelium (Read *et al.*, 1992; Rayner *et al.*, 1995). Induction of host-cell death at the mucosal epithelium is a proposed mechanism of bacterial invasion and ultimately dissemination within the host (Gao & Abu Kwaik, 2000; Kim *et al.*, 2010). Indeed *N. meningitidis* has been observed to cause greater loss of host cell viability than commensal *N. lactamica* in both endothelial and meningioma cells (Fowler *et al.*, 2006; Slanina *et al.*, 2011).

Necrosis is associated with host disease, predominantly resulting from chemical or mechanical disturbance and is recognised by swelling (oncosis), and lysis of, host cells (Kroemer *et al.*, 2009). The presence of necrotic cells can induce inflammation in neighbouring epithelial cells and immune cells (Lai *et al.*, 2009). The release of danger-associated molecular patterns (DAMPs) by necrotic host cells stimulates macrophages and dendritic cells that can in turn activate immune cells (Chen *et al.*, 2007). Although often considered an uncontrolled process, necrosis has recently been considered an orchestrated response by the host (Festjens, Vanden Berghe & Vandenabeele, 2006).

1.5.1 Apoptosis and the caspase cascade

Apoptosis is the organised process of self-initiated host-cell death that can regulate homeostasis within tissues and mucosal sites (Kerr, Wyllie & Currie, 1972). It is characterised by rounding up of the cell, reduced cell volume (pyknosis), nuclear fragmentation (karyorrhexis), and plasma membrane blebbing (Kroemer *et al.*, 2009). It is primarily induced by a family of cysteine proteases called caspases (Alnemri *et al.*, 1996). Caspases are found in the cell in inactive (pro-) forms consisting of a large and small subunit connected by a linker region with a connected pro-domain. Proteolytic cleavage of the pro-domain and the linker region results in an active caspase comprised of the large and small subunits (Nuñez *et al.*, 1998). Caspases have been divided into upstream or initiator caspases (caspases 2, 8, 9 and 10), and downstream or effector caspases (caspases 3, 6 and 7), depending on their position in the cascade (Nuñez *et al.*, 1998). Initiator caspases are specific, proteolytic activators of effector caspases. These in turn cleave host cell structural molecules such as actin to form cell blebs called apoptotic bodies (Communal *et al.*, 2002), as well as activating a range of further apoptotic molecules such as pro-poly(ADP-ribose) polymerase (PARP) to active PARP1 initiating DNA fragmentation (Williams, Little & Shipley, 1974; Tewari *et al.*, 1995). There are two main apoptotic pathways, the extrinsic and the intrinsic pathways (Figure 1.5).

The pore forming proteins (porins), pneumolysin from *S. pneumoniae* and PorB from *N. gonorrhoeae* induce apoptosis in host cells by translocating to, and causing damage to, host-cell mitochondria (Müller *et al.*, 1999; Braun *et al.*, 2007). Induction of apoptosis by *N. gonorrhoeae* could be suppressed by increasing Bcl2 (Müller *et al.*, 2000) an inhibitory protein of the intrinsic apoptotic pathway. *S. pneumoniae*-induced apoptosis was via activation of caspase 9, although this was induced through the MAPK pathways p38^{MAPK} and JNK^{MAPK} (N'Guessan *et al.*, 2005). Though conversely the PorB of Nmen has been shown to translocate to mitochondria, interact with voltage-dependent ionic channel (VDAC) of

mitochondria, and inhibit the intrinsic induction of apoptosis and subsequent induction of caspase 9 (Massari, Ho & Wetzler, 2000; Massari *et al.*, 2003).

Caspases 1, 4, 5, 11 and 12 are not involved with apoptosis, but rather the transduction of innate inflammation (Taylor, Cullen & Martin, 2008). Of these the best understood pathway involves caspase 1 activation that cleaves the pro-IL1 β and pro-IL18 cytokines to their active forms (Figure 1.5). These are then secreted from the host cell (Wilson *et al.*, 1994). This is followed by the activation of inflammasomes such as nucleotide-binding oligomerization domain-like receptor (NLR), pyrin domain-containing 3 (NLRP3) following stimulation by multiple TLRs including TLR4, 2 and 3 (He, Franchi & Núñez, 2013). *N. gonorrhoeae* has been shown to activate NLRP3 in macrophages (Duncan *et al.*, 2009) and pneumolysin expressing *S. pneumoniae* induce inflammation via caspase 1 and NLRP3 (Fang *et al.*, 2011). Additionally, stimulation of TLR4 by Gram-negative bacteria activates caspase 11-mediated inflammation via NLRP3 and caspase 1 activation during sepsis (Rathinam *et al.*, 2012).

In contrast to the induction of apoptosis, some intracellular pathogens protect the host to maintain the environment. For example, *Shigella flexneri* prevents the induction of intrinsic apoptosis in epithelial cells by preventing cytochrome c release and subsequent activation of caspase 9 (Clark & Maurelli, 2007). *Chlamydia trachomatis* and *C. pneumoniae* also inhibit apoptosis in host cells by preventing activation of BAX and BAK (Fischer *et al.*, 2004).

Recently, a new classification of host-cell death induced by invasive pathogens, pyroptosis, has been defined (Fink & Cookson, 2005). It combines the morphological features of necrosis; cell swelling, cell lysis and inflammation, with programmed cell death and crucially activation of caspase 1 and subsequently IL1 β (Fink & Cookson, 2005). It has been suggested to be a host survival mechanism to defend against intracellular bacteria, that has only been demonstrated in innate immune cells; macrophages, neutrophils (Fink & Cookson, 2006; Miao *et al.*, 2010) and in skin keratinocytes activated through TLR3 (Lian *et al.*, 2012).

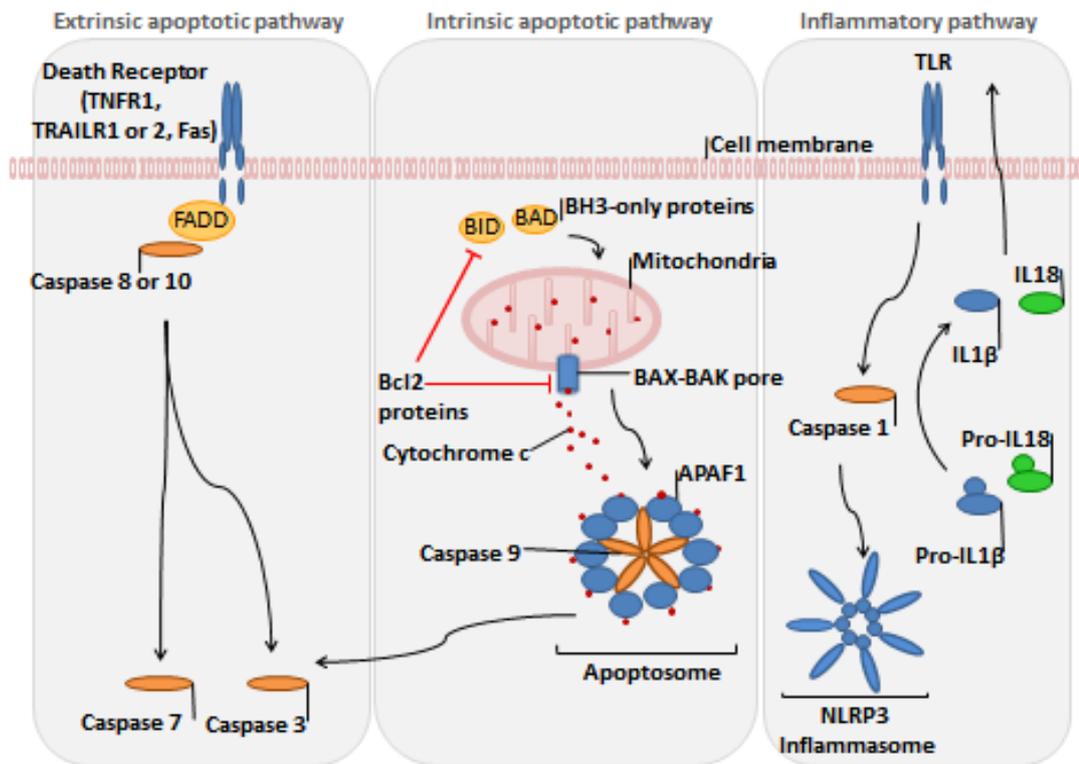


Figure 1.5 The caspase cascade pathways.

There are two canonical pathways that can each lead to induction of apoptosis in host cells. The extrinsic pathway is induced by extracellular signalling molecules interacting with host cell surface receptors, such as the death receptors, Fas (Itoh *et al.*, 1991; Bennett *et al.*, 1999), TNFR1 (Mangan, Mergenhagen & Wahl, 1993) and TRAIL (Oshima *et al.*, 2001) receptors. The extrinsic pathway is predominantly associated with activation of the initiator caspase 8, which mediates downstream activation of effector caspases such as caspase 3 (Van Herreweghe *et al.*, 2010; Neumann *et al.*, 2012). The intrinsic pathway involves intracellular activation of the BH3-only family of proteins, which inhibit the anti-apoptotic B cell CLL/lymphoma 2 (Bcl2) protein family. Inhibition of Bcl2 allows activation of Bcl2 homologous antagonist/killer protein (BAK) and Bcl2-associated X protein (BAX) to form pores within mitochondrial membranes allowing the release of intra-mitochondrial molecules, such as cytochrome c (Yin, Oltvai & Korsmeyer, 1994; Kluck *et al.*, 1997; Fletcher *et al.*, 2008). The Bcl2 family of proteins can inhibit intrinsic apoptosis by forming heterodimers with BAX, BAK or BH3-only proteins (Meijerink *et al.*, 1995). Cytosolic cytochrome c forms a complex (apoptosome) with APAF1 and pro-caspase 9 which is activated through this formation and initiates downstream activation of caspases 3 and 7 (Jiang & Wang, 2000). In addition to apoptosis innate inflammation can be induced via Toll-like receptor (TLR) activation of pro-caspase 1 that in turn activates the cytokines pro-IL1 β and pro-IL18 (Wilson *et al.*, 1994). Activation of caspase 1 leads to activation of the NLRP3 inflammasome and downstream NF κ B signalling (Bauernfeind *et al.*, 2009).

1.6 Commensal organisms

Probiotics are live microorganisms acting beneficially towards the host's health. They influence the stability of the surrounding microflora, inhibit colonisation by pathogens, influence the mucosal barrier and stimulate both adaptive and innate components of the immune system. The majority of research on probiotics has concentrated on commensal bacteria of the gastrointestinal (GI) tract and oropharynx. Several GI commensal bacteria have been identified that can attenuate pro-inflammatory responses in the intestinal epithelium (Kelly *et al.*, 2004), including non-pathogenic enteric *Salmonella* spp. (Figure 1.6; Neish *et al.*, 2000) and *Bacteriodes thetaiotaomicron* (Figure 1.6; Kelly *et al.*, 2004). As well as several strains and species of *Lactobacillus* and *Bifidobacterium* (Claes *et al.*, 2011). Additionally, commensal *E. coli* has been demonstrated to suppress agonist-induced apoptosis in the intestine of mice by inducing the production of IFN α by host cells (Mirpuri *et al.*, 2010), and the flagella of commensal bacteria suppress radiation-induced apoptosis in the gut epithelium by inhibition of the JNK^{MAPK} pathway (Jones *et al.*, 2011).

1.6.1 Oropharyngeal commensal and probiotic bacteria

It has been demonstrated that the most abundant genera of bacteria in the oral cavity of healthy individuals are *Streptococcus* (Kazor *et al.*, 2003) and *Neisseria* spp. (Belda-Ferre *et al.*, 2012) and that *Streptococcus* spp. isolated from healthy individuals inhibit the growth of cariogenic oral bacteria (Belda-Ferre *et al.*, 2012). More predominantly, the oral commensal *Streptococcus salivarius* strain K12 has been suggested as an oral probiotic strain as it has been shown to reduce halitosis due to the overpopulation of certain microorganisms (Burton, Chilcott & Tagg, 2005) and suppress inflammatory cytokines induced by *Pseudomonas aeruginosa* via suppression of NF κ B in human bronchial epithelial cells (Cosseau *et al.*, 2008).

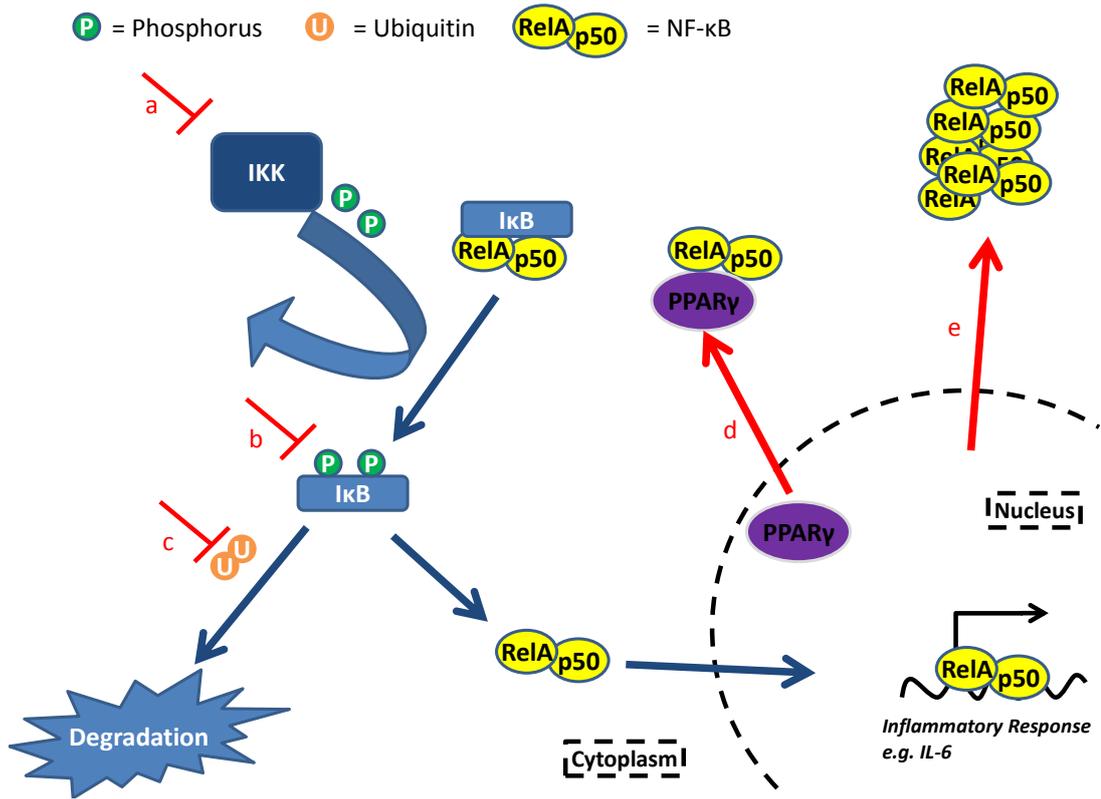


Figure 1.6 Mechanisms of attenuation of NFκB by bacteria include;

(a) blockade of IKK phosphorylation needed to release NFκB from IκB, as seen in pathogenic *Yersinia* (Schesser *et al.*, 1998) (b) dephosphorylation of IκB preventing release of NF-κB, seen in enteric *Salmonella* (Collier-Hyams *et al.*, 2002) (c) blockade of IκB ubiquitination and subsequent degradation, seen in enteric *Salmonella* (Collier-Hyams *et al.*, 2002) (d) nuclear export of proinflammatory transcription factor NFκB by the transcription factor PPARγ to the cytoplasm, seen in *Bacteriodes thetitaomicron* (Kelly *et al.*, 2004) (e) aggregation of NFκB subunit RelA within the cytoplasm inhibiting nuclear translocation as induced by the chronic pathogen *Bordetella bronchiseptica* (Yuk *et al.*, 2000).

The skin and occasional nasopharyngeal commensal *Staphylococcus epidermidis* has been shown to reduce inflammation in skin epidermal keratinocytes induced by *S. aureus* LTA via inhibition of NFκB signalling (Lai *et al.*, 2009, 2010). Furthermore, inhibition of necrotic host cell RNA-induced TLR3-mediated inflammation has been demonstrated via an inhibitory activation of TLR2 by *S. epidermidis* LTA. *S. epidermidis* has also been shown to inhibit biofilm formation by *S. aureus* and host colonisation in human subjects by the production of *S. epidermidis* serine protease (Esp) (Iwase *et al.*, 2010), and protects against group A

Streptococcus (GAS) in mice and human cell models by stimulating host production of β -defensins via TLR2 activation (Lai *et al.*, 2010). This reduction in inflammation is thought to help maintain barrier function and had been reported to reduce inflammatory bowel disease (Rembacken *et al.*, 1999). However, the majority of described microbial anti-inflammatory mechanisms revolve around preventing the release of active NF- κ B from its cytosolic inhibitor I κ B, or increasing export of active NF- κ B from the nucleus (Figure 1.6).

1.7 Commensal *Neisseria* spp.

Commensal *Neisseria* possess many genes associated with virulence in pathogenic *Neisseria* (Marri *et al.*, 2010) and transfer of genetic material has been demonstrated through natural competency and transformation (Saez-Nieto *et al.*, 1990; Marri *et al.*, 2010).

1.7.1 *N. lactamica*

MenB is known to colonise the URT of most healthy individuals without leading to invasive disease (Sim *et al.*, 2000). The well-studied *Neisseria* commensal *N. lactamica* (Nlac), lacks a polysaccharide capsule (Kim, Mandrell & Griffiss, 1989), though is genetically very closely related with Nmen, and occupies the same nasopharyngeal niche (Snyder & Saunders, 2006; Bennett *et al.*, 2012). It can be highly cross-reactive with anti-meningococcal antibodies (Saez-Nieto *et al.*, 1985), though can be differentiated by its ability to metabolise lactose (Hollis, Wiggins & Weaver, 1969). It colonises approximately 20-40% of infants and toddlers predominantly in males before and females after the age of 19 years (Gold *et al.*, 1978; Saez-Nieto *et al.*, 1985; Bennett *et al.*, 2005; Kristiansen *et al.*, 2012). With increased age there is an increase in Nmen carriage (Cartwright *et al.*, 1987) and a decrease in Nlac carriage (Gold *et al.*, 1978; Olsen *et al.*, 1991; Kristiansen *et al.*, 2012), particularly during teenage years, when a peak in Nmen invasive disease occurs.

Epidemiology has shown that prior colonisation of the URT with Nlac may correlate with a reduced susceptibility to invasive disease or carriage of MenB (Cartwright *et al.*, 1987; Guzzetta *et al.*, 2009; Evans *et al.*, 2011), and it has been suggested that horizontal gene transfer of a non-coding intergenic region from Nlac may produce noncapsular Nmen in the host (Claus *et al.*, 2002). It has also been suggested that the commensal Nlac could be used in the production of a meningococcal vaccine (Wyle *et al.*, 1972; Gorringer *et al.*, 2005).

There is a strong correlation between successful meningococcal vaccines and development of lysis-inducing serum bactericidal antibodies (SBA; Borrow, Balmer & Miller, 2005). It was postulated that the protection afforded by Nlac colonisation was due to the development of SBA to Nlac antigens that are cross reactive to MenB antigens (Gotschlich, Goldschneider & Artenstein, 1969; Kremastinou *et al.*, 1999; Troncoso *et al.*, 2002), which has previously been shown to be the case with new carriers of Nlac (Gold *et al.*, 1978). Outer membrane vesicles (OMVs) are portions of the bacterial membrane that bleb off to form extracellular membrane vesicles that contain many of the outer membrane proteins (OMPs) and other membrane components such as LPS or LOS. Intramuscular vaccination with outer membrane vesicles (OMVs) from Nlac (strain Y92-1009) with adjuvant have been shown to successfully protect mice against experimental invasive disease by MenB (Oliver *et al.*, 2002), as has intraperitoneal vaccination with live Nlac (strains L13 and Y92-1009) through stimulation of cross-reactive SBA production (Li *et al.*, 2006). A phase I clinical trial observed that intramuscular vaccination with Nlac OMVs in 18-55 year old men induced a weak, but broad immunogenic response, that included cross-reactive SBA to several strains of MenB (Gorringer *et al.*, 2009).

A study of tonsillar B cells has shown that *ex vivo* stimulation with Nlac OMVs induced a non-specific IgM response from infants under 5 years of age (Vaughan *et al.*, 2009), and proliferation of an innate B cell subset whereas Nmen OMV did not (Vaughan *et al.*, 2010). A small study demonstrated that nasal inoculation in humans, more representative of

natural immunity, with live Nlac induced nasal secretions that were able to inhibit attachment of MenB and MenA to primary oroepithelial cells (Andrade, Marques & de Santa Rosa, 1986). Recently, it has been shown that nasal inoculations with live Nlac (strain Y92-1009) in 18-45 year olds induced systemic IgG and salivary IgA at the nasal mucosa, but negligible cross-reactive SBA to Nmen tested (Evans *et al.*, 2011). This study also suggested that the presence of live Nlac, but not OMVs at the mucosa gives protection against acquiring colonising Nmen.

Pathogenic Nmen and commensal Nlac differ in their ability to adhere to, invade and stimulate inflammatory mediators from meningeal cells (Fowler *et al.*, 2006). More recent research conducted at the University of the West of England has confirmed that the differential ability of *Neisseria* spp. to stimulate the innate immune response extends to epithelial cells of the nasopharynx, where they colonise (Tezera *et al.*, 2011). Furthermore commensal Nlac (strain NL4.1) was found for the first time to be able to attenuate the inflammatory response induced by pathogenic MenB, via an intracellular mechanism that requires intact live bacteria; and attenuates signalling through the Toll-like receptor 2 and 1 (TLR2/1) heterodimer and involves PPAR γ . This is the first time that such a mechanism has been reported in the upper respiratory tract.

However, the anti-inflammatory effects of Nlac have not yet been evaluated against inflammation induced by other important upper respiratory tract pathogens. Furthermore, potential protective effects on mucosal barrier and host-pathogen association have not been measured.

1.7.2 *N. cinerea* and *N. polysaccharea*

Unlike Nlac, *N. cinerea* (Ncin) is commonly found in the oronasopharynx of approximately 20% of adults and occasionally isolated from urogenital sites (Knapp & Hook, 1988). It is

non-encapsulated and produces a negative result for biochemical sugar metabolism tests (glucose, maltose, fructose and lactose) and has been known to be mistaken for a glucose-negative *N. gonorrhoeae* (Knapp *et al.*, 1984; Boyce & Mitchell, 1985).

N. polysaccharea (Npoly) was first described in 1983 (Riou, Guibourdenche & Popoff, 1983), is non-encapsulated, and is distinguished by its ability to metabolise glucose and maltose, and crucially its ability to produce polysaccharide from sucrose (Riou & Guibourdenche, 1987). It has often been mischaracterised as Nmen or non-typeable Nmen due to its similar sugar metabolism and cross-reactivity with predominantly anti-MenB antibodies (Boquete, Marcos & Sáez-Nieto, 1986), and is a genetically diverse species (Anand *et al.*, 1991; Bennett *et al.*, 2012). It is found in approximately 3% of healthy infants (Boquete, Marcos & Sáez-Nieto, 1986; Cann & Rogers, 1989). Proteomic analysis suggests similarity to Nlac in this respect although it is the most genetically related commensal to Nmen (Bennett *et al.*, 2012). The effect of the commensals Ncin and Npoly on nasopharyngeal pathogen interactions with the host is currently poorly defined.

1.8 Aims and objectives

It has been hypothesised that commensal bacteria within the host environment give protection against colonisation and invasion by, and the inflammation induced by, pathogenic organisms that may colonise the same host environment. Specifically, commensal *N. lactamica* has previously been demonstrated to protect against inflammation induced by pathogenic *N. meningitidis in vitro*. Additionally, epidemiological studies have suggested *N. lactamica* may also protect against colonisation by *N. meningitidis* in the hosts.

The aim of this study was:

To identify if commensal *Neisseria* spp. give protection to the host from pathogenic bacteria commonly residing in the nasopharynx of humans, in the absence of an adaptive immune response.

Objectives of this study are:

To investigate if the commensal *N. lactamica* can attenuate the induction of inflammatory cytokines induced by human pathogens other than *N. meningitidis*, which commonly colonise the human nasopharynx and potentially cause meningitis.

To further define the inflammatory signalling pathways attenuated by *N. lactamica* in human nasopharyngeal epithelial cells following pro-inflammatory agonist stimulation.

To determine the effect of commensal *Neisseria* spp. including *N. lactamica*, on colonisation by *N. meningitidis* using an innate *in vitro* model of the host nasopharynx.

To investigate the host cell signalling pathways involved in *N. meningitidis*-induced host cell death, and to determine the roll of commensal *Neisseria* spp. in modulation of induced host cell death.

Chapter 2

Materials and Methods

2.1 Materials and Reagents

Unless otherwise stated in the text, all reagents were obtained from Sigma-Aldrich, UK. All tissue culture plastic ware was obtained from Corning, UK and equipment used in this study is listed in Table 2.1

Table 2.1 **Equipment used and manufacturer**

Equipment	Function	Software	Manufacturer
Biofuge Pico	Microcentrifuge	-	Kendro Laboratory Products, Germany
K40R	Centrifuge	-	CenturionScientific, UK
Consort E844	Electrophoresis Power Supply	-	Sigma-Aldrich, UK
FluoStar Optima	Fluorescent, Luminescent, Absorbance Microplate reader	Optima	BMG Labtech, Germany
MiniBis System	UV Imaging		DNR Bio Imaging Systems, Israel
Nanodrop 1000 Spectrophotometer	Microscale Absorbance Measurement	ND-1000	Thermo Scientific, UK
PTC-200 Peltier Thermal Cyclor	PCR Thermal Cyclor	-	MJ Research, Canada
Sonicator Ultrasonic Processor XL	Probe Sonicator	-	Misonix Inc, USA
SP50	Cuvette Spectrophotometer	-	Sanyo, Japan

2.2 Methods

2.2.1 Bacterial strains and growth conditions

Both commensal and pathogenic *Neisseria* spp. listed in Table 2.2 were grown on Brain Heart Infusion agar (BHI) (Fluka, Europe) supplemented with 10% heat-inactivated horse serum (HBHI) whereas the bioluminescent *N. meningitidis* strain MC58lux+ was grown on HBHI supplemented with 150 µg/ml kanamycin. All other strains of bacteria used in infection studies are listed in Table 2.3 of which strains of *S. pneumoniae* were grown in BHI broth (Oxoid, UK) containing 5% heat-inactivated FBS (PAA, UK). For enumerations (section 2.2.2) bacterial suspensions of *S. pneumoniae* were plated onto columbia agar containing 5% horse blood (blood agar). Strains of *H. influenzae* were grown on either columbia agar containing 5% horse blood heated to 70-80°C (chocolate agar) or on BHI agar supplemented with 2 µg/ml β-nicotinamide adenine dinucleotide hydrate (NAD) and 2 µg/ml hemin (sBHI). Liquid cultures were made in Levinthal's broth (Nutrient broth [Oxoid] containing 5% horse blood, heated to 90°C to produce a clear broth which is separated from the precipitate). *S. aureus* strain RN4220 was grown on nutrient agar at 37°C whereas the MRSA strain Mu50 was cultured on blood agar at 37°C in 5% CO₂. All cultures were established from frozen stocks stored at -80°C in cryopreservation medium (Pro-Lab Diagnostics, UK). All bacterial strains were grown overnight at 37°C in 5% CO₂ for 16-18 hours to reach mid-log phase of growth; except for *H. Influenzae* strains which were grown for 18-24 hours due to slower growth kinetics (Poje & Redfield, 2002).

2.2.2 Correlation of optical density and viable bacterial counts for rapid enumeration of bacterial concentration

Bacteria were grown overnight on the appropriate media (section 2.2.1) from which single colonies were then picked and suspended in 10mls Phosphate buffered saline (PBS) containing 0.5 mM calcium chloride and 0.9 mM magnesium chloride (PBS-B). The bacterial

suspension was left for 3 minutes to allow bacterial clumps to settle then the top 7mls were transferred to another universal for use.

A spectrophotometer was calibrated using 1 ml of PBS-B and the optical density of 1 ml of the bacterial suspension was then measured at a wavelength of 600nm (OD_{600}). If necessary the suspension was diluted to obtain an $OD_{600} < 0.5$ which was found to give greatest accuracy. A series of dilutions were prepared by doubling dilutions from initial stock in order to generate a graph to extrapolate viable bacterial counts from OD_{600} readings (Watson *et al.*, 1969).

In order to assess viable bacterial counts, 10-fold serial dilutions were made from 200 μ l of the above stock in a 96-well tissue culture plate from 10^0 to 10^{-7} . Then 10 μ l of each dilution was spread onto a third of an agar plate, in triplicate, and incubated overnight. Individual colonies were counted and multiplied by the dilution factor (df) to give colony-forming units (cfu) per ml (Miles, Misra & Irwin, 1938). This experiment was then repeated at least twice more with fresh cultures, to produce the final graphs for conversion of optical density (OD_{600}) into bacterial concentration (cfu/ml). Enumeration curves are presented in Appendix 1-4.

Table 2.2 The pathogenic and commensal *Neisseria* spp. used in this study

Abbr. Used	Bacterial Species	Bacterial Strain (Alias)	Serogroup	Isolated From	Sequence Type/ Clonal Complex	References
MC58 *	<i>N. meningitidis</i>	MC58 phenotypic variant #18.18 (Z7176)	Serogroup B	Human blood	ST-74/ cc32	(McGuinness <i>et al.</i> , 1991; Virji <i>et al.</i> , 1995a; Tettelin <i>et al.</i> , 2000)
C751 *	<i>N. meningitidis</i>	C751 (Z2491)	Serogroup A	Human cerebrospinal fluid	ST-4/ cc4	Achtman <i>et al.</i> , 1988; Crowe <i>et al.</i> , 1989; Parkhill <i>et al.</i> , 2000
Z4701 Δ	<i>N. meningitidis</i>	NG P20 (Z4701)	Serogroup B	Invasive (site unknown)	ST-11/ cc11	Caugant <i>et al.</i> 1986; Brehony <i>et al.</i> , 2009
Z6417 Δ	<i>N. meningitidis</i>	L93/4286 (Z6417)	Serogroup C	Invasive (site unknown)	ST-11/ cc11	Maiden <i>et al.</i> , 1998; Brehony <i>et al.</i> , 2009
Nlac *	<i>N. lactamica</i>	NL4.1 Derivative of NCTC 10617	Non-encapsulated	Human nose	ST-3787	(Hollis, Wiggins & Weaver, 1969; Serino & Virji, 2000)
Npoly †	<i>N. polysaccharea</i>	LNP 462 (NCTC 11858)	Non-encapsulated	Human throat	ST-3557	(Riou & Guibourdenche, 1987; Boquete, Marcos & Sáez-Nieto, 1986)
Ncin †	<i>N. cinerea</i>	194 (NCTC 10294)	Non-encapsulated	Human nasopharynx	ST-3579	(Berger & Paepcke, 1962)

* Provided as a generous gift from Professor Mumtaz Virji (University of Bristol, UK).

Δ Provided as a generous gift from Professor Dominique Caugant (University of Oslo, Norway).

† Obtained from the National Collection of Type Cultures (NCTC).

Table 2.3 Non-neisserial pathogenic bacteria used in this study

Abbr. Used	Bacterial Species	Bacterial Strain (Alias)	Serotype	Isolated From	References
D39 *	<i>S. pneumoniae</i>	D39 (NCTC 7466)	Serotype 2	unknown	(Avery, Macleod & McCarty, 1944; Lanie <i>et al.</i> , 2007)
Sp14 *	<i>S. pneumoniae</i>	unknown	Serotype 14	Human cerebrospinal fluid	Not published
Hib ‡	<i>H. influenzae</i>	Eagan	Type b	Human cerebrospinal fluid	(Anderson, Johnston & Smith, 1972)
Hia †	<i>H. influenzae</i>	620 (NCTC 8466)	Type a	Human cerebrospinal fluid	(Chapman & Osborne, 1942)
Mu50 Δ	<i>S. aureus</i>	Mu50 (ATCC 700699)	Serotype 5	Human abscess	(Hiramatsu <i>et al.</i> , 1997; Kuroda <i>et al.</i> , 2001)
RN4220 Δ	<i>S. aureus</i>	RN4220	Non-encapsulated	Laboratory Construct	(Kreiwirth <i>et al.</i> , 1983; Nair <i>et al.</i> , 2011)

* Provided as kind gifts from Professor Adam Finn (University of Bristol, UK).

‡ Provided as a kind gift from Professor J. Simon Kroll (Imperial College, UK).

† Obtained from the National Collection of Type Cultures (NCTC).

Δ University of the West of England Culture Collection.

2.2.3 Minimum inhibitory concentration and minimum death concentration of bacteria for antibiotics

To identify bacterial sensitivity to antibiotics, serial dilutions of antibiotic in appropriate broth for the bacteria were made (section 2.2.1). Broth cultures were seeded with 1×10^6 cfu/ml and cultured for 24 hours.

After 24 hours the turbidity of the liquid medium was compared against 1ml of that medium containing no bacteria as a reference. The lowest concentration of antibiotic that prevented turbidity was considered to be the minimum inhibitory concentration (MIC) of that antibiotic for the corresponding bacteria (modified from Andrews, 2001). Because the MIC may be bacteriostatic the minimum bactericidal concentration (MBC) was further determined by spreading 100 μ l of the non-turbid samples onto the corresponding solid medium in triplicate. The concentration of antibiotic that prevented growth of the bacteria was considered the minimum bactericidal concentration (MBC). Both MIC and MBC experiments were repeated in at least 2 separate experiments each performed in duplicate to confirm results.

2.2.4 Generation of bacterial lysates, heat-killed bacteria and source of OMVs

Overnight cultures of *N. meningitidis* or *N. lactamica* were suspended in PBS-B and adjusted to 2×10^9 colony forming units per ml (cfu/ml). For heat-killed bacteria, 1 ml aliquots of bacterial suspension were incubated in a pre-warmed 55°C waterbath for 35 minutes. Heat-killing was confirmed by viable colony counts and heat-killed bacteria were stored at -20°C until required and were freeze-thawed once only.

To generate bacterial lysates, 1ml aliquots of 2×10^9 cfu/ml bacterial suspension were sonicated (Ultrasonic Processor XL probe sonicator) on ice for 60 seconds, followed by 30 seconds wait, to prevent heating of the lysate and denaturation of proteins, until the lysate

cleared (approximately 7 cycles). Aliquots of bacterial lysate were centrifuged at 10,000xg for 15 minutes. The supernatant was then aliquoted and labelled as the soluble fraction of the lysate and the remaining pellet was re-suspended in PBS-B to the original volume of the lysate and labelled as the insoluble fraction. Aliquots were then stored at -20°C until required and were freeze-thawed once only. Lysates and heat-killed bacteria were used in experiments according to the original concentration of the bacterial suspension.

N. lactamica outer membrane vesicles (OMVs) were kindly provided by Professor Andrew Gorringe (Public Health England, UK) and were generated from strain Y92-1009.

2.2.5 Natural bioluminescent transformation of *N. meningitidis* strain MC58

Escherichia coli strain DH5 α containing plasmid pLKMp (Sjölinder & Jonsson, 2007) was provided as a generous gift from Professor Ann-Beth Jonsson and Assistant Professor Hong Sjolinder (Stockholm University, Stockholm, Sweden). Plasmid pLKMp contains the *luxCDABE* operon from the bioluminescent organism *Photobacterium luminescens*, the *Neisseria*-specific PorB promoter sequence from *N. meningitidis* strain FAM20, the *Neisseria* DNA uptake sequence ('5-GCCGTCTGAA-3') (Elkins *et al*, 1991), the *kanR* kanamycin resistant cassette and two DNA fragments homologous to non-coding regions of the FAM20 genome.

E. coli containing plasmid pLKMp was grown overnight in 10ml LB broth containing kanamycin (50 mg/L) to select for plasmid-containing bacteria. The pLKMp plasmid was isolated from *E. coli* using the Pureyield plasmid prep kit (Promega, UK) according to the manufacturer's instructions and stored at -20°C until required. Single colonies of overnight cultures of *N. meningitidis* strain MC58 were suspended in PBS-B. Meanwhile, 1 μ g pLKMp DNA was spotted onto a HBHI agar plate, marked and left to dry in aseptic conditions at room temperature. Then 5×10^7 *N. meningitidis* was spotted over the dried plasmid DNA and

incubated for 24 hours at 37°C and 5% CO₂. The resulting growth was suspended in 200 µl PBS-B and 100 µl was plated onto HBHI agar containing 150 mg/L kanamycin to select for transformants. Colonies that grew and were bioluminescent were designated MC58lux⁺ and were cultured on HBHI containing 150 mg/L kanamycin from frozen in all future experiments.

2.2.6 Identification of bioluminescent transformants as *N. meningitidis*

To confirm the transformed colonies as *N. meningitidis*, MC58lux⁺ colonies were picked and sub-cultured on HBHI containing 150 µg/ml kanamycin to select for bioluminescent transformants, with 20 µg/ml colistin to remove non-neisserial, Gram-negative bacteria. MC58lux⁺ colonies were also cultured onto chocolate agar and onto blood agar to select for *Neisseria* and other fastidious bacteria. Resulting growth was then applied to stab agar slants (2% peptone, 85 mM sodium chloride, 0.4% tryptone, 2.5% agar, pH 7.6, 0.08% phenol red) (modified from Cruickshank, 1975) containing 1% glucose, sucrose, maltose or lactose. Oxidation of specific sugars will cause a pH and thus colour change of the phenol red, and enabling confirmation of the bacteria as *N. meningitidis* by its ability to metabolise glucose and maltose, but not sucrose and lactose.

2.2.7 Purification of genomic DNA from *N. meningitidis* for further analysis

N. meningitidis strains MC58 and MC58lux⁺ were grown overnight in 5% CO₂ at 37°C on HBHI agar or HBHI agar containing 150 mg/L kanamycin respectively. Single colonies were then suspended in PBS-B to approximately 1-2x10⁹ cfu/ml. From this suspension, 1ml aliquots were made into 1.5 ml screw-cap micro-centrifuge tubes and centrifuged at 3500 rpm for 15 minutes to pellet the bacteria.

Bacterial genomic DNA was isolated and purified using the Wizard Genomic DNA Purification Kit (Promega, UK) following the manufacturers protocol. In brief, each bacterial pellet was gently re-suspended by pipetting in 600 µl Nuclei Lysis Solution. The suspension was incubated at 80°C for 5 minutes to lyse the bacteria followed by 30 minutes incubation at room temperature. Once cooled, 3 µl of RNase solution (4 mg/ml in TE buffer [10 mM Tris-HCL (ph 7.4) and 1 mM EDTA (ph 8.0)]) was added to the lysate and mixed by inversion 4 times, incubated at 37°C for 45 minutes, cooled for 20 minutes at room temperature. Then 200 µl of Protein Precipitation Solution was added to the lysate and the mixture was vortexed vigorously for 20 seconds and incubated on ice for 5 minutes. The mixture was then centrifuged at 13,000 rpm for 3 minutes and the supernatant was transferred to a new 1.5 ml micro-centrifuge tube containing 600 µl of room temperature isopropanol.

The supernatant-isopropanol solution was mixed by inversion until the DNA formed a visible mass. The mixture was centrifuged at 13,000 rpm for 2 minutes to pellet the DNA and the supernatant was removed. The DNA was washed by inversion in 600 µl of 70% ethanol and centrifuged at 13,000 rpm for 2 minutes and the ethanol was then aspirated. The tube was drained on clean absorbent paper and the pellet was air-dried for 15 minutes. The DNA was then rehydrated in 200 µl of nuclease-free water, incubated at 65°C for 1 hour and stored at 4°C.

To quantify the amount of DNA in solution and the purity of the DNA solution each sample was analysed using the Nanodrop 1000 spectrophotometer using nuclease-free water as a blank.

2.2.8 Confirmation of bioluminescent transformed MC58lux+ by standard PCR

The primers used in this study were manufactured by Eurofins MWG Operon, Germany, designed by Assistant Professor Hong Sjolinder (Stockholm University, Stockholm, Sweden)

and are listed in Table 2.4. Primers KS1 and luxEVS2 target sites within the designated integration sequence from plasmid pLKmp used to transform *N. meningitidis* strain MC58 to the *luxCDABE* expressing strain MC58lux+. Primers UsF and DsR target the chromosomal DNA of *N. meningitidis* strain MC58 (NCBI accession # NC_003112.2) outside the integration site.

Primers were re-suspended as per manufacturer's instructions to a final concentration of 100pmol/μl in nuclease-free water. PCR was performed using the GoTaq Hot Start Polymerase kit (Promega, UK) in a MJ Research PTC-200 Peltier Thermal Cycler according to the manufacturer's suggestions. In brief, 0.2 μg of bacterial genomic DNA was added to a final volume of 20μl in 1x Green GoTaq Flexi Buffer containing 2mM MgCl₂, 0.2 mM PCR Nucleotide Mix, 0.5 μM forward primer, 0.5 μM reverse primer and 1.25μM GoTaq Hot Start Polymerase. The PCR protocol included an initial denaturation step of 2 minutes at 95°C followed by 35 cycles of denaturation for 1 minute at 95°C, annealing for 1 minute at 52°C followed by an extension stage for 1 minute at 74°C before being refrigerated at 4°C until required.

Next, 18 μl of each sample or 5 μl of Easyladder 1 DNA ladder (Bioline, UK) was added to relevant wells on a 14-well, 2% agarose gel, containing 15ng/ml ethidium bromide to stain the PCR products, in TAE buffer. The gel was run at 120V and 400amps for approximately 30 minutes to separate PCR products. The gel was imaged with the MiniBis system. Standard PCR was repeated once to confirm results.

Table 2.4 List of primers used in this study

Primers	Sequence (5' – 3')	Direction	Tm (°C)
KS1	AATCATGCGAAACGATCCTCATC	Reverse	58.9
luxEVS2 (VS2)	GTGCTCGTTGAAATTTTACGTCG	Forward	58.9
UsF	ATTACGAAACCATTACTGCTGCC	Forward	58.9
DsR	AATAATCCAGAAATGGTTGCAGC	Reverse	57.1

2.2.9 Comparison of growth kinetics between *N. meningitidis* strain MC58 and the bioluminescent transformant MC58lux

N. meningitidis strains MC58 and MC58lux were grown overnight for 16-18 hours on HBHI agar and HBHI agar containing 150mg/L kanamycin, respectively, at 37°C in 5% CO₂. Resulting colonies were suspended in PBS-B as described previously (section 2.2.2). Bacterial cultures of each strain were made in both HBHI broth and M199 (2mM L-glutamine, 2% FBS, without phenol red and pH was adjusted to 7.2-7.4 with sodium bicarbonate) to a final concentration of approximately 1x10⁵ bacteria per 200µl. From each culture 200µl was added in triplicate to a 96-well µClear black-walled tissue culture plate (Griener-Bio One, UK, G655090) from which the optical density at 620nm (OD₆₂₀) was measured every 30 minutes in 5% CO₂ at 37°C using the Fluostar Optima (BMG Labtech) optical plate reader.

2.2.10 Correlation between optical density, viable counts and relative light units for the bioluminescent *N. meningitidis* strain MC58lux

Bioluminescent *N. meningitidis* strain MC58lux was grown overnight on HBHI containing 150 mg/L kanamycin at 37°C in 5% CO₂ for 16-18 hours. Resulting colonies were suspended in PBS-B as described previously (section 2.2.2). The bacterial solution was then added to Medium 199 without phenol red (M199) to a final OD₆₀₀ of approximately 1. From this bacterial solution several two-fold dilutions were made in M199. From each dilution 200 µl was added in triplicate to a 96-well µClear black-walled tissue culture plate (Griener-Bio One, UK, G655090) from which relative light units (RLU) were immediately measured using the Fluostar Optima (BMG Labtech) bioluminescent plate reader. Simultaneously, the OD₆₀₀ of 1ml of each dilution of bacterial solution was measured with the spectrophotometer calibrated using M199 alone, and viable counts were plated onto HBHI agar in triplicate as described previously (section 2.2.2).

2.2.11 Culture and maintenance of Detroit 562 nasopharyngeal epithelial cells

The human nasopharyngeal epithelial carcinoma cell line, Detroit 562 (D562), ECCAC# 87042205, was cultured in Eagles Minimum Essential Media (EMEM) supplemented with 10% heat-inactivated FBS, 0.1% lactalbumin hydrolysate (Oxoid, UK), 0.1mM non-essential amino-acids, 1mM sodium pyruvate and 2mM L-glutamine (complete media). Cells were cultured in T75 tissue culture flasks until $\geq 80\%$ confluent. The cells were then passaged by trypsinization whereby the cells were incubated in pre-warmed 0.05% trypsin-0.02% EDTA for 7 minutes or until $\geq 90\%$ of cells were in suspension. The cells were then washed by centrifugation at 500xg for 5 minutes and re-seeded at 3×10^6 per T75 flask for each passage of the cell line. For co-culture experiments, D562 cells were seeded into 24-well flat-bottomed tissue culture plates at 4×10^5 cells per well, or in 96-well tissue culture plates at 1×10^5 cells per well to establish confluent monolayers in 1-2 days.

2.2.12 Culture of Detroit 562 cells with pathogenic bacteria in the absence or presence of the commensal *Neisseria* spp.

D562 cells were cultured as described previously (section 2.2.11). Once D562 monolayers were grown to confluence in tissue culture plates the complete media was replaced with Medium 199 containing 2% FCS and 2mM L-glutamine (M199) to maintain epithelial cells and minimise bacterial growth during experiments.

D562 cells were incubated with pathogenic bacteria with or without commensal *Neisseria* spp. Bacteria were added to epithelial monolayers at a range of bacterial concentrations (0.2, 2, 20 to 200 bacteria per epithelial cell) referred to as multiplicity of infection (MOI). Following incubation at 37°C in 5% CO₂ for 3 hours, media and unbound bacteria were removed from each well, and monolayers were washed three times with PBS. Unless

otherwise stated, M199 containing 200 µg/ml gentamicin was then added to each well and incubated for a further 21 hours at 37°C in 5% CO₂ with humidity to kill the remaining bacteria, with the exception of experiments using *S. aureus* strain Mu50 where gentamicin was substituted for 100 U/ml penicillin and 100 µg/ml streptomycin for the final 21 hours of the incubation (Ellis, 2003). *S. aureus* strain Mu50 and *N. lactamica* strain NL4.1 co-cultures were found to have an MIC of less than 6.25 U/ml penicillin with 6.25 µg/ml streptomycin.

2.2.13 Culture of Detroit 562 nasopharyngeal cells with inflammatory stimuli in the absence or presence of *N. lactamica*

D562 cells were seeded in 96-well plates as previously described (section 2.2.11). D562 cells were cultured with and without *N. lactamica* strain NL4.1 at a range of bacterial concentrations (MOI 2- 2000) for 3 hours in M199, in the absence or presence of stimuli (Table 2.5). Following 3 hours incubation, D562 cells were washed with PBS to remove unbound bacteria and incubated for a further 21 hours in M199 containing gentamicin (without stimuli).

Table 2.5 Stimuli used in this study

Agonist name	Abbreviation	Agonist target	Concentration used
polyinosinic-polycytidylic acid sodium salt	poly(I:C)	Toll-like receptor 3 (TLR3)	0.05 µg/ml – 5 µg/ml
anisomycin	ANI	p38 ^{MAPK} /JNK ^{MAPK}	2.5 µg/ml-5 µg/ml
phorbol 12-myristate 13-acetate	PMA	ERK ^{MAPK}	0.1 µg/ml-1 µg/ml

2.2.14 Enzyme-linked immunosorbent assay for the quantification of secretory cytokines and chemokines from Detroit 562 cells

D562 cells were treated with bacteria or stimuli as described previously (sections 2.2.12 and 2.2.13). The resulting supernatants were collected after 24 hours incubation, aliquoted and stored at -80°C until required. The enzyme-linked immunosorbent assay (ELISA) recognising human IL6, IL8 or TNF α was used according to the manufacturer's instructions (R&D Systems, UK) with minor modifications, to quantify the amount of cytokine/chemokine in the culture supernatants generated previously. In brief, 100 μ l of capture antibody (360 μ g/ml mouse anti-human IL6 capture antibody, or 720 μ g/ml mouse anti-human IL8 or 720 μ g/ml mouse anti-human TNF α) in PBS was added to each well of a 96-well immunoplate and incubated at room temperature (RT) overnight in order to coat the immunoplate. The immunoplate was then washed three times with an excess of wash buffer (0.05% Tween 20 in PBS (0.1M sodium chloride, 8mM disodium hydrogen orthophosphate 2-hydrate, 50mM potassium phosphate, 2.5mM potassium chloride, pH 7.4)) to remove unbound antibody, and non-specific antibody binding was blocked by incubation in 300 μ l of reagent diluent (1% bovine serum albumin in PBS, pH 7.4) for 1 hour.

Cytokine/chemokine standards were made by doubling dilutions of recombinant protein of 600pg/ml to 4.7 pg/ml (IL6), 2000 pg/ml to 15.3 pg/ml (IL8) or 1000 pg/ml to 7.6 pg/ml (TNF α). All supernatants were freshly thawed and diluted appropriately prior to testing to bring the levels of cytokine/chemokine into the detectable range, in reagent diluent. Then 100 μ l of samples or standards were added to each well and incubated at 4°C overnight to allow cytokine/chemokine in the samples to bind to the capture antibody.

Each immunoplate was washed as before, to remove unbound cytokine/ chemokine then 100 μ l biotinylated goat anti-human IL6 (36 μ g/ml), goat anti-human IL8 (20 ng/ml) or goat anti-human TNF α (250 ng/ml) secondary antibody in reagent diluent was added to each well and incubated at RT for 4 hours to bind to captured cytokine/chemokine. The wells

were washed as before to remove any unbound detection antibody and 100 µl of streptavidin-conjugated horseradish-peroxidase (HRP) (1:200 dilution) was added to each well. Each immunoplate was kept in the dark to protect the light-sensitive HRP and was incubated at RT for 20 minutes. A final wash was performed to remove excess unbound HRP and 100µl of peroxidise substrate solution was added to each well to start the enzymatic reaction and incubated at RT in the dark for a further 30 minutes. Finally 50µl of stop solution (2N H₂SO₄) was added to each well and the optical density (OD) was read at 450nm using the Fluostar Optima (BMG Labtech) absorbance plate reader, and at 540nm for correction. A standard 4-parameter logistic curve of log₁₀OD against known cytokine/chemokine concentration was generated from the standards and used to calculate the levels of cytokine/chemokine in the supernatant using Graphpad Prism 5 software (Graphpad Software, San Diego, USA). These were then multiplied by the dilution factor to give the final concentrations of cytokine/chemokine.

2.2.15 Fluorescent stains to monitor induction of cell death in nasopharyngeal epithelial cells and possible protection by commensal *Neisseria* spp.

D562 monolayers were infected with pathogenic bacteria in the presence and absence of commensal *Neisseria* spp. as described previously (section 2.2.12). D562 cells were permeabilised with 1% saponin to allow maximal staining of DNA. In optimisation experiments D562 cells were also treated for 24 hours with either 10x PBS to induce necrosis or 1µM staurosporine (STRP) to induce apoptosis.

To determine commensal *Neisseria* spp. effect on apoptotic inducers, D562 cells were incubated with commensal *Neisseria* spp. for 3 hours in the presence or absence of 1µM STRP or 20 ng/ml TNFα (Peprotech, UK) with 20 µg/ml cycloheximide (chx) to induce intrinsic or extrinsic apoptosis respectively (Liu, Wetzler & Massari, 2008; Deghmane *et al.*,

2009). Epithelial cells were then washed in PBS, and then incubated in M199 containing 200 µg/ml gentamicin with either STRP or TNF α /CHX for a further 21 hours.

To quantify D562 cells present, after a total of 24 hours incubation, a final concentration of 5 µg/ml of the DNA stain Hoechst 33342 was added to each well and incubated for 1 hour at 37°C in 5% CO₂. The treatments were duplicated in wells stained with a final concentration of 1µM of the apoptotic marker Yopro-1 (Idziorek *et al.*, 1995);(Plantin-Carrenard *et al.*, 2003) and 5 µg/ml of the necrotic marker propidium iodide (PI) was added for 30 minutes and 15 minutes respectively. The fluorescent intensity from each stain was measured using a Fluostar Optima plate reader (BMG Labtech, UK), using 350nm excitation and 460nm emission filters to measure Hoechst 33342 staining, 485nm excitation and 570nm emission filters to measure Yopro-1 staining and 540nm excitation and 620nm emission filters to measure PI staining.

2.2.16 Analysis of apoptotic cell death in Detroit 562 epithelial cells by detecting activated caspase-3 by flow cytometry

D562 cells were cultured in 24-well tissue culture plates and challenged by bacteria as described previously (section 2.2.12). Following 18 hours incubation in M199 containing gentamicin, activated caspase 3 was labelled using the CaspGlow Fluorescein Active Caspase 3 Staining Kit (eBioscience, UK, 88-7004) according to the manufacturer's instructions. In brief, a fluorescein (FITC)-labelled activated caspase inhibitor (FITC-DEVD-FMK) that binds irreversibly and specifically to activated caspase 3, was added directly to each well (1:1000 dilution) and incubated at 37°C in 5% CO₂ for 45 minutes. To include non-adherent cells the supernatants were removed and transferred to labelled centrifuge tubes, then each well was washed in PBS and the PBS was also transferred to the corresponding centrifuge tubes. D562 cells were next incubated for 20 minutes in PBS containing 4mM EDTA at 37°C to disassociate epithelial cells, then transferred to centrifuge tubes containing

both supernatant and PBS wash. Cell suspensions were centrifuged at 500 xg for 5 minutes to pellet the cells and re-suspended in PBS to wash the cells, which was then repeated. The pelleted cells were re-suspended in chilled PBS containing 4% PFA. Following 20 minute incubation for fixation, the cells were centrifuged as before and re-suspended in PBS to be stored at 4°C until required. Where possible 10,000 events were collected per sample and FITC-caspase 3-labelled cells were then analysed using the Accuri C6 flow cytometer.

2.2.17 Surface expression of Toll-like receptor 2 on Detroit 562 epithelial cells following bacterial challenge

D562 cells were culture in the presence of *N. meningitidis* in the absence or presence of commensal *Neisseria* spp. for 3 hours as described in section 2.2.12. D562 cells were washed three times with PBS and disassociated with EDTA (4mM in PBS) for 20 minutes at 37°C in 5% CO₂. D562 cells were then pipette washed in the EDTA solution to remove cells and transferred to screw-capped microcentrifuge tubes and pelleted by centrifugation at 500xg for 5 minutes. The EDTA solution was poured off and the remaining pellet was re-suspended in chilled paraformaldehyde (PFA) (4% in PBS), incubated at room temperature for 20 minutes then topped up to 1ml with PBS. The PFA fixed cell suspension was stored at 4°C until required.

Half of the fixed cell suspension (approximately 2×10^5 cells) was transferred to a new microcentrifuge tube, pelleted by centrifugation and re-suspended in PBS. The D562 cells were again pelleted then re-suspended in blocking buffer (1% BSA, 0.1% NaN₃ in PBS) and incubated at room temperature for 10 minutes to block non-specific binding. D562 cells were further incubated in blocking buffer containing a 1:250 dilution of mouse anti-human TLR2 (clone TL2.1, eBioscience, UK) antibody for 1 hour at room temperature. To remove unbound antibody, cells were washed twice by centrifugation followed by re-suspension in blocking buffer, then incubated for 1 hour at room temperature with a 1:500 dilution of

goat anti-mouse Alexa-fluor 647-conjugated secondary antibody (Life Technologies, UK). D562 cells were then washed twice by centrifugation as before, followed by re-suspension in PBS. The cells were analysed for fluorescence using the Accuri C6 flow cytometer.

2.2.18 Dual-labelling of cell death and *N. meningitidis* in nasopharyngeal epithelial cells following challenge

To determine if direct association by *N. meningitidis* with D562 cells is required for induction of cell death, D562 cells were challenged with an MOI of 2 or 20 bacteria per epithelial cell with *N. meningitidis* strain MC58. Unlike previous experiments, following a 3 hour challenge with bacteria, D562 cells were washed with PBS and M199 (without gentamicin) was added for a further 18 hours. D562 cells were then labelled for the apoptotic marker, activated caspase 3 as described in section 2.2.16 according to manufacturer's instructions. Cells were then washed three times by centrifugation in chilled PBS and fixed in 4% PFA for 20 minutes at room temperature. Cells were then labelled for adherent bacteria as described in section 2.2.21 then analysed using the Accuri C6 flow cytometer.

2.2.19 Bioluminescent reporter for the detection of activated caspase in Detroit 562 epithelial cells challenged with *N. meningitidis* with and without commensal *Neisseria* spp.

To determine the effect of commensal *Neisseria* spp. on *N. meningitidis*-induced activation of caspase cell death pathways, D562 cells were seeded into 96-well, black-walled, clear-bottomed tissue culture plates (Greiner Bio-One, UK). Following a 3 hour challenge with *N. meningitidis* strain MC58 (MOI 20) alone or in combination with commensal *Neisseria* spp. (MOI of 200 bacteria per D562 cell), each well was washed with PBS and M199 containing

200 µg/ml gentamicin (without phenol red) was added for the remainder of the assay. At indicated time points the caspase-glo 8 (Promega, UK) or the caspase-glo 9 (Promega, UK) assay reagent was added to separate wells and incubated at 37°C in 5% CO₂ for 30 minutes. Each assay reagent contains a caspase-specific substrate that when cleaved by activated caspase, luminesces. Bioluminescence (relative light units; RLU) was measured using the Fluostar Optima. D562 cells were incubated with 1µM STRP for the duration of the experiment as positive controls for activated caspase 9, or 20 ng/ml TNFα as a positive control for activated caspase 8.

2.2.20 Viable bacterial counts to measure association and invasion of *Neisseria* spp. with Detroit 562 epithelial monolayer

To monitor association and invasion of bacteria to D562 cells, bacterial suspensions of *N. meningitidis* and *N. lactamica* were made (section 2.2.2). The bacteria were re-suspended in M199 to a final concentration of 200 bacteria per epithelial cell (MOI) and added to D562 cell monolayers in triplicate wells of a 96-well tissue culture plate, per treatment. To confirm bacterial concentrations added to monolayers, bacteria were enumerated by serial 10-fold dilutions and spreading onto HBHI agar to determine viable colony counts. To distinguish between *N. meningitidis* and *N. lactamica*, bacteria were spread onto HBHI agar containing 100 mg/L 5-Bromo-4-chloro-3-indoyl B-D-galactopyranoside (x-gal) (Bennett *et al.*, 2005), a lactose derivative that is selectively metabolised by *N. lactamica* resulting in a blue colony colouring compared to off-white colony colouring of *N. meningitidis*. Following culture of D562 cells with bacteria for 3 hours at 37°C in 5% CO₂, growth of bacteria was monitored by viable counts from the culture supernatant.

Bacterial association to D562 monolayers was determined as described previously (Virji *et al.*, 1992c). Briefly, cells were washed vigorously by pipetting with PBS-B to remove unassociated bacteria, and then incubated for 30 minutes at 37°C in 5% CO₂ in PBS-B

containing 1% saponin to lyse epithelial cells. Duplicate wells containing bacteria without D562 cells were used to correct for non-specific binding to the exposed well area. Saponin at 1% was found not to affect viability of *Neisseria* by viable colony counts.

To examine invasion of bacteria into D562 cells, a gentamicin protection assay was used (Shaw & Falkow, 1988; St Geme & Falkow, 1990) with minor modification (Virji *et al.*, 1992c). Monolayers were washed vigorously by pipetting with PBS-B prior to a 90 minute incubation in M199 containing 200 µg/ml gentamicin to eliminate extracellular bacteria (Vaudaux & Waldvogel, 1979). The cells were then washed in PBS-B, lysed with 1% saponin, and viable counts were made.

To confirm that gentamicin effectively eliminates only extracellular bacteria, in selected experiments, D562 monolayers were pre-incubated for 1 hour with 2 µg/ml cytochalasin-D, to inhibit phagocytic internalization of *Neisseria*, prior to bacterial challenge (Bessen & Gotschlich, 1986; Virji *et al.*, 1992c; Merz & So, 1997). After 3 hours incubation D562 cells were washed, incubated with gentamicin and lysed with saponin.

2.2.21 Flow cytometry analysis of *N. meningitidis* adhesion to Detroit 562 epithelial cells in the presence and absence of commensal *Neisseria* spp.

D562 cells were challenged with either *N. meningitidis* strain MC58 or C751 alone or in the presence of the commensal *Neisseria* spp; *N. lactamica*, *N. cinerea* or *N. polysaccharea*, (MOI 20 or 200 bacteria per epithelial cell) for 3 hours at 37°C in 5% CO₂. D562 cells were then washed three times with PBS and incubated in PBS containing 4mM EDTA for 20 minutes at 37°C in 5% CO₂. D562 cells were then pipette washed in the EDTA solution to remove cells and transferred to screw-capped microcentrifuge tubes and pelleted by centrifugation at 500 xg for 5 minutes. The remaining pellet was re-suspended in chilled paraformaldehyde (PFA) (4% in PBS) for fixation, incubated at room temperature for 20

minutes then topped up to 1ml with PBS and stored at 4°C until required. The D562 cells were again washed by centrifugation in PBS to remove PFA and then re-suspended in blocking buffer (1% BSA, 0.1% NaNH₃ in PBS) and incubated at room temperature for 10 minutes to block non-specific antibody binding. D562 cells were further incubated in blocking buffer containing mouse anti- *N. meningitidis* serosubtype P1.7 antibody (1:250 dilution) (NIBSC, UK) for 1 hour at room temperature. To remove unbound antibody, cells were washed twice by centrifugation in blocking buffer, then incubated for 1 hour at room temperature with goat anti-mouse Alexa-fluor 647 conjugated or goat anti-mouse Alexa-fluor 488 conjugated secondary antibody (1:500 dilution) (Life Technologies, UK) in blocking buffer. D562 cells were then washed twice by centrifugation as before, followed by re-suspension in PBS. Untreated, antibody-labelled cells were used as a background control. The cells were analysed for fluorescence using the Accuri C6 flow cytometer.

2.2.22 Monitoring invasion into Detroit 562 nasopharyngeal epithelial cells by the pathogen, *N. meningitidis*, using a bioluminescent reporter

To determine the effect of commensal *Neisseria* spp. on the ability of *N. meningitidis* to invade nasopharyngeal epithelial cells, D562 cells were seeded into 96-well black-sided, clear-bottomed tissue culture plate (Greiner Bio-one, UK) until 100% confluent as described in section 2.2.11. Complete media was replaced with M199 (without phenol red) to reduce background luminescence (Flentie *et al.*, 2009). MC58lux⁺ at a MOI of 200 bacteria per epithelial cell was used to challenge D562 cells in the presence and absence of commensal *Neisseria* spp at a concentration of 20, 200 or 2000 bacteria per epithelial cell (MOI). Relative light units (RLU) emitted by the bioluminescent MC58lux⁺ were monitored every 60 minutes using the Fluostar Optima plate reader (BMG Labtech). After 3 hours incubation at 37°C in 5% CO₂ appropriate wells were washed and M199 containing 200 µg/ml gentamicin was added to kill the extracellular bacteria. Following a further 90 minutes incubation at

37°C in 5% CO₂, a final concentration of 1% saponin was added to permeabilise cells, and allow gentamicin mediated killing of intracellular bacteria. RLU's from gentamicin and saponin treated wells were compared against treatments with gentamicin alone to give a measure of invasion. Untreated D562 cells were used to measure the lower limit of detection.

2.3 Statistical analysis

For correlation between bacterial concentration (cfu/ml) as determined by viable colony counts and OD₆₀₀ or relative light units (RLU) a single order polynomial line of 95% best fit was generated for x/y scatterplots and significance was analysed by Spearman correlation. For all other statistical comparisons when comparing two treatments, such as the effect of single concentrations of commensal neisseriae on pathogen-induced host cell death, paired student t-tests were used. When comparing growth curves between *N. meningitidis* strains unpaired student t-tests were used. When comparing three or more treatments, such as inflammatory or cell death response to a range of concentrations of bacteria or stimuli, a one-way ANOVA with Bonferroni's post-test was used to compare between treatments.

Values stated represent means \pm standard error of the mean (SEM) unless otherwise stated and p-values less than or equal to 0.05 ($p \leq 0.05$) were considered statistically significant. All statistical tests were conducted using Graphpad prism version 5 (Graphpad Software, San Diego, California, USA).

Chapter 3

Modulation of the inflammatory response of nasopharyngeal epithelial cells by *N. lactamica*

3.1 Introduction

The human mucosa plays a pivotal role in recruiting innate and adaptive immune cells to the site of infection and modulating the systemic response towards homeostasis or inflammation. For instance, epithelial cells exposed to DNA from commensal or pathogenic bacteria induce different responses in antigen presenting cells (Campeau *et al.*, 2012). Conditioned media from nasopharyngeal epithelial cells exposed to bacterial or viral proteins influenced the proliferation of T cells and the production of specific IgA and IgG antibodies by human peripheral blood lymphocytes (Yeh *et al.*, 2013).

The meningitis-causing pathogens *N. meningitidis* and *H. influenzae* are potent inducers of inflammatory cytokines in meningeal cells, in contrast to *S. pneumoniae* (Fowler *et al.*, 2004). Despite comparable levels of association with meningeal cells, *N. lactamica* is poorly inflammatory compared to *N. meningitidis* (Fowler *et al.*, 2006).

Previous work by this group at the University of the West of England has demonstrated that *N. meningitidis* (strain MC58) is also a potent inducer of innate inflammation in human nasopharyngeal epithelial cells, and that this inflammation can be attenuated by *N. lactamica* (strain NL4.1) in a dose-dependent manner (Tezera *et al.*, 2011). Further to this, *N. lactamica* was shown to attenuate inflammation induced via the Toll-like receptor (TLR) 2/1 heterodimer and could also attenuate inflammation induced by the inflammatory cytokines IL1 β and TNF α . Together this suggests that the ability of *N. lactamica* to attenuate inflammation was downstream of bacteria-receptor interaction (Tezera *et al.*, 2011).

Therefore the ability of *N. lactamica* to extend attenuation of pathogen-induced innate inflammation induced by the meningitis-causing Gram-negative pathogen *H. influenzae*, as well as the Gram-positive pathogens *S. pneumoniae* and *S. aureus* in nasopharyngeal epithelial cells was investigated.

TLR2 mediated inflammation induced by PAMPs activates the MAPK pathway via ERK1/2 in human airway epithelial cells (Martel, Bérubé & Rousseau, 2013). In mice, TLR2 agonists activate ERK^{MAPK} and p38^{MAPK} pathways leading to induction of IL6 and IL1 β , whereas, JNK^{MAPK} enhanced agonist-induced inflammation (Peroval *et al.*, 2013). Neisserial PorB induces activation of ERK1/2^{MAPK} in murine B cells (MacLeod, Bhasin & Wetzler, 2008) and in transfected HEK cells overexpressing TLR2 (Toussi *et al.*, 2012), whereas the activation of JNK1/2^{MAPK} and p38^{MAPK} have been shown respectively to be involved in invasion and induction of cytokine responses by whole bacteria in human brain endothelial cells (Sokolova *et al.*, 2004). Whether the presence of *N. lactamica* could affect innate inflammation induced by intracellular MAPK pathway agonists was therefore studied.

Previous work by this group identified that *N. lactamica* could attenuate inflammation induced by specific agonists that are known to stimulate inflammation through surface bound receptors, namely TLR4, TNF receptor and IL1 receptor. TLR3 is a receptor found within the host cell cytoplasm bound to endosomes (Alexopoulou *et al.*, 2001) and is therefore not associated with cell surface expression. Activation of TLR3 by viral or synthetic double-stranded ribonucleic acids (dsRNAs) or extracellularly derived RNAs from damaged host cells induces inflammation via MyD88 and TRAF6 in a similar fashion to TLR4 (Lai *et al.*, 2009). Hence it was determined to investigate the effect of *N. lactamica* on TLR3-induced innate inflammation in this study.

3.2 Results

3.2.1 Modulation of innate inflammatory response induced by ERK1/2^{MAPK} and p38^{MAPK} pathway ligands by *N. lactamica*

To assess the effect of the commensal *N. lactamica* on innate inflammatory response in nasopharyngeal epithelial cells induced via activation of the mitogen-activated protein kinase (MAPK) pathways, levels of pro-inflammatory cytokine IL6 secreted from D562 cells were measured using an enzyme-linked immunosorbant assay (ELISA) in the presence and absence of agonists to different arms of the MAPK pathway.

Epithelial cells were incubated with *N. lactamica* at concentrations of 20 and 200 bacteria per epithelial cell (MOI) (Figure 3.1). *N. lactamica* induced IL6 in a dose-dependent manner, and at a high dose (MOI 200) induced modest levels of IL6 (158pg/ml) compared to untreated monolayers (23 pg/ml). Equivalent monolayers were treated with the agonist phorbol 12-myristate 13-acetate (PMA) at 0.1 µg/ml and 1µg/ml to stimulate the extracellular signal-regulated kinase (ERK) 1 and 2 (ERK1/2) MAPK pathway (Figure 3.1a). Both concentrations of PMA poorly induced IL6 secretion (mean 66 and 73 pg/ml respectively), at less than 50% of the levels of IL6 induced by high dose of *N. lactamica*. When PMA at either 0.1 or 1 µg/ml was co-incubated with *N. lactamica* there was a dose-dependent, additive increase in levels of IL6 induced by *N. lactamica*.

To evaluate the effect of *N. lactamica* on inflammation induced via the p38^{MAPK} /JNK^{MAPK} pathway, monolayers were similarly incubated in the presence of the p38^{MAPK} /JNK^{MAPK} agonist anisomycin (ANI) (Figure 3.1b) at concentrations of 2.5 µg/ml and 5 µg/ml. ANI potently induced the secretion of IL6 from epithelial monolayers (means of 1103 and 2568 pg/ml respectively). When ANI was co-incubated with *N. lactamica* there was a dose-dependent, synergistic increase in IL6 secreted from epithelial cells above ANI alone. This result suggests that *N. lactamica* agonises this pathway in the presence of a stimulant.

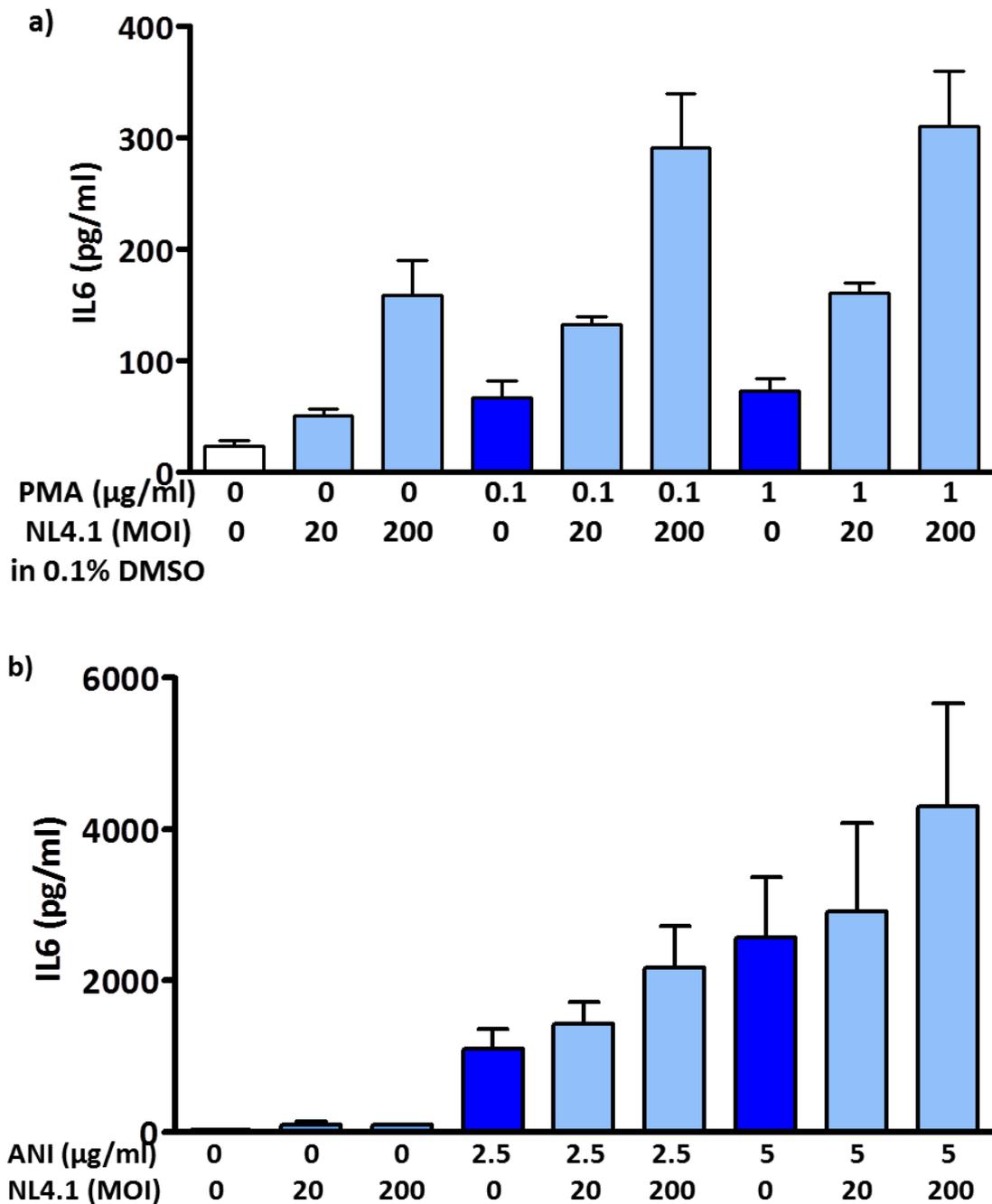


Figure 3.1 The effect of *N. lactamica* on secretion of IL6 induced by ERK^{MAPK} and p38^{MAPK} /JNK^{MAPK} agonists on Detroit 562 epithelial cells

Epithelial monolayers were incubated for 3 hours with the MAPK pathway agonists a) ERK^{MAPK} agonist (PMA) or b) p38^{MAPK} /JNK^{MAPK} agonist (ANI), in the absence and presence of *N. lactamica* (NL4.1). Supernatants were collected and concentrations of IL6 were measured by ELISA following a further 21 hours incubation in gentamicin supplemented media (200 µg/ml). The data represents the mean ± SEM from two independent repeats each replicated in triplicate.

3.2.2 The effect of *N. lactamica* on IL6 induced by the TLR3 agonist poly(I:C)- in Detroit 562 epithelial cells

To identify the effect of *N. lactamica* on innate inflammation induced via the TLR3 pathway, the agonist polyinosinic:polycytidylic acid (poly(I:C)) was used in the absence or presence of *N. lactamica* (Figure 3.2). As shown previously, *N. lactamica* alone induced little IL6 at MOI 2 and MOI 20 with a modest induction of IL6 at MOI 200. Similarly, poly(I:C) at concentration of 0.05 µg/ml and 0.5 µg/ml induced moderate levels of IL6. However, the highest concentration of poly(I:C) (5 µg/ml) significantly increased ($p < 0.001$) levels of IL6 (mean 708 µg/ml) compared to untreated epithelial cells. When 5 µg/ml poly(I:C) was co-incubated with *N. lactamica* at MOI 2 and 20 there was a dose-dependent reduction in levels of IL6, with a significant reduction ($p < 0.001$) in levels of IL6 secreted in the presence of *N. lactamica* at MOI 20.

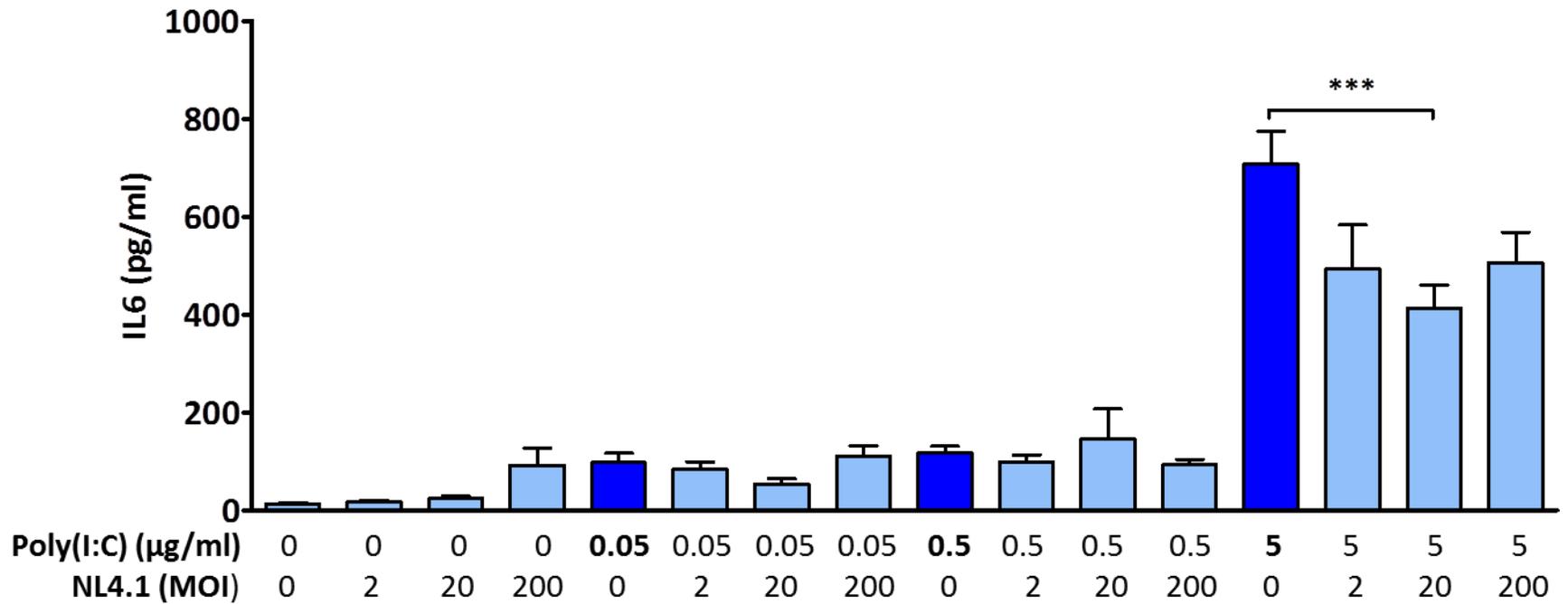


Figure 3.2 The effect of *N. lactamica* on IL6 induced by a TLR3 agonist from nasopharyngeal epithelial cells

D562 nasopharyngeal epithelial monolayers were incubated with the TLR3 agonist polyinosinic:polycytidylic acid (poly(I:C) in the absence and presence of *N. lactamica* (NL4.1) at concentrations (MOI) of 2-200 bacteria per epithelial cell for 3 hours. Epithelial cells were incubated in media containing gentamicin (200 µg/ml) for a further 21 hours, then supernatants were collected and IL6 concentrations were measured by ELISA. The data represents the mean ± SEM from three independent repeats each replicated in triplicate. ***p<0.001.

3.2.3 The effect of *N. lactamica* and *H. influenzae* on the induction of IL6 from Detroit 562 epithelial cells

To extend on previous findings that *N. lactamica* (strain NL4.1) attenuates *N. meningitidis* serogroup B (strain MC58)-induced IL6 stimulation from nasopharyngeal epithelial cells, responses to another Gram-negative pathogen, *H. influenzae*, was also examined for comparison (Figure 3.3).

Stimulation with the commensal *N. lactamica*, at MOI 0.2 to MOI 20 did not significantly induce levels of IL6 above those of epithelial cells untreated with bacteria; although there was a trend towards a weak stimulation of IL6 with increased bacterial concentration. *N. lactamica* at MOI 200 stimulated a modest, but significant increase in IL6 ($p < 0.001$) compared to untreated cells. In contrast, challenge with *H. influenzae* type a (Hia; Figure 3.3a) or type b (Hib; Figure 3.3b) did not induce IL6 compared to untreated epithelial cells. In the presence of *N. lactamica* MOI 200 all concentrations (MOI 0.2-200) of *H. influenzae* type a significantly reduced *N. lactamica*-induced IL6 by approximately 50% ($p < 0.01$). In contrast, only MOI 2 and 20 of *H. influenzae* type b reduced *N. lactamica*-induced IL6, though not significantly. These results suggest that *H. influenzae* type a, but not type b consistently reduces *N. lactamica*-induced innate inflammation. Differences were seen in levels of IL6 between assays (Figure 3.3a and b) with untreated epithelial cells producing 122 $\mu\text{g/ml}$ and 36 $\mu\text{g/ml}$, respectively, and high dose *N. lactamica*-treated epithelial cells producing 392 $\mu\text{g/ml}$ and 133 $\mu\text{g/ml}$, respectively.

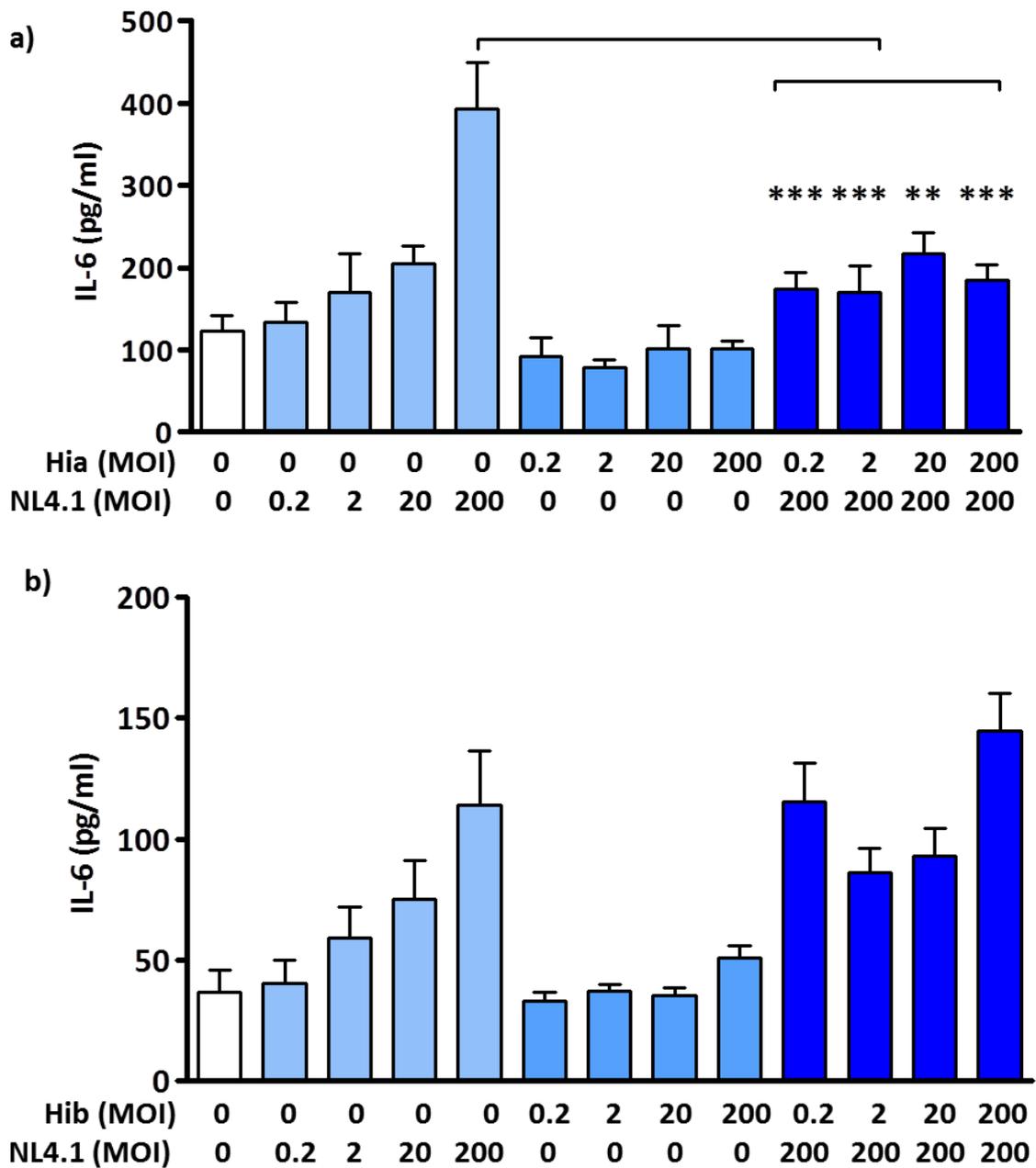


Figure 3.3 Levels of IL6 from Detroit 562 epithelial cells following stimulation with invasive *H. influenzae* and *N. lactamica*

D562 epithelial cells were incubated for 3 hours in the presence of the pathogen *H. influenzae* a) type a strain 620 (Hia) or b) type b strain Eagan (Hib), at a range of concentrations from 0.2 to 200 bacteria per epithelial cell (MOI), with and without *N. lactamica* (NL4.1). Monolayers were further incubated for 21 hours in media containing gentamicin (200 µg/ml). Supernatants were then collected and concentrations of pro-inflammatory IL6 (pg/ml) were measured by ELISA. The data represents the mean ± SEM from three independent repeats each replicated in triplicate. ***p<0.001 **p<0.01.

3.2.4 The effects of *N. lactamica* on *S. aureus*-induced IL6 secretion from Detroit 562 cells

The present study was extended to examine the effect of *N. lactamica* on the innate inflammatory response to Gram-positive pathogens that colonise the nasopharynx in humans. *S. aureus* (strain RN4220) (Figure 3.4a) stimulated monolayers showed a weak dose-dependent increase in secretion of IL6 that became significant ($p < 0.05$) at MOI 200. Co-cultures of *N. lactamica* with *S. aureus* MOI 200 had comparable results to *S. aureus* alone. In comparison, *S. aureus* strain Mu50 (Figure 3.4b) did not induce significant levels of IL6 above untreated epithelial cells and was unable to reduce IL6 induced by *N. lactamica* MOI 200. Once again, differences were seen in levels of IL6 between assays (Figure 3.4a and b) with untreated epithelial cells producing 132 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$, respectively, and high dose *N. lactamica*-treated epithelial cells producing 402 $\mu\text{g/ml}$ and 126 $\mu\text{g/ml}$, respectively.

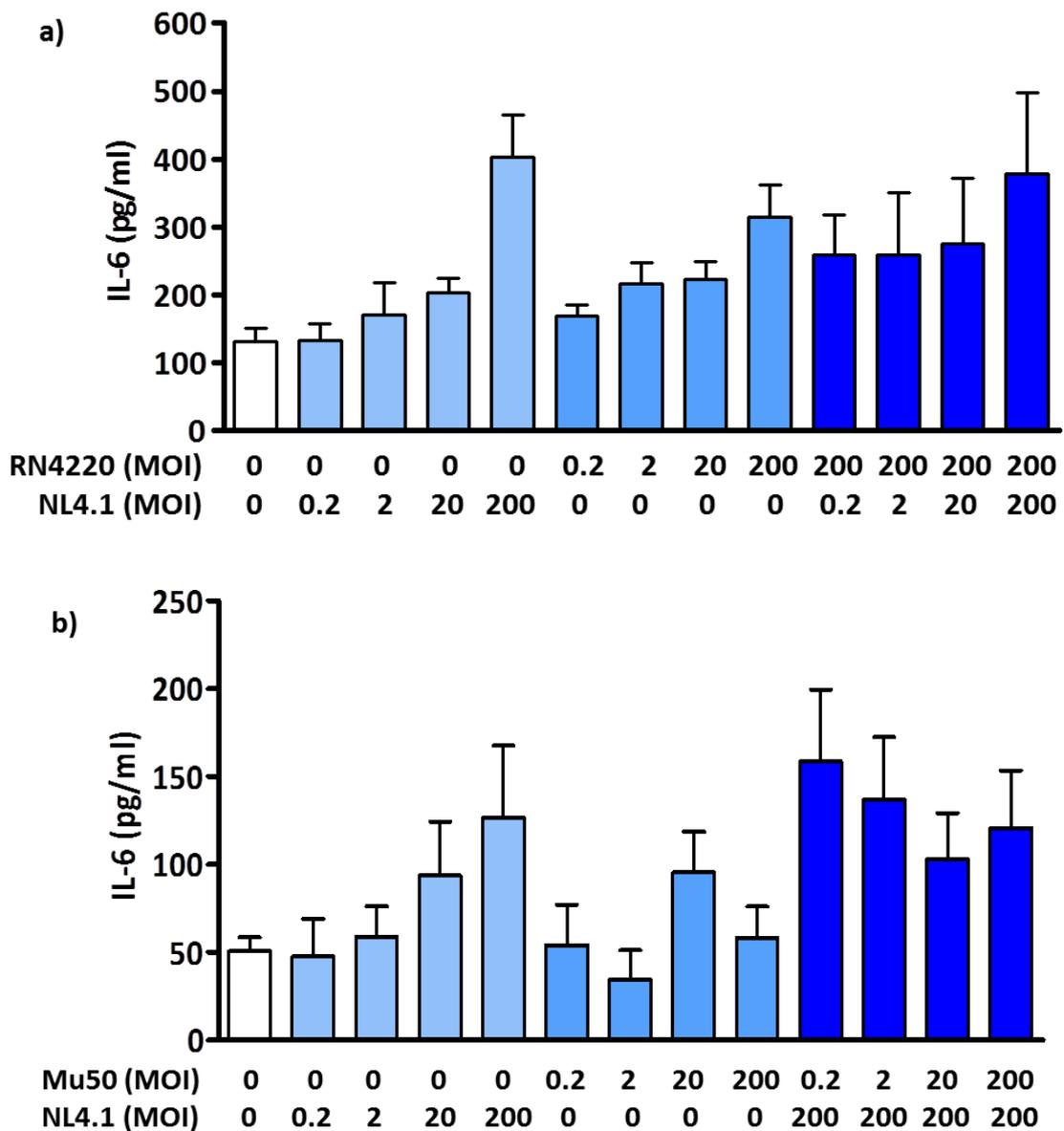


Figure 3.4 Secretion of pro-inflammatory cytokine IL6 following challenge by *S. aureus* in the absence and presence of *N. lactamica*

Epithelial cell monolayers were incubated for 3 hours with the commensal *N. lactamica* (strain NL4.1) or the pathogen *S. aureus* a) strain RN4220 or b) strain Mu50 at a range of concentrations from 0.2 to 200 bacteria per epithelial cell (MOI). Following a 21 hour incubation in media containing a) gentamicin (200 µg/ml) or b) penicillin (100 U/ml) and streptomycin (100 µg/ml), the supernatants were collected and levels of the secreted pro-inflammatory cytokine IL6 (pg/ml) were measured by ELISA. The data represents the mean ± SEM from a) three or b) two independent repeats each replicated in triplicate.

3.2.5 The effects of *N. lactamica* on *S. pneumoniae*-induced innate inflammatory response by Detroit 562 epithelial cells

The study was further extended to examine the effect of *N. lactamica* on the innate inflammatory response to the unrelated Gram-positive upper respiratory tract, human pathogen *S. pneumoniae* (Figure 3.5). Detroit 562 (D562) epithelial cells challenged with *S. pneumoniae* strain D39 (Figure 3.5a) did not secrete levels of IL6 above untreated epithelial cells at any dose of bacteria (MOI 0.2-200). In co-cultures *N. lactamica* at MOI 200 with *S. pneumoniae* (MOI 0.2-20) there was a slight, but not significant reduction in *N. lactamica*-induced IL6. To assess if the innate epithelial response was strain specific, the serotype 14 *S. pneumoniae* isolate Sp14 was used (Figure 3.5b). *S. pneumoniae* at MOI 0.2-20 did not induce IL6, but in contrast, at MOI 200 there was a significant increase in IL6 ($p < 0.001$) compared to untreated epithelial cells. When *S. pneumoniae* was co-cultured at MOI 200 with *N. lactamica*, all doses except MOI 2 of *N. lactamica* significantly reduced *S. pneumoniae*-induced IL6.

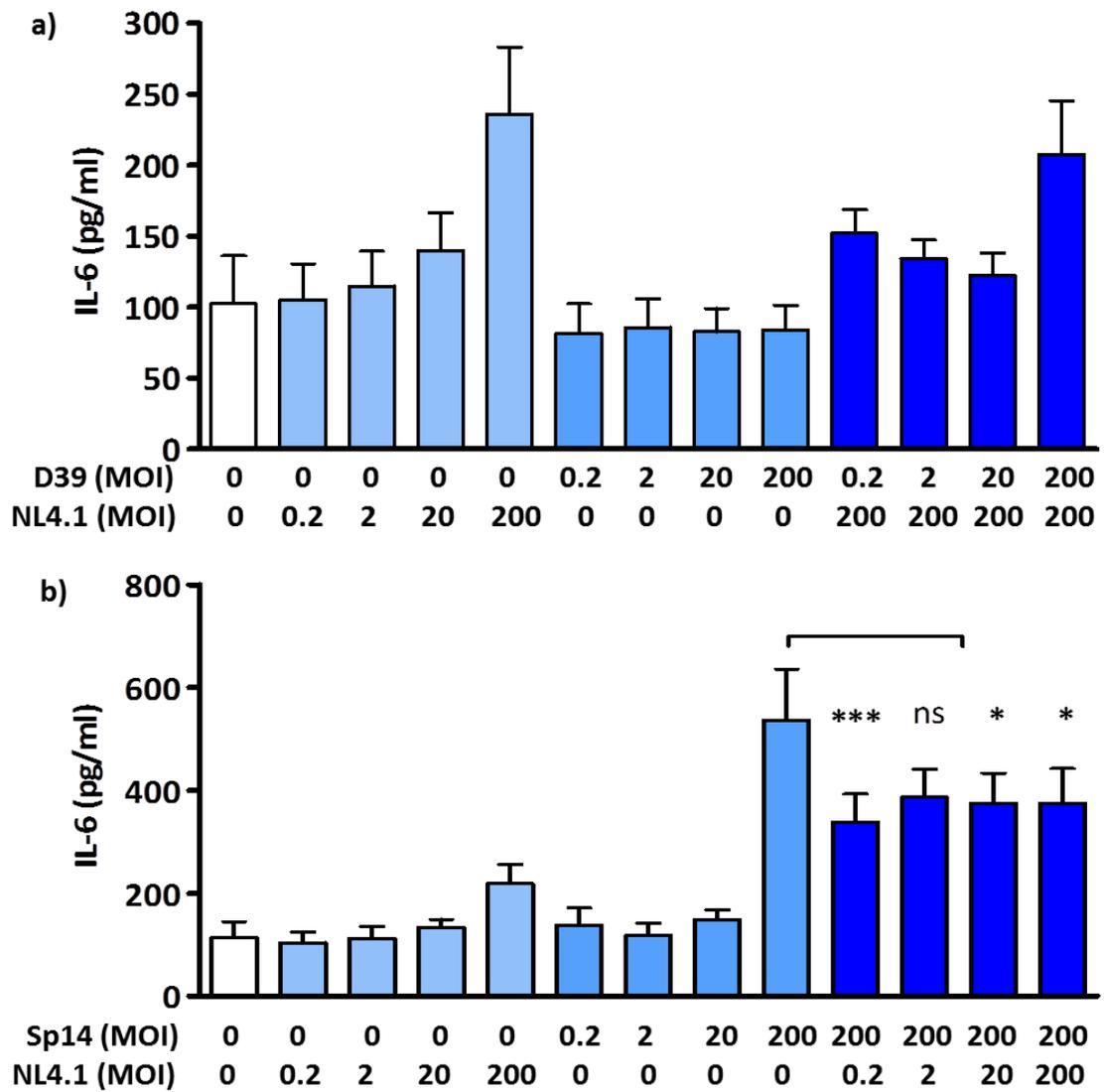


Figure 3.5 IL6 secretion following challenge with pathogenic *S. pneumoniae* in the absence or presence of *N. lactamica*

Epithelial cell monolayers were challenged for 3 hours with *S. pneumoniae* a) strain D39 or b) isolate Sp14 in the presence or absence of *N. lactamica* (NL4.1) at 0.2 to 200 bacteria per epithelial cell (MOI). Epithelial monolayers were further incubated for 21 hours in media containing gentamicin (200 µg/ml). Supernatants were collected at 24 hours and levels of the pro-inflammatory cytokine IL6 (pg/ml) were measured by ELISA. The data represents the mean ± SEM from three independent repeats each replicated in triplicate. ***p<0.001 *p<0.05 ns= non-significant.

To further assess the induction of innate inflammation by *S. pneumoniae* isolate Sp14 and attenuation by *N. lactamica*, the secretion of pro-inflammatory cytokine TNF α (Figure 3.6a), and the chemokine IL8 (Figure 3.6a) secreted from nasopharyngeal epithelial cells was examined. Neither untreated nor *N. lactamica* alone treated cells induced detectable levels of TNF α in these experiments. In contrast, *S. pneumoniae* at MOI 200 strongly induced TNF α secretion from epithelial cells (91 pg/ml; Figure 3.6b). When epithelial cells were co-incubated with *S. pneumoniae* and *N. lactamica*, all concentrations of *N. lactamica* except MOI 0.2 significantly reduced *S. pneumoniae*-induced TNF α secretion by 40% or more ($p < 0.05$).

Similarly, *S. pneumoniae* at MOI 200 strongly induced IL8 that was significantly increased compared to untreated epithelial cells (mean 21 ng/ml and 3 ng/ml respectively; $p < 0.01$; Figure 3.6b). However, there was no significant change in the levels of *S. pneumoniae*-induced IL8 by *N. lactamica* despite up to a 40% reduction in with MOI 0.2.

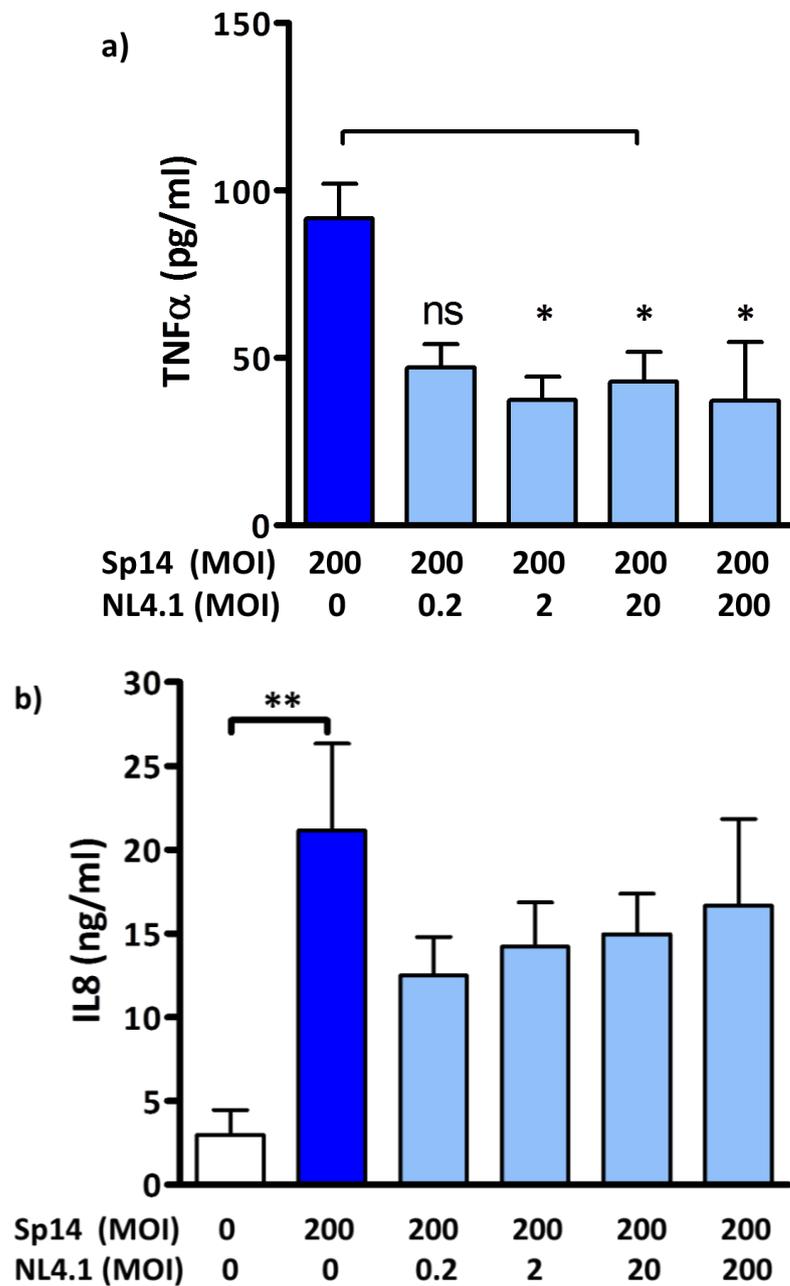


Figure 3.6 *N. lactamica* mediated suppression on *S. pneumoniae*-induced cytokine and chemokine secretion from Detroit 562 epithelial cells

Epithelial cell monolayers were incubated for 3 hours with the commensal *N. lactamica* (NL4.1) at MOI 0.2 to 200 in the absence or presence of *S. pneumoniae* (Sp14) at MOI 200. Following a 21 hour incubation in media containing gentamicin (200 µg/ml) the supernatants were collected and levels of the secreted pro-inflammatory a) cytokine TNFα (pg/ml) or b) chemokine IL8 (ng/ml) were measured by ELISA. The data represents the mean ± SEM from three independent repeats each replicated in triplicate. **p<0.01 *p<0.05 ns=non-significant.

3.3 Discussion

It has previously been demonstrated that *N. lactamica* (strain NL4.1) suppresses inflammatory cytokine secretion from human nasopharyngeal epithelial cells stimulated with *N. meningitidis* challenge. Agonists that stimulate inflammation through TLR2, TNF receptor or IL1 receptor have shown a similar suppression (Tezera *et al.*, 2011). In the experimental model used (medium containing 2% FBS), LPS poorly induces cytokine secretion suggesting TLR4 cannot be optimally stimulated.

N. meningitidis-induced TNF α secretion in cell culture models has been implicated in the autocrine stimulation of IL6 (Lapinet *et al.*, 2000). However, TNF α is poorly induced (≤ 30 pg/ml) by *N. meningitidis* relative to the levels (>1 ng/ml) required to induce IL6 secretion from epithelial cells (Tezera *et al.*, 2011). *N. meningitidis* stimulates inflammation via the activation of TLR2 and TLR4 in peripheral blood mononuclear cells (PBMCs), and to a lesser extent via TLR9 (Mogensen *et al.*, 2006a). It was therefore hypothesised that the main TLR involved in the model used within this study was TLR2.

N. lactamica suppresses inflammation induced by the TLR2 agonist PAM3Cys (Tezera *et al.*, 2011). Activation of TLR2 induces downstream activation of MAPK pathways in a diverse range of cell types (Peroval *et al.*, 2013). Activation of MAPK pathways are involved in induction of inflammatory cytokine secretion from (p38^{MAPK} or ERK^{MAPK}), and invasion by (JNK^{MAPK}), *N. meningitidis* into host cells (Sokolova *et al.*, 2004; MacLeod, Bhasin & Wetzler, 2008). However, the hypothesis that *N. lactamica* could suppress inflammation induced by intracellular p38^{MAPK} or ERK^{MAPK} pathway agonists was not supported in these experiments. ERK^{MAPK} activation appears to poorly stimulate secretion of IL6 in Detroit 562 (D562) epithelial cells when induced by an ERK^{MAPK} agonist (PMA). It would therefore be unlikely to be involved in *N. meningitidis*-induced inflammation, which is consistently induced to a far greater extent. However this does not rule-out its possible involvement in other *N.*

meningitidis-mediated events in epithelial cells, such as invasion or activation of host cell death.

Anisomycin, in contrast to PMA, potently induced IL6 secretion from epithelial cells suggesting a more active pathway for induction of inflammation in the cell line used. However, this is not only suggestive of the involvement of p38^{MAPK} in IL6 secretion as anisomycin has been found to also induce JNK^{MAPK} (Ogawa *et al.*, 2004) and ERK^{MAPK} (Shafer & Slice, 2005) although to lesser extents, depending on the cell line or type used. Anisomycin could be interpreted as being a broad activator of the MAPK pathway, which *N. lactamica* was unable to suppress in epithelial cells when stimulated, and indeed appeared additive to either anisomycin or *N. lactamica* alone. This suggested that *N. lactamica* is unlikely to suppress inflammation induced via the MAPK pathway in this model regardless of any possible involvement of these pathways in *N. meningitidis* derived induction. If further investigation of the involvement of the MAPK pathways was warranted, then the use of specific inhibitors during challenge by pathogen or agonist, or analysis of the phosphorylation status of the MAPK components would be recommended.

As well as surface exposed receptors such as TLR2 and TLR4, D562 cells produce a functional form of the intracellular receptor TLR3 (Matijevic, Marjanovic & Pavelic, 2009; Rydberg *et al.*, 2009), which is stimulated by intracellular dsRNA typically from viruses or neighbouring necrotic host cells (Lai *et al.*, 2009). Activation of TLR3 by dsRNA, such as the synthetic ligand poly(I:C), can induce both host cell death (Rydberg *et al.*, 2009; McAllister *et al.*, 2013) and inflammation (Matijevic, Marjanovic & Pavelic, 2009).

As found in a previous study, poly(I:C) induced significant levels of IL6 in D562 cells at the same concentration used here (Matijevic, Marjanovic & Pavelic, 2009). However, concentrations of IL6 in this experiment were several times higher than reported previously. This discrepancy may be explained by a higher concentration of cells in this

experiment to that used in the previous study (1×10^5 cells compared to 2.5×10^4 cells per well, respectively).

In this study there was a strong correlation between the concentration of *N. lactamica* and the inhibition in inflammation induced through TLR3. This could suggest the involvement of an intracellular mechanism of *N. lactamica*-induced suppression of inflammation as has been put forward (Tezera *et al.*, 2011). However, the lipoteichoic acid (LTA) of the Gram-positive bacteria *S. epidermidis* has been demonstrated to reduce TLR3-mediated inflammation by interacting with surface TLR2 on keratinocytes (Lai *et al.*, 2009, 2010). This is perhaps supported by the finding that strong activation of TLR2 activates the toll-like signalling molecule TIRAP (MAL), but is not dependent on the activation of TIRAP (MAL). However, activation of TIRAP (MAL) potentially by TLR2, suppresses the induction of IL6 stimulated by the activation of TLR3 (Kenny *et al.*, 2009).

The hypothesis that *N. lactamica* inhibits inflammation via an inhibitory signal through TLR2 may be supported by the finding that purified PorB from *N. lactamica* physically associates with TLR2 (Liu *et al.*, 2010) and *N. lactamica* inhibits inflammation induced through TLR2 (Tezera *et al.*, 2011). This is in contrast to *N. meningitidis* PorB that associates with a different binding site on TLR2 receptor (Massari *et al.*, 2006; Liu *et al.*, 2010). An anti-TLR2 antibody (clone TL2.1) can also inhibit TLR2 activation (Lien *et al.*, 1999) possibly by binding to another binding site on TLR2 receptor to pro-inflammatory ligands (Tsukamoto *et al.*, 2012). There could also be a reduction in surface TLR2 by shedding of the receptor as found with TNF receptor (Deghmane *et al.*, 2009) or internalisation, although this hypothesis would not explain the suppression of inflammation induced through other receptors (Tezera *et al.*, 2011) unless it was a global mechanism.

An unexpected finding in this study was the lack of induction of inflammation, as monitored by IL6 secretion, induced by common pathogens that reside in the upper respiratory tract of humans. Although *H. influenzae* type b (Hib) strain Eagan induces IL6 in human

meningioma cells (Fowler *et al.*, 2004), neither this strain nor the *H. influenzae* type a (Hia) strain 620 was able to induce IL6 secretion from nasopharyngeal cells in this study. This may be explained by poor association of encapsulated *H. influenzae* with mucosal epithelial cells (Read *et al.*, 1992) or by paracellular invasion of *H. influenzae* with epithelial monolayers (van Schilfgaarde *et al.*, 1995), potentially avoiding inflammatory receptors localised to the apical surface.

In contrast, the lack of induction of IL6 by *S. pneumoniae* strain D39 observed by Fowler *et al.* (2004) was supported in this study. As with *H. influenzae* this may be explained by the poor adherence (Gould & Weiser, 2002) and paracellular invasion (Beisswenger *et al.*, 2007) of strain D39 to D562 cells, though these hypotheses would require further evidence to support them.

For the first time herein *N. lactamica* was demonstrated to suppress inflammation induced by the unrelated, Gram-positive *S. pneumoniae* isolate Sp14. Although this isolate has not been characterised, the species is known to induce inflammation through both TLR2 and TLR4. However induction through TLR4 is dependent on the expression of pneumolysin, which may or may not be present in this isolate (Beisswenger, Lysenko & Weiser, 2009). Although further work is required to characterise the means by which *N. lactamica* is able to suppress *S. pneumoniae*-induced inflammation, this study does demonstrate that the ability of *N. lactamica* to suppress inflammation is not limited to one strain or species of pathogen.

Chapter 4

Characterisation of bioluminescent *N. meningitidis* strain MC58lux

4.1 Introduction

A key characteristic of pathogenic neisseriae is the ability to enter host cells. Brief exposure of cells with an antibiotic such as colistin, kanamycin or gentamicin (Tang *et al.*, 1993), that does not enter host cells can be used to exclude extracellular bacteria. A conventional method to determine the numbers of internalised or invading bacteria into host cells is to count viable bacterial colonies from lysed host cells (St Geme & Falkow, 1990). To be able to measure any effect on invasion by *N. meningitidis* by commensal *Neisseria* spp., it is necessary first to distinguish between colonies derived from each species. Due to the strong similarity between colony morphology of both species cultured on HBHI recovery medium it would be necessary to modify this method. It is possible to distinguish between *N. meningitidis* and *N. lactamica* by introducing 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (xgal) into the media (Bennett *et al.*, 2005) producing blue/green *N. lactamica* colonies compared to off-white *N. meningitidis* colonies (Figure 4.1), as xgal is a lactose derivative that is metabolised by *N. lactamica*, but not *N. meningitidis* (Hollis, Wiggins & Weaver, 1969). However, this method cannot be used to differentiate *N. meningitidis* from the commensals *N. polysaccharea* and *N. cinerea*, and subsequent identification of colonies by other tests, such as antibiotic susceptibility (Margolis, Yates & Levin, 2010) or colony immunoblots (Serino & Virji, 2000) is impractical due to cost and time.

The bacterial derived *luxCDABE* operon has been utilised to construct light-emitting (bioluminescent) bacteria producing the luciferase enzyme, which do not require specific exogenous substrates to produce light. Bioluminescent transformed bacteria have previously been utilised as biosensors for rapid, sensitive, real-time measurement of the effect of antibiotics on bacterial growth (Salisbury *et al.*, 1999; Beard *et al.*, 2002), dissemination of invasive bacteria within animal models (Contag *et al.*, 1995; Sjölander &

Jonsson, 2007) and invasion by bacteria in cell culture models (Forde, Parton & Coote, 1998; Nelson *et al.*, 2003; Flentie *et al.*, 2009).

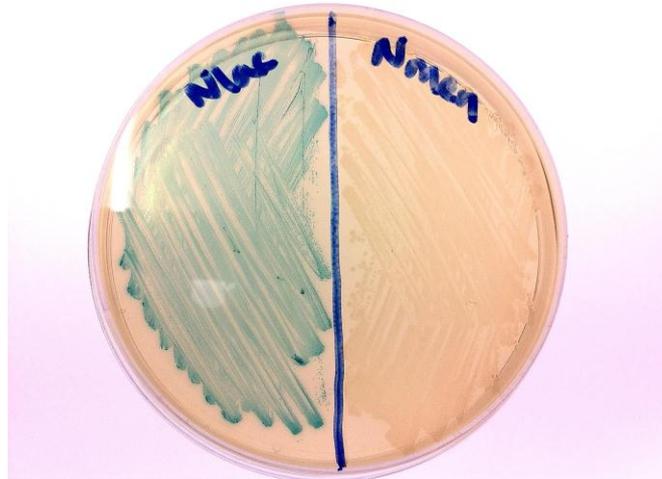


Figure 4.1 Differentiation of *N. lactamica* and *N. meningitidis* on HBHI agar containing 5-bromo-4-chloro-indolyl- β -D-galactopyranoside

N. lactamica (Nlac) and *N. meningitidis* (Nmen) were grown overnight (16-18 hours at 37°C in 5%CO₂) on HBHI agar then a single colony was streaked onto one half of a HBHI agar plate containing 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (xgal; 100 μ g/ml) and incubated for 24 hours (at 37°C in 5%CO₂). *N. lactamica* colonies can be seen to have developed a blue/green colour, compared to *N. meningitidis* that remain uncoloured.

Transformation of *N. meningitidis* with a constitutively active bioluminescent operon to produce a bioluminescent reporter, has previously been performed for *N. meningitidis* serogroup C strain FAM20 (Sjölinder & Jonsson, 2007) and a non-encapsulated derivative of serogroup A strain C751 (unpublished Robinson, 2006). It was determined that construction of a stably transformed *N. meningitidis* serogroup B organism, could allow rapid determination of invasion by *N. meningitidis* in the presence of otherwise phenotypically similar commensal *Neisseria* spp. in real-time, without utilising viable counts. One mechanism to achieve this utilises the natural competency of *Neisseria* and its recognition of a 10 base pair DNA uptake sequence (DUS) that increases the transformation frequency

of *Neisseria* when present on exogenous DNA (Elkins *et al.*, 1991). In this case the pLKMp plasmid containing the *luxCDABE* operon previously developed and utilised by Sjölander & Jonsson (2007) was used to construct a *N. meningitidis* serogroup B organism (reference strain MC58). Once constructed the genetic and phenotypic characteristics of transformants need to be confirmed and compared to the untransformed parent strain.

4.2 Results

4.2.1 Bioluminescent transformation of *N. meningitidis*

N. meningitidis serogroup B (strain MC58) was transformed using the pLKMp plasmid containing the *luxCDABE* operon by natural transformation with the spotting method (section 2.2.5) to produce the bioluminescent derivative MC58lux. As expected, the resulting colonies were confirmed as a single culture of Gram-negative cocci when visualised by light microscopy following Gram-staining (Figure 4.2a). Transformants were further verified as *N. meningitidis* by confirming metabolism specifically of maltose and glucose. This was visualised by a red to yellow colour change of phenol red in sugar agar slopes containing 1% maltose or 1% glucose (Figure 4.2c), with no colour change observed for 1% lactose or 1% sucrose when compared to negative controls containing no bacteria (Figure 4.2b). Similar results were obtained for both the transformed (MC58lux) and parent strain (MC58). As future assays were to use gentamicin for extracellular killing of *N. meningitidis*, a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay was conducted. The parental MC58 and the bioluminescent derivative MC58lux did not differ in sensitivity to gentamicin with a MIC of ≤ 12.5 $\mu\text{g/ml}$ and an MBC of ≤ 25 $\mu\text{g/ml}$.

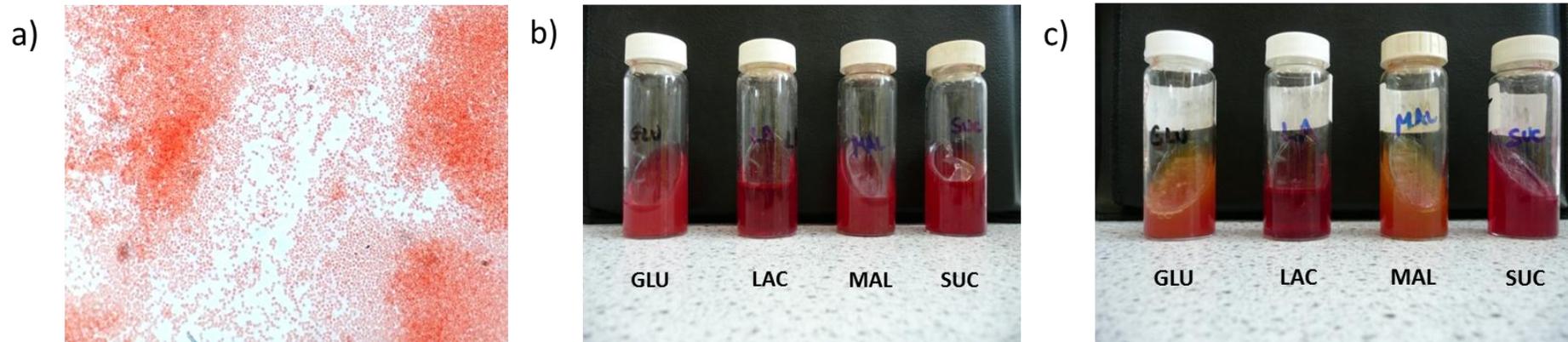


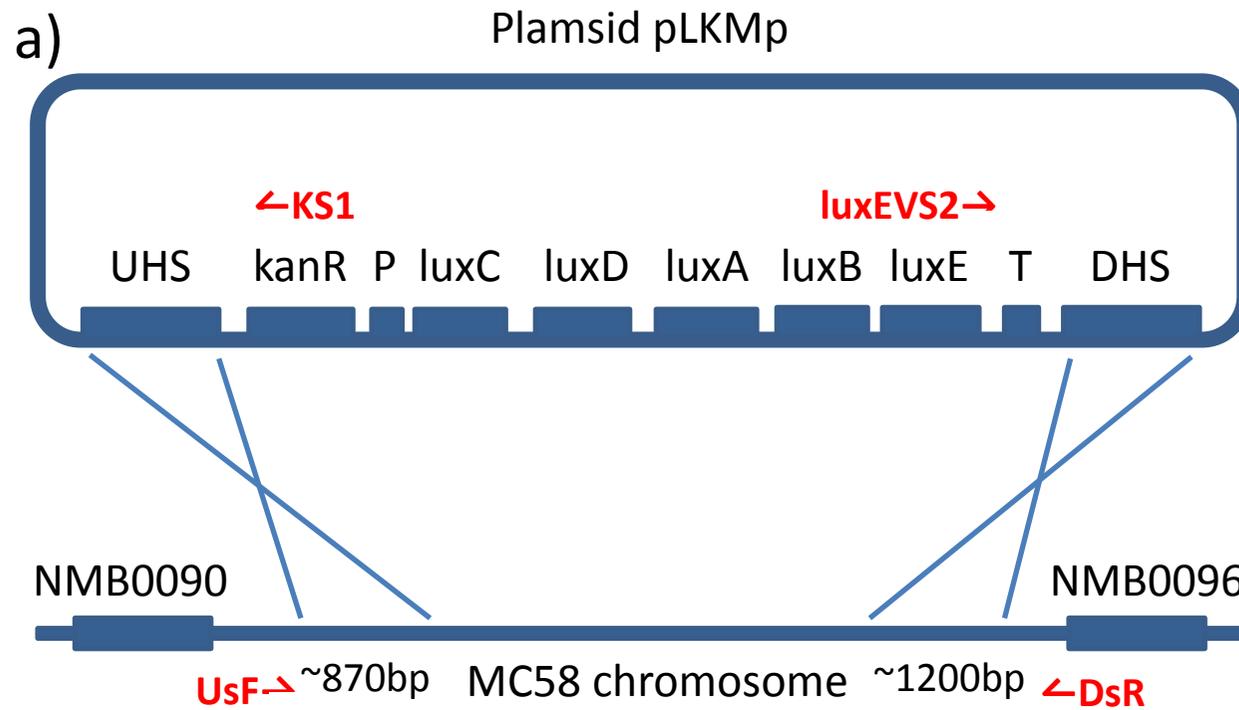
Figure 4.2 Gram-stain and results of sugar oxidation test to identify bioluminescent transformants as *N. meningitidis*

Bioluminescent *N. meningitidis* strain MC58lux was cultured overnight (16-18 hours) on HBHI agar containing kanamycin (150µg/ml). Single colonies were (a) Gram-stained and imaged at x100 magnification with oil immersion showing Gram negative cocci. Agar slopes containing 1% glucose (GLU), 1% lactose (LAC), 1% maltose (MAL) or 1% sucrose (SUC) were incubated overnight at 37°C (b) without bacteria (control) or (c) with streaking of a single colony of MC58lux. Agar slopes containing the parental strain MC58 were used as a positive control and showed identical results to transformed strain MC58lux.

4.2.2 Chromosomal integration of *luxCDABE* cassette into *N. meningitidis* strain MC58

Integration and positioning of the *luxCDABE* operon-containing cassette from plasmid pLKMp was confirmed within the chromosomal DNA of the bioluminescent transformed *N. meningitidis* strain MC58lux by PCR (section 2.2.8).

The hypothesised insertion site was predicted from corresponding sequences within the genome of strain MC58 (NCBI accession # NC_003112.2) to the upstream (UHS) and downstream (DHS) homologous sequence from non-coding regions of *N. meningitidis* strain FAM20. The UHS and DHS represent two complementary sequences in the region between genes NMB0090 and NMB0096 in MC58 genome. To confirm the position within the chromosome of MC58lux, the forward primer UsF and reverse primer DsR corresponding to DNA sequences from the chromosome of MC58lux either side of the hypothesized insertion site were used. To confirm integration of the *luxCDABE* operon, the reverse primer KS1 and the forward primer luxEVS2 corresponding to sequences within the bioluminescent cassette from plasmid pLKMp were used (Figure 4.3a). The PCR product of primers UsF and KS1 was hypothesised to be approximately 870bp and the product of primers of luxEVS2 and DsR were hypothesised to be approximately 1200bp. Both PCR fragments were able to be amplified from the bioluminescent derivative MC58lux and were absent in the non-bioluminescent parent strain MC58 (Figure 4.3b). A DNA ladder with known sequence lengths was used to confirm the size of the PCR fragments as approximately 870bp and 1200bp, respectively. PCR confirmed the integration of the *luxCDABE* plasmid pLKMp by homologous recombination into the genome of strain MC58lux between the genes designated NMB0090 and NMB0096.



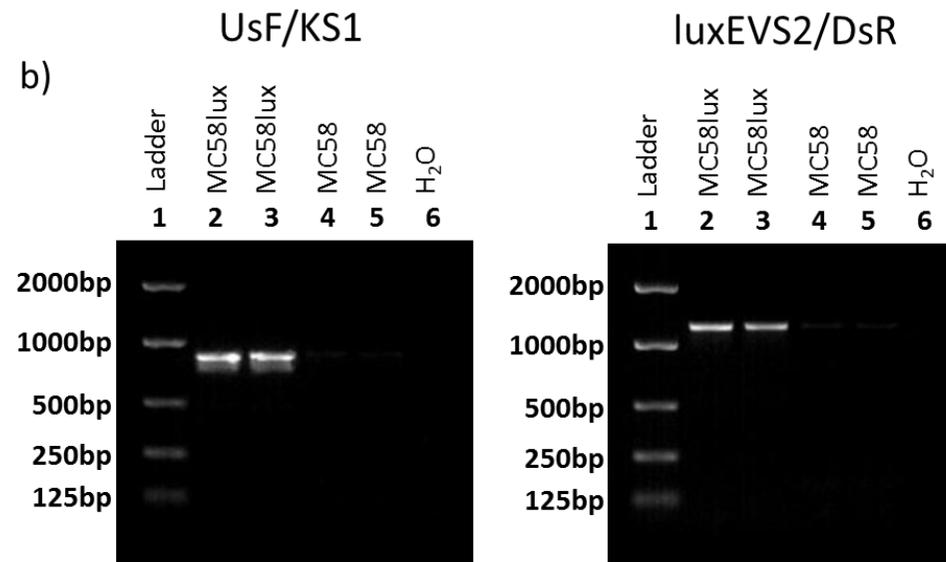


Figure 4.3 Chromosomal integration of the bioluminescent cassette from plasmid pLKMp into the genome of *N. meningitidis* strain MC58

The bioluminescence encoding lux cassette of plasmid pLKMp contains (a) the *luxCDABE* operon, flanked by the *Neisseria* specific *porA* promoter (P) and *gapdh* terminator (T) sequences to allow constitutive luciferase expression. The kanamycin resistance gene, *kanR*, allows antibiotic selection of transformed bacteria. The upstream (UHS) and downstream homologous sequences (DHS) represent two complementary sequences in the region between genes NMB0090 and NMB0096 in the MC58 genome. (b) DNA was extracted from overnight cultures of *N. meningitidis* strain MC58 and MC58lux and the primer sets (UsF/KS1 or luxEVS2/DsR) were used to amplify sequences from the transformed strain MC58lux (lane 2 and 3). Strain MC58 was used as comparison (lane 4 and 5) and nuclease-free H₂O was used as a non-specific control (lane 6). A DNA ladder with known sequence lengths was included to confirm the size of PCR products (lane 1). Images are representative of two independent repeats.

4.2.3 Correlation between bioluminescence, optical density and viable bacterial counts for *N. meningitidis* strain MC58lux

To determine the relationship between bioluminescence, optical density and viable bacterial counts, *N. meningitidis* strain MC58lux was suspended in medium 199 (M199). This medium is suitable for use in bacteria-epithelial cell co-culture assays. To compare bioluminescence with conventional bacterial enumeration methods, optical density was determined by spectrophotometric readings of samples at 600nm (OD_{600}) with concurrent enumeration of viable bacterial colony counts (cfu/ml). MC58lux showed a strong correlation between optical density and viable counts (R^2 0.99; Figure 4.4a), which was accurate between a range of approximately 0.1 to 1.0 (OD_{600}). Optical density was compared to MC58lux light output, measured in relative light units (RLU) (Figure 4.4b). Strong correlation was seen with optical densities between approximately 0.05 and 0.3 (OD_{600}), and light output between approximately 1×10^5 and 8×10^5 RLU (R^2 0.96). The relationship between light output from MC58lux and viable bacteria similarly showed correlation (R^2 0.91; Figure 4.4c) within the range 1×10^4 to 7×10^4 RLU, continuing to approximately 3.5×10^{10} cfu/ml.

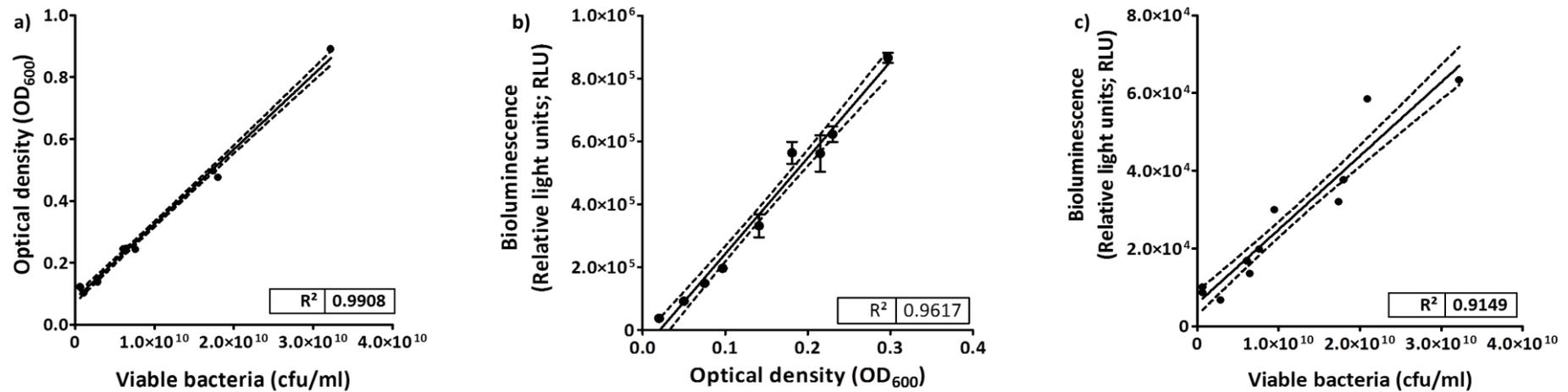


Figure 4.4 Correlation between optical density, viable bacteria and bioluminescence from *N. meningitidis* strain MC58lux

The bioluminescent *N. meningitidis* strain MC58lux was grown overnight (16-18 hours) on HBHI agar containing kanamycin (150 µg/ml) and single colonies were initially suspended in PBS-B. From this stock suspension serial dilutions were prepared in medium M199 (without phenol red). Each suspension the optical density was measured at a wavelength of 600nm (OD₆₀₀) using a spectrophotometer (SP50). Viable bacterial concentrations were determined by colony counts from overnight cultures on HBHI agar and expressed as colony forming units per ml (cfu/ml). Bioluminescence from the bacterial suspension was measured using a plate reader (Fluostar Optima). Correlation between a) optical density and viable bacteria, (b) bioluminescence and optical density, or (c) bioluminescence and viable counts were assessed. Each point represents the mean ±SEM of triplicate values gathered from three independent repeats. Solid lines represent the line of best fit, whilst dotted lines represent the 95% confidence interval.

4.2.4 Comparison of growth between *N. meningitidis* strain MC58 and bioluminescent derivative MC58lux measured by optical density

To identify whether integration of the *luxCDABE* operon into the genome of *N. meningitidis* incurred a fitness cost, growth curves were generated for both *N. meningitidis* parental strain (MC58) and bioluminescent derivative (MC58lux) (Figure 4.5). MC58lux consistently produced a higher optical density than MC58 in both media used. To correct for differing quantities in the starting culture, the growth rate was compared during the linear part of each growth curve, between 4-12hr in M199 medium (Figure 4.5a) and 4-18hr in HBHI broth (Figure 4.5b). In M199 the growth rate of MC58 ($0.031 \pm$ standard deviation of 0.001) and MC58lux ($0.032 \pm$ SD of 0.002) did not differ significantly when compared by unpaired student t-test. Similarly, in HBHI broth the growth rate of MC58 ($0.028 \pm$ SD of 0.0017) and MC58lux ($0.025 \pm$ SD of 0.0013) did not significantly deviate thus demonstrating comparable growth of both parental and transformed *N. meningitidis* in the media tested.

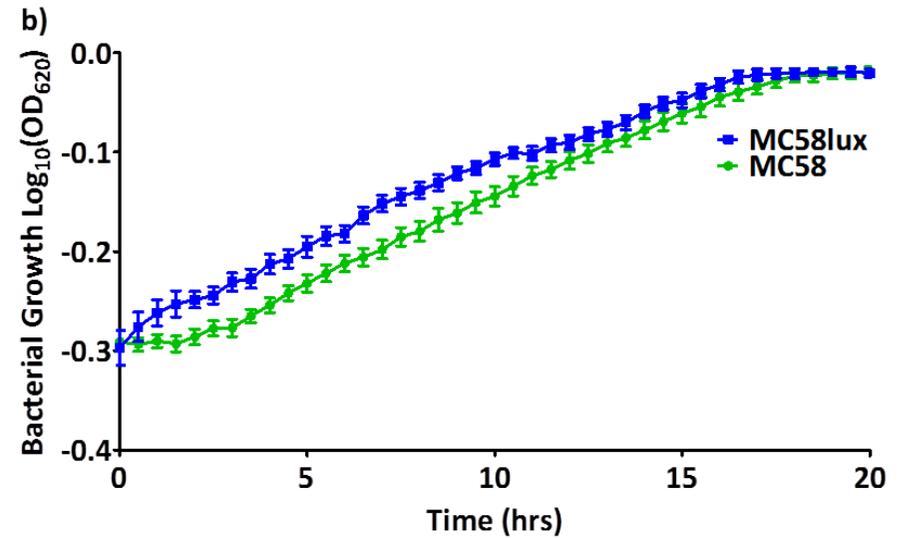
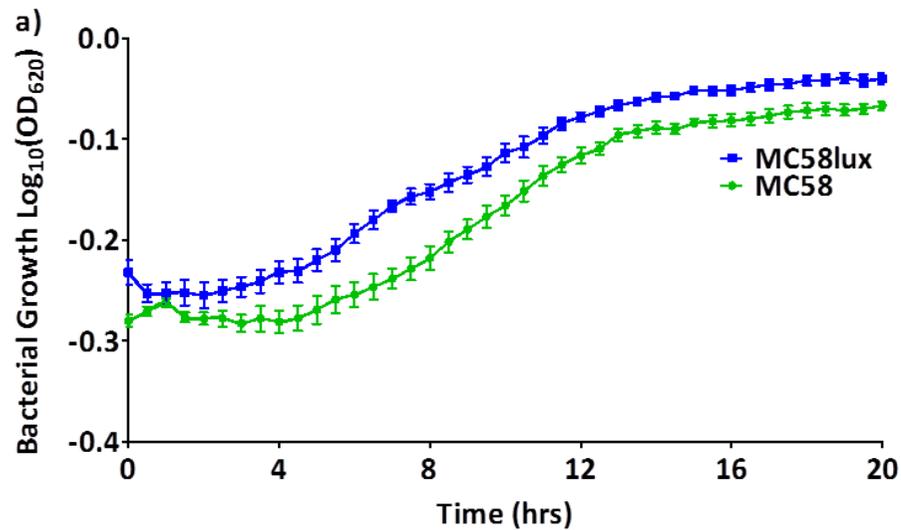


Figure 4.5 Growth of *N. meningitidis* strains MC58 and the bioluminescent derivative MC58lux

N. meningitidis strains MC58 and MC58lux were grown overnight (16-18 hours) on HBHI agar in the absence or presence of kanamycin (150 µg/ml), respectively, and single colonies were suspended in PBS-B. Approximately 1×10^5 cfu/ml of bacteria was added to each well in either (a) M199 (without phenol red) or (b) HBHI broth. The optical density was read at 620nm (OD₆₂₀) for each well every 30 minutes using an optical plate reader (Fluostar, Optima). Each half-hourly time point represents the mean \pm SEM from three independent repeats each replicated in triplicate.

4.2.5 Comparison of association and invasion of D562 cells by *N. meningitidis* strains MC58 and MC58lux

To evaluate the effect of the bioluminescent transformation of *N. meningitidis* on bacterial interaction with nasopharyngeal epithelial cells, association and invasion assays of Detroit 562 (D562) epithelial monolayers were performed in order to compare the *N. meningitidis* parental strain (MC58) with the bioluminescent transformant (MC58lux; Figure 4.6).

Strain MC58 and MC58lux were added to separate monolayers as single cultures. The concentration of the initial inoculum (Time 0 hr) added to epithelial monolayers was shown to be comparable between MC58 (3.6×10^7 cfu/monolayer) and MC58lux (3.2×10^7 cfu/monolayer). Following 3 hour incubation, the growth (Time 3 hr) of the two cultures showed a slight, but not significant increase for MC58 (1.3×10^8 cfu/monolayer) compared to MC58lux (9.2×10^7 cfu/monolayer). To determine association of bacteria with epithelial monolayers, each well was washed vigorously by pipetting and viable counts were performed using saponin lysed monolayers (section 2.2.20). At 3 hours post-infection there was a strong association of both MC58 and MC58lux with D562 cells (2.7×10^7 cfu/monolayer for each) showing no significant difference between parent and transformed strains. Invasion of D562 cells by *N. meningitidis* was assessed by the gentamicin protection assay. There was a slight, but not significant reduction in invasion by MC58lux (3.2×10^4 cfu/monolayer) compared to MC58 (4.2×10^4 cfu/monolayer). Overall there was no reduction in the ability of *N. meningitidis* strain MC58 to associate with, or invade epithelial monolayers following integration of the *luxCDABE* cassette.

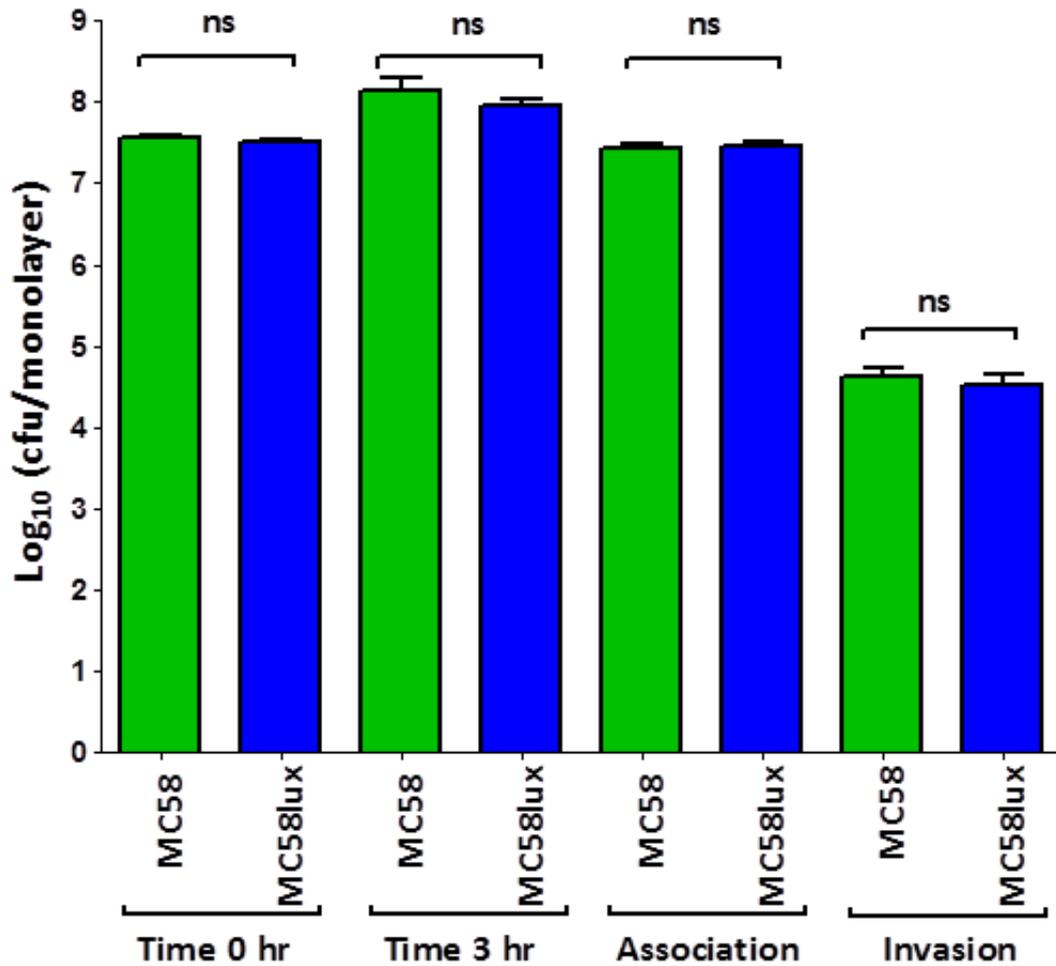


Figure 4.6 Association and invasion of *N. meningitidis* strain MC58 or MC58lux with monolayers of Detroit 562 epithelial cells

Confluent monolayers of D562 cells were challenged with *N. meningitidis* (MOI 200) for 3 hours. Levels of associated bacteria were measured by viable counting. Invasion was assessed using the gentamicin protection assay. As controls viable counts were made at 0 hours and at 3 hours. Data represents mean \pm SEM from three independent repeats each replicated in triplicate. ns= non-significant.

4.3 Discussion

The *N. meningitidis* serogroup B (MenB) reference strain MC58 was transformed in this study by homologous recombination using the pLKMp plasmid (Sjölinder & Jonsson, 2007) to create the derivative MC58lux. The transposable element of plasmid pLKMp contains the *luxCDABE* operon encoding the luciferase enzyme and fatty acid reductase complex required for the production of bioluminescent light in metabolically active organisms (Meighen, 1991). This is preceded by the constitutively expressed *porA* promoter sequence from *N. meningitidis* strain FAM20 that facilitates increased bioluminescent light output (Sjölinder & Jonsson, 2007). The transposable element was inserted into the genome of MC58 within a putative gene sequence as confirmed by PCR (Figure 4.3).

Optical density of bacterial cultures has long been established as a reliable microbiological method for rapid determination of bacterial numbers when correlated with viable counts (Koch, 1961). Bioluminescence has since been demonstrated to have strong correlation with viable counts and optical density when constitutively expressed (Nelson *et al.*, 2003). This was also demonstrated here with the newly constructed MC58lux (Figure 4.4), although there was a greater correlation between optical density and viable counts than between bioluminescence and viable counts. There was still a strong correlation between the latter demonstrating bioluminescence is a viable reporter for the presence of MenB as utilised in further invasion assays in the presence of otherwise largely indistinguishable *Neisseria* commensal spp. (chapter 5).

Following the insertion of new genomic DNA and subsequent expression of novel protein complexes the transformed MenB was compared to the parent strain for both growth kinetics and interactions with host cells by conventional methods. Whether in medium for D562 epithelial cell co-cultures (M199) or medium for bacterial growth (HBHI broth), the growth rate of MC58lux did not deviate from parental MC58 (Figure 4.5). However, MC58lux consistently gave higher optical density values suggesting either consistently

higher quantities of bacteria in starting cultures or an increased mass of the bacterium. Perhaps this may be due to the addition of protein complexes within the bioluminescent construct. However, strongly comparable association and invasion of D562 cells, a process involving a diverse range of bacterial ligands and signalling pathways, suggests no lack of fitness due to bioluminescent transformation under these experimental conditions (Figure 4.6).

Therefore this MenB bioluminescent reference strain can be a useful tool for the rapid, real-time evaluation of the presence and the kinetics of MenB in a variety of conditions that would otherwise be difficult or impossible to monitor, such as determination of invasion of host cells by MenB in mixed cultures of bacteria (Chapter 5).

Chapter 5

Bacterial association and invasion of nasopharyngeal epithelial cells by commensal and pathogenic neisseriae

5.1 Introduction

The lining of the nasopharyngeal mucosa comprises an epithelial layer of mostly ciliated columnar epithelial cells with occasional mucus secreting goblet cells joined by tight junctions, that provide a protective barrier, and produce inflammatory substances, overlaying a submucosal layer (Sahin-Yilmaz & Naclerio, 2011). Polymicrobial communities of bacteria are known to survive within the mucosa of healthy individuals without causing disease (Rudney, Chen & Sedgewick, 2005). *N. meningitidis* can be found within the epithelial mucosa of healthy individuals at the nasopharyngeal niche (Greenfield, Sheehe & Feldman, 1971). *N. meningitidis* interacts mainly with non-ciliated epithelial cells (Stephens, Hoffman & McGee, 1983), maintains close association with epithelial cells of the human nasopharynx and has been hypothesised to survive within the mucosa, potentially avoiding killing by immune cells (Sim *et al.*, 2000).

To cause systemic disease *N. meningitidis* must passage through the epithelial barrier, and additionally the endothelium, to enter the bloodstream (bacteraemia) and disseminate to the cerebrospinal fluid (CSF) and meninges to cause meningitis (Stephens, Hoffman & McGee, 1983). However, routes of infection via the olfactory nerve to the brain (Sjölander & Jonsson, 2010), and carriage to the CSF by attachment to neutrophils (Söderholm *et al.*, 2011; Criss & Seifert, 2012) have more recently been proposed. Invasion into epithelial cells is preceded by microcolony formation on the apical surface of the host cell (Stephens, Hoffman & McGee, 1983). It has been demonstrated that unlike *S. pneumoniae* and *H. influenzae* that traverse epithelial monolayers between the epithelial cells via a paracellular route (van Schilfgaarde *et al.*, 1995; Beisswenger *et al.*, 2007), *N. meningitidis* moves by transcytosis through the epithelial cells by internalisation into vesicles (Stephens, Hoffman & McGee, 1983; Sutherland *et al.*, 2010) similar to the mechanism used by *N. gonorrhoeae*, following recruitment of host cell receptors and cortical plaque formation (Higashi *et al.*, 2007). These cortical plaques are comprised of host cell membrane protrusions called

microvilli that are the result of polymerisation of host cytoskeleton associated molecules such as actin and ezrin, and are predominantly induced by bacterial pili (Merz & So, 1997; Merz, Enns & So, 1999). This route is dependent on expression of pili and the presence of the polysaccharide capsule (Sutherland *et al.*, 2010) despite down-regulation of both during invasion (Deghmane *et al.*, 2002), perhaps suggesting the origins of *N. meningitidis* as a commensal with a small subpopulation being pathogenic. The expression of bacterial pili and Opa has been demonstrated to be important for neisserial association with epithelial cells, where Opa bind to host CEACAM1 receptor (Griffiths *et al.*, 2007) and is important in invasion of epithelial monolayers (Wang *et al.*, 1998).

This mechanism of invasion into epithelial cells is in contrast to *N. meningitidis* invasion into endothelial cells. In endothelial cells *N. meningitidis* interacts with the host β 2-adrenoceptor which recruits host cell to host cell adhesion molecules from tight junctions within the cortical plaque (Coureuil *et al.*, 2010). This allows microvilli formation to surround *Neisseria* microcolonies protecting them from blood flow shear stress within blood vessels (Mikaty *et al.*, 2009). The re-recruitment of cell adhesion molecules loosens host cell-cell contact allowing paracellular invasion following endothelial barrier dysfunction (Coureuil *et al.*, 2012).

It has been suggested that the loss of commensal organisms, even those that may become pathogenic, from the human microbiota may be detrimental in the long-term and that we should learn to control rather than eliminate them (Blaser & Falkow, 2009). Even potentially pathogenic colonisers of the nasopharyngeal niche can establish harmonious communities or prevent future colonisation by unrelated species or even strains of the same species (Margolis, Yates & Levin, 2010). A possible role for commensal organisms within the microbiota is to protect the host.

The oral commensal *Streptococcus salivarius* is one of the most studied oral commensals and has been shown to maintain a reduction in oral malodour-causing bacteria on the

tongue (Burton, Chilcott & Tagg, 2005). *S. salivarius* also prevents adhesion of the fungal pathogen *Candida albicans* by binding directly to the hyphae of the pathogen, and can protect against candidiasis in mouse models (Ishijima *et al.*, 2012). Additionally *S. salivarius* protects against adhesion of the pathogen *Streptococcus pyogenes* to nasopharyngeal cells and has thus been suggested as an oronasopharyngeal probiotic (Guglielmetti *et al.*, 2010). In a study of adults, oral administration of a probiotic *Lactobacillus*, *Bifidobacterium* and *Streptococcus* cocktail reduces the nasal carriage of Gram-positive pathogens *S. aureus* and *S. pneumoniae* (Glück & Gebbers, 2003).

To date, comparative studies have been undertaken to look at adhesion and invasion of pathogenic *N. meningitidis* and commensal *N. lactamica* individually, and there have been two studies observing the potential protective properties of live *N. lactamica* on colonisation by *N. meningitidis* in the host (Andrade, Marques & de Santa Rosa, 1986; Evans *et al.*, 2011). It was therefore decided to evaluate the effect of commensal *Neisseria* spp; *N. lactamica* and *N. cinerea* and *N. polysaccharea* as comparison, on the interaction of *N. meningitidis* with nasopharyngeal epithelial cells.

5.2 Results

5.2.1 *N. meningitidis* serogroup B strain MC58 and *N. lactamica* strain NL4.1 adhesion and invasion of Detroit 562 nasopharyngeal epithelial monolayers

Detroit 562 (D562) epithelial monolayers were challenged with *N. meningitidis* serogroup B (MenB; strain MC58), *N. lactamica* (Nlac; strain NL4.1) or equivalent numbers of both to assess the effect of mixed cultures on the ability of each strain to associate with, and invade human nasopharyngeal epithelial cells (Figure 5.1). The initial inoculum (Time 0 hr) was enumerated and showed comparable numbers of *N. meningitidis* (Figure 5.1a) and *N. lactamica* (Figure 5.1e) when added alone or in mixed culture. After 3 hours' incubation (Time 3 hr) there was a slight, but not significant reduction in *N. meningitidis* (Figure 5.1b) and *N. lactamica* (Figure 5.1f) numbers when in mixed culture relative to numbers when cultured alone.

There was approximately 50% of the initial inoculum (Figure 5.1a) of *N. meningitidis* associated with epithelial cells at 3 hours (Figure 5.1c), which was approximately 300 times greater than *N. lactamica* (Figure 5.1g). When in mixed culture, there was a significant reduction ($p < 0.001$) of more than 60% in *N. meningitidis* association relative to *N. meningitidis* alone. Conversely, there was a significant increase ($p < 0.01$) in *N. lactamica* association in the presence of *N. meningitidis* compared to in the absence, though still approximately 300 times less than *N. meningitidis* (Figure 5.1g).

N. meningitidis (Figure 5.1d) displayed approximately 40-fold greater invasion into epithelial cells than *N. lactamica* (Figure 5.1h). In the presence of *N. lactamica*, *N. meningitidis* invasion decreased significantly ($p < 0.01$; Figure 5.1d) by approximately 90%, whereas invasion by *N. lactamica* increased ($p < 0.001$; Figure 5.1h) by approximately 80% in the presence of *N. meningitidis*. This increase in invasion by *N. lactamica* when in mixed culture was however still approximately 30 times lower than that of *N. meningitidis* invasion when in single culture.

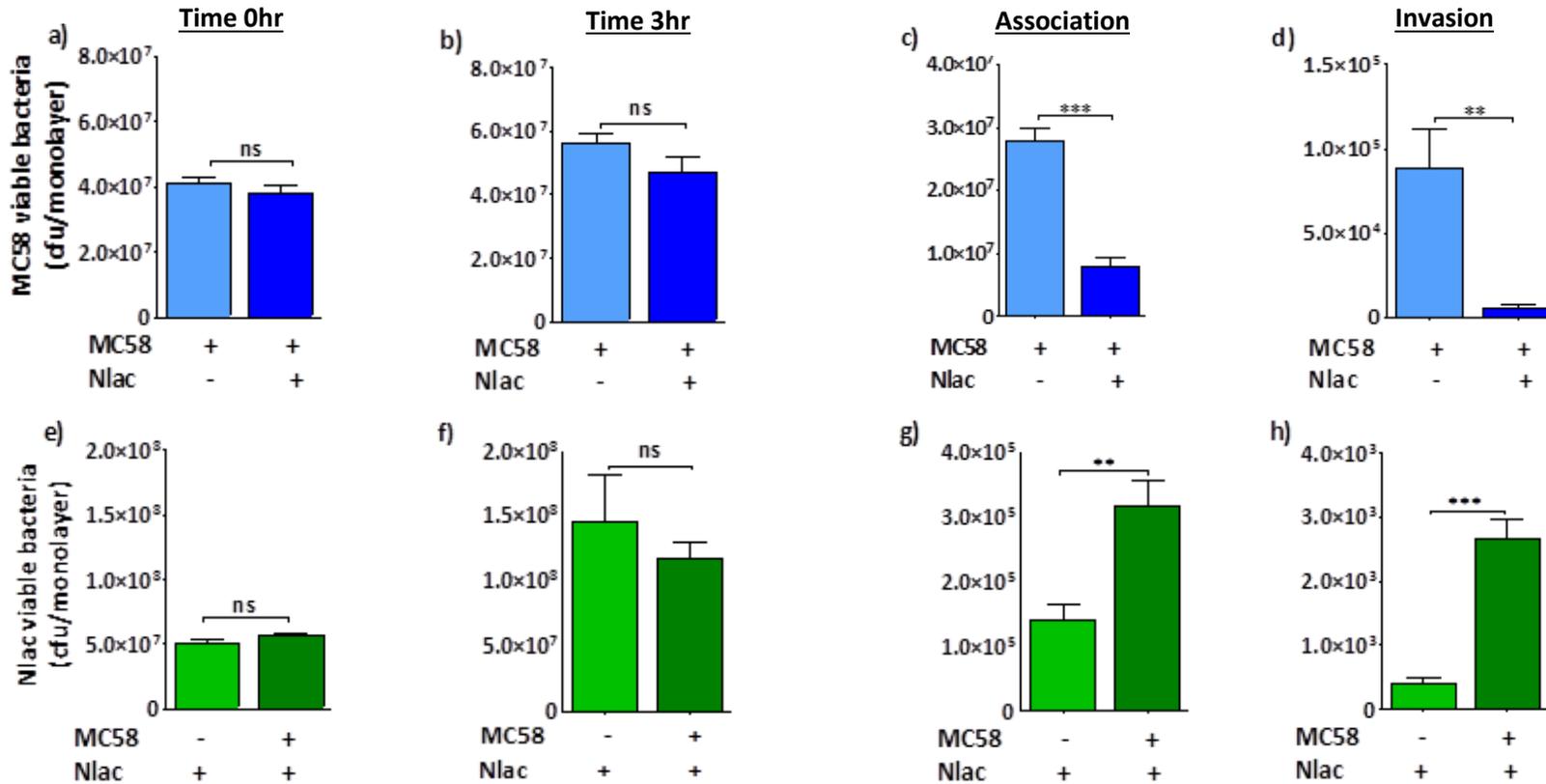


Figure 5.1 Association and invasion of Detroit 562 epithelial cells by *N. meningitidis* strain MC58 and *N. lactamica* alone and in mixed culture

D562 monolayers were challenged for 3 hours with MenB (strain MC58) or *N. lactamica* (strain NL4.1; Nlac) alone or in mixed culture at equivalent doses. Viable MC58 (a, b, c and d) or Nlac (e, f, g and h) were enumerated by viable counts and differentiated on HBHI agar containing xgal (100 µg/ml). Controls for the amount of bacteria initially added (Time 0hr) and bacterial growth during the assay (Time 3hr) are included. Associated bacteria were enumerated from washed and lysed monolayers, and invaded bacteria were enumerated using the gentamicin protection assay (section 2.2.20). Data represents the mean ± SEM of three independent repeats each replicated in triplicate. *** p<0.001 ** p<0.01 ns= non-significant.

5.2.2 The effect of heat-killed *N. lactamica* and *N. lactamica* outer membrane vesicles on association of *N. meningitidis* with Detroit 562 epithelial cells

The influence of dead (heat-killed) *N. lactamica* or *N. lactamica* outer membrane vesicles (OMVs) to the association of *N. meningitidis* (strain MC58) with D562 epithelial cells was assessed (Figure 5.2). The initial inoculum (Time 0 hr) was enumerated and although there were consistently lower numbers of *N. meningitidis* in the presence of OMVs and heat-killed *N. lactamica* relative to *N. meningitidis* alone, there was no significant difference in the amount of *N. meningitidis* in each treatment. Following 3 hours' incubation (Time 3 hr), again numbers of *N. meningitidis* were slightly reduced in the presence of OMVs and heat-inactivated *N. lactamica* compared to *N. meningitidis* alone, though this was not statistically significant. Consistent with previous experiments (Figure 5.1) approximately 50% of *N. meningitidis* incubated with host cells associated with them following 3 hours' incubation. Although there was a slight increase in association of *N. meningitidis* in the presence of heat-killed *N. lactamica*, *N. meningitidis* association was not significantly influenced in the presence of either *N. lactamica* OMVs or heat-killed *N. lactamica*.

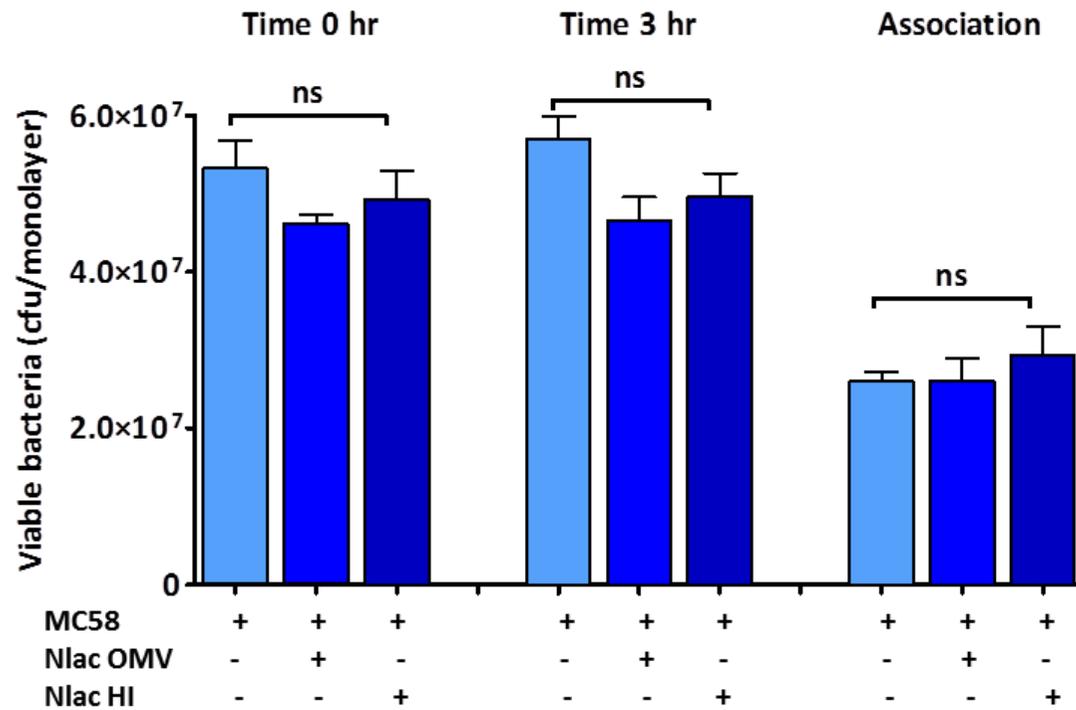


Figure 5.2 Association of *N. meningitidis* with Detroit 562 epithelial cells in the absence and presence of heat-inactivated *N. lactamica* or outer membrane vesicles

Confluent monolayers of D562 cells were challenged with MenB (strain MC58) alone or in the presence of *N. lactamica* outer membrane vesicles (Nlac OMV) or heat-inactivated *N. lactamica* (Nlac HI) for 3 hours. Controls for the concentration of bacteria initially added (Time 0hr), growth during the assay (Time 3hr) and levels of bacteria associated with monolayers were measured by viable counts. Data represents mean ±SEM from three independent repeats each replicated in triplicate. ns= non-significant.

5.2.3 The effect of *N. lactamica* on association of a clonal complex ST-11 *N. meningitidis* serogroup B strain with Detroit 562 epithelial cells

To confirm the ability of *N. lactamica* (Nlac; strain NL4.1) to inhibit *N. meningitidis* association with D562 epithelial cells, *N. meningitidis* serogroup B (MenB; strain Z4701) of the ST-11 clonal complex was used (Figure 5.3). The initial inoculum of *N. meningitidis* (Figure 5.3a) and *N. lactamica* (Figure 5.3d) were comparable when alone and in mixed culture. After 3 hours' incubation (Time 3 hr) numbers of *N. meningitidis* in single culture (Figure 5.3b) increased by more than three times compared to the initial inoculum (2.3×10^7 cfu/monolayer to 7.2×10^7 cfu/monolayer), whereas *N. lactamica* (Figure 5.3e) more than doubled (4.1×10^7 cfu/monolayer to 8.7×10^7 cfu/monolayer). Numbers of *N. meningitidis* and *N. lactamica* in mixed culture also increased following three hours incubation, however significantly fewer organisms were recovered compared to when cultured alone ($p < 0.05$ and $p < 0.01$, respectively).

N. meningitidis strain Z4701 (Figure 5.3c) adhered poorly with less than 0.1% of the initial inoculum found to be associated with epithelial cells (2×10^4 cfu/monolayer) following three hours incubation. In the presence of *N. lactamica* there was a significant reduction ($p < 0.001$) of approximately 25% in *N. meningitidis* association (1.5×10^4 cfu/monolayer). Similarly, *N. lactamica* association (Figure 5.3f), though more than 10-fold greater than *N. meningitidis* (2.5×10^5 cfu/monolayer) was significantly reduced ($p < 0.05$) in the presence of *N. meningitidis* (9.6×10^4 cfu/monolayer).

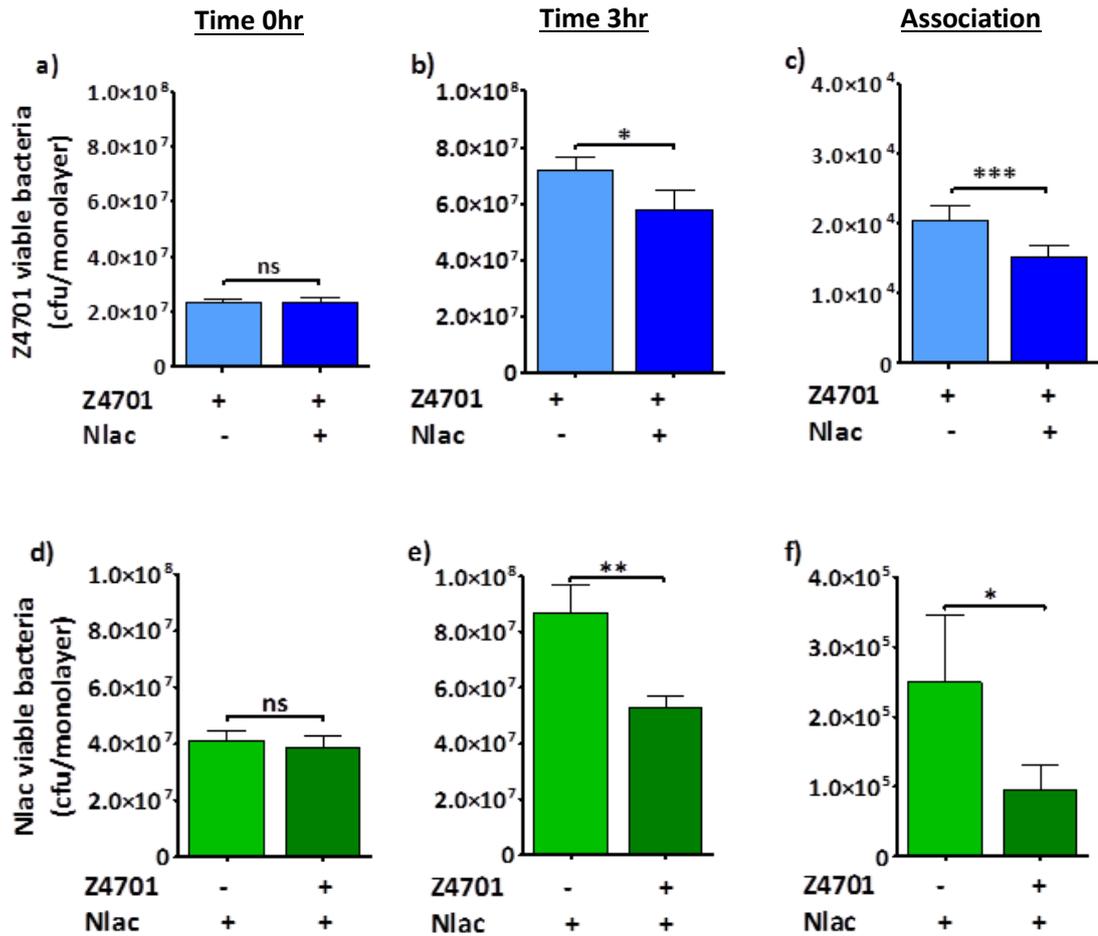


Figure 5.3 *N. meningitidis* serogroup B strain Z4701 and *N. lactamica* association with Detroit 562 epithelial monolayer, alone and in mixed culture

D562 monolayers were challenged for 3 hours with either *N. meningitidis* (strain Z4701) or *N. lactamica* (strain NL4.1; Nlac) alone or in mixed culture. Viable Z4701 (a, b and c) or Nlac (d, e and f) were enumerated by viable counts and differentiated on HBHI agar containing xgal (100µg/ml). Controls for the amount of bacteria initially added (Time 0hr) and growth during the assay (Time 3hr) are included. Associated bacteria were enumerated from washed and lysed monolayers. Data represents the mean ± SEM of three independent repeats each replicated in triplicate. *** p<0.001 ** p<0.01 * p<0.05 ns= non -significant.

5.2.4 The effect of *N. lactamica* on association of a clonal complex ST-11 *N. meningitidis* serogroup C strain with Detroit 562 epithelial cells

This study was extended to investigate the effect on *N. lactamica* on adhesion of another serogroup of *N. meningitidis*. The serogroup C strain Z6417, which is of the ST-11 clonal complex, was utilised (Figure 5.4). Epithelial monolayers were challenged with a comparable inoculum (Time 0 hr) of *N. meningitidis* (Figure 5.4a) and *N. lactamica* (Figure 5.4d) whether alone or in mixed culture. Following 3 hours' incubation, growth (Time 3 hr) of cultures was measured. When alone, the amount of *N. meningitidis* (Figure 5.4b) almost tripled compared to the initial inoculum (2.4×10^7 CFU/monolayer to 6.8×10^7 CFU/monolayer). In the presence of *N. lactamica* there was a significant inhibition of growth ($p < 0.01$) in viable *N. meningitidis* at 3 hours of approximately 30% compared to bacteria cultured alone. Similarly, there was a significant inhibition in growth ($p < 0.05$) of *N. lactamica* at 3 hours in the presence of *N. meningitidis* (Figure 5.4e) compared to single culture. *N. meningitidis* poorly adhered to epithelial cells (Figure 5.4c) with approximately 0.01% of the initial inoculum associated. Unlike previous strains of *N. meningitidis* assayed, there was a significant increase ($p < 0.05$) in association with host cells in the presence of *N. lactamica*. Conversely, *N. lactamica* association with host cells (Figure 5.4f) was significantly reduced ($p < 0.01$) in the presence of *N. meningitidis*.

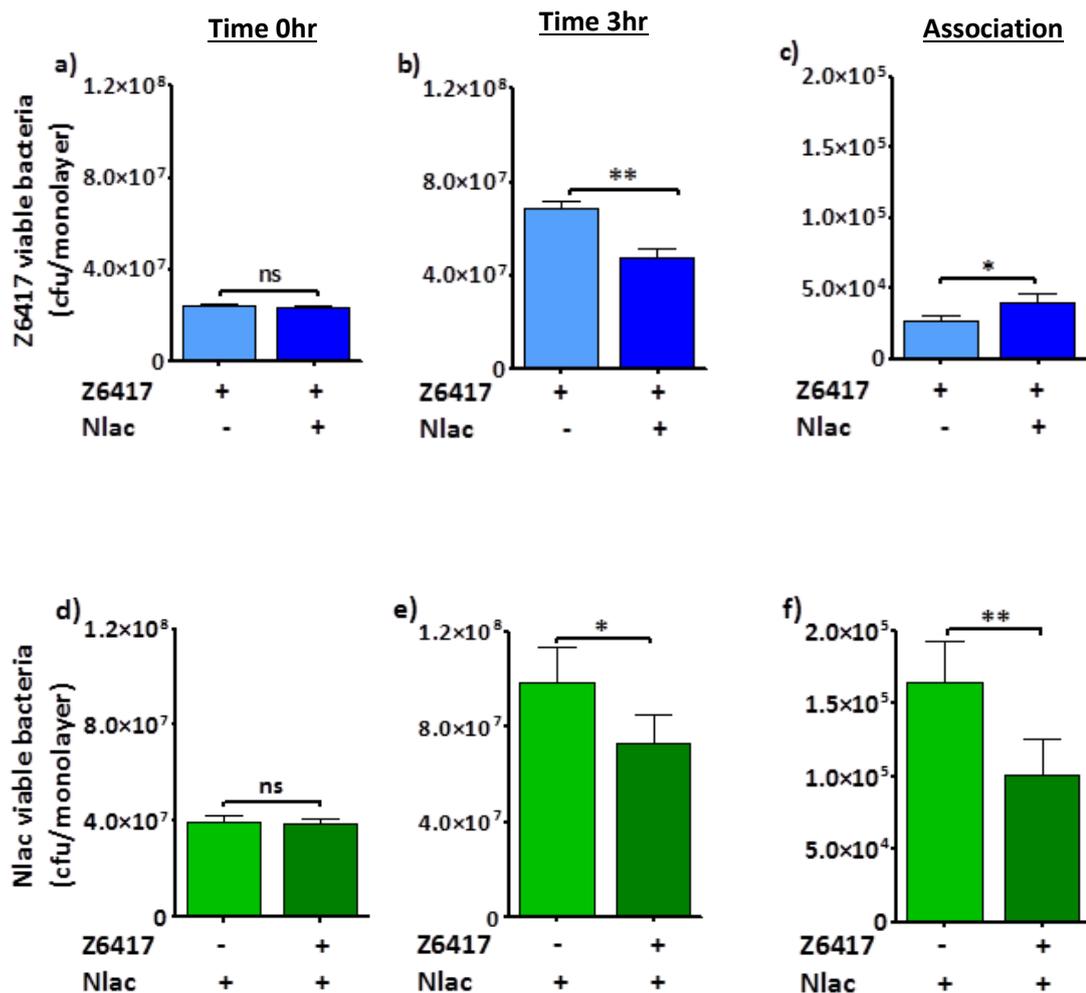


Figure 5.4 *N. meningitidis* serogroup C strain Z6417 and *N. lactamica* association with Detroit 562 epithelial monolayer, alone and in mixed culture

D562 monolayers were challenged for 3 hours with either *N. meningitidis* (strain Z6417) or *N. lactamica* (strain NL4.1; Nlac) alone or in mixed culture at equal doses. Viable Z6417 (a, b and c) or Nlac (d, e and f) were enumerated by viable counts and differentiated on HBHI agar containing xgal (100µg/ml). Controls for the amount of bacteria initially added (Time 0 hr) and growth during the assay (Time 3 hr) are included. Associated bacteria were enumerated from washed and lysed monolayers. Data represents the mean ± SEM of three independent repeats each replicated in triplicate. ** p<0.01 * p<0.05 ns= no significance.

5.2.5 The effect of other commensal *Neisseria spp.* on adhesion by serogroup B *N. meningitidis* with Detroit 562 nasopharyngeal epithelial cells

This study has demonstrated the presence of *N. lactamica* inhibits association of *N. meningitidis* strain MC58, and to a lesser extent *N. meningitidis* strain Z4701, with D562 nasopharyngeal epithelial cells. To identify if this inhibition is shared by other commensal *Neisseria spp.* adhesion of *N. meningitidis* serogroup B (strain MC58) at low (MOI 20) and high dose (MOI 200) was compared in the absence and presence of *N. lactamica* (Nlac), *N. cinerea* (Ncin) or *N. polysaccharea* (Npoly) at similar doses and analysed by flow cytometry (Figure 5.5).

When *N. meningitidis* was incubated in the presence of *N. lactamica* there was a dose-dependent reduction of both low (Figure 5.5a) and high dose (Figure 5.5d) *N. meningitidis*. This reduction in *N. meningitidis* adhesion became significant with the highest dose of *N. lactamica* ($p < 0.01$ and $p < 0.05$, respectively), and was greatest when *N. lactamica* was at a 10-fold higher dose than *N. meningitidis* (MOI 200 and MOI 20, respectively; Figure 5.5a).

In the presence of *N. cinerea* there was no significant change in adhesion of either low (Figure 5.5b) or high dose (Figure 5.5e) *N. meningitidis*. However, similarly to *N. lactamica*, *N. polysaccharea* showed a dose-dependent reduction in low (Figure 5.5c) and high dose (Figure 5.5f) *N. meningitidis* adhesion that was significant with the highest dose of *N. polysaccharea* ($p < 0.05$).

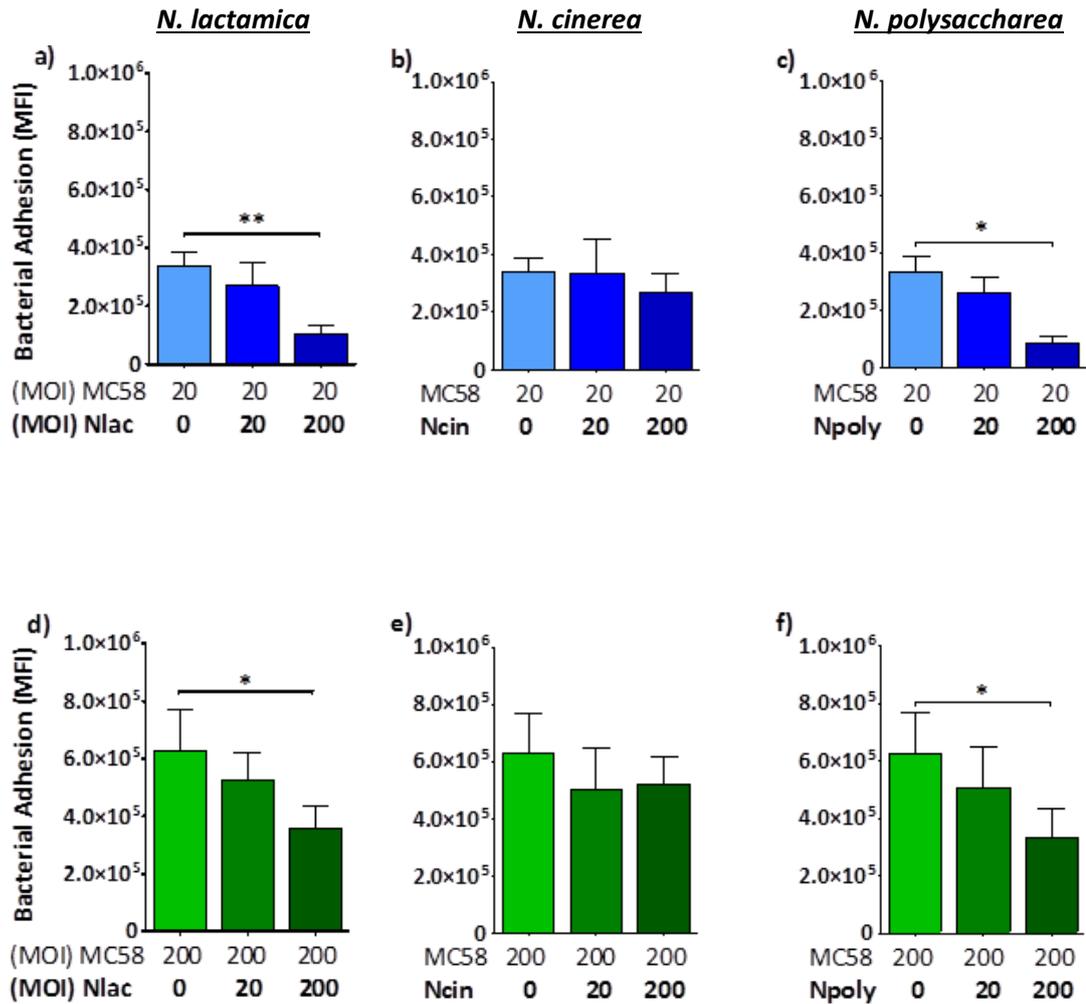


Figure 5.5 *N. meningitidis* serogroup B strain MC58 adhesion to Detroit 562 epithelial cells in the absence and presence of commensal *Neisseria* spp.

Detroit 562 (D562) epithelial cells were challenged with *N. meningitidis* (strain MC58) at 20 (a, b and c) or 200 bacteria per epithelial cell (MOI; d, e and f) in the absence or presence of *N. lactamica* (strain NL4.1; Nlac; a and d), *N. cinerea* (strain 194; Ncin; b and e) or *N. polysaccharea* (strain LNP 462; Npoly; c and f) at MOI 20 or 200. After 3 hours epithelial cells were washed and dissociated with EDTA (4mM). MC58 was labelled with mouse anti-*N. meningitidis* serosubtype P1.7 primary antibody and goat anti-mouse Alexa-fluor 647 secondary antibody. When possible 10,000 events were analysed by flow cytometry using the Accuri C6 flow cytometer and median fluorescent intensity (MFI) was measured. Data represents the mean \pm SEM of three independent repeats ** $p < 0.01$ * $p < 0.05$.

5.2.6 The effect of commensal neisseriae on adhesion of *N. meningitidis* serogroup A with Detroit 562 nasopharyngeal epithelial cells

This study was further extended to investigate the effect of commensal neisseriae on adhesion of *N. meningitidis* serogroup A (strain C751) with D562 epithelial cells using antibody labelling of *N. meningitidis* and analysis by flow cytometry (Figure 5.6). D562 epithelial cells were challenged with *N. meningitidis* (MOI 200) alone or in the presence of *N. lactamica* (Nlac; Figure 5.6a), *N. cinerea* (Ncin; Figure 5.6b) or *N. polysaccharea* (Npoly; Figure 5.6c) at MOI 20 or MOI 200.

N. meningitidis showed inconsistent levels of adhesion in these experiments as represented by SEM. Never-the-less in the presence of *N. lactamica* (Figure 5.6a) at MOI 200 but not MOI 20, there was a strong, though not significant reduction in *N. meningitidis* adhesion to host cells compared to single cultures. *N. cinerea* (Figure 5.6b) however did induce a dose-dependent reduction in *N. meningitidis* adhesion at both MOI 20 and MOI 200. *N. polysaccharea* at both MOI 20 and MOI 200 reduced adhesion of *N. meningitidis* with both concentrations being comparable in reducing adhesion to host cells. MOI 200 of each commensal *Neisseria* spp. tested showed a large, but not significant reduction in *N. meningitidis* adhesion with host cells (mean MFI 3.2×10^4 to mean MFI $< 1.4 \times 10^4$).

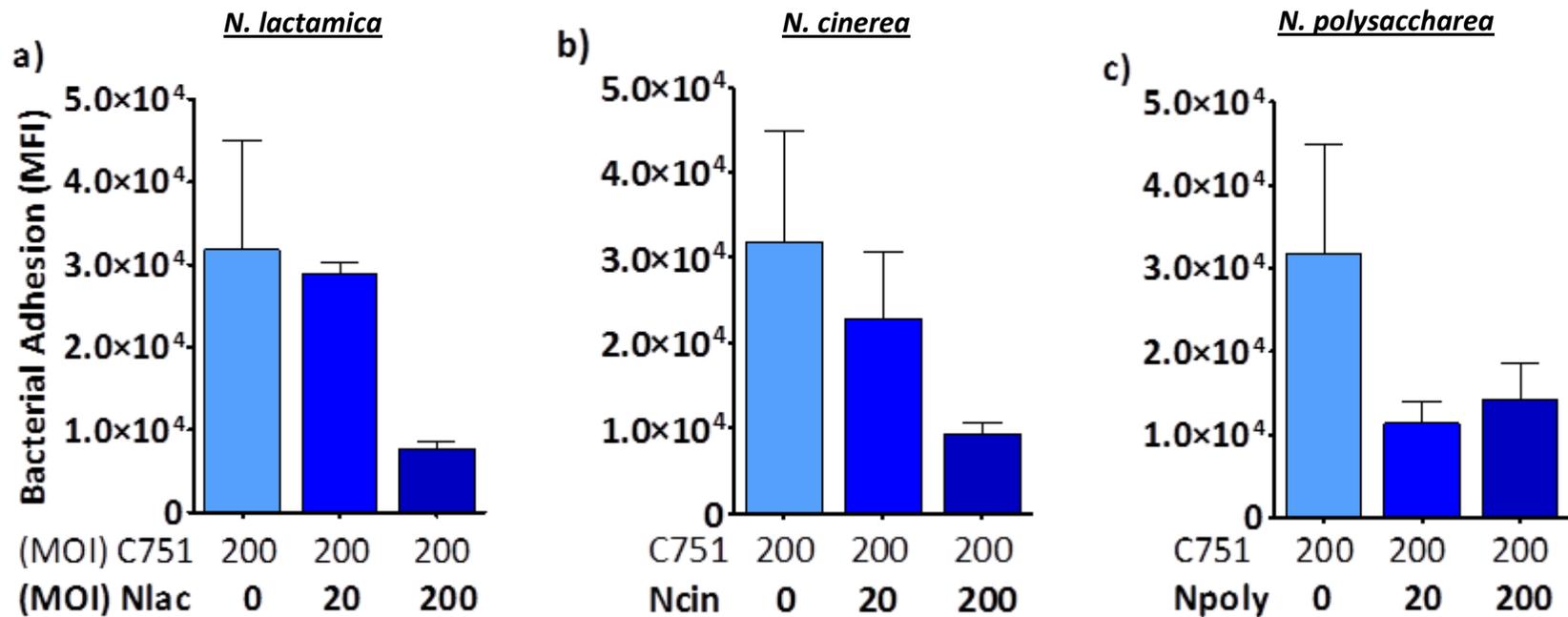


Figure 5.6 *N. meningitidis* strain C751 adhesion to Detroit 562 epithelial cells in the absence and presence of commensal *Neisseria* spp. analysed by flow cytometry

Detroit 562 (D562) epithelial monolayers were challenged with *N. meningitidis* (strain C751) at 200 bacteria per epithelial cell (MOI) alone or in the presence of *N. lactamica* (strain NL4.1; Nlac; a), *N. cinerea* (strain 194; Ncin; b) or *N. polysaccharea* (strain LNP 462; Npoly; c) at MOI 20 or 200. After 3 hours epithelial cells were washed and dissociated with EDTA (4mM). C751 was labelled with mouse anti- *N. meningitidis* serosubtype P1.7 primary antibody and goat anti-mouse Alexa-fluor 647 secondary antibody. When possible, 10,000 events were analysed by flow cytometry using the Accuri C6 flow cytometer and median fluorescent intensity (MFI) was measured. Data represents the mean ± SEM of three independent repeats

5.2.7 The effect of commensal neisseriae on invasion of Detroit 562 epithelial cells by serogroup B *N. meningitidis*

To monitor the effect of the commensal neisseriae on invasion of nasopharyngeal epithelial cells by *N. meningitidis*, the bioluminescent *N. meningitidis* construct (strain MC58lux) was utilised in a gentamicin protection assay, since *N. cinerea* and *N. polysaccharea* cannot be differentiated on x-gal containing medium. Epithelial monolayers were challenged with bioluminescent *N. meningitidis* at MOI for 3 hours, alone or in the presence of the non-bioluminescent commensal *Neisseria* spp; *N. lactamica*, *N. cinerea* or *N. polysaccharea* (Figure 5.7).

In the presence of *N. lactamica* (Figure 5.7a) at MOI 20 there was no significant change in *N. meningitidis* invasion within host cells compared to *N. meningitidis* alone. In the presence of *N. lactamica* at MOI 200 and 2000 there was a significant, dose-dependent reduction ($p < 0.01$ and $p < 0.001$, respectively) in invasion by *N. meningitidis* of approximately 40% and 60%, respectively. Lower levels of bioluminescence were detected from *N. meningitidis* in experiments with *N. lactamica* compared to those detected in experiments with *N. cinerea* (Figure 5.7b) and *N. polysaccharea* (Figure 5.7c) due to a change in the type of tissue culture plate used.

When epithelial cells were challenged with *N. meningitidis* in the presence of *N. cinerea* (Figure 5.7b) at MOI 20 and 200, there was no significant change in *N. meningitidis* invasion within host cells. However, when *N. cinerea* was at MOI 2000 there was a significant reduction ($p < 0.001$) in invasion of epithelial cells by *N. meningitidis* of approximately 60% relative to *N. meningitidis* alone.

Similar to that seen with both previous *Neisseria* commensals, in the presence of *N. polysaccharea* (Figure 5.7c) at MOI 20 there was no significant change in invasion by *N. meningitidis* compared with *N. meningitidis* alone. However, at MOI 200 and MOI 2000 there was a significant, dose-dependent reduction ($p < 0.001$) in invasion by 40% and 75%,

respectively. These results indicate that both *N. lactamica* and *N. polysaccharea* are capable of inhibiting invasion by *N. meningitidis* at an equal number of bacteria, but all commensal *Neisseria* tested were able to inhibit *N. meningitidis* invasion when at a 10-fold higher concentration than *N. meningitidis*.

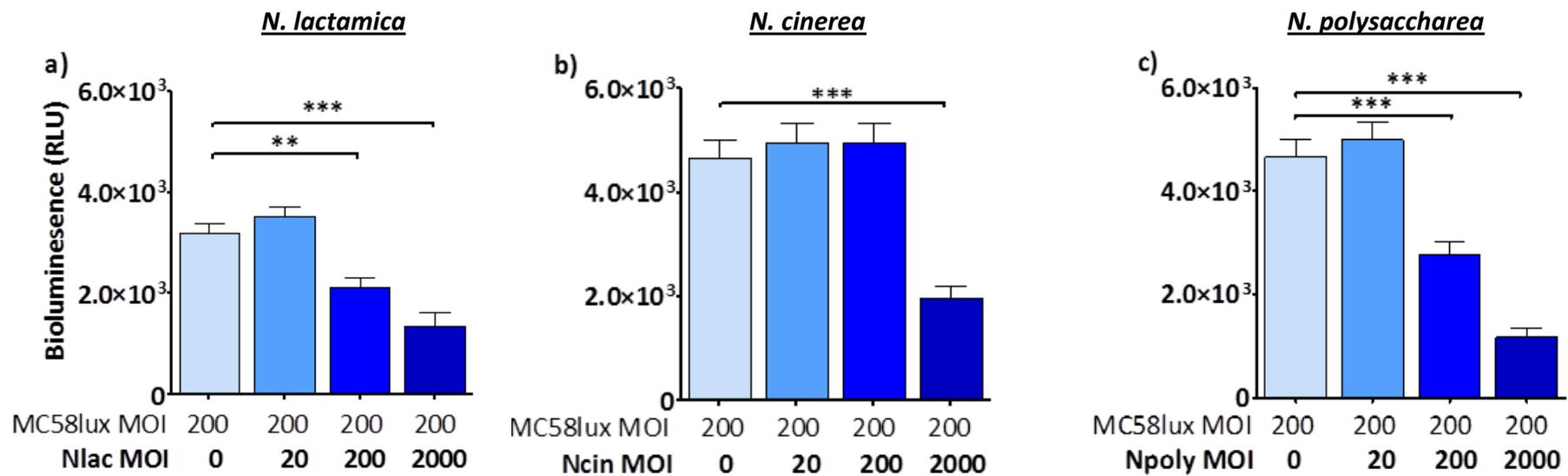


Figure 5.7 Bioluminescence as a reporter for invasion of *N. meningitidis* into Detroit 562 epithelial monolayers in the absence or presence of commensal *Neisseria* spp.

Detroit 562 epithelial monolayers were challenged with bioluminescent *N. meningitidis* (strain MC58lux) at 200 bacteria per epithelial cell (MOI) alone or in the presence of commensal a) *N. lactamica* (strain NL4.1; Nlac), b) *N. cinerea* (strain 194; Ncin) or c) *N. polysaccharea* (strain LNP 462; Npoly) at MOI 20, 200 or 2000. After 3 hours, monolayers were washed and M199 containing gentamicin (200µg/ml) was added for a further 90 minutes to kill extracellular bacteria. Saponin (final 1%) was then added to permeabilise epithelial cells facilitating gentamicin-mediated killing of invaded bacteria. Bioluminescence (relative light units; RLU) from MC58lux was compared between gentamicin treated, and gentamicin with saponin treated cells at 5 hours, to measure invasion. Data represents the mean ± SEM of three independent repeats replicated in triplicate. *** p<0.001 ** p<0.01.

5.2.8 The effect of *N. lactamica* on viability of *N. meningitidis* in co-cultures

In previous experiments with mixed cultures of *N. meningitidis* (strain MC58) and *N. lactamica*, at 3 hours when invasion was measured, there was no difference in numbers of bacteria whether alone or in mixed culture (Figure 5.1). However, bioluminescent traces using the *luxCDABE* expressing *N. meningitidis* (strain MC58lux) showed a reduction in bioluminescence when *N. meningitidis* was cultured in the presence of *N. lactamica* after approximately 8 hours, when compared to *N. meningitidis* alone (Figure 5.8a).

To confirm the reduction in bioluminescence was both an indication of loss of viable bacteria, and to confirm the effect was representative of parental *N. meningitidis* (strain MC58), the cultures were enumerated by viable counts. The initial inoculum (Time 0 hr) was enumerated to confirm a comparable quantity of bioluminescent *N. meningitidis* (Figure 5.8b) and parental *N. meningitidis* (Figure 5.8c) were added in single cultures and in mixed cultures with *N. lactamica*. The same was shown for *N. lactamica* (Figure 5.8d) whether in single cultures or mixed cultures with parental or bioluminescent *N. meningitidis*.

Following 18 hours incubation (Time 18 hr) there were comparable numbers of both bioluminescent (Figure 5.8e) and parental (Figure 5.8f) *N. meningitidis* with approximately a 10-fold increase in viable bacteria. In the presence of *N. lactamica* there was a substantial and significant decrease ($p < 0.001$) in viable *N. meningitidis* that was comparable between bioluminescent and parental *N. meningitidis*.

In the presence of bioluminescent and parental *N. meningitidis* there was a significant reduction ($p < 0.001$ and $p < 0.01$) in viable *N. lactamica*, compared to *N. lactamica* alone (Figure 5.8g). However, this reduction in viable *N. lactamica* was a greater in the presence of bioluminescent *N. meningitidis* (60%) compared to the presence of parental *N. meningitidis* (30%) which was also statistically significant ($p < 0.05$).

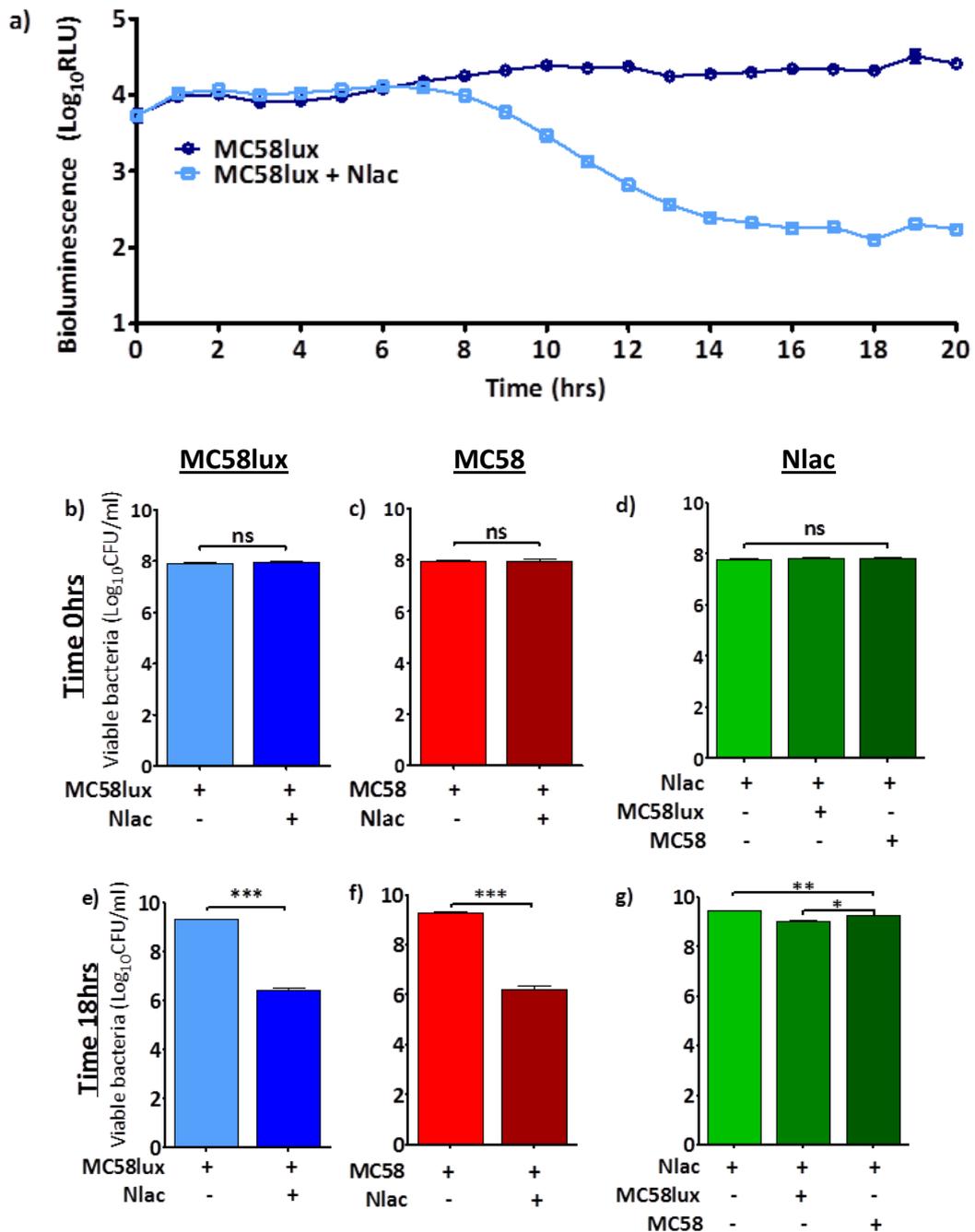


Figure 5.8 Viability over time of *N. meningitidis* and *N. lactamica* in mixed culture, measured with a bioluminescent reporter and viable counts

Parental *N. meningitidis* (strain MC58) and bioluminescent derivative (strain MC58lux) were incubated in M199 alone or in mixed culture with *N. lactamica* (strain NL4.1; Nlac). (a) Bioluminescence (Log₁₀ relative light units; RLU) from MC58lux was monitored over 20 hours and representative luminometer traces are shown. Viable counts (log₁₀ cfu/ml) were taken at 0 hours for (b) MC58lux, (c) MC58 and (d) Nlac, and at 18 hours (e, f and g respectively), alone and in mixed culture. Data represents mean ± SEM from three independent repeats each replicated in triplicate. *** p<0.001 ** p<0.01 *p<0.05 ns= non-significant.

5.3 Discussion

Epidemiological data indicates that nasopharyngeal carriage of *N. lactamica* reduces the incidence of *N. meningitidis* serogroup B (MenB) carriage and subsequently invasive disease (Cartwright *et al.*, 1987). This has been hypothesised to be due to an adaptive immune response acquired during *N. lactamica* carriage that cross-reacts with *N. meningitidis* to protect against systemic invasion (Oliver *et al.*, 2002). However, a recent study suggests that nasopharyngeal carriage of live *N. lactamica* rather than the development of a cross-reactive immune response, may confer protection against *N. meningitidis* carriage (Evans *et al.*, 2011).

The aim of this work was to investigate the effect of *N. lactamica* and as a comparison, other commensal *Neisseria* spp; *N. cinerea* and *N. polysaccharea*, on the interaction of *N. meningitidis* with nasopharyngeal epithelial cells. *N. lactamica* colonies can be differentiated from *N. meningitidis* colonies by the inclusion of xgal in the media (Bennett *et al.*, 2005), so viable counts were used with the gentamicin protection assay to measure association and invasion of both pathogenic and commensal bacteria in mixed cultures. However, flow cytometry and the newly constructed and characterised bioluminescent *N. meningitidis* were required to investigate the effect of *N. cinerea* and *N. polysaccharea* on *N. meningitidis* association and invasion.

The MenB strain MC58 associated and invaded epithelial cells to approximately the same extent as has been found in previous studies (Virji *et al.*, 1992a, 1995a; Tezera *et al.*, 2011). This study has demonstrated for the first time that *N. lactamica* inhibits both association (approximately 60%) and invasion (approximately 90%) of nasopharyngeal epithelial cells by MenB strain MC58 when measured by viable counts. Conversely, *N. lactamica* association increased in the presence of MC58, however approximately 100-fold more *N. meningitidis* were displaced than were replaced by *N. lactamica*. This could suggest prevention of *N. meningitidis* association with a crucial epithelial cell receptor and subsequent micro-colony

formation. For instance *N. gonorrhoeae* has been demonstrated to form microcolonies over clusters of host CD46 receptor, which has been putatively implicated in meningococcal association with host cells (Weyand *et al.*, 2006).

Alternatively, the upregulation of CEACAM1 receptor on host cells increases association and invasion of Opa-expressing neisseriae with host cells including *N. meningitidis* strains MC58 and C751 (Griffiths *et al.*, 2007; Rowe *et al.*, 2007). Opa is an adhesin known to also be expressed by a range of commensal *Neisseria* spp. including *N. lactamica* (Toleman, Aho & Virji, 2001). It has however been suggested that a second unknown neisserial adhesin also associates with host CEACAM1 (Kuespert, Roth & Hauck, 2011).

The porB of both *N. meningitidis* and *N. lactamica* interacts with host TLR2/1 receptor (Toussi *et al.*, 2012), and inflammation induced via this receptor is inhibited by *N. lactamica* (strain NL4.1; Tezera *et al.*, 2011). Inhibition of TLR2/1 signalling could be a mechanism of reducing internalisation of *N. meningitidis*-bound TLR2/1 or subsequent clustering of host receptors and bacterial microcolony formation (Triantafilou *et al.*, 2006). However what role TLR2/1 receptor plays in association, invasion or merely in inflammation is speculative at this point.

Another mechanism to prevent association of *N. meningitidis* with host cells could also be bacteria-bacteria association such as co-aggregation of both bacteria (Cisar, Kolenbrander & McIntire, 1979; Kolenbrander & Andersen, 1986). Neisseriae and other bacteria are known to associate strongly with each other via physical association particularly via pili-pili interaction in neisseriae (Virji *et al.*, 1995a). Whatever the mechanism may be, the ability of *N. lactamica* to inhibit *N. meningitidis* association with epithelial cells is dependent on live organisms as there was no inhibition of MC58 association when in the presence of either heat-killed *N. lactamica* or outer membrane vesicles from *N. lactamica*. In the model used in this study, both *N. lactamica* and *N. polysaccharea* predominantly are able to inhibit association and therefore potentially colonisation in the whole host by a mechanism not

dependent on acquisition of adaptive immunity and development of antibodies as has been suggested previously by a small scale human experiment (Andrade, Marques & de Santa Rosa, 1986). However, this does not exclude the modulation of other secretory products from epithelial cells by commensal neisseriae as a means of preventing colonisation by *N. meningitidis*, such as defensins (McGillivray *et al.*, 2009), mucins (Brodeur *et al.*, 1986), exogenous vesicles (Kesimer *et al.*, 2009) or host receptors (Deghmane *et al.*, 2009).

The inhibition of association by *N. meningitidis* to epithelial cells was further expanded to include the serogroup B ST-11 strain Z4701 using viable counts, and the serogroup A strain C751 by flow cytometry. Flow cytometry analysis confirmed the results obtained using viable counts, illustrating the inhibition of *N. meningitidis* strain MC58 association (approximately 50%) with epithelial cells by *N. lactamica*. The variation in levels of inhibition by *N. lactamica* when analysed by viable counts or flow cytometry could be due to viable counts only measuring viable bacteria, whereas flow cytometry does not discriminate between viable and non-viable bacteria.

However, when the ST-11 MenC strain Z6417 was used to challenge D562 cells in the presence of *N. lactamica*, converse to findings herein with the previously mentioned Nmen strains, there was a slight but significant increase in association of MenC compared with MenC alone. This increase in association by MenC was accompanied by a decrease in association with D562 cells of *N. lactamica* compared with *N. lactamica* alone. The decrease in association by *N. lactamica*, as measured by viable counts, was approximately 4 times as great as the increase in association by MenC. As with the opposite results found with MenB strain MC58, this suggests a mechanism other than direct competition for receptors between the two strains. In previous studies, the effect of meningococcal capsule on interaction with host cells has been investigated using capsule-deficient mutant strains (Unkmeir, 2002; Sokolova *et al.*, 2004). Down-regulation of capsule by meningococci is associated with carriage strains of meningococci rather than invasive strains (Claus *et al.*,

2002; Beddek *et al.*, 2009). The polysaccharide capsule of Nmen has previously been thought to be poorly antigenic, yet more recent findings have demonstrated the polysaccharide capsule may stimulate host cells through Toll-like receptor pathways (Zughaier, 2011). The use of capsule deficient mutants could be used to identify if the reduction or increase in association of meningococci by *N. lactamica* is dependent on the presence of particular polysaccharide capsule in the meningococcal strains used in this thesis.

This work demonstrated for the first time that the occasional human coloniser *N. polysaccharea* inhibited association of MenB strain MC58 and MenA strain C751 to an equal or greater extent than did *N. lactamica*. In both instances the greatest inhibition was in the presence of 10-fold greater ratio of commensal neisseriae to pathogenic *N. meningitidis*. The inhibition was dose-dependent and significant in the presence of equal doses of each species at MOI 200. Additionally, the related commensal *N. cinerea* in contrast did not inhibit association by MenB strain MC58 even when at a 10-fold greater ratio to MC58, though *N. cinerea* did inhibit association of the MenA strain C751. This suggests *N. lactamica* and *N. polysaccharea* may share a common mechanism to inhibit association of *N. meningitidis* to epithelial cells that is not shared by *N. cinerea*, which primarily inhibits association of some strains of *N. meningitidis* only at 10-fold greater ratio to *N. meningitidis*. To identify if competition for receptors is involved in the inhibition of *N. meningitidis* association a comprehensive inventory of adhesins present on the commensal *Neisseria* strains used in this study would be required. Alternatively, blocking antibodies or ligands could be used to elucidate the role of epithelial cell receptors involved.

The newly constructed bioluminescent derivative of strain MC58 was utilised to monitor invasion by MenB in the presence of each commensal *Neisseria* spp. As demonstrated by viable counts with MenB strain MC58, *N. lactamica* inhibited invasion by the bioluminescent derivative MC58lux, though not to the extent monitored by viable colony

counts (approximately 90% and 40% respectively) or by inhibition of metabolism in invading *N. meningitidis*. This may be attributable to the real-time monitoring of bioluminescence versus the long-term monitoring by viable counts. However, the inhibition was dependent on concentration of *N. lactamica* and as with association; this phenomenon was mirrored in the presence on *N. polysaccharea*. Both *N. lactamica* and *N. polysaccharea* were able to inhibit association or invasion by *N. meningitidis* at lower or equal numbers during culture, whereas, in these experiments only a ten-fold greater ratio of *N. cinerea* was able to inhibit invasion by MC58lux.

Furthermore, *N. lactamica* was able to reduce the viability of MenB strain MC58 and the bioluminescent derivative MC58lux during co-cultures. Using the bioluminescent MC58lux, *N. lactamica* at an equal concentration reduced MenB bioluminescence after 8 hours co-culture. By 18 hours following co-culture *N. lactamica* had significantly reduced viable MenB compared to single cultures by approximately a third, in the absence of epithelial cells. This could be due to competition for nutrients, production of toxic by-products or secretion of antibacterial substances. The use of *N. lactamica* conditioned media could help to identify if the factor is produced by *N. lactamica* in contrast to competition for nutrients. Interestingly, although viable *N. lactamica* only slightly, but significantly reduced in the presence of MenB, there was a greater reduction in viable *N. lactamica* in the presence of the bioluminescent strain MC58lux. This raises the possibility that *N. lactamica* is sensitive to either metabolic by-products of bioluminescent metabolism or to the light emanating from MenB itself.

In conclusion, both *N. lactamica* and *N. polysaccharea* suppress association of, and invasion by MenB strain MC58 with nasopharyngeal epithelial cells. Additionally, *N. lactamica* was able to suppress association of the ST-11 MenB strain Z4701 to human epithelial cells. This suggests a shared mechanism of inhibition. However, both commensal *Neisseria* spp. with the addition of *N. cinerea*, were able to suppress association of MenA strain C751,

suggesting more than one mechanism may be utilised by commensal *Neisseria* to protect against colonisation by potentially pathogenic *N. meningitidis*.

Chapter 6

Induction of host-cell death by pathogenic bacteria and modulation by commensal neisseriae

6.1 Introduction

Induction of host cell death by pathogenic bacteria is a proposed mechanism for invasion through the mucosal barrier. Using proliferation and microscopy assays, it has been demonstrated that both commensal *N. lactamica* (strain NL4) and pathogenic *N. meningitidis* (strains MC58 and C311) induce cytotoxicity in endothelial cells in a LPS and type IV pili-dependent manner, though this was in the presence of a high (5%) serum concentration in culture medium (Dunn, Virji & Moxon, 1995), with a requirement for TLR4 signalling in conjunction with soluble CD14 and LPS binding protein (LBP). In contrast, experiments with meningeal cell lines showed low induction of host cell death with *N. lactamica* (NCTC 10617), compared to higher levels by *N. meningitidis* (strain MC58) despite equivalent levels of adhesion and invasion between bacteria in a low (1%) serum concentration (Fowler *et al.*, 2006).

It has been identified that the outer membrane porin protein PorB of *N. gonorrhoeae* plays a major role in the induction of host cell death in cervical epithelial cells (HeLa cells) through induction of apoptosis by localising with host mitochondria (Müller *et al.*, 1999, 2000). This suggests that neisserial induction of apoptosis in host cells is via the intrinsic apoptotic pathway, which was supported by a reduction in apoptosis when the suppressor of intrinsic apoptosis Bcl2, was overexpressed in host cells (Müller *et al.*, 2000).

In contrast, in the same cell line the purified PorB of *N. meningitidis* did not induce apoptosis (Massari, Ho & Wetzler, 2000), but localised to host mitochondria and inhibited host cell intrinsic apoptosis induced by staurosporine, but not extrinsic apoptosis induced by TNF α with cycloheximide (Massari *et al.*, 2003, 2010). There was also a reduction in caspase 3 and 9 activation that was not dependent on Bcl2 expression.

However, comparison of carriage and pathogenic isolates of *N. meningitidis* found that pathogenic isolates induced apoptosis in host cells via autocrine activation of TNF receptor, which did not require invasion by *N. meningitidis*, but was reduced in the absence of pili

(Deghmane *et al.*, 2009). This suggests that association with host cells is required for pathogenic *N. meningitidis*-induced apoptosis. However, carriage isolates of *N. meningitidis* did not induce apoptosis and were found to suppress both pathogen and TNF α -induced host cell death through inducing shedding of TNF receptor from host cells and subsequent lack of autocrine stimulation.

The effect of commensal neisseriae and pathogenic *N. meningitidis* on host cell death has previously been investigated, but the effect of both in co-culture has not. The role of the extrinsic TNF α and intrinsic staurosporine-induced apoptotic pathways will be investigated, as will the involvement of the caspase cascade and the effect of commensal *N. lactamica*, *N. cinerea* and *N. polysaccharea* on epithelial cell death. *S. pneumoniae* has also been included as a Gram-positive comparison of pathogen-induced epithelial cell death.

6.2 Results

6.2.1 Host cell death induced by *N. meningitidis* or *S. pneumoniae*, in the absence or presence of commensal *Neisseria* spp

To investigate the ability of pathogenic *N. meningitidis* (strain MC58) or *S. pneumoniae* (isolate Sp14) to induce cell death in Detroit 562 (D562) epithelial cells in the absence or presence of commensal *Neisseria* spp; *N. lactamica* (Nlac), *N. cinerea* (Ncin) or *N. polysaccharea* (Npoly), three fluorescent nucleic acid stains were used (Figure 6.1).

To determine if any significant loss of epithelial cells occurred between treatments the cell permeable nucleic acid stain Hoechst was used. There was no significant difference in epithelial cell numbers when challenged with any of the commensal *Neisseria* spp. alone (Figure 6.1a). There was however approximately a 25% reduction compared to untreated epithelial cells when challenged with *N. meningitidis* alone ($p < 0.05$) or in the presence of *N. polysaccharea* ($p < 0.01$; Figure 6.1a). No significant loss of cells was observed when epithelial cells were challenged with *N. meningitidis* in the presence of *N. lactamica* or *N. cinerea*. Similarly, *S. pneumoniae* challenge significantly reduced epithelial cell number ($p < 0.05$) by 20% compared to untreated epithelial cells, however this reduction was also seen when in the presence of *N. lactamica* ($p < 0.01$) and *N. cinerea* ($p < 0.05$; Figure 6.1b).

To assess the levels of apoptosis in epithelial cells the cell impermeable, nucleic acid stain Yopro-1 was used. None of the commensal *Neisseria* spp. induced detectable levels of apoptosis above untreated epithelial cells (Figure 6.1c). *N. meningitidis* in contrast strongly induced apoptosis ($p < 0.01$). In the presence of *N. lactamica* and *N. polysaccharea* there was a significant reduction ($p < 0.05$) in apoptosis induced by *N. meningitidis*, which was not seen in the presence of *N. cinerea* (Figure 6.1c). Similarly, *S. pneumoniae* strongly induced apoptosis in epithelial cells ($p < 0.001$), which was significantly reduced in the presence of *N. lactamica* ($p < 0.001$), and to a lesser extent *N. cinerea* ($p < 0.01$) and *N. polysaccharea* ($p < 0.01$; Figure 6.1d).

The live cell impermeable, nucleic acid stain propidium iodide (PI) was used to assess necrosis in treated epithelial cells. None of the commensal *Neisseria* spp. alone induced necrosis in epithelial cells compared to untreated (Figure 6.1e). However, *N. meningitidis* strongly induced necrosis ($p < 0.001$). This was reduced in the presence of *N. lactamica* and *N. cinerea* ($p < 0.05$), and strongly reduced ($p < 0.001$) in the presence of *N. polysaccharea* (Figure 6.1e). Necrosis was also significantly induced in epithelial cells by *S. pneumoniae* alone ($p < 0.01$), and was significantly reduced ($p < 0.01$) when in the presence of *N. lactamica*, *N. cinerea* and *N. polysaccharea* (Figure 6.1f).

Though consistent, high levels of apoptosis and necrosis were shown for untreated epithelial cells (approximately 25%). This may have been due to sub-optimal permeabilisation by 1% saponin, as triton-x 100 was later used and produced greater total staining with Yopro-1 and PI.

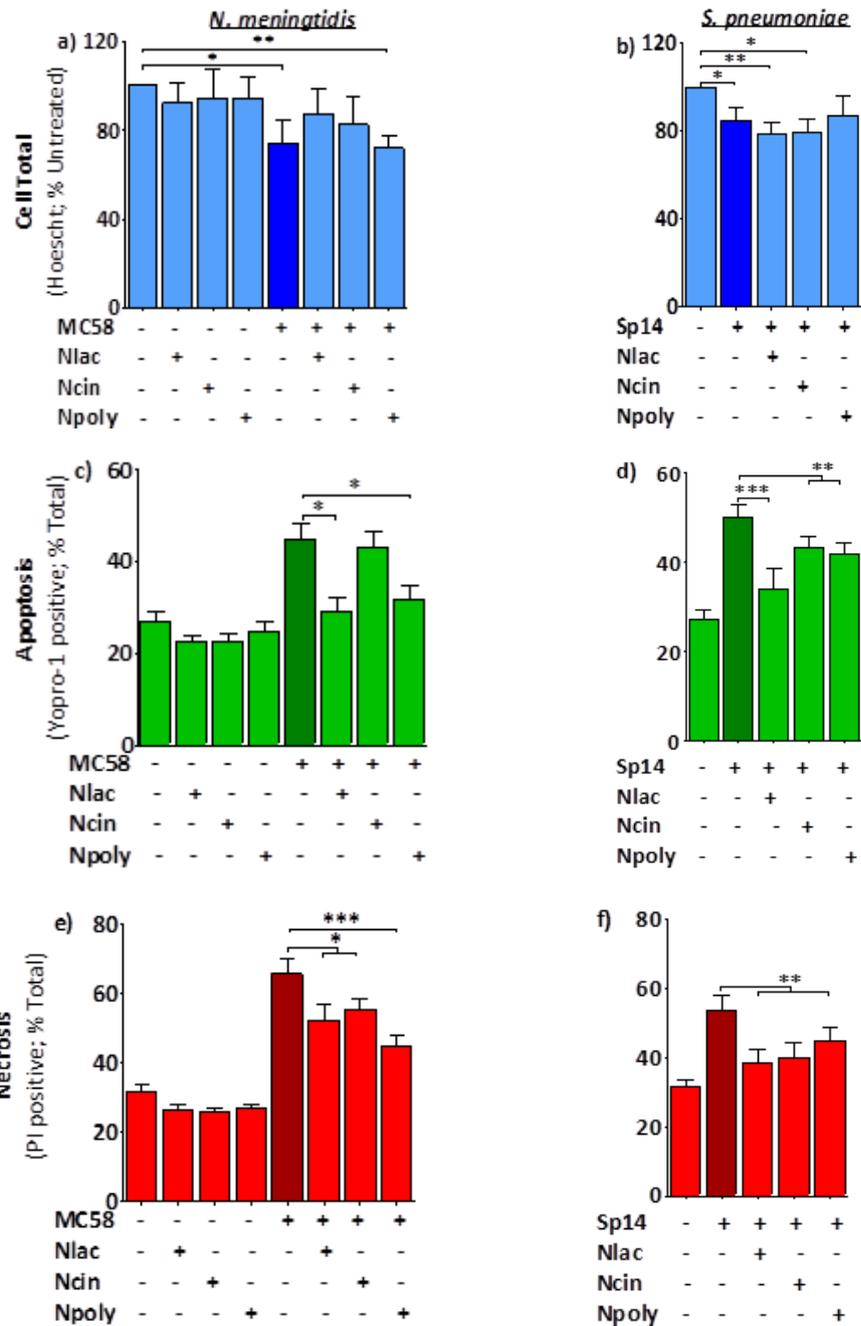


Figure 6.1 The effect of commensal neisseriae on *N. meningitidis* and *S. pneumoniae*-induced host cell death

Detroit 562 (D562) epithelial cells were challenged for 3 hours with *N. meningitidis* strain MC58 (a, c and e) or *S. pneumoniae* isolate Sp14 (b, d and f) in the absence or presence of *N. lactamica* (Nlac), *N. cinerea* (Ncin) or *N. polysaccharea* (Npoly) at 200 bacteria per epithelial cell (MOI). D562 cells were incubated in M199 media containing gentamicin (200µg/ml) for a further 21 hours. Fluorescence was measured after D562 epithelial cells were stained with Hoechst (a and b), the apoptotic stain Yopro-1 (c and d) or the necrotic stain propidium iodide (PI; e and f) on a microplate reader (Fluostar Optima). Data represents mean ± SEM from three independent repeats each replicated in triplicate. *** p<0.001 ** p<0.01 *p<0.05.

6.2.2 The effect of commensal *Neisseria* spp. on *N. meningitidis*-mediated activation of caspase 3 in Detroit 562 epithelial cells

To further investigate the apoptotic pathways stimulated in the presence of *N. meningitidis* activation of the downstream effector caspase 3 was analysed by flow cytometry.

N. lactamica at low (MOI 20) and high dose (MOI 200) did not activate caspase 3 above untreated epithelial cells, whereas *N. meningitidis* showed a strong, dose-dependent activation (Figure 6.2a). Caspase 3 activation by low dose *N. meningitidis* was significantly reduced ($p < 0.05$) in the presence of high dose *N. lactamica*. Caspase 3 activation by high dose *N. meningitidis* was reduced by *N. lactamica* in a dose-dependent manner, though not significantly.

Similarly, *N. cinerea* did not activate caspase 3 above untreated epithelial cells (Figure 6.2b). In the presence of *N. cinerea* there was a dose-dependent, though not significant, reduction in both low and high dose *N. meningitidis*-mediated activation of caspase 3.

N. polysaccharea alone did not increase activation of caspase 3 compared to untreated epithelial cells (Figure 6.2c). In the presence of both low and high dose *N. polysaccharea* there was a significant reduction ($p < 0.05$ and $p < 0.01$, respectively) in activation of caspase 3 by low dose *N. meningitidis*. In the presence of high dose *N. meningitidis* there was a dose-dependent decrease in caspase 3 activation by *N. polysaccharea* that was significant ($p < 0.05$) at high dose.

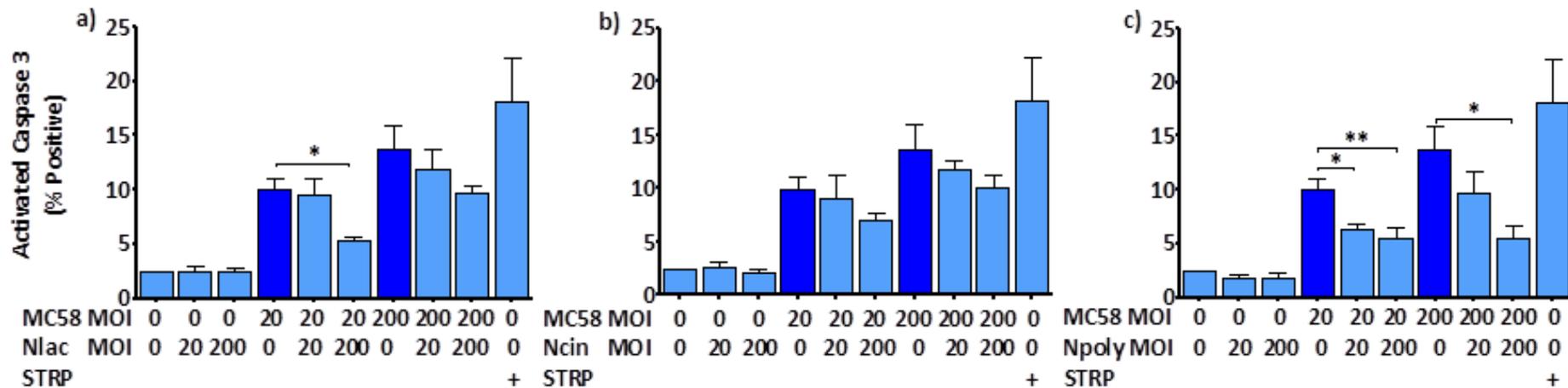


Figure 6.2 Activated caspase 3 as a marker for apoptosis in host cells challenged by *N. meningitidis* in the absence and presence of commensal neisseriae

Detroit 562 (D562) cells were challenged for 3 hours with *N. meningitidis* strain MC58 at 20 or 200 bacteria per epithelial cell (MOI) for 3 hours in the absence or presence of commensal neisseriae; a) *N. lactamica* (Nlac), b) *N. cinerea* (Ncin) or c) *N. polysaccharea* (Npoly) at MOI 20 or 200, followed by 18 hours incubation in M199 media containing gentamicin (200µg/ml). Staurosporine (STRP; 1µM) was used as a positive control. D562 cells were dissociated with EDTA (4mM) and activated caspase 3 was stained with the FITC-conjugated caspase 3 inhibitor (FITC-DEVD-FMK). Fluorescence was analysed by flow cytometry with the Accuri C6 flow cytometer. Data represents the mean ± SEM from three independent repeats. **p<0.01 *p<0.05.

6.2.3 The effect of commensal *Neisseria* spp. on *N. meningitidis*-induced activation of caspase 8 and 9 in Detroit 562 epithelial cells

To investigate the apoptotic pathways involved with *N. meningitidis*-induced host cell death in Detroit 562 (D562) epithelial cells, activation of the extrinsic death receptor associated initiator caspase 8 was measured by bioluminescent reporter assay. After 3 hours' challenge with the positive control TNF α (with cycloheximide) there was a significant increase ($p < 0.001$) in activation of caspase 8 (Figure 6.3a) compared to untreated epithelial cells. *N. meningitidis* at MOI 20 induced a significant increase ($p < 0.01$) in caspase 8 activation compared to untreated epithelial cells. This was reduced ($p < 0.01$) almost to the level of untreated epithelial cells in the presence of *N. polysaccharea*, but not *N. lactamica* or *N. cinerea* at MOI 200. However, by 6 hours (Figure 6.3b) *N. meningitidis*-induced caspase 8 levels were no longer significantly different even in the presence of commensal *Neisseria* spp.

After 9 hours (Figure 6.3c) activated caspase 8 was still significantly increased in epithelial cells challenged with *N. meningitidis* ($p < 0.001$), compared to untreated cells. However, in the presence of all commensal neisseriae levels were reduced; there was a significant reduction in the presence of *N. lactamica* ($p < 0.01$) and *N. polysaccharea* ($p < 0.001$) with levels reduced approximately to that of untreated cells. However, at all time-points measured *N. meningitidis* poorly induced activation of caspase 8 (mean 680 RLU) compared to the positive control TNF α (mean 3800 RLU).

To further investigate the apoptotic pathways involved in *N. meningitidis*-induced host cell death the intrinsic mitochondrial associated initiator caspase 9 was measured by bioluminescent reporter assay. After 3 hours' challenge (Figure 6.3d) there was a significant increase ($p < 0.001$) in activated caspase 9 by the positive control staurosporine (STRP) compared to untreated epithelial cells, but not in epithelial cells challenged with bacteria. After 6 hours' (Figure 6.3e) although *N. meningitidis*-induced activation was not

significantly increased compared to untreated cells, there was a significant reduction in the presence of *N. lactamica* ($p < 0.005$) and *N. polysaccharea* ($p < 0.01$), but not *N. cinerea*. By 9 hours' post challenge with *N. meningitidis* there was a significant increase ($p < 0.01$) in activated caspase 9 compared to untreated cells. Levels were reduced to that of untreated cells in the presence of both *N. lactamica* ($p < 0.001$) and *N. polysaccharea* ($p < 0.001$), but were unaffected by the presence of *N. cinerea* (Figure 6.3f).

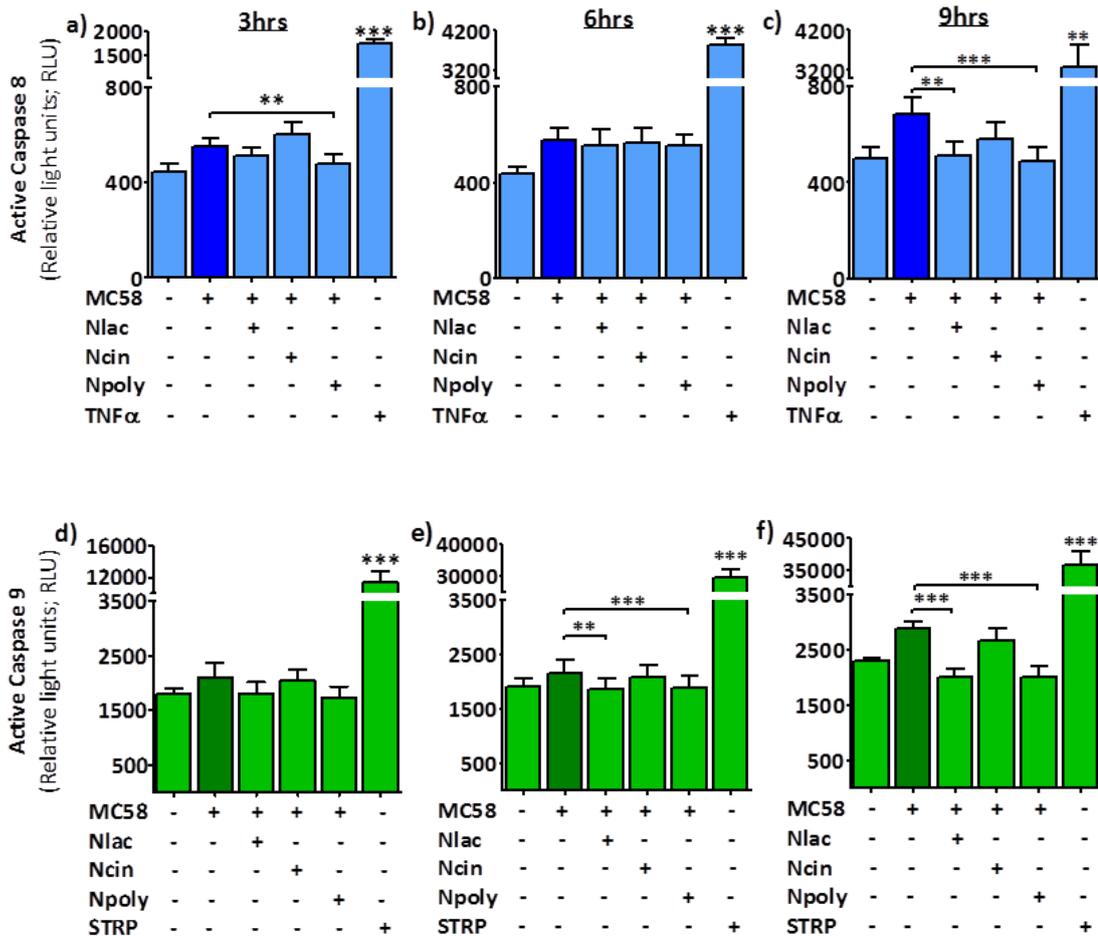


Figure 6.3 Activated caspase 8 and 9 in Detroit 562 cells challenged with *N. meningitidis* in the absence and presence of commensal neisseriae

Detroit 562 (D562) cells were challenged with *N. meningitidis* strain MC58) at 20 bacteria per epithelial cell (MOI) for 3 hours in the absence or presence *N. lactamica* (Nlac), *N. cinerea* (Ncin) or *N. polysaccharea* (Npoly) at MOI 200. D562 cells were then incubated in M199 media containing gentamicin (200µg/ml). Activated caspase 8 (a, b and c) and caspase 9 (d, e and f) were measured with the respective caspase-glo bioluminescent reporter assay kits (Promega, UK) at 3 hours (a and d), 6 hours (b and e) and at 9 hours (c and f) post challenge. TNFα (20ng/ml) with cycloheximide (chx; 20µg/ml), or staurosporine (STRP; 1µM) was used as positive control. Relative light units (RLU) were measured with a microplate reader (Fluostar Optima). Data represents mean ± SEM from three independent repeats each replicated in duplicate. *** p<0.001 *p<0.05

6.2.4 The effect of commensal neisseriae on the induction of host cell death by extrinsic or intrinsic inducers

As shown previously, all of the commensals *N. lactamica*, *N. cinerea* and *N. polysaccharea* challenged epithelial cells showed comparable Hoechst staining to untreated epithelial cells (Figure 6.4a and b). Treatment of epithelial cells with the extrinsic host cell death inducer TNF α with cyclohexamide (chx; Figure 6.4a), or the intrinsic host cell death inducer staurosporine (Figure 6.4b) resulted in a significant reduction of Hoechst staining ($p < 0.01$ and $p < 0.001$, respectively) by up to 20% in epithelial cells. This was unaltered by the presence of commensal *Neisseria* spp.

TNF α /chx significantly induced levels of apoptosis ($p < 0.001$) of approximately 30% compared to untreated epithelial cells at approximately 10% in these experiments (Figure 6.4c). There was a significant increase ($p < 0.001$) in TNF α /chx-induced apoptosis in the presence of each of the commensal *Neisseria* spp. with the greatest increase observed in the presence of *N. lactamica* to approximately 40%. Staurosporine also significantly induced apoptosis ($p < 0.01$) in epithelial cells compared to untreated to nearly 40% (Figure 6.4d). Staurosporine-induced apoptosis was unaltered in the presence of *N. lactamica*.

There was a slight, but significant induction of necrosis with both TNF α /chx ($p < 0.01$; Figure 6.4e) and staurosporine ($p < 0.05$; Figure 6.4f). When in the presence of commensal *Neisseria* spp. there were comparable levels of necrosis to either TNF α /chx or staurosporine alone. Commensal neisseriae did not have any suppressive effect on these extrinsic or intrinsic pathways.

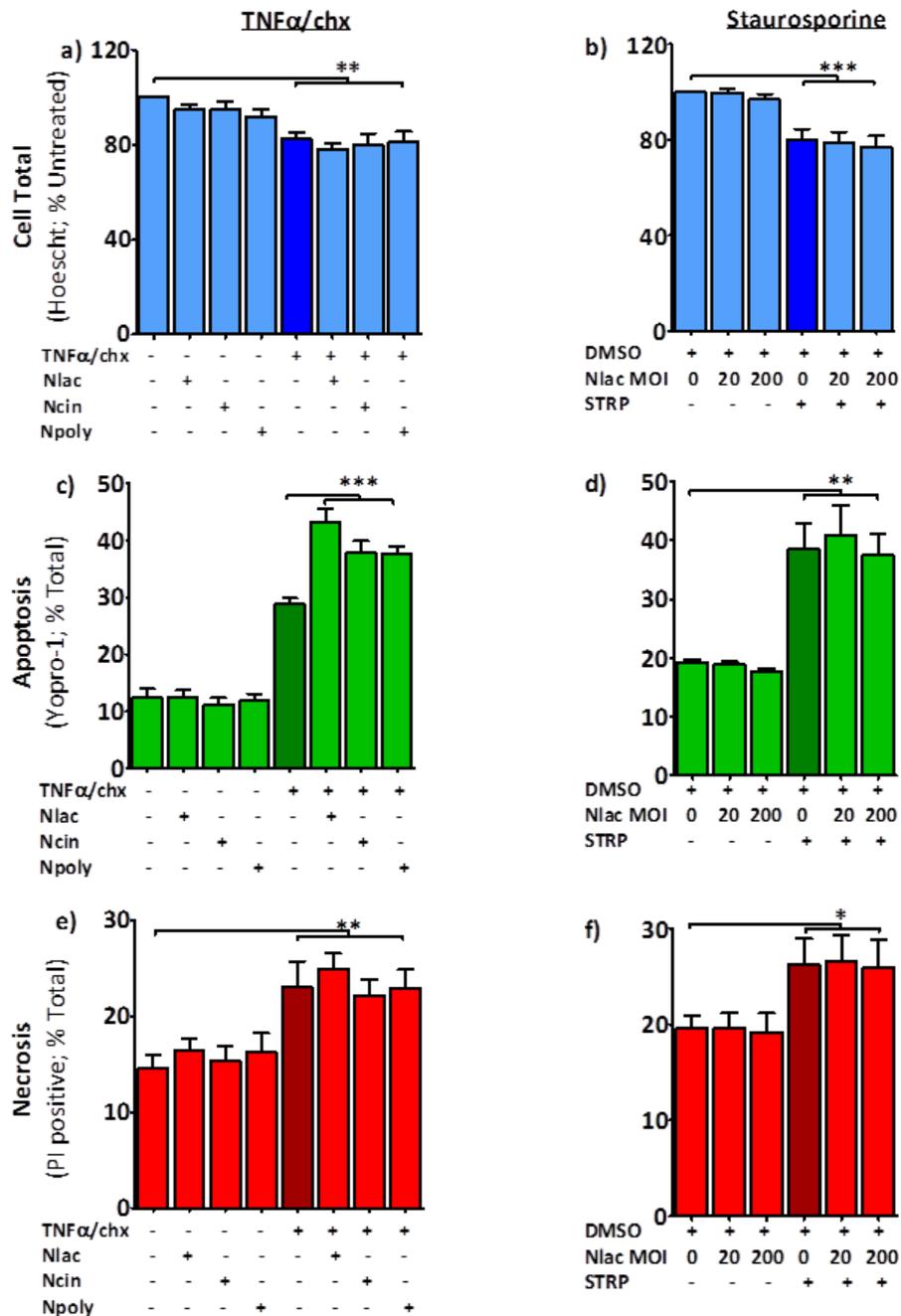


Figure 6.4 The host cell death induced by TNFα with cyclohexamide, or staurosporine in the absence or presence of commensal *Neisseria* spp.

Detroit 562 (D562) epithelial cells were incubated for 3 hours with TNFα (20ng/ml) and cyclohexamide (chx; 20μg/ml; a, c and e) in the absence or presence of commensal neisseriae; *N. lactamica* (Nlac), *N. cinerea* (Ncin) or *N. polysaccharea* (Npoly) at 200 bacteria per epithelial cell (MOI), or staurosporine (STRP; 1μg/ml; b, d and f) in the absence and presence of *N. lactamica* at MOI 20 or 200. Fluorescence was measured after D562 cells were stained with the nucleic acid stain Hoechst (a and b), the apoptotic stain Yopro-1 (c and d) or the necrotic stain propidium iodide (PI; e and f) using a microplate reader (Fluostar Optima). Data represents mean ± SEM from three independent repeats each replicated in triplicate. *** p<0.001 ** p<0.01 *p<0.05.

6.2.5 The effect of cytochalasin D on *N. meningitidis*-induced host cell death, and protection by *N. lactamica*

To investigate the involvement of invasion on *N. meningitidis*'s ability to induce host cell death, Detroit 562 (D562) epithelial cells were pre-incubated with the actin inhibitor cytochalasin D (CytoD) that prevents invasion by *Neisseria* spp. As the carrier solution for cytochalasin D, DMSO was used in controls. Host cell death was assessed using the fluorescent nucleic acid stains Hoechst, Yopro-1 and propidium iodide (PI).

Epithelial cells were challenged with *N. meningitidis* (strain MC58) at low dose (MOI 20) in the absence and presence of *N. lactamica* at high dose (MOI 200). Hoechst staining showed comparable levels of epithelial cells following treatment with *N. lactamica*, *N. meningitidis* or both together (Figure 6.5a). Neither DMSO nor cytochalasin D affected epithelial cells.

As seen previously, *N. lactamica* did not induce apoptosis, compared to DMSO control, even in the presence of cytochalasin D (Figure 6.5b). *N. meningitidis* significantly induced apoptosis ($p < 0.001$) to approximately 35% compared to DMSO control (20%), and showed a slight yet significant increase in apoptosis ($p < 0.001$) in the presence of cytochalasin D. As expected, there was a significant reduction ($p < 0.001$) in apoptosis induced by *N. meningitidis* in the presence of *N. lactamica*. The presence of cytochalasin D did not affect *N. lactamica*'s ability to suppress *N. meningitidis*-induced apoptosis in epithelial cells.

As expected, *N. meningitidis* significantly induced necrosis ($p < 0.001$) to approximately 50%, which was synergistically and significantly increased ($p < 0.001$) in the presence of cytochalasin D (Figure 6.5c). *N. lactamica*, once again reduced *N. meningitidis*-induced necrosis ($p < 0.001$). *N. meningitidis*-induced necrosis was still reduced to near DMSO control levels by *N. lactamica* when in the presence of cytochalasin D, suggesting *N. lactamica* does not require invasion into host cells to suppress *N. meningitidis*-induced host cell death.

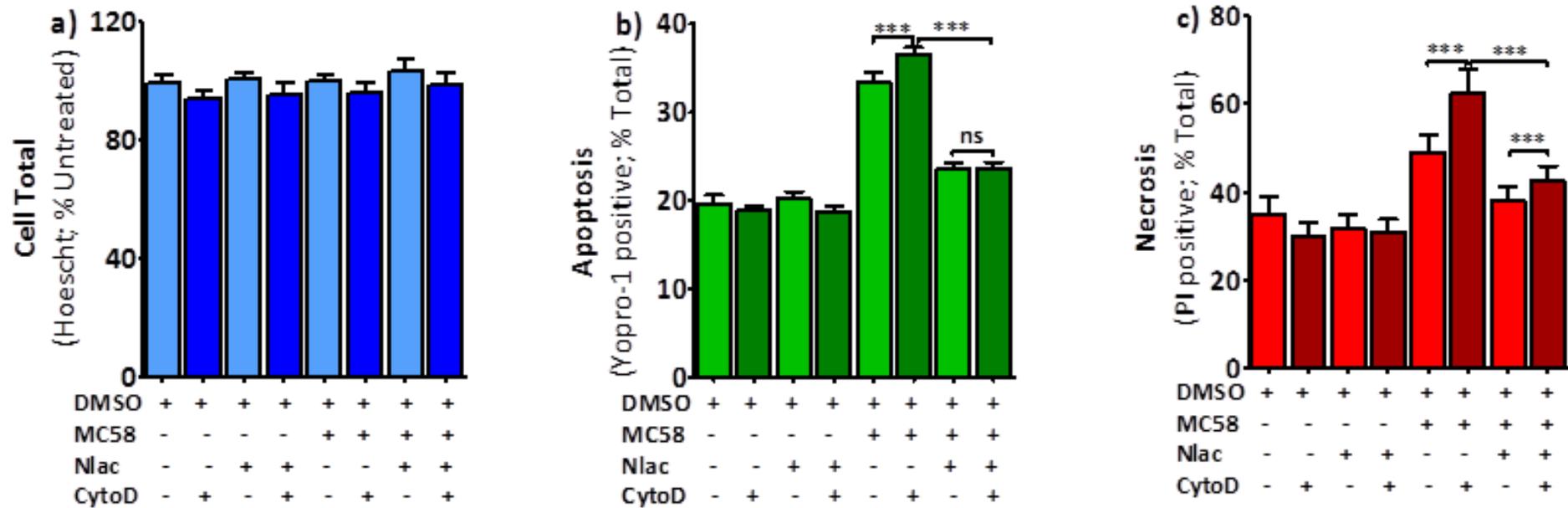


Figure 6.5 Host cell death induced by *N. meningitidis* in the presence of cytochalasin D

Detroit 562 (D562) epithelial cells were pre-incubated for 1 hour with cytochalasin D (CytoD; 2µg/ml) in DMSO. D562 cells were then challenged with *N. meningitidis* strain MC58 at 20 bacteria per epithelial cell (MOI), *N. lactamica* (Nlac) at MOI 200 or both for 3 hours, followed by 21 hours incubation in M199 media containing gentamicin (200µg/ml). D562 cells were stained with the nucleic acid stain Hoechst (a), the apoptotic stain Yopro-1 (b) or the necrotic stain propidium iodide (PI; c). Fluorescence was measured with microplate reader (Fluostar Optima). Data represents mean ± SEM from three independent repeats each replicated in triplicate. *** p<0.001 ** p<0.01 *p<0.05 ns= non-significant.

6.2.6 The effect of heat-killed *N. lactamica* and *N. lactamica* lysates on *N. meningitidis*-induced host cell death

To ascertain if live *N. lactamica* was required to suppress *N. meningitidis*-mediated host cell death, Detroit 562 (D562) epithelial cells were challenged with *N. meningitidis* (strain MC58) at a low dose (MOI 20) in the absence or presence of either heat-killed *N. lactamica* (Nlac HI) at an equivalent of low (MOI 20) and high dose (MOI 200), or *N. lactamica* lysates (Nlac Lys). Both the soluble (Sol) and insoluble (InS) fractions of lysates were tested.

As seen previously, there was a slight, but significant reduction ($p < 0.01$) in Hoechst staining of about 10% from *N. meningitidis* challenged cells (Figure 6.6a and b). Hoechst staining was further reduced ($p < 0.001$) when epithelial cells were challenged by *N. meningitidis* in the presence of heat-killed *N. lactamica* (Figure 6.6a) or *N. lactamica* lysates (Figure 6.6b).

As demonstrated previously with live *N. lactamica*, there was no induction of apoptosis by heat-killed *N. lactamica* or *N. lactamica* lysates compared to untreated epithelial cells. As expected, compared to untreated cells *N. meningitidis* significantly induced apoptosis ($p < 0.001$) in epithelial cells. However, in contrast to previous experiments with live *N. lactamica*, neither heat-killed *N. lactamica* (Figure 6.6c) nor *N. lactamica* lysates (Figure 6.6d) were able to affect *N. meningitidis*-induced apoptosis.

Similarly, heat-killed *N. lactamica* (Figure 6.6e) and *N. lactamica* lysates (Figure 6.6f) alone failed to induce necrosis. *N. meningitidis* induced significant levels of necrosis ($p < 0.001$) of nearly 30% compared to untreated epithelial cells at approximately 15%. *N. meningitidis*-induced necrosis was not affected by low dose heat-killed *N. lactamica*. However there was a slight, but significant increase ($p < 0.01$) in the presence of high dose heat-killed *N. lactamica*. *N. lactamica* lysates had no observable effect on *N. meningitidis*-induced necrosis in epithelial cells.

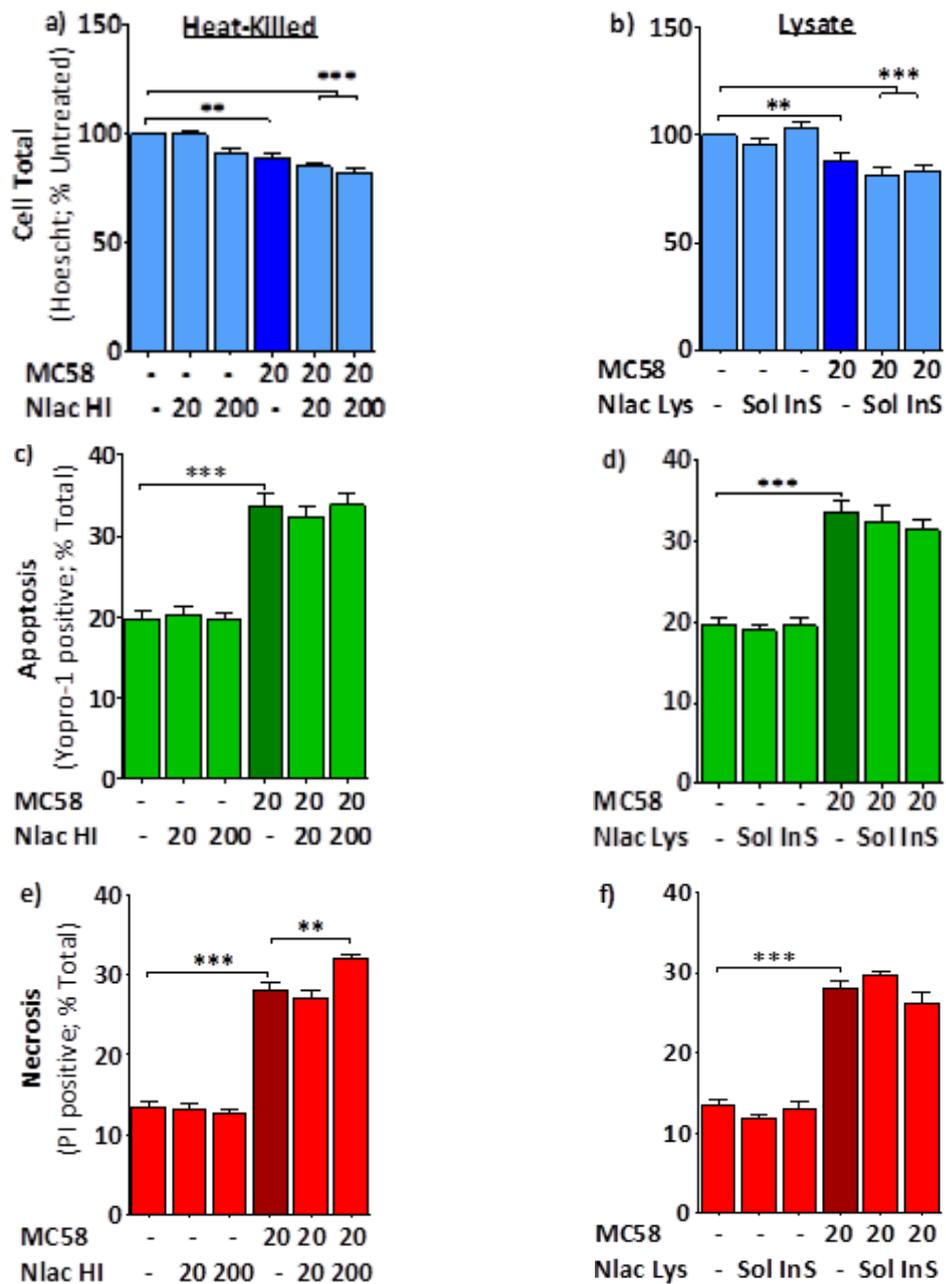


Figure 6.6 Host cell death induced by *N. meningitidis* in the absence or presence of heat-killed *N. lactamica* and *N. lactamica* lysates

Detroit 562 (D562) cells were challenged for 3 hours with *N. meningitidis* (strain MC58) at 20 bacteria per epithelial cells (MOI) in the absence or presence of heat-killed *N. lactamica* at an equivalent MOI 20 or 200 (Nlac HI; a, c and e), or the soluble (Sol) or insoluble (InS) fraction of *N. lactamica* lysates (Nlac Lys; b, d and f) at an equivalent MOI 200. Following 21 hours incubation in M199 containing gentamicin (200µg/ml) epithelial cells were stained with the nucleic acid stain Hoechst (a and b), the apoptotic stain Yopro-1 (c and d) or the necrotic stain propidium iodide (PI) (d and f). Fluorescence was measured with a microplate reader (Fluostar Optima). Data represents mean ± SEM from three independent repeats each replicated in triplicate. *** p<0.001 ** p<0.01.

6.2.7 Adhesion of *N. meningitidis* induces cell death of Detroit 562 epithelial cells

To identify if induction of apoptosis in host cells was only induced in epithelial cells associated with *N. meningitidis*, challenged epithelial cells were dual-labelled and analysed by flow cytometry.

Following 21 hours incubation approximately 5% of untreated D562 epithelial cells were labelled positively for apoptosis using the active caspase 3 fluorescent-labelled inhibitor (Figure 6.7a). When challenged with *N. meningitidis* (strain MC58) MOI 2 (Figure 6.7b) apoptotic positive epithelial cells increased by approximately 15%, of which all were also labelled positive for *N. meningitidis*. When challenged with *N. meningitidis* MOI 20 (Figure 6.7c) approximately 80% of epithelial cells were labelled positive for *N. meningitidis*. Apoptotic epithelial cells increased to approximately 80%, of which more than two thirds were also labelled positive for adherent *N. meningitidis*. Overall, this data demonstrates that there is an increase in apoptosis positive epithelial cells with a greater dose of *N. meningitidis*, and that this increase is associated with adhesion by *N meningitidis*.

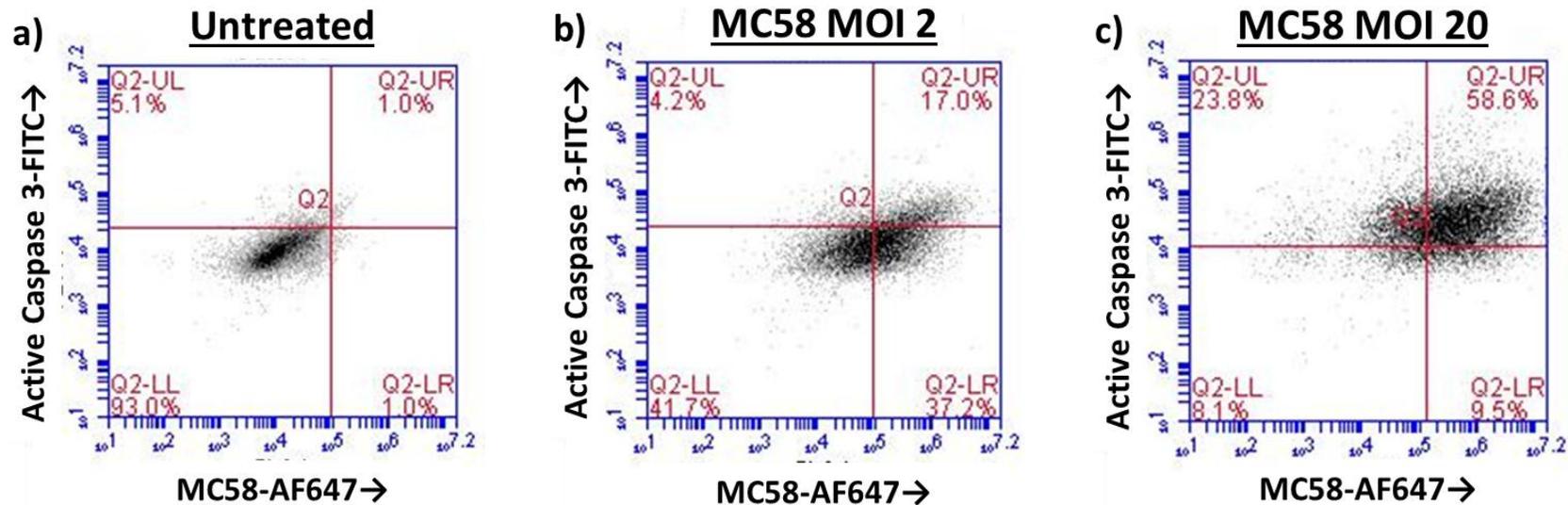


Figure 6.7 Dual-labelling for adherent *N. meningitidis* and activated caspase 3

Detroit 562 (D562) epithelial cells were unchallenged (a) or challenged for 3 hours with *N. meningitidis* (strain MC58) at a concentration of 2 (b) or 20 (c) bacteria per epithelial cell (MOI). D562 cells were then washed and incubated for a further 18 hours in M199 media (without gentamicin). D562 cells were then disassociated with EDTA (4mM) then labelled with the FITC-labelled caspase 3 inhibitor FITC-DEVD-FMK and adherent *N. meningitidis* was labelled with mouse anti-*N. meningitidis* P1.7 antibody and rabbit anti-mouse Alexa-fluor 647 (AF647) conjugated secondary antibody. Fluorescence was analysed by flow cytometry (Accuri C6). Active caspase 3-FITC was measured in FL1 and MC58-AF647 was measured in FL4. Representative dotplots of two independent repeats. Q2-LL=unlabelled Q2-UL=active caspase 3-labelled Q2-LR= MC58-labelled Q2-UR= MC58 and active caspase 3-labelled.

6.3 Discussion

The purpose of this section of this study was to investigate the induction of host cell death by upper respiratory tract dwelling pathogens and the potential effects of commensal *Neisseria* spp. on modulation of this induction of host cell death. Both viability stains and specific stains were used to analyse this.

N. meningitidis induces host cell death by multiple pathways depending on the cell type or line used. *N. meningitidis* induces apoptosis by stimulating TNF α secretion from host cells that induces autocrine-TNF α -induced apoptosis (Deghmane *et al.*, 2009), or through toxicity induced through pili or LPS interactions with the host cells (Dunn, Virji & Moxon, 1995).

Unlike a previous study also using endothelial cells in higher serum concentration medium (Dunn, Virji & Moxon, 1995), *N. lactamica* did not induce host cell death in epithelial cells in this study, nor did the *Neisseria* commensals *N. cinerea* and *N. polysaccharea*, when measured by viability stains and activation of caspase 3. However, *N. meningitidis* and *S. pneumoniae* were both potent inducers of apoptosis and necrosis using viability stains. These observations suggest that induction of host cell death within the nasopharynx is possibly a mechanism for invasion of the mucosa by pathogenic bacteria, but is not a mechanism employed by commensal bacteria.

All three commensal *Neisseria* spp. reduced apoptosis and necrosis induced by *S. pneumoniae*, with the greatest reduction in apoptosis by *N. lactamica* when measured after 24 hours. This was however still in the presence of a reduction in total epithelial cells. All three commensal *Neisseria* spp. also reduced necrosis induced by *N. meningitidis*, though *N. polysaccharea* was most effective at reducing necrosis.

N. meningitidis was a potent inducer of apoptosis in nasopharyngeal epithelial cells when measured by the viability stain Yopro-1 and activation of caspase 3. *N. lactamica* and *N. polysaccharea* were able to reduce Yopro-1 staining nearly to the level of untreated

epithelial cells which did not occur in the presence of *N. cinerea*. Both *N. lactamica* and *N. polysaccharea* were also able to reduce activation of caspase 3 when *N. meningitidis* was at low concentration (MOI 20), but only *N. polysaccharea* was able to reduce activation of caspase 3 induced by a high concentration (MOI 200) of *N. meningitidis*. *N. cinerea* was not able to reduce the induction of host cell death by *N. meningitidis*. These observations suggest that only *N. lactamica* and *N. polysaccharea* may share a common mechanism to prevent *N. meningitidis*-induced host cell death.

The extrinsic induction of apoptosis by agonisation of surface receptors such as TNF α involves activation of caspase 8 (Mangan, Mergenhagen & Wahl, 1993; Van Herreweghe *et al.*, 2010), whereas intrinsic induction of apoptosis via the mitochondrial pathway leads to activation of caspase 9 (Jiang & Wang, 2000). Activation of either initiator caspase leads to activation of the effector caspase 3 in host cells. The same pattern of suppression by *N. lactamica* and *N. polysaccharea* was found with activation of the initiators caspase 8 and caspase 9 after 9 hours. This suggests *N. meningitidis* may induce apoptosis via both activation of surface receptors and via the mitochondrial pathway. However, TNF α -, staurosporine- and *N. meningitidis*- treated epithelial cells gave similar Yopro-1 staining in separate experiments, whereas TNF α and staurosporine induced 4 times and 10 times greater activation of caspase 8 and caspase 9, respectively. This suggests activation of these caspases is a minor component of *N. meningitidis*-induced apoptosis in epithelial cells.

This is further supported by experiments involving activation of host cell death by TNF α or staurosporine. Massari *et al* (2010) demonstrated protection by meningococcal porB against apoptosis induced by the potent intrinsic inducer staurosporine through its interaction with mitochondria. In this study *N. lactamica* was unable to modulate host cell death induced by staurosporine suggesting a different mechanism was employed. Also carriage strains of *N. meningitidis* protect epithelial cells from invasive meningococci by inducing shedding of TNF receptor and thus prevention of autocrine TNF α signalling

(Deghmane *et al.*, 2009). Again *N. lactamica*, as well as *N. cinerea* and *N. polysaccharea*, were unable to suppress host cell death induced by TNF α (with cyclohexamide), and even synergistically enhanced apoptosis in the presence of TNF α . This enhancement may be due to the synergistic effect of death receptors such as TNF receptor with Toll-like receptors to activate caspase 1 and caspase 11 (Hisahara *et al.*, 2000), of which TLRs can be activated by commensal as well as pathogenic bacteria.

To identify if invasion by *N. meningitidis* was required to induce cell death in epithelial cells, cytochalasin D, an inhibitor of actin re-arrangement and subsequently neisserial invasion, was used. Interestingly in the presence of cytochalasin D there was a significant increase in both apoptosis and necrosis induced by *N. meningitidis* suggesting that prevention of invasion by the pathogen enhances its ability to kill epithelial cells and that damaging the mucosal barrier in this manner is an alternative means of invading the host to transcellular invasion. It may also suggest that modifications to surface ligands of the minor invasive subpopulation of bacteria that facilitates invasion may also be required for host cell death pathways activation.

The protection by *N. lactamica* on *N. meningitidis*-induced host cell death was dependent on live *N. lactamica* as has been previously found with the suppression of inflammation induced by *N. meningitidis* (Tezera *et al.*, 2011). As neither heat-killed nor lysates of *N. lactamica* protected against *N. meningitidis*-induced epithelial cell death, the induction of apoptosis and interaction of *N. meningitidis* with epithelial cells was investigated. Using lower doses (MOI 2 and 20) of *N. meningitidis* over 21 hours without antibiotic, there was a dose-dependent increase in both association of bacteria and apoptosis measured by activated caspase 3 with the largest population of epithelial cells being dual-labelled for both association of bacteria and active caspase 3. This lends argument to the hypothesis that *N. meningitidis* interaction with the surface receptors of epithelial cells signals the induction of apoptosis by these bacteria. This may involve signalling through both autocrine

activation of TNF receptor and TLRs as putatively suggested by TNF α cell death experiments, or some other mechanism that involves caspase 8 and caspase 9 in a minor role.

To summarise; *N. lactamica* and *N. polysaccharea*, but not *N. cinerea* prevent association of *N. meningitidis* with nasopharyngeal epithelial cells. Association with epithelial cells is required for induction of apoptosis by *N. meningitidis*. Both *N. lactamica* and *N. polysaccharea*, but not *N. cinerea* reduce apoptosis induced by *N. meningitidis* in epithelial cells, suggesting the suppression in association is the mechanism involved in suppression of apoptosis by commensal *Neisseria* spp.

To further evaluate the mechanism of protection by both *N. lactamica* and *N. polysaccharea* the involvement of caspase 1 should be further investigated. A recent study has revealed caspase 1 is required for induction of apoptosis by the related pathogen *N. gonorrhoeae* (Duncan *et al.*, 2009) potentiating its involvement in *N. meningitidis*-mediated apoptosis. The mechanism by which commensal *Neisseria* prevent association of *N. meningitidis* with epithelial cells whether agglutination, aggregation or competition for receptors, should also be investigated.

Chapter 7

General discussion and further work

7.1 General discussion

Experiments herein were designed to investigate the host-bacterial interactions during colonisation by pathogenic and commensal bacteria whose natural habitat is the human nasopharynx (Cartwright *et al.*, 1987; Oh *et al.*, 2008; Sleeman *et al.*, 2001; Kluytmans, van Belkum & Verbrugh, 1997). Although many studies include both epithelial cells and endothelial cells from multiple human tissues, a study by Virji *et al.* (1992a) identified differential association of *N. meningitidis* with epithelial and endothelial cells. This has more recently been investigated and found to utilise different mechanisms of host cell signalling (Lécuyer, Nassif & Coureuil, 2012). Even TLR expression differs between upper and lower respiratory epithelial cells (Rydberg *et al.*, 2009) and therefore responses to bacteria and bacterial ligands would be expected to also. For example, microarray analysis of bronchial epithelial cells challenged with *N. lactamica* (strain Y92-1009) or *N. meningitidis* (strain MC58) showed an upregulation in transcriptional activation of pro-inflammatory cytokines following challenge with *N. lactamica*, but not *N. meningitidis* (Wong *et al.*, 2011). This contrast in findings to other studies (Griffiths *et al.*, 2007; Tezera *et al.*, 2011) could be tissue specific, though a study on another bronchial epithelial cell line (BEAS-2B) suggests that it is time, strain or cell line-dependent (Massari *et al.*, 2010).

To this end, many studies to investigate the colonisation of *N. meningitidis* in particular, with the human nasopharynx, have used primary human nasopharyngeal cells (de Vries *et al.*, 1996) and explants of mucosal tissues (Stephens, Hoffman & McGee, 1983; Rayner *et al.*, 1995; Read *et al.*, 1999). These are often difficult to source and create greater variability in results than do cell lines. Tumourigenic epithelial cell lines undergo genetic and epigenetic changes, such as upregulation of the transcription factor NFκB (Li *et al.*, 2006a), which can affect their behaviour compared to non-tumourigenic cells (Rydberg *et al.*, 2009). Two human pharyngeal epithelial cell lines are commercially available; the Detroit 562 (D562) and Fadu cell lines. The Fadu cell line has previously been used to characterise

N. meningitidis colonisation and invasion (Sutherland *et al.*, 2010) primarily due to its origin and characteristics of polarizing and forming tight junctions. However, unlike both primary epithelial cells and the D562 cell line, Fadu cells do not possess a functional TLR3 (Matijevic, Marjanovic & Pavelic, 2009; Rydberg *et al.*, 2009) and D562 cells have a similar though greater response to TLR agonists (Rydberg *et al.*, 2009). The D562 cell line has been more frequently used in meningococcal studies (Bradley *et al.*, 2005; Griffiths *et al.*, 2007; Sa E Cunha *et al.*, 2009; Tezera *et al.*, 2011). Therefore, all experiments herein that required host-bacterial interactions were conducted with the human nasopharyngeal carcinoma cell line D562.

N. lactamica strain NL4.1 has previously been demonstrated to suppress the transcription and secretion of inflammatory cytokines and chemokines (IL6, TNF α and IL8) induced by the closely related pathogenic *N. meningitidis* strain MC58 in D562 human nasopharyngeal epithelial cells. Additionally, strain NL4.1 has been demonstrated to suppress the induction of cytokines induced by the host cell surface receptor agonists IL1 β , TNF α and the TLR2/1 agonist PAM3Cys, in the absence of pathogenic bacteria (Tezera *et al.*, 2011). This suggests that strain NL4.1 possesses the capacity to suppress inflammation induced in host cells via differing signalling pathways. This suppression correlated with a reduction in the transcriptional activation of NF κ B and involved, in an undefined capacity, the nuclear receptor PPAR γ . However, microarray analysis of bronchial epithelial cells challenged with *N. lactamica* (strain Y92-1009) or *N. meningitidis* (strain MC58) identified an increase in inflammatory mediators only following *N. lactamica* challenge (Wong *et al.*, 2011), though there was also an increase in genes that regulate and prevent proinflammatory responses. This could suggest host site-specific responses or perhaps differing roles for *N. lactamica* strains in the human microbiota, to induce inflammation and stimulate a cross-reactive immune response (strain Y92-1009), or to reduce inflammation induced by pathogens to protect the mucosal barrier (strain NL4.1).

Induction of inflammation via TLR2, a key meningococcal inflammatory receptor in D562 cells, is induced through activation of multiple MAPK pathways and involves NFκB activation (Rydberg *et al.*, 2009). It was therefore hypothesised that NL4.1 suppresses some if not all MAPK pathways in D562 cells. Conversely, data presented in chapter 3 of this thesis suggests NL4.1 synergistically enhances MAPK pathway-induced inflammation. However, NL4.1 did suppress inflammation induced through the intracellularly localised TLR3. Inflammation induced through TLR3 in D562 cells does not require the MAPK pathways, but does require NFκB activation, further supporting the findings of Tezera *et al.* (2011). The notion that NL4.1 possesses a global anti-inflammatory mechanism may also be supported by suppression of TLR3-mediated inflammation. The TLR3 receptor is not found on the host cell surface, avoiding bacterial interaction, and has not been implicated in neisserial inflammation (Figure 7.1).

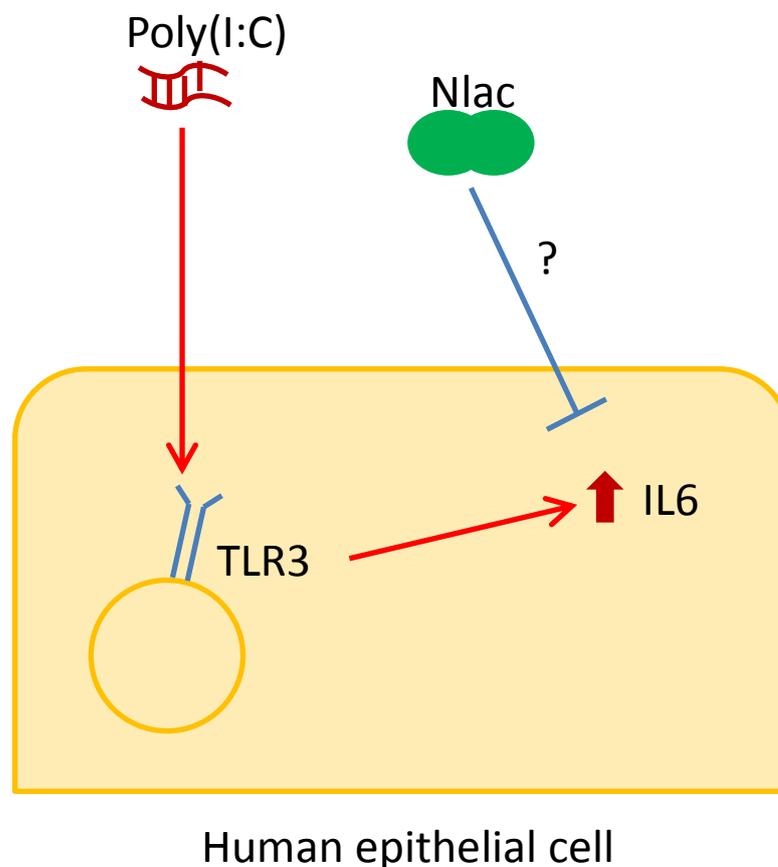


Figure 7.1 Representation of suppression of IL6 induced via TLR3 by *N. lactamica*.

N. lactamica suppressed inflammation measured by the proinflammatory cytokine IL6, induced by the intracellular TLR3 agonist poly(I:C), in human nasopharyngeal epithelial cells by as yet an unknown mechanism.

Both *N. meningitidis* and *N. lactamica* express porB, which is a ligand for TLR2. Differences in the molecular structure of porB from each species can modify the specific site on TLR2 to which each species interacts. TLR2 has both inflammatory and inhibitory signalling pathways depending on the ligand interaction, which has been demonstrated to inhibit inflammation induced through TLR3 for example (Lai *et al.*, 2009). Modulated surface labelling of TLR2 (Appendix 5) in the presence of *N. lactamica* (as well as other commensal *Neisseria* spp.) could suggest modulation of surface receptors as a means of *N. lactamica*-mediated inflammatory suppression. Alternatively, it may suggest competition for a TLR2 binding site, which is also recognised by the antibody used to label TLR2, a known inhibitor of TLR2-induced inflammation (Flo *et al.*, 2000). However, this data is merely suggestive on its own and requires further evaluation. It could suggest commensal *Neisseria* spp. that are poorly inflammatory and do not cause invasive or localised disease in the host, may have adapted (or retained) molecular structures to conserved neisserial proteins to this end, in contrast to invasive *Neisseria* spp. which have adapted (or retained) alternate molecular structures.

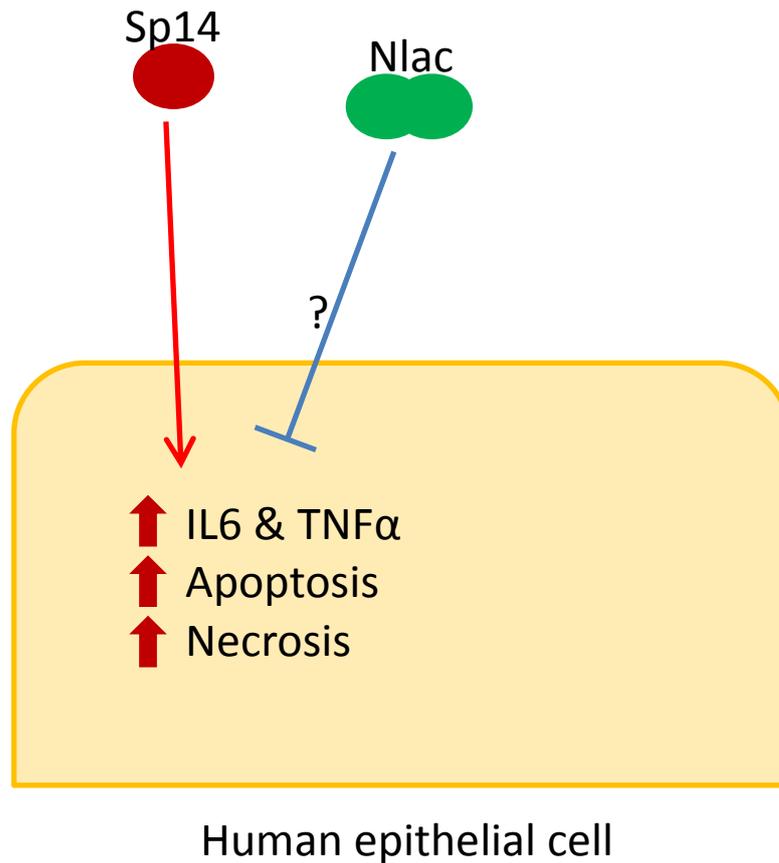


Figure 7.2 Representation of host cell responses to *S. pneumoniae* by *N. lactamica*.

N. lactamica suppressed inflammation measured by the proinflammatory cytokines IL6 and TNF α , and host cell death induced *S. pneumoniae* isolate Sp14, in human nasopharyngeal epithelial cells by as yet unknown mechanisms.

Additionally, *N. lactamica* has been observed in this study to suppress inflammation induced by an unrelated Gram-positive human pathogen for the first time (Figure 7.2). Although this project did not extend to demonstrate the pathways involved in the induction of inflammation in D562 cells by the *S. pneumoniae* isolate used, other strains of *S. pneumoniae* have been demonstrated to induce inflammation through TLR2, and to a lesser extent TLR4 (Mogensen *et al.*, 2006a).

Both pathogenic *N. meningitidis* and commensal *N. lactamica* exclusively colonise humans with a preference for the nasopharyngeal niche. It has long been hypothesised that *N. lactamica* may confer protection to the host against carriage and/or invasive disease elicited by *N. meningitidis* (Gold *et al.*, 1978; Cartwright *et al.*, 1987; Oliver *et al.*, 2002). This

was often considered to be due to the generation of antibodies by the host to *N. lactamica* that cross-reacted with *N. meningitidis* as has been observed previously (Gold *et al.*, 1978). However, recent findings suggest that it is primarily a non-specific IgM antibody response that is generated by *N. lactamica* (Vaughan *et al.*, 2010), and that live *N. lactamica* or OMV induce antibody responses that are poorly cross-reactive with the meningococcus and do not significantly induce increased serum bactericidal antibody in human participants (Evans *et al.*, 2011). It has been proposed that live *N. lactamica* is required within the nasopharyngeal niche of the host to give protection via a means other than the induction of adaptive immunity (Tezera *et al.*, 2011; Evans *et al.*, 2011).

In chapter 5 of this thesis, several methods were utilised to measure the association and invasion of *N. meningitidis* to D562 nasopharyngeal epithelial cells in the absence of immune cells, and the effect of *N. lactamica* primarily on this *in vitro* model of colonisation. *N. lactamica* strain NL4.1 reduced both association and invasion by two strains of *N. meningitidis* serogroup B, the reference strain MC58 and the ST-11 strain Z4701, but not the ST-11 serogroup C strain Z6417. Though Z4701 was poorly adherent, the hyper-adherent strain MC58 was suppressed from adhering to D562 cells by up to 60% and invasion was reduced by 90% in the presence of *N. lactamica*. However, neither *N. lactamica* adherence nor invasion increased by equivalent amounts when measured by the sensitive colony count method, suggesting direct competition for host receptors was not involved, but a more complicated or indirect means of suppression was employed by *N. lactamica*. Additionally, the involvement of bacterially-induced secretion of host factors to protect against association by *N. meningitidis* was not evaluated in this study and may warrant further work (Andrade, Marques & de Santa Rosa, 1986).

Initially as comparison to *N. lactamica* the neisserial commensals *N. cinerea* and *N. polysaccharea* were also used to modulate *N. meningitidis* association and invasion of host nasopharyngeal cells. To this end *N. meningitidis* serogroup B strain MC58 was manipulated

by homologous recombination to express the *luxCDABE* operon allowing rapid measurement by the production of bioluminescent light and thus discrimination from commensal *Neisseria*. The bioluminescent construct (strain MC58lux) was compared to the parental strain for evaluation as an appropriate reporter in chapter 4.

Along with antibody labelling of *N. meningitidis* and flow cytometry analysis, modulation of *N. meningitidis* invasion and association, respectively, was measured. Using viable colony counts obtained from *N. meningitidis* and *N. lactamica* co-cultures mentioned above, comparisons could be made between methods to support the validity of the results obtained by bioluminescent reporter and antibody labelled assays.

Using the bioluminescent reporter of *N. meningitidis*, there was less suppression of invasion in the presence of *N. lactamica*. This could be accounted for by the real-time measure of bioluminescence measuring metabolically active bacteria present as opposed to viable counts measuring bacteria that survive after 24hr culture. None-the-less, a similar and dose-dependent suppression of *N. meningitidis* invasion of epithelial cells was measured. Similarly, *N. polysaccharea* was also able to suppress *N. meningitidis* invasion of epithelial cells in a dose dependent manner. *N. polysaccharea* has not previously been implicated in protection from *N. meningitidis* though these experiments suggest there may be a role for it in host protection. This protection was further demonstrated by a reduction in association of *N. meningitidis* in the presence of *N. polysaccharea*. This strongly suggests that some, but not all commensal *Neisseria* spp. have the ability to suppress association and possibly therefore invasion of pathogenic *N. meningitidis* with the host.

In chapter 6 both viability stains and reporters for activation of specific host caspases were used to evaluate host cell death induced by *N. meningitidis* serogroup B and to a lesser extent *S. pneumoniae* serotype 14. Both *N. meningitidis* and *S. pneumoniae* were potent inducers of host cell death in contrast to commensal *Neisseria* spp. that did not elicit cytotoxicity in host cells. This supports the hypothesis that pathogenic bacteria of the

nasopharynx may use induction of host cell cytotoxicity as a mechanism of invasion through the mucosa.

This study demonstrates for the first time that all three commensal *Neisseria* spp. are capable of suppressing cytotoxicity induced by the unrelated Gram-positive pathogen *S. pneumoniae* in nasopharyngeal epithelial cells. This suggests that commensal organisms that normally occupy the same niche as pathogens can give cross-species protection, that is not dependent on immunological ligand similarities due to genetic relatedness.

In contrast only *N. lactamica* and *N. polysaccharea* were able to effectively suppress epithelial cell cytotoxicity induced by *N. meningitidis*, as measured by both viability stains and monitoring activation of specific host caspases, in particular the activator caspase 3. The inability of *N. cinerea* to inhibit *N. meningitidis*-induced host cell death suggests that a common mechanism for suppression of host cell death by both Gram-negative and Gram-positive pathogens may not be present. Or that the mechanism utilised by *N. cinerea* is less effective against *N. meningitidis* than *S. pneumoniae*. In the case of *N. lactamica*, the ability to suppress host cell death was reliant on the live organism, as neither whole heat-killed *N. lactamica*, nor homogenised lysates were effective at suppressing *N. meningitidis*-induced host cell death.

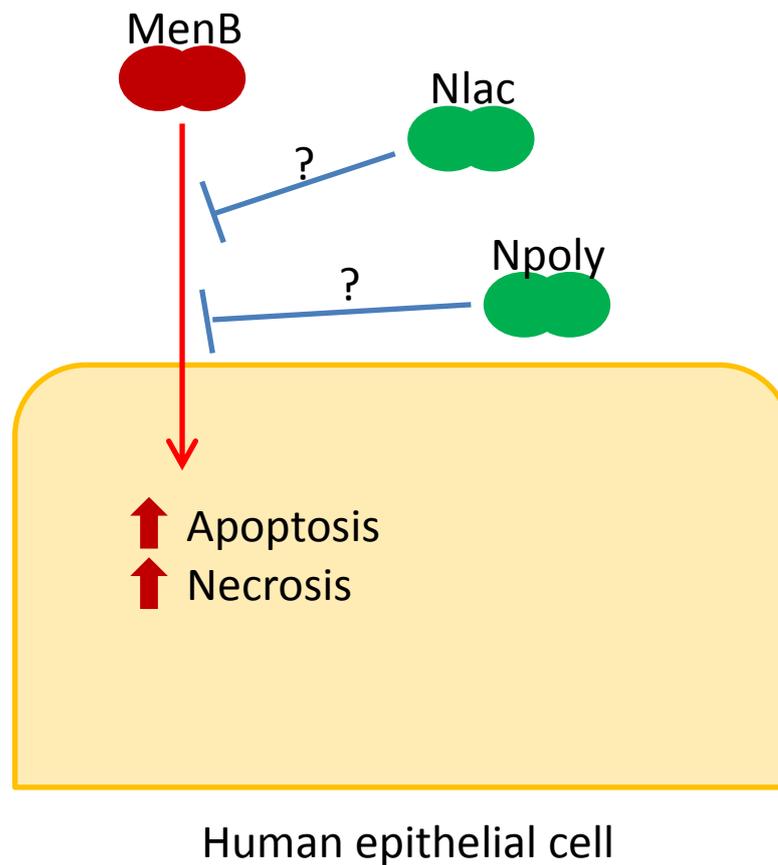


Figure 7.3 Representation of commensal neisseriae protection against *N. meningitidis* serogroup B-induced host cell death.

Both *N. lactamica* (Nlac) and *N. polysaccharea* (Npoly) reduced association of *N. meningitidis* serogroup B (MenB) with host cells by a currently unknown mechanism. Both commensals also protected against host cell death induced by MenB, which required MenB association with host cells.

An apparent similarity in the trend in suppression of both association with, and induction of host cell death, by *N. meningitidis* in the presence of commensal *Neisseria* spp. was observed. Therefore induction of host cell death and association by *N. meningitidis* was evaluated by flow cytometry. The majority of host cells induced to undergo apoptosis, as measured by active caspase 3, were also found to have *N. meningitidis* association. This suggests a strong correlation and that meningococcal interaction with host cell receptors is required for induction of cell death, as opposed to invasion by meningococci or secretion of bacterial products such as LPS (Figure 7.3). Therefore the mechanism of suppression of

meningococcal-induced host cell death by commensal *N. lactamica* and *N. polysaccharea* is most probably tied to their ability to reduce association of the pathogen with host cells.

The data herein supports the hypothesis that a single commensal *Neisseria* spp. such as *N. lactamica* or *N. polysaccharea*, or a combination of species could be utilised as potential probiotics for the prevention of meningococcal colonisation and carriage.

7.2 Future work

One significant limitation of this body of work is the use of one carcinoma cell line for all host-bacterial interaction studies. The limitation has largely been due to the limited availability of epithelial cell lines of the nasopharynx, which the primary organisms of this study colonise. To further corroborate this data it is vital that other pharyngeal cells lines such as Fadu (Rangan, 1972; Sutherland *et al.*, 2010), primary or immortalised oral or nasopharyngeal epithelial cells, such as those used in cancer studies (Sun *et al.*, 1992; Li *et al.*, 2006a) be used to replicate this work. This is particularly important since differences between primary cells and cell lines have previously been reported (Rydberg *et al.*, 2009).

Additionally, microarrays have been utilised by other researchers to profile the induction or suppression of relevant genes, such as those involved with apoptosis and inflammation, often to reveal opposing results to those found herein (Linhartova *et al.*, 2006; Wong *et al.*, 2011). This could be due to differences in host cell type or bacterial strains used, but could also be due to methodological differences, so could warrant further investigation, especially as mixed bacterial co-cultures were not conducted.

In the same vein, it has previously been reported that vast differences have been seen in the responses from host cells between different strains of meningococci (Deghmane *et al.*, 2009). Therefore it is reasonable to assume that differences would be found both between different species of commensal neisseriae, as has been found in this work, and between

different strains of each species. For instance the *N. lactamica* strain used herein belongs to the ST-3787 clonal complex and is therefore unique among isolates that have been MLST typed. Whereas, ST-624, ST-613, ST-640 and ST-595 have been found to be more commonly isolated from infants (Bennett *et al.*, 2005), and therefore strains of these clonal complexes could be worth investigating, such as the *N. lactamica* reference strains Y92-1009 (ST-613) and 020-06 (ST-640). If differences in host cell responses and suppression of meningococcal association with host cells were found between different strains of *N. lactamica* or *N. polysaccharea*, then further proteomic and genomic studies could be conducted. Two-dimensional electrophoresis or far-western blots could be used to identify variation in proteomic expression between strains of protective and non-protective commensal *Neisseria*, and to potentially identify meningococcal ligands, if indeed any do exist. To identify genetic factors differentiating protective and non-protective strains, subtractive hybridization has previously been used with success (Lewis *et al.*, 2005) and may be useful to further this work.

Aside from identifying potential commensal neisserial bacterial products that may give protection against meningococcal infection, the mechanisms and dynamics of commensal protection against meningococcal colonisation should be further studied. Sequential blockade and identification of host cell receptors and bacterial ligands should be conducted to ascertain the crucial interactions in this model. A systematic analysis of neisserial proteins on the strains of *N. cinerea* and *N. polysaccharea* used in this study, and comparisons to the *N. lactamica* strain used, would be an effective route to evaluating the interactions. Furthermore, herein the ability of *N. lactamica* to inhibit association by *N. meningitidis* to D562 cells was not globally recognised by all meningococcal strains tested as *N. lactamica* increased association of MenC strain Z6417. However, as no vaccines are currently in use against MenB, but are against other serogroups it could be of interest to expand the study of *N. lactamica*'s inhibition of meningococcal association with host cells to

other common meningococcal clonal complexes, in particular ST-41/44 being the most commonly isolated in Europe.

Association and invasion assays presented in this thesis were all conducted with mixed cultures of *N. meningitidis* and commensal neisseriae added to host cells at the same time point. Therefore coagglutination and coaggregation assays may be a recommended choice of assay to help identify potential mechanisms of commensal protection against meningococci (Kolenbrander & Andersen, 1986; Whittaker, Klier & Kolenbrander, 1996). Additionally the dynamics of protection should be investigated. Do protective commensal neisseriae give greater protection when pre-associated with host cells, or do they disassociate meningococci that have already associated with host epithelial cells? The answers to these questions will help to evaluate the effectiveness of these commensal *Neisseria* spp. for use as live probiotics to protect against colonisation and potentially disrupt carriage of meningococci in the human nasopharynx.

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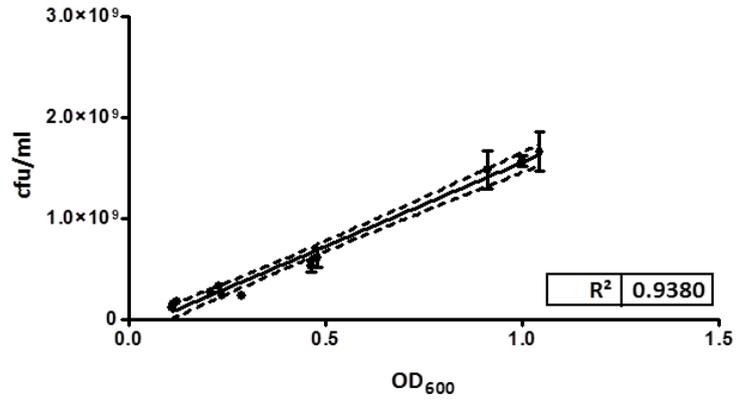
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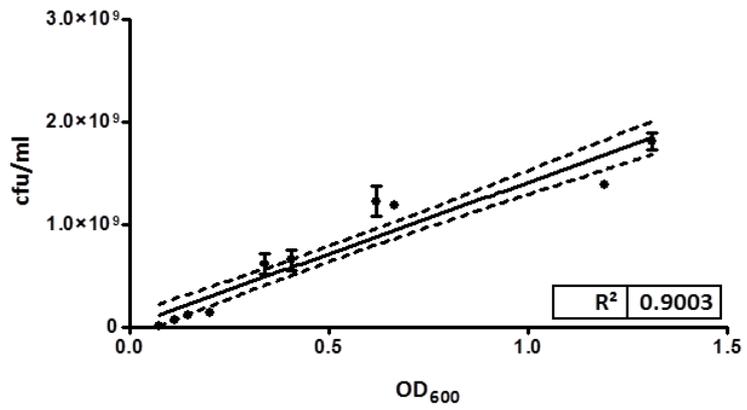
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Appendices

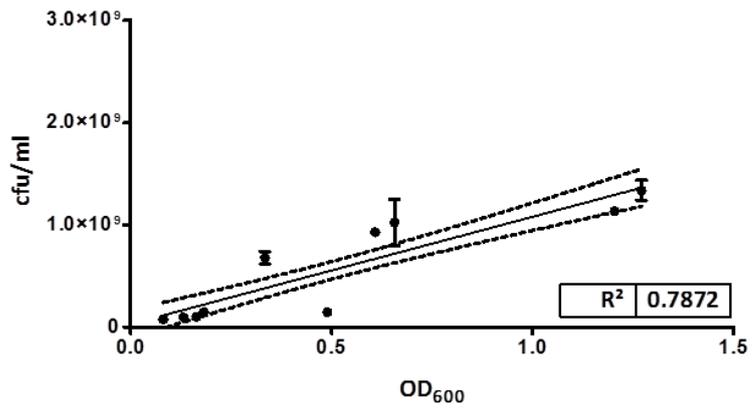
N. lactamica strain NL4.1 (Nlac)



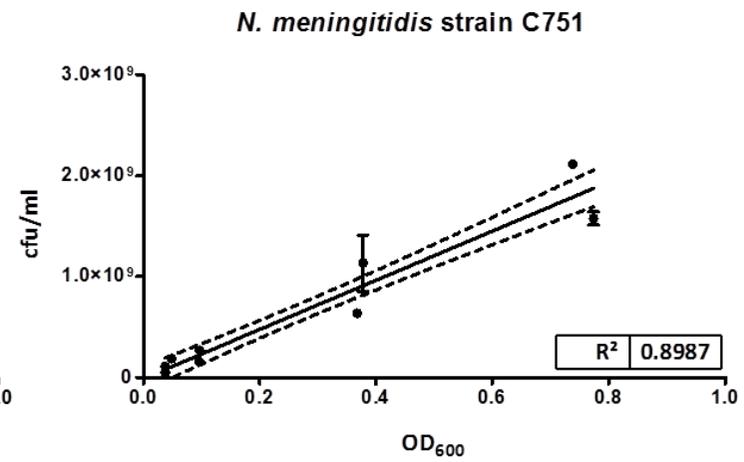
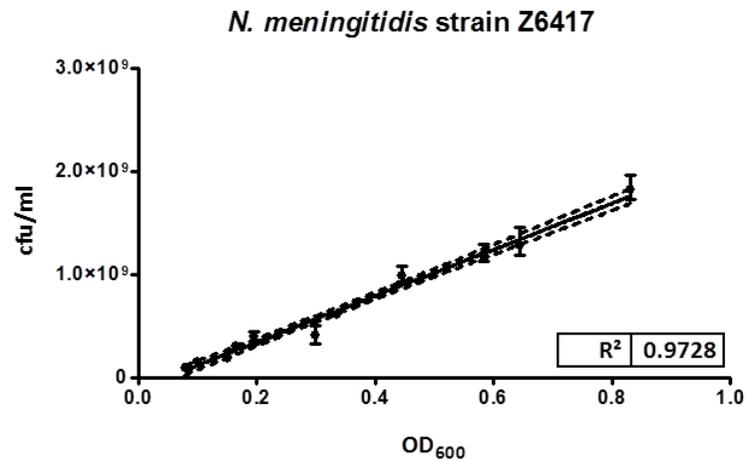
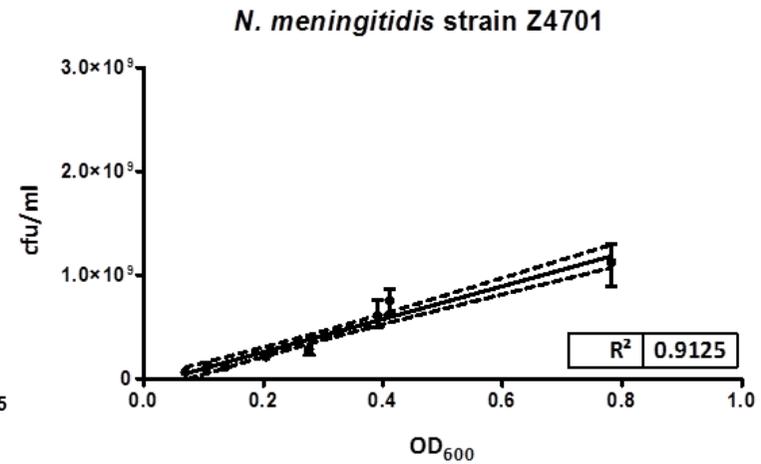
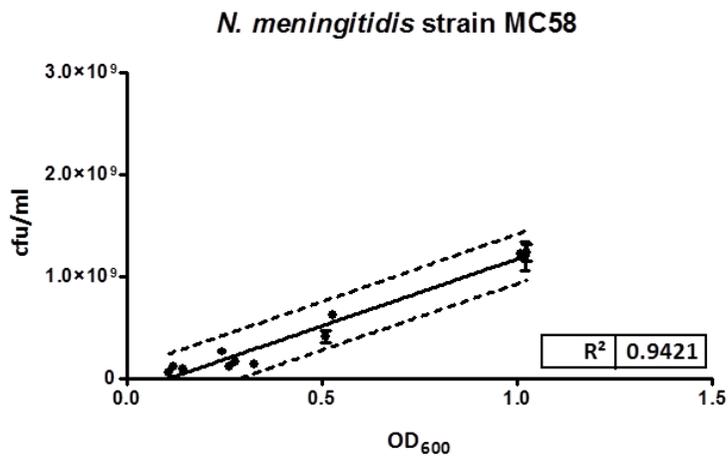
N. cinerea strain 194 (Ncin)



N. polysaccharea strain LNP 462 (Npoly)

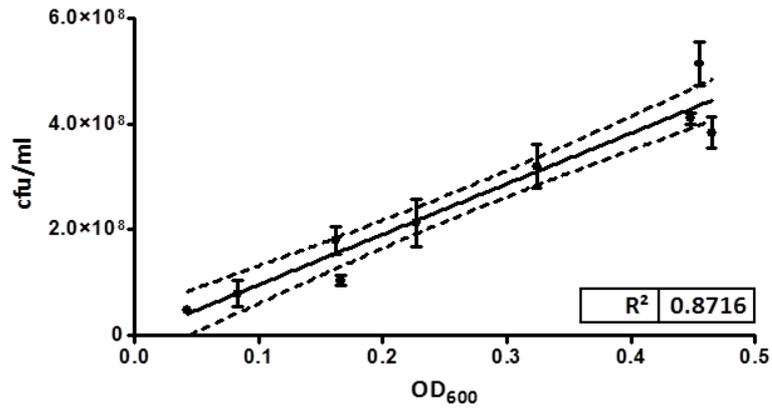


Appendix 1 Enumeration curves from strains of commensal *Neisseria*

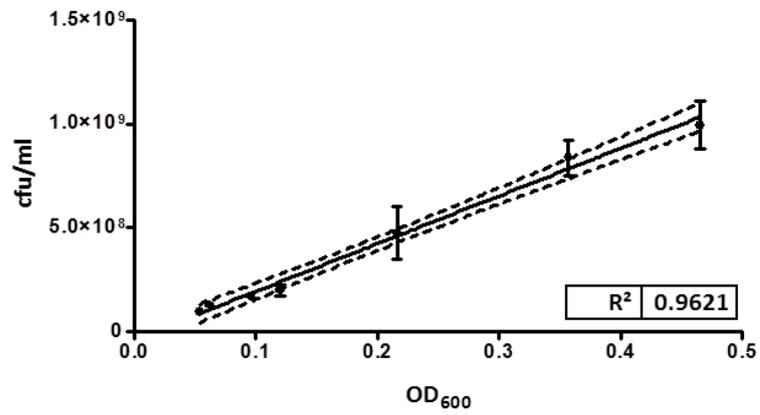


Appendix 2 Enumeration curves from strains of *N. meningitidis*

H. influenzae type a strain 620 (Hia)

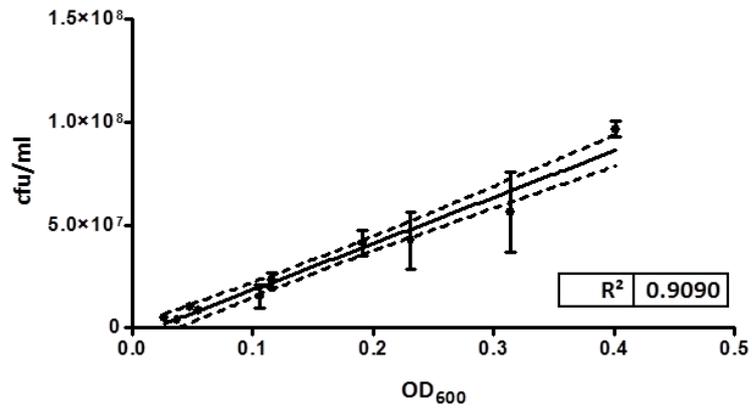


H. influenzae type b strain Eagan (Hib)

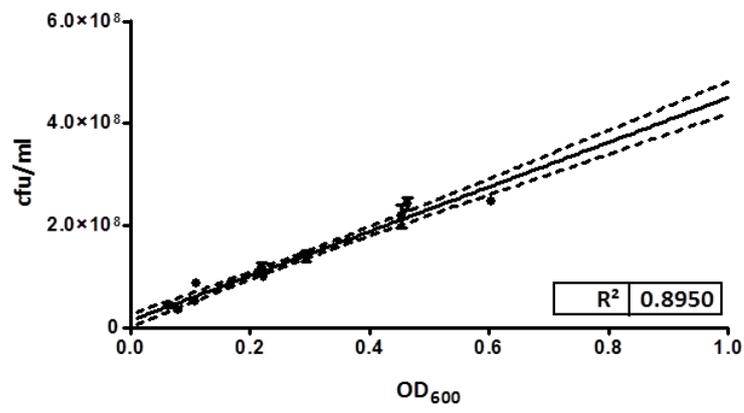


Appendix 3 Enumeration curves from strains of *H. influenzae*

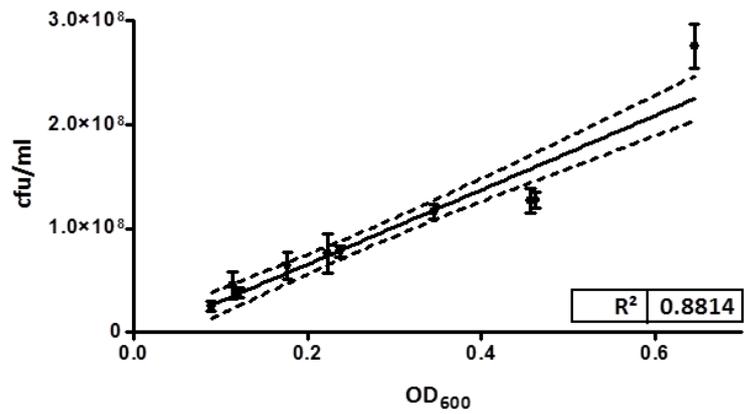
S. aureus strain RN4220



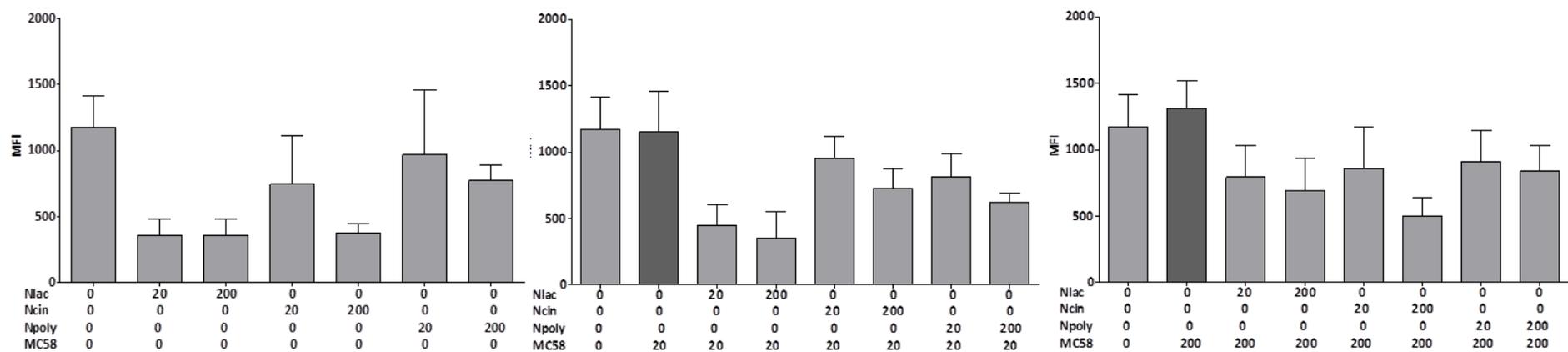
S. aureus strain Mu50



S. pneumoniae serotype 14 isolate (Sp14)



Appendix 4 Enumeration curves from strains of *S. aureus* and *S. pneumoniae*



Appendix 5 Surface labelling of TLR2 following bacterial challenge

Detroit 562 (D562) cells were challenged for 3 hours with *N. meningitidis* strain MC58 at 20 or 200 bacteria per epithelial cell (MOI) for 3 hours in the absence or presence of commensal Neisseriae; a) *N. lactamica* (Nlac), b) *N. cinerea* (Ncin) or c) *N. polysaccharea* (Npoly) at MOI 20 or 200. D562 cells were disassociated with EDTA (4mM) in PBS and surface TLR2 was labelled with a mouse anti-human TLR2 primary antibody and rabbit anti-mouse Alexa-Fluor 647 conjugated secondary antibody. Fluorescence was analysed by flow cytometry with the Accuri C6 flow cytometer. Data represents the mean \pm SEM from three independent repeats in treatments involving MC58 and two independent repeats with treatments with commensals only.