The Immunomodulation of Dendritic Cells by *Neisseria meningitidis*

by

Alastair Copland

This thesis is submitted for the degree Doctor of Philosophy at University College London

---

UCL Institute of Child Health

December 2014

Abstract

Neisseria meningitidis is a pervasive bacterial coloniser of the human nasopharynx with up to 40% carriage in the population. Paradoxically, invasive disease occurs in only ~1 in 100,000 individuals. Sophisticated immune evasion mechanisms allow the bacterium to persist in the host, yet these adaptations may also enable its transition from a harmless commensal to an invasive pathogen. Dendritic cells (DC) are principal controllers of mucosal immunity, and bacteria can exploit their maturation process to evade the immune system. In this study, it is reported for the first time that live serogroup B meningococcus (NmB) impedes DC maturation by disrupting STAT1 through dephosphorylation of tyrosine 701 (Y701). DC were therefore refractory to interferon stimulation—which is essential for DC function—and had low levels of maturation markers and other STAT1-dependent genes. Interestingly, infected DC retained the activity of other major signalling pathways, including the MAP kinases, which induced PD-L1^{hi} DC that were CD4^{+} T-cell suppressive. Disruption of STAT1 also had the major effect of dampening *SOCS1* and *CISH* expression, which are critical for homeostatic control of DC inflammatory cytokine production, and correlated with enhanced inflammatory cytokine production. Restoration of STAT1-Y701 phosphorylation reprogrammed DC towards a typical CD86^{hi} maturation state, normalised cytokine profiles and T-cell responses. These data establish an unconventional inflammatory pathway for the meningococcus, but also link immune evasion and pathogenesis via a shared mechanism. These findings elucidate how NmB may undermine immunity within the normal mucosa but

also inflict damage in the context of septicaemia due to uncontrolled inflammation.

Word count: 59,942

Acknowledgements

I'd like to thank my supervisors Hannah Jones and Garth Dixon for their support during this thesis. I received a great PhD project from them that I've really enjoyed. Sadly, Hannah and I didn't win the EuroMillions despite numerous attempts, but at least it made me finish my thesis.

I'd also like to thank people who've helped with the project along the way: Mona Bajaj-Elliott for suggesting a number of good experiments; Philip Dusart for general help on the project; Sarah Eisen for teaching me how to bleed people (and letting me practice on her!); David Escors for tips regarding T-cell assays and signalling pathways; Karine Breckpot for friendly advice and protocols concerning DC lentiviral transduction; Holly Stephenson for helping with the luciferase assays; Mark Lucas for showing me how to do Westerns; Emma Chan for kindly donating some of her lentiviruses; Dagmar Alber and Katja Brunner for providing bacterial strains; Nayani Pramanik for providing mouse cells; and David Bending for teaching me how to do real-time PCR.

I'd also like to thank other people on the 5th and 6th floors, especially: Anna F, Ariane, Jose, Nazila, Julia, Sarah, Mario, Anne P, Enea, Elaine, Ronan, Shaz, Lisa, Vania, Marina, Mitch, Joanna, Hannah L, Ying and Despina for all being so friendly and supportive.

List of Commonly Used Abbreviations and Terms

APC	Antigen presenting cell
CD	Cluster of differentiation
DC	Dendritic cell(s)
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
H/m/s	Hours/minutes/seconds
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase (ERK, p38, JNK)
Meningococcus	*Neisseria meningitidis* (no specific serogroup)
MOI	Multiplicity of infection
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa B
Nl	*Neisseria lactamica*
NmB	*Neisseria meningitidis* serogroup B
Opa	Opacity protein
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-L1/PD-L2	Programmed death receptor ligand-1/2
PFA	Paraformaldehyde
PMA	Phorbol-12-myristate-13-acetate
PorA/PorB	Porin A/Porin B

PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RT-PCR	Real-time PCR
SEB	*Staphylococcus* enterotoxin B
SOCS	Suppressors of cytokine signalling
STAT	Signal transducer and activators of transcription
T_H1	Type 1 $CD4^+$ T-helper cell
T_H17	Type 17 $CD4^+$ T-helper cell
T_H2	Type 2 $CD4^+$ T-helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

Peer-reviewed publications produced during work on this thesis:

- **Stephenson HN, Mills DC, Jones H, Milioris E, Copland A, Dorrell N, Wren BW, Crocker PR, Escors D, Bajaj-Elliott M.** Pseudaminic Acid on *Campylobacter jejuni* Flagella Modulates Dendritic Cell IL-10 Expression via Siglec-10 Receptor: A Novel Flagellin-Host Interaction.

  Journal of Infectious Diseases. (2014) 210 (9): 1487-1498.

- **Jones HE, Copland A, Hamstra HJ, Cohen J, Brown J, Klein N, van der Ley P, Dixon G.** LOS oligosaccharide modification enhances dendritic cell responses to meningococcal native outer membrane vesicles expressing a non-toxic lipid A.

  Cellular Microbiology. (2014) Apr;16(4):519-34

Index

1. Chapter 1: Introduction

1.1 Meningococcal Disease

Meningococcal disease was first described in the 16th Century and the causative infectious agent, *Neisseria meningitidis* (the meningococcus), was first isolated in 1887 (Rosenstein et al., 2001). Meningococcal disease clinically manifests as septicaemia or haematological bacteraemia, which may or may not be accompanied by cerebral meningitis, i.e. inflammation of the meninges. Symptoms include headache, confusion, fever and haemorrhagic rash. Even with prompt medical treatment, the mortality rate is typically estimated to be 8-10% in developed countries (Thorburn et al., 2001). Long-term sequealae in survivors are a cause of significant—and possibly underestimated—morbidity. Memory loss, cognitive deficits, deafness and behavioural problems have been reported several years post-discharge from healthcare services (Chandran et al., 2011). Moreover, meningococcal disease predominantly occurs in infants, adolescents, the elderly and the immunocompromised. As such, meningococcal disease presents a major current global health challenge that warrants further research into disease pathophysiology.

1.1.1 Epidemiology

Rates of meningococcal disease can vary greatly depending on geographical location. In sub-Saharan Africa, epidemics due to the infectious transmission

of *N. meningitidis* can affect up to 1% of the population. This has led to the region being called the 'meningitis belt'. Conversely, the incidence in European countries has recently been reported to be approximately 1 per 100,000 (Harrison et al., 2009). Several factors have been identified that may contribute to the large variation in epidemiological rates observed between developed and developing nations. Vaccination strategies in African countries have effectively reduced the burden of meningococcal disease, although socioeconomic factors and their effects on healthcare provisions have significantly mitigated these preventative efforts (Woods et al., 2000). Crowding of persons in the household or community has also been associated with meningococcal disease outbreaks, and this is strongly associated with lower-income areas (Baker et al., 2000; Deutch et al., 2004). Immunocompromisation by pathogens such as human immunodeficiency virus (HIV) may also vitally contribute towards the spread of meningococcal disease (Stephens et al., 1995), which is most prevalent in impoverished African countries.

In developed nations, outbreaks of meningococcal disease have been shown to proceed directly after reported seasonal increases in cases of acute respiratory infections, e.g. influenza (Makras et al., 2001). It is thought that co-infection may allow *N. meningitidis* to more easily spread between individuals and may also prelude infection and dissemination in the host. Smoking, sexual contact and alcohol consumption are also risk factors for developing disease (Harrison, 2010). Despite the disparity in incidence between geographical regions, the healthcare costs in containing specific epidemics of

meningococcal disease can be substantial for both low- and high-income countries (Anonychuk et al., 2013).

1.2 *N. meningitidis* microbiology

N. meningitidis is the etiological cause of meningococcal disease. The bacterium is a fastidious, diplococcal Gram-negative coloniser of the human nasopharynx and upper respiratory tract, however it may colonise other mucosal sites in certain demographics (Judson et al., 1978). In healthy individuals, *Neisseria* spp. can represent over 20% of the total microbial diversity in its ecological niche (Park et al., 2014). Indeed, up to 40% of the population may be carrying *Neisseria meningitidis* at any one time. There is no known natural reservoir of the meningococcus outside of the human mucosae, for reasons that will be discussed. *N. meningitidis* is a proteobacterium that belongs to the genus *Neisseria*; other members of the genus include the *N. lactamica*, *N. sicca*, *N. elongata* and *N. gonorrhoeae*. Many species of the *Neisseria* genus colonise the human nasopharynx asymptomatically and are rarely associated with disease, therefore they are usually considered commensal organisms. Only *N. gonorrhoeae* (which colonises the urethra) and *N. meningitidis* have pathogenic potential in humans, although *N. lactamica* may cause disease in exceptional circumstances (Zavascki et al., 2006).

The genome of *N. meningitidis* was first sequenced in 2000, and revealed a highly compact genome of 2,272,351 base-pairs (Tettelin et al., 2000), which is approximately 50% fewer genes than laboratory strains of *Escherichia coli*

(Lo et al., 2009). Importantly, the analysis also revealed a very dynamic genome with many genes subject to high phase-shift variation. This is the process by which genes are activated and deactivated by site-specific recombination, epigenetic modification or slipped-strand mispairing. Thus, the genome of *N. meningitidis* possesses enormous plasticity compared to other bacteria. Virulence genes may be switched-on, -off or modified by mutation, due to selective pressures.

N. meningitidis is also notable in its ability to readily uptake foreign DNA — a process that is called genetic transformation. Examination of foreign sequences within the meningococcal genome has revealed that many of these nucleotides have been acquired via horizontal gene transfer from co-habiting bacteria within the human host. More surprisingly, some of these sequences appear to have been acquired from the human host, thus emphasizing the significant plasticity of the meningococcal genome (Putonti et al., 2013). To facilitate the uptake of DNA from the environment, *N. meningitidis* possesses over 2,000 DNA-uptake sequences (DUS) that enable the bacterium to efficiently incorporate DNA into its own genome (Vogel et al., 2010). Genetic comparisons of meningococcal isolates between carriage and disease strains has revealed that this mechanism of horizontal gene transfer in the nasopharyngeal microbial reservoir is likely to be responsible for the acquisition of key virulence factors, hence the emergence of the bacterium as a pathogen of the human host (Schoen et al., 2008). It is likely that the dynamic genome and natural competence of *N. meningitidis* is likely to be the

most significant factor contributing towards the pathogenic potential of the organism.

1.2.1 *N. meningitidis* surface structure

The surface structure of *N. meningitidis* is subject to large variation due to the aforementioned dynamic genome. However, there are many conserved structures that have been found to contribute towards colonisation and virulence.

As a Gram-negative bacterium, the meningococcus contains a relatively high amount of lipooligosaccharide (LOS) in its outer membrane. LOS is comprised of an inner core containing the immunogenic lipid A moiety bound to an oligosaccharide core with branching sugars. The LOS serves a structural purpose in the bacterium by supporting the architecture of the outer membrane, but the lipid A moiety is also an important virulence factor that binds to human immunological receptors and induces inflammation. Meningococcal LOS also protects the bacterium against the human immune system by the post-translational addition of sialic acid. Furthermore, natural variants in the lipid A moiety have been identified. LOS is typically hexa-acylated (i.e. six fatty acid chains), however penta-acylated variants have been found in patients with less severe disease, establishing the lipid A moiety a major determinant of LOS pathogenicity (Fransen et al., 2009). Despite its importance in structural integrity and host-pathogen interactions, LOS is not strictly essential for meningococcal viability or growth *in vitro*, as has been demonstrated with LOS-deficient mutants (Steeghs et al., 1998).

N. meningitidis also contains porins in the outer membrane that are essential for survival. These proteins form β-barrel structures that allow the translocation of ions and molecules between the outer membrane (OM) and the periplasmic space (PS). The meningococcus contains two major porins: porin A (PorA) and porin B (PorB), which are immunodominant bacterial epitopes and typically generate robust immune responses in some cell types (Singleton et al., 2005). However, these porins can also modulate host immunity by preventing antimicrobial responses such as phagocyte activation (Bjerknes et al., 1995) and phagosome maturation (Mosleh et al., 1998). Furthermore, porins are implicated in the development of antibiotic resistance in Neisserial species, by allowing efflux of antimicrobial compounds (Olesky et al., 2006).

Pili are another important meningococcal virulence factor. These are filamentous protein structures composed of polymeric pilin subunits; the dominant subunit is PilE, however other subunits (PilC, PilV and PilX) can be incorporated under various conditions (Virji, 2009). Pili are essential for mediating the attachment of the bacterium to the host tissue; in this role, they act as 'hook-like' molecules that allow colonisation of the human nasopharynx. As the pili are subject to intense antigen variation, it has been noted that pili from different strains of bacteria possess varying attachment affinities for human epithelial tissue (Nassif et al., 1993). Accordingly, comparisons between asymptomatic colonising and invasive strains of *N. meningitidis* have found that pili undergo significant phase variation — therefore, pili are heavily

implicated in the ability of the bacterium to transition from a harmless commensal to invasive pathogen (Rytkönen et al., 2004).

Upon invasion, pili are directly toxic to human endothelial cells and also induce high levels of inflammatory mediators that result in vascular damage to the host. Importantly, the pro-inflammatory and toxic effects of pili can synergise with LOS to amplify the pathogenic effect of the bacteria (Dunn et al., 1995). Xenografts of human vasculature in a murine model of meningococcal disease have demonstrated the necessity of pili for vascular adhesion of the bacteria, tissue damage and an inflammatory response (Melican et al., 2013). Furthermore, the human receptor responsible for meningococcal adherence to endothelial cells has now been identified, and experimental evidence suggests that it is a ligand for type IV pili (Bernard et al., 2014). Thus, the evidence suggests that pili are critical for both colonisation and pathogenesis in the human host.

Opacity-associated (Opa) proteins are expressed on the bacterial surface of Neisserial species. These proteins have a role in colony formation and bacterial aggregation (Dehio et al., 1998), thus affording resistance to human serum (Bos et al., 1997). Furthermore, gonococcal Opa proteins can modulate host immune cells. Notably, Opa can bind to human carcinoembryonic antigen-related cell adhesion molecules (CEACAM) proteins, which are expressed on many cell types, and modulate cellular signalling events. The Opa-CEACAM interaction can effectively disable many immune cells and override their normal function (Boulton and Gray-Owen, 2002). Although Opa proteins have been implicated in host attachment—in a

similar fashion to pili—*in vivo* colonisation experiments have found that they are not strictly necessary for this process (Jerse et al., 1994). However, many Opa variations are nevertheless associated with invasive disease. Opa proteins may therefore enhance adhesion, infection and invasive disease.

Lastly, the meningococcal capsule is a sugar coat that surrounds the outer membrane of the bacterium. Encapsulated meningococcal are broadly grouped according to their capsular serotype, which is determined by the sugar structure of the capsule matrix. Serogroups A, B, C, W-135 and X are all associated with clinical disease. In the U.K. and many other developed countries, *N. meningitidis* serogroup B (NmB) remains the predominant disease-causing strain due to successful vaccination efforts against other strains; while serogroup A is rampant in the meningitis belt of sub-Saharan Africa.

The capsule serves the function of protecting the bacterium from environmental insults and the human host. Since almost all invasive clinical strains are encapsulated, the capsule is considered to be the prime virulence factor for meningococcal disease (van Deuren et al., 2000). There is growing genetic evidence that the meningococcus began as an ordinary commensal of the human nasopharynx, but emerged as a pathogen upon acquisition of capsule genes from co-habiting organisms in its local environment (Schoen et al., 2008).

The capsule is heavily hydrated, containing up to 95% water (Costerton et al., 1981). The heavy water content is thought to enable environmental

transmission in the form of exhaled water droplets, therefore allowing the bacterium to temporarily survive between hosts while airborne. The capsule also covers and protects many protein structures on the surface that may be exposed to the many defences of the human immune system, by forming a physical barrier. Sialic acid within the capsule is extremely effective at neutralising the human complement system, which presents a major barrier to colonisation and invasive disease. Sialic acid is ordinarily present on the surface of human vascular tissue where it recruits the host molecule factor H (fH), which in turn prevents auto-reactions of the complement system — therefore the incorporation of this molecule into the capsule is a critical immune evasion strategy to avoid bacterial killing (Kugelberg et al., 2008). Complement is exceptionally abundant in oral and nasal mucous secretions (Lai A Fat et al., 1973; Ap et al., 2005), and so the evolution of the biochemical pathways allowing the subversion of this system are thought to be necessary for meningococcal colonisation.

NmB is unique in that the capsule contains structural homology to human neural cell glycopeptides. This was first discovered in 1983 by researchers who incubated human brain tissue with NmB anti-sera and found cross-reactivity with isolated bacterial capsule (Finne et al., 1983). Successful vaccines for other pathogenic serogroups have been based upon recombinant capsular polysaccharide to generate an immune response, but serogroup B capsule is poorly immunogenic, owing to the molecular mimicry of its structure. Furthermore, there are concerns that any induced immunogenic reaction would have the theoretical potential to induce an autoimmune

reaction, although experimental evidence for this is severely limited (Robbins et al., 2011).

The overarching theme with nearly all meningococcal components is that the bacterium has evolved a myriad of refined mechanisms with the purpose to intricately adapt to its microenvironment in the human mucosae. These molecules are important for survival by serving physiological functions, e.g. the porins. However, many of these molecules have a dual function in modulating the host, especially the immune system. This bacterial multitasking or 'moonlighting' has been observed in many other bacteria, including *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* (Henderson and Martin, 2011). Due to the limited size of the average bacterial genome, this dichotomy of function is often an evolutionary necessity and advantage. Table 1-1 is a summary of Neisserial virulence factors and their dual functions. The common element to these molecules is that they provide a clear cellular function within the bacterium itself (e.g. nutrient acquisition, structural support), but also serve a major toxic or host-modulating role. The importance for bacteria to modify host function is demonstrated by the fact that some microbes encode essential proteins solely for this purpose. For example, *M. tuberculosis* contains tyrosine phosphatases without encoding any tyrosine kinases (Whitmore and Lamont, 2012). Hence, some bacterial proteins are solely involved in host modulation, and are essential for virulence.

Table 1-1. A list of meningococcal virulence factors and their role in pathogenesis.

Virulence factor	Cellular and physiological function	Host modulation and pathogenesis
Lipooligosaccharide (LOS)	1. Colony formation (Albiger et al., 2003); 2. Membrane structural integrity (Raetz, 1990)	1. Invasion of endothelial cells (Lambotin et al., 2005); 2. Adherence to epithelial cells (Albiger et al., 2003); 3. Inflammatory response (Dixon et al., 2001)
Porin	1. Antibiotic efflux (Olesky et al., 2006); 2. Nutrient acquisition (Achouak et al., 2001)	1. Anti-apoptotic effect on lymphocytes (Massari et al., 2000); 2. Inflammatory response (Singleton et al., 2005)
Pilus	1. Motility (Mattick, 2002)	1. Adherence to epithelial cells (Virji et al., 1993); 2. Adherence to

		endothelial cells (Melican et al., 2013)
Capsule	1. Survival outside the host (Swain and Martin, 2007)	1. Survival in host cells (Spinosa et al., 2007); 2. Mitigation of complement (Schneider et al., 2007)
Opacity protein (Opa)	1. Colony formation (Dehio et al., 1998)	1. Attenuation of adaptive immune responses (Yu et al., 2013); 2. Invasion of epithelial cell barriers (Wang et al., 1998)

1.3 The Immune System

The immune system is a collection of cells, processes and molecules that protect the host from bacteria, viruses, fungi, protozoans and tumours. To achieve this, the immune system must be able to distinguish 'self' from 'non-self' and respond accordingly. When a pathogen (e.g. influenza) triggers an immune reaction, this typically results in clearance or control of the invading

microorganism. Under normal circumstances, the immune system does not mount an immunological defence to host cells and tissues due to specificity in recognising non-self antigens: molecular signatures that induce cellular and humoral immune responses. The non-responsiveness of the immune system to self-antigens is termed immunological tolerance. However, this equilibrium can be disrupted for various reasons in instances of autoimmune disease.

Since this model was first proposed, our understanding of the immune system has come to appreciate that there is also a normal diversity of microflora occupying epithelial surfaces across the human body. The human mucosa contains billions of bacteria, viruses and fungi that constitute the microbiome. These microorganisms mostly exist in a symbiotic relationship, but can also confer many benefits to the host, while the host provides an environment rich in nutrients. For example, mucosal bacteria of the gut are responsible for generating metabolic by-products such as menaquinones (vitamin K) that are essential for human blood coagulation (Conly and Stein, 1992). The presence of certain commensal bacteria—those that are not normally associated with disease—can protect against allergy (Stefka et al., 2014) and viral infection (Abt et al., 2012). Moreover, more recent evidence has suggested that the microbiome can even influence brain development and behaviour in mice (Heijtz et al., 2011). Therefore, the prime importance of the microbiome to human health has added another level of complexity to the paradigm of self and non-self: that of the distinction between commensal and pathogenic microorganisms.

1.3.1 Innate Immunity

The immune system may be divided into two broad elements: innate and adaptive immunity. Innate immunity is evolutionarily older than adaptive immunity, being an ancient form of host defence that is thought to have originated at approximately the same time as the origin of life over 3 billion years ago (Hoffmann and Reichhart, 2002). The innate immune system contains no immunological memory, i.e. repeated exposure to the same antigens will not generate any specific, protective immunity. This is due to the fact that the components of the innate immune system are germline-encoded, and have evolved to recognise constant threats in the form of conserved molecular signatures.

1.3.1.1 Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are germline-encoded receptors that recognise pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), also known as 'danger signals' (O'Neill et al., 2013). There are several different classes of PRRs, but the most extensively studied are the Toll-like receptors (TLRs), RIG-like receptors (RLRs) and NOD-like receptors (NLRs) (Creagh and O'Neill, 2006). These receptors are present on the surface of the cell (TLRs) and also within intracellular compartments and the cytosol itself (TLRs, RLRs and NLRs). When triggered by the necessary PAMPs, these PRRs induce intracellular signalling events that result in the production of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β). Though these receptors have diverse

ligands (RLRs recognise intracellular viral antigens; NLRs recognise bacterial components), the common downstream cellular event is the direct or indirect activation of nuclear factor kappa-b (NF-κB) and the transcription of immunity-related genes. PRRs are expressed on a wide array of immune and non-immune cells.

1.3.1.1.1 Toll-like receptors

To date, there are ten identified TLRs in humans (see Table 1-2), which are type I integral membrane-bound receptors capable of sensing PAMPs and DAMPs. The events leading to the discovery of TLRs in the early 1990s were triggered by sequence comparisons between the interleukin 1 receptor-1 (IL-R1; also known as the Toll–IL-1-resistence (TIR) domain) and the Toll proteins of *Drosophila melagonaster* (Gay and Keith, 1991). Toll proteins were hitherto only implicated in morphological changes in *D. melagonaster*, however the common element of NF-κB activation led to the discovery that these receptors were critical in the innate immune response (Lemaitre et al., 1996). Eventually, human homologues were identified and constitutively-active mutants were subsequently cloned into human cells, therefore proving their role in the innate immune response (Medzhitov et al., 1997). The ligands for all the TLR receptors—with the exception of TLR10—have now been discovered, and a summary of these interactions is provided in Table 2. Furthermore, it is now understood that TLRs can also recognise danger signals, adding another class of molecules to their repertoire of ligands. A well-characterised example of this is the binding of endogenous fatty acids to

TLR4 (Shi et al., 2006), which can act as released inflammatory mediators to prime the immune response before encountering a pathogen.

Table 1-2 TLRs and their respective ligands - adapted from (Akira and Takeda, 2004).

Toll-like Receptor	Ligand
TLR1	Triacyl lipopeptides
TLR2	Peptidoglycan, porins, lipoteichoic acid, glycolipids
TLR3	Double-stranded RNA
TLR4	LPS, fatty acids
TLR5	Flagellin
TLR6	Zymosan
TLR7	Single-stranded RNA
TLR8	Single-stranded RNA

TLR9	CpG DNA
TLR10	Unknown

X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy have revealed the molecular structures of many TLRs, such as TLR3, TLR4 and TLR9. Despite the differences in ligand specificity, the common element in TLR structure appears to be the extracellular domain, which forms hydrophobic leuceine-rich repeats that gain the quaternary structure of a horseshoe-like shape (Botos et al., 2011). Upon recognition of the appropriate ligands, TLRs will homo- or hetero-dimerise, leading to conformational changes that allow intracellular signalling through the TIR domain (Kawai and Akira, 2006). Most TLR pairs are homodimers, e.g. TLR4, but TLR2 will heterodimerise with TLR6 to recognise bacterial wall components.

The TIR domain associates with adaptor molecules immediately downstream of the TLR receptor. Upon dimerisation, TIR will initiate a signalling cascade by binding to the adaptor molecules myeloid differentiation primary response gene 88 (MyD88) (in the case of TLR-1, -2, -4, -7, -8 and -9) or TIR domain-containing adaptor protein inducing interferon (IFN)-β (TRIF) (in the case of TLR-3 and -4) (Kawai and Akira, 2006). MyD88 activation leads to the activation of interleukin-1 receptor-associated kinase (IRAK) 4 and IRAK2. In turn, IRAK2 phosphorylates TRAF6 that leads to the activation of NF-κB and the transcription of NF-κB-dependent genes. Furthermore, TRAF6 can also

activate the mitogen activated protein kinases (MAPKs) that activate activating protein 1 (AP-1) (O'Neill et al., 2013).

TRIF activation, on the other hand, leads to recruitment of TANK-binding kinase-1 (TBK1) and the subsequent phosphorylation of interferon regulatory factor 3 (IRF3). Upon phosphorylation, IRF3 is activated and begins to transcribe IFN-β. Importantly, TRIF can also recruit the molecule receptor-interacting protein 1 (RIP1), that also mediates activation of NF-κB (O'Neill et al., 2013).

These signalling events are usually divided into two branches: the MyD88-dependent and the TRIF-dependent signalling axes. It should be noted, however, that there is considerable overlap between the two pathways. For example, the TRIF-dependent pathway can activate the NF-κB pathway indirectly: once transcribed, autocrine IFN-β can induce NF-κB signalling via the janus kinase (JAK) family (Kim et al., 2002). Indeed, modern approaches to studying intracellular signalling have revealed complex cascades that challenge traditional linear models (Lu et al., 2007). Genomic approaches to cell signalling have expanded the paradigm considerably by highlighting the crucial contribution of spatio-temporal dynamics to signalling outcomes (Kholodenko, 2006). Thus, a single stimulus can have seemingly paradoxical effects, dependent on the spatio-temporal organisation of the signalling network.

1.3.1.1.2 RLRs, NLRs and other C-type lectins

While TLRs recognise a diverse array of PAMPs, there are other specialised receptors that act as sensors for microbial molecular patterns. NLRs exist in the cytosol; these are a family of more than ten proteins that contain a shared three-fold structure of (i) leucine-rich repeats (similar to TLRs), (ii) a nucleotide-binding oligomerisation (NOD) domain and (iii) a caspase recruitment domain (CARD) (Chen et al., 2009). NLRs mainly recognise bacterial cell wall components, such as peptidoglycan (PGN). Once they recognise PGN, NLRs dimerise via their CARD domains and then activate the NF-κB pathway to drive the transcription of pro-IL-1β. Conversely, RLRs are PRRs that recognise viral ligands such as single-stranded and double-stranded RNA (ssRNA; dsRNA). Engagement of RLRs with their required ligand induces both the NF-κB and IRF3 pathways, to induce pro-IL-1β and IFNs, in a similar fashion to TLR ligation (Yoneyama and Fujita, 2009).

C-type lectin receptors (CLRs) are widely expressed on myeloid immune cells. These receptors are responsible for recognising carbohydrate motifs which are shared among viral, bacterial and fungal pathogens (Osorio and Reis e Sousa, 2011). The defining feature of CLRs is the presence of a C-type lectin-like domain (CTLD), also known as the carbohydrate-recognition domain (CRD). These domains are rich in hydrophobic β-sheet structures and amino acids to coordinate Ca^{2+} binding, which facilitates recognition of carbohydrate sugars (Drickamer, 1999). Although the structure is highly evolutionarily conserved, there are known Ca^{2+}-independent CTLDs which

instead utilise loop structures in carbohydrate recognition (Drickamer, 1999; Llera et al., 2001).

Antigen recognition by CLRs leads to both signalling and endocytosis events. CLRs contain immune-modulating regions known as immunoreceptor tyrosine-based activation/inhibition motifs (ITAM/ITIM). Binding of a carbohydrate ligand to the transmembrane CLR results in a conformational change and the recruitment of intracellular phosphatases, namely SHP-1 and SHP-2. These phosphatases carry out diverse immunological functions by modulating critical mitogen-activated protein kinases (MAPKs), which are important in immune cell activation (Geijtenbeek and Gringhuis, 2009). Significantly, pathogens have exploited the ability of CLRs to modulate important steps in immune cell signalling. It was discovered that viral and bacterial pathogens could target the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor to interfere with TLR signalling. HIV-1 binds to DC-SIGN by the viral glycoprotein gp120 which induces the phosphorylation of key proteins to prevent immune cell activation (Hodges et al., 2007).

1.3.1.2 Cells of the Innate Immune System

Innate immune cells ultimately originate from $CD34^+$ haematopoetic precursor cells in the bone marrow and differentiate according to the environmental stimuli and physiological milieu that determine cell fate. These precursor cells may differentiate into further precursors such as the myeloid progenitor and the lymphoid progenitor. With important exceptions, the myeloid progenitor

gives rise to the cells of the innate system, while the lymphoid progenitor gives rise to cells of the adaptive immune system (Huang and Terstappen, 1994). Innate immune cells express a wide array of PRRs that enable them to adequately respond to microbial threats.

1.3.1.2.1 Granulocytes

Granulocytes are immune cells with lobed nuclei containing granules filled with histamines, leukotrienes, enzymes and other inflammatory mediators. Granulocytes differentiate from precursor cells by stimulation with the cytokines IL-1, IL-6 and granular macrophage-colony stimulating factor (GM-CSF). In humans, there are three types of granulocyte: basophils, eosinophils and neutrophils (Duffin et al., 2010). Basophils and eosinophils are heavily loaded with histamines, but also secrete cytokines that contribute towards allergic responses, such as IL-4. However, they have a critical role in protecting against helminths and other parasites (Chirumbolo, 2012). Neutrophils have been more extensively studied than other granulocytes, partly owing to their higher abundance in the peripheral circulation. Neutrophils are able to secrete their granular contents, and also form neutrophil extracellular traps (NETs): structures made of protein and DNA that are able to trap and disable extracellular bacteria (Brinkmann et al., 2004). Neutrophils are often the first cells to respond to bacterial infection. Their importance in this regard is highlighted by the fact that patients with primary neutrophil immunodeficiencies are often much more susceptible to bacterial infection (Dinauer, 2007).

1.3.1.2.2 Monocytes and Macrophages

Monocytes derive from the macrophage and dendritic cell precursors (MDPs) in the bone marrow. MDPs give rise to $Ly6C^+$ monocytes. These monocytes express a range of chemokine receptors, such as chemokine receptor 2 (CCR2) and so respond to constitutively-expressed chemokines in the blood and lymphatic systems (Shi and Pamer, 2011). Therefore, monocytes are able to constitutively circulate and patrol the tissues of the body before encountering danger signals. Upon bacterial infection, the local release of a unique inflammatory cytokine and chemokine signature acts as an attractant to circulating monocytes. Chemokine ligands 2 and 7 (CCL2, CCL7) and IL-23 have been shown to be particularly important for monocytes to quickly respond to *Listeria monocytogenes* and *Salmonella typhumurium* infection *in vivo* (Indramohan et al., 2012; Rydström and Wick, 2009). At the site of infection, monocytes can phagocytose and kill bacteria, albeit at a much reduced rate compared to neutrophils (Peterson et al., 1977; Steigbigel et al., 1974).

The epigenetic and cellular plasticity of monocytes gives rise to their second major function: a large reservoir of cells able to differentiate into macrophages or dendritic cells (DC) (Sica and Mantovani, 2012). Monocytes are specially adapted to respond to cytokine cues from their local environment. They are therefore able to differentiate into macrophages, which gain more honed functions in combating microbial infection. Upon stimulation with GM-CSF, macrophage-colony stimulating factor (M-CSF) or other inflammatory cytokines, monocytes begin to acquire a macrophage-like phenotype. This

differentiation process is accompanied by vast changes in gene expression, with an up-regulation in genes involved in lipid metabolism (e.g. lipases), cytokine recognition (e.g. interleukin receptors) and the inflammatory response (e.g. prostaglandins) (Hashimoto et al., 1999; Martinez et al., 2006). Therefore, macrophages are much more efficient at phagocytosing and killing bacteria than monocytes (Nagl et al., 2002). Depending on the exact combination of cytokines that monocytes receive, they may differentiate into classical (M1) or alternatively activated (M2) macrophages (Sica and Mantovani, 2012). The former are typical bactericidal macrophages, while the latter are markedly more anti-inflammatory.

Notably, there are some specialised macrophages that do not derive from monocytes. Kupffer cells of the liver and yolk sac-derived macrophages do not derive from MDPs, but from separate lineages. Indeed, modern approaches to monocyte ontogeny have questioned the extent to which conventional monocytes contribute towards steady-state macrophage replenishment in tissues (Ginhoux and Jung, 2014). Nonetheless, monocytes and macrophages have overlapping yet specialised functions, and in cases of active infection, there is considerable experimental evidence that monocytes may replenish the macrophage pool.

1.3.1.2.3 Dendritic cells

Dendritic cells (DC) are professional antigen presenting cells (APCs) that act as sentinels of the immune system, bridging innate and adaptive immunity. DC are distributed throughout the body but are relatively more abundant at

mucosal sites. In the mucosa, DC are able to sample whole bacteria and antigens in the lumen by forming transepithelial dendrites (Rescigno et al., 2001). In contrast to macrophages, once DC phagocytose and process antigens, they are able to migrate to lymph nodes and activate naive $CD4^+$ T-cells. DC are also the most potent cells at inducing T-cell proliferation and differentiation (Steinman and Cohn, 1974). Therefore, DC are critical for generating immune responses at mucosal sites.

In the mucosa, DC must migrate to the mucosal-associated lymphoid tissue (MALT) in order to present antigen. In the human nasopharynx, tonsils are the primary sites of lymphoid tissue. These are part of a larger structure called the Waldeyer's ring, which is analogous to the nasopharynx-associated lymphoid tissue (NALT) in mice (Brandtzaeg, 2011). Importantly, lymphocytes primed in the tonsils may egress to cervical lymph nodes and the peripheral circulatory system.

1.3.1.2.4 Dendritic cell subsets

Dendritic cells are a heterogeneous group of cells with several lineages of ancestry. Like the T-cell field, the rapid speed at which technology has progressed has meant that there has been enormous diversification in DC subsets. This has been further complicated by differences in the methods used to isolate these cells, which can result in phenotypic artefacts and therefore erroneous classification of DC ontogeny (Collin et al., 2013). Indeed, there is considerable controversy as to whether many proposed subsets are *bona fide* DC— i.e. cells that are sufficiently distinct from macrophages

(Mabbott et al., 2010). However, very recent work by the Immunological Genome Consortium has underscored the significant transcriptional and phenotypic differences between DC and macrophages *in vivo* (Miller et al., 2012).

In humans, DC may be divided into two broad categories: (i) plasmacytoid DC (pDC) and (ii) myeloid DC (mDC; also called conventional or classical DC (cDC) in mice) (Ueno et al., 2010). $CD123^+$ pDC are found in the blood and the mucosal tissues—including the tonsils—and can produce vast quantities of IFNs in response to viral antigens. Accordingly, pDC express high levels of TLR7, which recognises viral RNA (Rajagopal et al., 2010). Therefore, it has been suggested that pDC are primarily antiviral in function. pDC have a unique origin compared to mDC since they arise from a lymphoid progenitor, although the exact hierarchy of cell fate and ontogeny remains poorly defined in humans (Collin et al., 2011).

mDC—as the name would suggest—arise from myeloid progenitor cells. These DC typically express CD1a and CD11c and can be further sub-divided into separate subsets such as Langerhans cells, $CD14^+$ DC and interstitial DC (Chu et al., 2011). mDC are also expressed ubiquitously in tissues and the blood, although at a much lower frequency than macrophages and monocytes. Whereas pDC favour the development of antiviral and anti-helminth type 2 immune responses (for which they were originally called 'DC2'), mDC (also called 'DC1') favour the development of type 1 antibacterial responses (Banchereau et al., 2000; Ueno et al., 2010). Therefore, mDC are critical for controlling bacterial infections. Physiologically, monocytes can give

rise to mDC *in vivo* by the addition of an appropriate stimulus such as LPS, although as yet there is no evidence that monocytes can generate DC under steady-state conditions (Shi and Pamer, 2011). It should be noted that there is considerable plasticity among DC populations; it has been reported that under certain conditions, mDC can become pDC-like cells (del Hoyo et al., 2002) and *vice versa* (Zuniga et al., 2004).

The study of human dendritic cell biology is largely dominated by the use of monocyte-derived DC (moDC). These cells are differentiated *in vitro* by the addition of IL-4 and GM-CSF to freshly isolated $CD14^+$ monocytes extracted from peripheral blood (Sallusto and Lanzavecchia, 1994). Primary DC are extremely rare in human blood, necessitating the need for moDC in order to perform functional studies. Since monocytes are highly abundant in peripheral blood, high numbers of moDC can be generated with relative ease and convenience. Like primary DC, fully differentiated moDC can readily secrete pro-inflammatory cytokines and induce antigen-specific naive $CD4^+$ T-cell responses *in vitro* (Langenkamp et al., 2000). Gene array analyses have revealed that moDC most closely resemble tonsillar mDC among all tested human ex-vivo subsets, thus making them an appropriate model for investigating DC responses to pathogens of the nasopharynx (Lundberg et al., 2013).

An alternative to moDC in the study of host-pathogen interactions is the use of tonsillar DC (tDC). However, this has several important disadvantages. Tonsillectomies are usually performed due to chronic inflammation and infections. Therefore, tDC may be aberrantly activated even before isolation.

Secondly, standard protocols involve aggressive techniques in order to remove the tDC from the dense tonsillar tissue (Cameron and Stent, 2001). As DC are exquisitely sensitive to their environment, any excess laboratory manipulation may lead to pre-activation. Studies examining tDC responses to standard immuno-activators (LPS, poly(I:C) and whole bacteria) have also been in disagreement. A prominent study found that tDC can produce high levels of cytokines but not up-regulate surface markers (Polak et al., 2008); on the other hand, another laboratory independently found that tDC can up-regulate surface markers but secrete negligible levels of cytokines (Hallissey et al., 2014), despite using a similar protocol. The discrepancies in the available data may be partly explained by the great heterogeneity of tDC subsets (Stent et al., 2002; Summers et al., 2001). Alternatively, the clinical context and etiology of the tonsillectomy may influence the resulting tDC phenotype and function.

1.3.1.2.5 Dendritic cell maturation

DC express a wide variety of TLRs, NLRs, RLRs and CLRs in order to respond effectively to microbial challenges. Hence, DC become activated by various PAMPs or DAMPs. The most well-studied of these responses is the response of DC to LPS, which serves as a model pathway with which to study DC maturation. Pivotally, DC can respond differently to specific TLR ligands, such as the immunological outcome would warrant. For example, viral RNA binds to DC-expressed TLR3 and induces potent IRF3 activity, inducing a robust antiviral response characterised by large quantities of the antiviral cytokine IFN-β (Vercammen et al., 2008). Commensal bacteria, on the other

hand, typically activate TLR2 via immunomodulatory glycoproteins (Tunis and Marshall, 2014). This results in production of IL-10 that suppresses the DC inflammatory response (Re and Strominger, 2004).

DC maturation is a complex process involving cascades of intracellular signalling events that lead to vast phenotypic, morphological and functional changes. In an immature state DC are highly phagocytic but express low levels of 'maturation markers'. These are surface receptors involved in $CD4^+$ T-cell co-stimulation, inhibition and antigen presentation. For example, immature human DC express low levels of co-stimulatory receptors CD40, CD80 and CD86; low levels of co-inhibitory receptors programmed death ligand receptor-1/2 (PD-L1 and PD-L2); and relatively low levels of antigen presentation molecules human leukocyte antigen (HLA) Class I and HLA-DR. Furthermore, immature DC produce negligible amounts of pro-inflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α. Immature DC are thus poor inducers of $CD4^+$ T-cell proliferation or differentiation, and they also fail to undergo chemotaxis in response to many chemokine signals (e.g. CCL19) (Banchereau et al., 2000). Therefore, immature DC are considered to be somewhat immunologically inert until activation. When DC mature, maturation makers are up-regulated and cytokine production ensues.

Numerous gene array studies on DC maturation have revealed a process involving thousands of different genes and hundreds of various transcription factors (Türeci et al., 2003a). This complexity allows for enormous flexibility and fine-tuning with regard to handling microbial threats. However, the process of maturation may be reduced to two major signalling axes: the

MyD88-dependent and TRIF-dependent. Using mouse knock-out (KO) mutants, numerous groups have found that both MyD88 and TRIF-dependent signalling is fundamental for full DC maturation. Deletion of either of these pathways results in a immature or semi-mature DC upon stimulus with LPS or another immuno-adjuvant (Hoebe et al., 2003; Shen et al., 2008).

Upon LPS stimulation, TLR4 signalling leads to activation of the MyD88 pathway and the induction of NF-κB signalling. NF-κB is a heterodimeric transcription factor comprising of two subunits, p50 and p65, that are retained in the cytoplasm by the inhibitor IκB-α. MyD88 signalling results in the activation of the kinase IKKγ and phosphorylation of IκB-α. Phosphorylated IκB-α is targeted for ubiquitination and subsequent proteasomal degradation. Thereafter, NF-κB is able to translocate to the nucleus and transcribe target genes (Hayden and Ghosh, 2004). High levels of pro-inflammatory cytokine secretion by activated DC depends on the MyD88-NF-κB signalling axis, and either MyD88 deletion (Kawai et al., 1999) or pharmacological inhibition of NF-κB can significantly dampen cytokine production (O'Sullivan and Thomas, 2002). Since this signalling pathway is rapidly induced after LPS stimulation, cytokine transcription is one of the first events to occur in DC maturation.

LPS-TLR4 signalling also induces TRIF-dependent signalling, resulting in phosphorylation of the transcription factor IRF3 and transcription of IFN-β. IFN-β is then secreted by the DC and acts as an autocrine signalling loop that constitutes a late-phase maturation signal (Gautier et al., 2005; Honda et al., 2003; Longman et al., 2007; Montoya et al., 2002; Pollara et al., 2006; Severa et al., 2006). In certain circumstances, autocrine IFN-γ can also fulfill this

function (Pan et al., 2004). IFN-β binds to IFN alpha receptor-1 (IFNAR1) expressed on the surface of the DC and signals via the receptor kinases JAK1 and Tyk2. These kinases phosphorylate the transcription factors signal transducer and activator of transcription-1 (STAT1) and STAT2, which heterodimerise and then bind to IFN-stimulated response elements (ISREs) in gene promoters. DC surface maturation markers—such as HLAs, CD40 and CD86—have ISRE and STAT1-binding elements in their promoters (Lefebvre et al., 2001; Qin et al., 2005; Shi et al., 2006). Therefore, deletion of TRIF (Hoebe et al., 2003), blockade of IFNAR1 (Pollara et al., 2006), neutralisation of the autocrine IFN-β (Longhi et al., 2009), inhibition of JAK proteins (Kubo et al., 2013) or gene deletion of STAT1 (Johnson and Scott, 2007) can prevent the full up-regulation of DC maturation markers.

Additionally, IFN-β can act as an amplification signal for DC maturation because it can also activate the NF-κB pathway by STAT1-independent IFN-β signalling. Upon IFN-β stimulation, there is also STAT-independent activation of MAPKs, PI3K and Akt, leading to a reinforcement signal amplifying NF-κB-dependent cytokine production (Pollara et al., 2006). Thus, inhibition of JAK proteins in stimulated DC can also dampen pro-inflammatory cytokine production (Kubo et al., 2013), and even STAT1-KO APCs can up-regulate IL-1β in response to IFN-β (Joshi et al., 2006), illustrating the dual function of the autocrine IFN loop. Given this central role of IFNs in DC biology, it is therefore unsurprising that the addition of exogenous IFN-β alone is sufficient to induce fully functional, mature DC (Pantel et al., 2014).

Importantly, TLR signalling also induces signalling by the three MAPKs: ERK, p38 and JNK. These kinases coordinate central aspects of DC maturation. For example, p38 activation induces phosphorylation of histone deacetylases that unwind heterochromatic regions and establish a chromatin landscape permissive to gene expression (Seguín-Estévez et al., 2014). JNK is important in the generation of reactive oxygen species (ROS) and together with ERK, the induction of certain co-inhibitory molecules (Fife et al., 2009). MAPKs function in ancillary roles in DC maturation, such as augmenting cytokine secretion or maturation marker expression, and inhibition of these kinases can modulate the resulting phenotype (Mitchell et al., 2010).

Lastly, MAPKs functionally bridge TRIF and MyD88 signalling. STAT1 depends on two critical phosphorylation events: serine 727 (S727) phosphorylation, and tyrosine 701 (Y701) phosphorylation. S727 phosphorylation is necessary for full transcriptional activation of STAT1 and depends on p38, which is activated very early upon LPS stimulation; conversely, Y701 phosphorylation depends on JAK family kinases, which are optimally activated by the later IFN signal (Kovarik et al., 1999). Early MAPK signalling therefore 'primes' STAT1 for transcriptional activity, in preparation for the late-phase autocrine IFN loop.

In summary, DC maturation requires cascades of signals that have distinct, yet overlapping functions. These signals may often amplify each other at different phases of the maturation process. Many transcription factors are necessary for DC maturation, yet it is less clear which are sufficient. However, this paradigm may be crudely simplified into two important points: (i) that

inflammatory cytokine production is largely dependent on the MyD88-dependent pathway, and (ii) up-regulation of surface maturation markers is more dependent on the TRIF-dependent pathway.

1.3.2 The Adaptive Immune System

In evolutionary terms, the adaptive immune system is newer than innate immunity, and is less widespread across multicellular organisms. The adaptive immune system belongs only to jawed vertebrates, and is thought to have originated around 500 million years ago (Flajnik and Kasahara, 2010).

During the course of an infection, the innate immune response responds quickly in the acute phase, while the adaptive immune system takes several days to respond (Miao et al., 2010). This is because the adaptive immune system has more specificity in recognising antigen, but also because there is usually clonal expansion of lymphocytes — the cells that mediate adaptive immunity. The expansion in antigen-specific lymphocytes leads to immunological memory, which is an absent feature in the innate immune system.

1.3.2.1 T-cells

T-cells originate from lymphoid progenitor cells in the bone marrow and then egress to the thymus to undergo thymic education (Sedwick, 2006). T-cells are unique in that they possess a T-cell receptor (TCR) that is able to recognise specific antigens when presented by HLA. TCRs are extremely diverse in their specificity, principally due to VDJ recombination, driven by non-homologous end-joining (NHEJ) which is utilised to rearrange TCR

segments into a high multiplicity of combinations (Malu et al., 2012). In the thymus, TCR^+ T-cells undergo a selection process that eliminates those cells whose TCR binds too strongly or too weakly to antigen, thereby selecting the useful and rejecting the harmful T-cell clones (Klein et al., 2014).

The TCR is co-expressed with CD4 or CD8, which separates T-cells into two major lineages (Germain, 2002). $CD4^+$ T-cells are called T helper cells (T_H) and recognise HLA Class II, which is only expressed on APC and a handful of other cell types. $CD4^+$ T-cells secrete cytokines such as IL-4, IFN-γ and IL-17 to modulate and coordinate the immune response. $CD8^+$ T-cells recognise HLA Class I, which is more ubiquitously expressed. $CD8^+$ T-cells are also called cytotoxic T-cells (T_C) because they can induce cellular death to virally infected cells, or those expressing tumour antigens.

Mature DC are able to migrate to the lymph nodes and present antigen to T-cells. Since mature DC express high levels of HLAs and co-stimulatory molecules, they can present antigen on their surface and effectively stimulate lymphocytes to proliferate and differentiate into certain subsets. Like DC and monocytes, both naive and memory T-cells can patrol the body in a steady-state, but they can also hone to sites of infection and lymph nodes (Worbs and Förster, 2009). Therefore, although most T-cells reside in lymphoid tissue, there are many opportunities for T-cell to encounter DC and undergo activation (Lewis et al., 2007)

1.3.2.1.1 T-cell Activation

For DC to activate and polarise T-cells, they need to provide three critical signals (Escors et al., 2013). Signal one is the binding of peptide-loaded HLA (called major histocompatibility complex (MHC) in mice) to TCR. To do this, a DC physically associates with the T-cell and forms an immunological synapse (Huppa and Davis, 2003). CD4 or CD8 forms a co-receptor that helps to bridge the HLA-TCR interaction, and allows for specificity with regards to HLA Class I or II binding. On the T-cell, the TCR exists in a complex with CD3: the latter then signals via the intracellular kinases ZAP70 and Lck upon TCR stimulation, providing a positive signal for T-cell growth and division (Brownlie and Zamoyska, 2013).

However, signal one is normally insufficient to induce T-cell activation. Indeed, the induction of signal one alone is likely to result in T-cell anergy. To fully commit a T-cell to activation (i.e. IL-2 production and proliferation), a second signal is required. The second signal is co-stimulation, which is induced by a variety of molecules such as CD40, CD80, CD86, ICOSL and OX40L (Chen and Flies, 2013). The best studied of these molecules is the B7 family. Mature DC have the phenotype $CD80^{hi}$ ($B7\text{-}1^{hi}$)/$CD86^{hi}$ ($B7\text{-}2^{hi}$) and these highly expressed molecules bind to CD28 expressed on the T-cell at the immunological synapse. This delivers a second PI3K/NF-κB-dependent signal to the T-cell that ensures activation (Logue and Sha, 2004). Importantly, it is now appreciated that the ultimate fate of DC-mediated T-cell activation depends on the subtle interplay of the whole repertoire of co-stimulatory and co-inhibitory receptors, many of which have uncharacterised functions (Reis e

Sousa, 2006). Indeed, DC can also express co-inhibitory receptors such as programmed death ligand-1 (PD-L1) and PD-L2. These restrict the T-cell response by providing a negative signal that induces SHP-1 and SHP-2 phosphatase activity in the T-cell to counteract ZAP70 and Lck tyrosine phosphorylation (i.e. activation) from signal one (Sheppard et al., 2004).

Signal three is the cytokine milieu that influences T-cell differentiation. Naive $CD4^+$ T-cells (sometimes called T_H0) can further differentiate into T_H1, T_H2, T_H17 and T_{REG} cell-types (Luckheeram et al., 2012). These subsets are defined by a hallmark transcription factor and cytokine signature: T_H1 cells express the transcription factor T-bet, secrete IFN-γ and are important for immune responses to intracellular bacteria and viruses; T_H2 cells express the transcription factor GATA-3, secrete IL-4 and are important in allergy and anti-helminth responses; T_H17 cells express the transcription factor RORC, secrete IL-17 and are important for immunity to extracellular bacteria and fungi; T_{REG} express the transcription factor FOXP3, secrete IL-10 and are important for immunoregulation (Zhu and Paul, 2009). The cytokines secreted by mature DC influence the resulting $CD4^+$ T-cell phenotype. For example, IL-12 secretion by DC will promote the induction of T-bet and IFN-γ production in the T-cell (i.e. T_H1 differentiation), whereas TGF-β secretion by the DC will promote the induction of FOXP3 and IL-10 production in the T-cell (i.e. T_{REG} differentiation).

$CD4^+$ T-cells are critical for generating vigorous and protective immune responses to invading pathogens. Indeed, this is highlighted by the opportunistic bacterial infections associated with chronic HIV infection. $CD4^+$

T-cells are especially important for anti-bacterial immunity at mucosal sites (Hagiwara et al., 2003; Kadioglu et al., 2004). Significantly, impairment of $CD4^+$ T-cells is associated with markedly increased microbial translocation across mucosal epithelial barriers (Perreau et al., 2014) and bacterial sepsis (Hawkins et al., 2006). In summary, the generation of useful $CD4^+$ T-cell responses by DC is critical for protecting the host from microbial threats.

1.3.2.2 B-cells and Plasma Cells

Helper T cells are important for directing the immune response. However, B-cells are able to differentiate into plasma cells, which are the effector cells of antibody production. Immunoglobulins such as IgA are secreted in gram quantities in the human mucosa on a daily basis (Brandtzaeg and Pabst, 2004), highlighting the importance of B-cells in mucosal immunity. Primary B-cell deficiency in humans results in a dramatic increase in morbidity associated with a higher rate of opportunistic infections (Conley et al., 2009).

B-cells recognise antigen directly by the expression of the B-cell receptor (BCR), which is a receptor belonging to the immunoglobulin family. Unlike T-cells, B-cells do not require antigen to be presented on MHC. The antigens that can induce direct B-cell proliferation are termed T-independent (T-I) antigens. T-I antigens are usually polysaccharides or TLR ligands such as LPS, and these antigens can induce B-cell responses and antibody production independently of any $CD4^+$ T-cell help by binding directly to the BCR and TLR receptors (Mond et al., 1995). Notably, this predominantly results in the 'default' production of IgD or IgM antibodies.

B-cells can produce many different classes of immunoglobulin, these include: IgA, IgD, IgE, IgG and IgM. To undergo class switching, B-cells often require $CD4^+$ T-cell help. B-cells can recognise T-cell dependent (T-D) antigen via the BCR. Once recognised by the B-cell, T-D antigens are internalised, endocytosed and presented on MHC in a similar way to DC. Hence, B-cells are also considered to be APCs. Once endocytosed, B-cells can present T-D antigens to the cognate $CD4^+$ T-cell, and the B-cell-expressed co-stimulatory molecule CD40 can induce T-cell proliferation and cytokine production (Zubler, 2001). The T-cell derived cytokines can then induce B-cell class-switching, i.e. from IgM to IgA/IgG production (Cerutti, 2008). B-cells can also then become memory B-cells or plasma cells — the latter of which are able to produce vast quantities of antibodies compared to the smaller effector B-cell.

1.4 Pathophysiology of *N. meningitidis*

Pathogenesis occurs by two related routes, the first being bacteria-mediated tissue destruction and the second being immune cell-mediated inflammation. Studies *in vitro* using human endothelial cells have shown that meningococcus can cause direct injury to the endothelial monolayer by causing cellular detachment and exerting direct cytopathic effects (Schubert-Unkmeir et al., 2010). It has been found that *N. meningitidis* utilises the CD147 receptor to bind to endothelial cells, and this leads to colony formation *in* vitro and *in vivo,* resulting in vascular damage and dysfunction (Bernard et al., 2014).

Invasion of meningococci into the bloodstream causes a 'cytokine storm' that is caused by the elicitation of pro-inflammatory cytokines by bacterial mediators (Brandtzaeg et al., 2001). LOS is a major determinant of this reaction, since it binds to TLR4 and induces the production of these molecules. LOS can be found in high quantities in the blood of meningococcal disease patients, as well as multiple cytokines such as IL-1β, IL-6, IFN-γ and TNF-α (Pathan et al., 2003). These cytokines derive from many sources, including monocytes and neutrophils, and correlate with disease severity and organ dysfunction (Bozza et al., 2007; Lewis et al., 2013). Studies investigating meningococcal infection have also established the contribution of activated neutrophils to tissue destruction, finding a direct correlation between neutrophil adhesion to the endothelial layer and cellular apoptosis (Klein et al., 1996).

The inflammation that accompanies invasive meningococcal disease ultimately results in severe vascular damage to the host, coagulation and endothelial dysfunction.

1.4.1 Natural Immunity to *N. meningitidis*

Natural immunity to *N. meningitidis* is a complex and little-understood subject, principally due to the lack of a good animal model. One of the major reasons behind this fact is the adaptation of Neisserial iron-acquiring transferrin proteins for the human host. These meningococcal transferrin proteins are not able to efficiently sequester iron from a murine host, and therefore growth of *N. meningitidis* in mice is significantly impaired (Holbein, 1981). As such, mice

can clear extremely high doses of non-replicative haematogenous bacteria in a way that does not truly imitate natural infection or disease (Oftung et al., 1999). Therefore, studies relying on human data have been necessary to understand natural immunity to the meningococcus.

N. meningitidis can colonise up to 40% of the population largely asymptomatically, yet disease only occurs in approximately 1 in 100,000 invididuals in Western nations (Harrison, 2010). It has been inferred from this observation that natural immunity must offer reasonable protection against meningococcal disease. Indeed, colonisation of the human nasopharynx by *N. meningitidis* induces the production of IgA in the saliva which coincides with the presence of bactericidal IgG in the sera of colonised subjects (Robinson et al., 2002). Given its role in complement-mediated killing, IgG appears to be critical for protection against invasive meningococcal disease.

Critically, colonisation with *N. meningitidis* offers some protection against invasive disease, but only offers moderate cross-protection against different strains (Reller et al., 1973). Colonisation with *N. meningitidis* also does not appear to prevent the re-acquisition of the same strain at a later date, or indeed a different strain to the one first encountered (Ala'Aldeen et al., 2000). It can hence be concluded that colonisation usually results in an immune response that offers some protection against invasive disease, but does not lead to (at least immediate) bacterial clearance.

It is thought that the commensal *N. lactamica* (Nl) may provide cross-protection to the meningococcus. Nl is unencapsulated, and so its pathogenic

potential is extremely limited. Various epidemiological studies have suggested that Nl acts as a protective commensal in the nasopharynx. Early studies in young children noted that Nl carriage was associated with a decreased risk of meningococcal disease (Gold et al., 1978). Mathematical modelling of epidemiological data has enforced this hypothesis, illustrating a reciprocal relationship between Nl carriage and incidence of meningococcal disease (Coen et al., 2000).

Carriage of Nl in humans induces cross-reactive antibodies which have opsonophagocytic — but not bactericidal — activity against NMB (Evans et al., 2011; Gold et al., 1978). Animal immunisation models have further revealed that intraperitoneal inoculation with Nl induces cross-protective antibodies of sufficient titre to protect against lethal meningococcal challenge (Li et al., 2006). Furthermore, inoculation with OMVs derived from various strains of Nl also provides protective antibodies against meningococcal infection (Oliver et al., 2002).

The question then remains: how does *N. meningitidis* transition from an asymptomatic commensal to an invasive pathogen? This central issue is largely unresolved, however the answer is likely to lie in the interplay between host, bacterial and environmental factors. For instance, it is known that hyper-virulent (i.e. hyper-invasive) strains have caused outbreaks and epidemics of meningococcal disease. It is believed that this is due to the expression of certain Opa proteins that may allow tighter attachment of the bacterium to the epithelial surfaces of the nasopharynx (Callaghan et al., 2006). Indeed, hyper-invasive strains of *N. meningitidis* that are associated with a higher incidence

of disease are genetically less diverse than carrier isolates (Read, 2014). There is thus strong evidence of bacterial determinants that may determine the ability of *N. meningitidis* to cause disease.

Nonetheless, there are also many host factors that may play a role in meningococcal pathogenesis. Complement is abundant in the bloodstream and offers protection against the haematogenous spread of many invading microorganisms (Brown et al., 1983). In lieu of this, patients with complement and neutrophil deficiencies are statistically more likely to develop meningococcal disease than healthy individuals (Figueroa and Densen, 1991). Large genome-wide association studies have confirmed that mutations in the complement pathway are significantly associated with risk of meningococcal disease (Davila et al., 2010). Neutrophils—which have high expression of complement receptors—have been shown to phagocytose and kill meningococci *in vitro*, therefore they may have a critical role in front-line defence against the bacterium (Ross et al., 1987). Neutropenia is an identified risk factor for invasive meningococcal disease, thus supporting the notion that neutrophils are central to host protection from *N. meningitidis* invasion (Peters et al., 2001; Smith et al., 2013).

Other cells besides neutrophils are associated with protection from meningococcal disease. There is some evidence that adults with susceptibility to meningococcal disease have subtle B-cell immunodeficiencies (Foster et al., 2009). Similarly, primary and secondary T-cell immunodeficiencies have been linked with an increased risk of meningococcal disease (Cohen et al.,

2010; Foster et al., 2010). Together, these data implicate both innate and adaptive immune responses in providing protection against invasive disease.

Environmental factors can also modulate the risk of invasive meningococcal disease. Smoking has been noted as a risk factor, and so has domestic crowding (Fischer et al., 1997). Furthermore, influenza co-infection has long been associated with an increased risk of meningococcal disease (Makras et al., 2001). A mechanism has recently been proposed to account for this: it was found by Loh et al. that during elevated temperatures, *N. meningitidis* up-regulates host resistance factors such as capsule and LOS (Loh et al., 2013). Therefore, *N. meningitidis* may exist in certain state of equilibrium with the host, which is disturbed in certain circumstances.

1.4.1.1 The interaction of NmB with DC

It was previously found by our laboratory that live NmB could inhibit DC maturation by a contact-dependent mechanism. Whereas killed NmB and its components (e.g. LOS) can robustly activate dendritic cells (Al-Bader et al., 2004a; Dixon et al., 2001; Jones et al., 2008; Kolb-Maurer et al., 2001; Singleton et al., 2005; Uronen-Hansson et al., 2004a) as indicated by the up-regulation of maturation markers and the production of pro-inflammatory cytokines, the culturing of DC with viable bacteria suppressed the phenotypic maturation of these cells (Jones et al., 2007). Since DC are critical for controlling immune responses, this phenomenon was investigated in the present study, providing the premise of the thesis, with the aim to uncover the mechanism behind the modulation of DC maturation by live NmB. Since

natural immunity to the meningococcus is observed in humans and is mostly protective from invasive disease, it was an interesting paradox that DC (which are vitally important for adaptive immune responses) should be inhibited by the bacterium.

The thesis begins in Chapter 3 by exploring the background literature surrounding the interaction between NmB and DC, and also the experimental investigation of the basic interaction between NmB and DC, by interrogating the DC surface phenotype and also pro-inflammatory cytokine production induced by the bacterium. Previous results (i.e. the suppression of DC maturation) are re-capitulated, but the novel finding of enhanced pro-inflammatory cytokine secretion is discovered, revealing a de-coupling of two fundamental aspects of DC maturation induced by NmB. This led to the analysis of DC signalling pathways in Chapter 4, with the aim to discover if NmB was targeting a major signalling pathway. The results show that NmB precisely de-activated a single transcription factor. This transcription factor (STAT1) was previously shown to be required for DC maturation. In Chapter 5, the functional outputs of this effect are explored by using DC migration and T-cell co-culture assays. The hypothesis that the inhibition of DC maturation would result in impaired immunological functions is experimentally validated. In Chapter 6, the de-activation of STAT1 is pharmacologically reversed, which restores all aspects of DC function after infection with live NmB, thus providing good evidence that this transcription factor was being targeted by the bacterium to evade immunity. Furthermore, a candidate bacterial effector molecule is proposed. Finally in Chapter 7, the implications of these findings

and how they relate to our understanding of natural immunity to the meningococcus are discussed, particularly with relevance to vaccine development and paradigms of host-pathogen interactions.

2 Chapter 2 - Materials and Methods

Ethics Statement

Peripheral blood used for this study was obtained from healthy adult volunteers with written and informed consent. Consent and donations were recorded in accordance with the Institute of Child Health's Research Governance and Ethical Regulations.

2.1 Materials

2.1.1 General Consumables

Below is a list of general consumables and their suppliers used in this study:

Table 2-1 General consumables

Reagent	Supplier
2-mercaptoethanol	Sigma-Aldrich
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich
5(6)-Carboxyfluorescein diacetate	Sigma-Aldrich

N-succinimidyl ester (CFSE)	
BD CellFix	Becton Dickinson
BD Fix Buffer I	Becton Dickinson
BD Perm Buffer III	Becton Dickinson
Brefeldin A	Sigma-Aldrich
Bright-Glo	Promega
Bromthenol blue	Sigma-Aldrich
CD14 Monocyte Isolation beads	Miltenyi Biotech
CD19 B-cell Isolation Kit	Miltenyi Biotech
Chloramphenicol powder	Sigma-Aldrich
CL-XPosure X-ray Film	Pierce
Deoxyribonucleotide phosphates (dNTPs)	Life Technologies

Dithiothreitol	Life Technologies
DMSO	Sigma-Aldrich
ECL Prime western blotting detection reagent	Amersham
Endotoxin-low bovine serum albumin (BSA)	Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
First strand buffer	Life Technologies
Glutamine x100	Life Technologies
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
Gonococcal agar	Becton Dickinson
Heparin sulfate	

Ionomycin	Sigma-Aldrich
Lipopolysaccharide (LPS) from *E. coli*	Sigma-Aldrich
LS magnetic columns	Miltenyi Biotech
Lymphoprep	StemCell Technologies
Marvel dried skimmed milk powder	Tesco
Methanol	Sigma-Aldrich
Molecular biology grade ethanol	Sigma-Aldrich
Naive CD4 Isolation Kit	Miltenyi Biotech
Pan-CD4 Isolation Kit	Miltenyi Biotech
Paraformaldehyde powder	Sigma-Aldrich
PBS tablets	Oxoid
Penicillin/streptomycin x100	Life Technologies

Peptidoglycan from *S. aureus*	Sigma-Aldrich
Phorbol myristate acetate (PMA)	Sigma-Aldrich
Phosphate buffered saline (PBS)	Life Technologies
Plasticware	Corning/BD
Polyinosinic-polycytidylic acid (poly(I:C))	Sigma-Aldrich
Polyvinylidene difluoride (PVDF) membrane	Pierce Biotechnology
Protamine sulfate	Sigma-Aldrich
Protease Inhibitor Cocktail Tablets	Roche
Random hexamer primers	GE Healthcare
Ready-SET-Go ELISA Human Kits (TNF-α, IL-1β and IL-6)	eBioscience
RNAse Out	Life Technologies

RNEasy Mini Kit	Qiagen
Roche PhosStop Tablets	Roche
Roswell Park Memorial Institute medium-1640, (RPMI-1640)	Life Technologies
Saponin	Sigma-Aldrich
Sodium azide	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Staphylococcal enterotoxin B (SEB)	Sigma-Aldrich
Superscript II Reverse Transcriptase	Life Technologies
SYBR Green Mastermix	Bio-Rad
Triton-X 100	Sigma-Aldrich

Trypan Blue 0.4% solution	Sigma-Aldrich
Tween-20	Sigma-Aldrich
Vitox	Oxoid
X-VIVO 15 media	Lonza

2.1.2 Cytokines and Chemokines

Below is a list of cytokines and chemokines that were used in this study:

Table 2-2 Cytokines and chemokines

Cytokine	Supplier
Human C-C motif chemokine 19 (CCL19)	PeproTech
Human granular-macrophage colony stimulating factor (GM-CSF)	PeproTech / R&D
Human interferon beta (IFN-β)	PeproTech

Human interferon gamma (IFN-γ)	PeproTech
Human interleukin-2 (IL-2)	PeproTech
Human interleukin-4 (IL-4)	PeproTech / R&D

2.1.3 Antibodies

Below is a list of antibodies (**FC**: Flow cytometry; **F**: Functional; **WB**: Western blot) that were used in this study:

Table 2-3 List of antibodies

Antigen	Clone	Application	Supplier
CD1a	HI149	FC	eBioscience
CD14	M5E2	FC	Becton Dickinson
CD3	UCHT1	FC	Biolegend
CD3	OKT3	F (co-culture)	Biolegend
CD4	OKT4	FC	Biolegend

CD40	5C3	FC	Becton Dickinson
CD45RA	HI100	FC	Becton Dickinson
CD45RO	UCHL1	FC	Becton Dickinson
CD83	HB15e	FC	Becton Dickinson
CD86	IT2.2	FC	Biolegend
DC-SIGN	LWC06	FC	eBioscience
HLA Class I	W6/32	FC	eBioscience
HLA-DR	L203	FC	R&D
IFN-γ	25723.11	FC	Becton Dickinson
Isotype Control (IgG1, IgG2)	n/a	FC	eBioscience
IL-1β	CRM56	FC	eBioscience
IL-4	8D4-8	FC	Becton Dickinson

IL-6	MQ2-13A5	FC	eBioscience
IκB-α	Polyclonal	WB	CellSignaling
JAK1	6G4	WB	CellSignaling
JNK	Polyclonal	WB	CellSignaling
p-Akt	D9E	WB	CellSignaling
p-ERK	D13.14.4E	WB	CellSignaling
p-GSK-3	Polyclonal	WB	CellSignaling
p-JAK1	Polyclonal	WB	CellSignaling
p-JAK2	C80C3	WB	CellSignaling
p-JNK	81E11	WB	CellSignaling
p-NF-κB	93H1	FC	CellSignaling
p-p38	D3F9	WB	CellSignaling

p-p65	93H1	WB	CellSignaling
p-S727 STAT1	Polyclonal	WB	CellSignaling
p-S727 STAT1	K51-856	FC	Becton Dickinson
p-Src	Polyclonal	WB	CellSignaling
p-Tyk2	Polyclonal	WB	CellSignaling
p-Y690 STAT2	Polyclonal	WB	CellSignaling
p-Y701 STAT1	Polyclonal	WB	CellSignaling
p-Y701 STAT1	4a	FC	Becton Dickinson
p-Y705 STAT3	4/P-STAT3	FC	Becton Dickinson
PD-L1	29E.2A3	FC	Biolegend
PD-L1	Polyclonal	F (blocking)	Biolegend
PD-L2	24F.10C12	FC	Biolegend

SOCS1	A156	WB	CellSignaling
STAT1	Polyclonal	WB	CellSignaling
α-rabbit IgG HRP	n/a	WB	Cellsignaling
β-actin	13E5	WB	CellSignaling

2.1.4 Inhibitors

Below is a list of inhibitors that were used in this study:

Table 2-4 List of inhibitors

Inhibitor	Supplier
JAK inhibitor (Tofacitinib)	Tocris
JNK inhibitor (SP 600125)	Sigma-Aldrich
MEK/ERK inhibitor (PD 98059)	Sigma-Aldrich
NF-κB inhibitor (BAY 117082)	Sigma-Aldrich

p38 inhibitor (SB 203580)	Sigma-Aldrich
SHP-2 inhibitor (NSC 87877)	Tocris
Sodium orthovanadate (pre-activated)	JENA Bioscience

2.1.5 General Buffers and Media

Below is a list of buffers and media that were used in this study, including recipes for their composition:

Table 2-5 General buffers and media

Buffer	Composition
Complete media	RPMI containing 10% (v/v) FCS, 100 U/mL streptomycin/penicillin and 2.4 mM L-glutamine
Cryopreservation media	FCS containing 10% (v/v) DMSO
ELISA wash buffer	PBS containing 0.05% (v/v) Tween

Flow cytometry buffer	PBS containing 0.2% (w/v) BSA and 0.02% (v/v) sodium azide
Laemmli buffer	20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (v/v) bromthenol blue, 0.2M Tris-HCl
Lysis buffer	1% (v/v) Triton X-100, 50 mM Tris, 100 mM NaCl and x1 Complete Roche PhosStop and Protease inhibitor cocktail tablets
MACS buffer	PBS containing 2% (v/v) FCS and 2 mM EDTA
Permeabilisation buffer	Flow cytometry buffer containing 0.2% (w/v) saponin
Running buffer	25mM Tris, 192mM glycine, 1% (w/v) SDS
Stripping buffer	10% (w/v) SDS, 0.5 M Tris-HCl, 0.1 2-mercaptoethanol, pH 6.8

Transfection media	X-VIVO 15 media supplemented with 10% (v/v) FCS, 100 U/mL streptomycin/penicillin and 2.4 mM L-glutamine, 100 ng/mL GM-CSF and 50 ng/mL IL-4
Transfer buffer	20% (v/v) methanol, 25mM Tris, 192mM glycine

2.1.6 Foetal calf serum

FCS is known to vary depending on supplier and batch. This is largely due to variations in the background levels of contaminants and inflammatory factors (Opitz et al., 1977). Therefore, different FCS batches were tested for immunogenicity by a DC activation assay (for methodology of DC culture and activation, see sections 2.2.3.2 and 2.2.4). DC were cultured in the presence of 10% FCS from multiple suppliers, and then stimulated for 24h with PFA fixed (killed) 10 MOI (multiplicity of infection) NmB, in order to check normal activation. Levels of DC maturation were then measured by flow cytometric analysis of surface expression of CD40 and CD86, using MFI (median fluorescence intensity). FCS batches that induced high levels of background cell activation (i.e. high CD40 and CD86 expression) were excluded from the study. For all experiments, it was decided that endotoxin-low FCS from South America (Life Technologies/Gibco) would be used, as this serum gave

consistent DC activation profiles for all three batches tested (Figure 2-1). Prior to cell culture use and aliquoting, all FCS was heat-inactivated by incubation at 56°C for 30 mins.

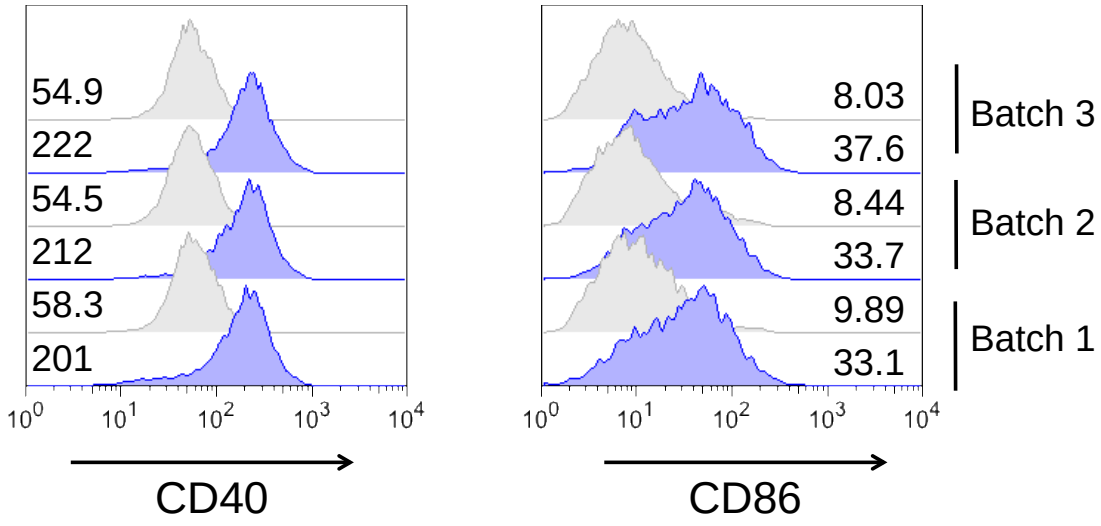

Figure 2-1 *Batch-testing of FCS.* To measure background levels of DC activation, FCS was batch tested to ensure consistency between experiments. FCS quality is essential for the growth and activation of DC. To do this, DC were cultured in various heat-inactivated endotoxin-low FCS batches from South America (Gibco). DC were then either stimulated with 10 MOI killed NmB for 24h (blue histograms) or left unstimulated (grey histograms). CD40 and CD86 were then measured as indicators of DC maturation. MFI values are displayed on the histogram.

2.2 Methods

2.2.1 Bacterial methods

2.2.1.1 Strains

Bacteria used in this study were predominantly of the strain *N. meningitidis* serogroup B strain H44/76 (B:15:P1.7,16), which was first isolated from a meningococcal disease patient in Norway in 1978 (Holten, 1979). This strain possesses a capsule and expresses pili. For one experiment, strain MC58 was used, which is a serogroup B strain first isolated from a patient in the U.K. in 1991 (McGuinness et al., 1991). For other experiments, H44/76 and MC58 isogenic mutants were constructed using allelic replacement with an antibiotic resistance cassette in the *SiaD*, *RmpM* and *PilE* genes, as previously described (Steeghs et al., 1998). These mutants were kind gifts from Professor Peter van der Ley (Netherlands Vaccine Institute, Netherlands).

Campylobacter jejuni, *Streptococcus pneumoniae* and *Lactobacilli reuteri* were kindly provided and prepared by Dr Dagmar Alber (UCL Institute of Child Health, UK) and Ms Katja Brunner (UCL Institute of Child Health, UK).

2.2.1.2 Bacterial culture

Bacteria were grown from frozen 10% glycerol stocks overnight on gonococcal plates made of BD GC Agar supplemented with 1% Vitox growth nutrients in a humidified incubator at 37°C in 5% CO_2. Bacterial number was determined by spectrophotometry at $O.D._{600}$. Bacteria were adjusted to O.D. =

1, and viability counts were used to determine that $O.D._{600}$ = 1 was equal to 1 x 10^9/mL bacterial cells. This was used to calculate MOI for DC infection assays. Bacteria were killed by fixation in 0.5% paraformaldehyde (PFA) for 20 mins at room temperature, and then washed extensively in PBS to ensure removal of PFA. Viability was checked by plating a sample of the killed bacteria stock on GC agar and incubating at 37°C in 5% CO_2 overnight. Fresh stocks of killed bacteria were regularly made and stored at 4°C for no more than 1 week.

2.2.2 Cell counting

All cells were counted using a Neubauer hemocytometer and a light microscope. Briefly, small samples of cell suspensions were mixed in 1:1 ratios with trypan blue and then a 10 µL aliquot was placed in the hemocytometer. All viable cells (i.e. non-blue) were counted within three diagonal 1 mm^2 squares; an average of the three values was used in the calculation. Cell counts in the original sample were calculated according to the following formula:

$$Total\ Cells\ = (Cells\ Counted\ Per\ Square\ x\ 10{,}000\ x\ 2)\ x\ Volume\ (mL)$$

2.2.3 Isolation of Peripheral Blood Mononuclear Cells from Human Blood

To isolate PBMCs, healthy donors were bled using 50 mL syringes after 70% ethanol sterilisation of the skin. Peripheral blood was decanted into a 50 mL tube containing 10 U/mL heparin sulfate, and then inverted gently. Blood was then split in a 1:1 ratio with 37°C pre-warmed complete media.

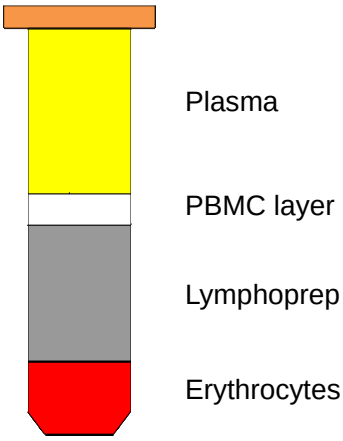

Figure 2-2 *Density separation of PBMCs from peripheral blood.* Above is a diagram of heparin-treated blood that had been layered on Lymphoprep and centriuged at 800 x g for 20 mins at room temperature. The PBMC layer was then delicately extracted from the tube, with careful attention paid in order to remove minimal lymphoprep or plasma, as these contained contaminating cells (e.g. platelets and erythrocytes).

Diluted blood was then gently layered onto lymphoprep at a 3:1 ratio and centrifuged at 800 x *g* for 20 mins at room temperature. The PBMC layer (see Figure 2-2) was then carefully extracted from the tube and washed twice with complete media by centrifugation at 500 x *g* for 10 mins each. The supernatant was discarded after each wash. The remaining pellet represented the extracted PBMC.

2.2.3.1 Enrichment of Monocytes from PBMCs

To isolate monocytes from PBMCs, the PBMC pellet from the previous step was re-suspended in 30 mL ice-cold MACS buffer at 500 *g* for 10 mins. The supernatant was then discarded. The PBMC pellet containing <7 x 10^7 cells was then re-suspended in 300 µL MACS buffer, and 100 µL Miltenyi CD14 Positive Selection Beads were added to the suspension. Cells were then incubated at 4°C for 30-60 mins. At the end of the incubation, 30 mL MACS buffer were added to the suspension, and cells were centrifuged at 500 x *g* for 10 mins. The supernatant was discarded, and cells were re-suspended in 5 mL ice-cold MACS buffer. Meanwhile, an LS magnetic column was attached to the Miltenyi Magnet system, and primed with 3 mL MACS buffer. The 5 mL cell suspension was then run through the column, which was subsequently washed with 3 x 3 mL aliquots of MACS buffer to ensure high purity of collected monocytes. The LS magnetic column containing the monocytes was then removed from the magnet, and the monocytes were extracted by plunging 5 mL of MACS buffer through the LS column into a fresh container.

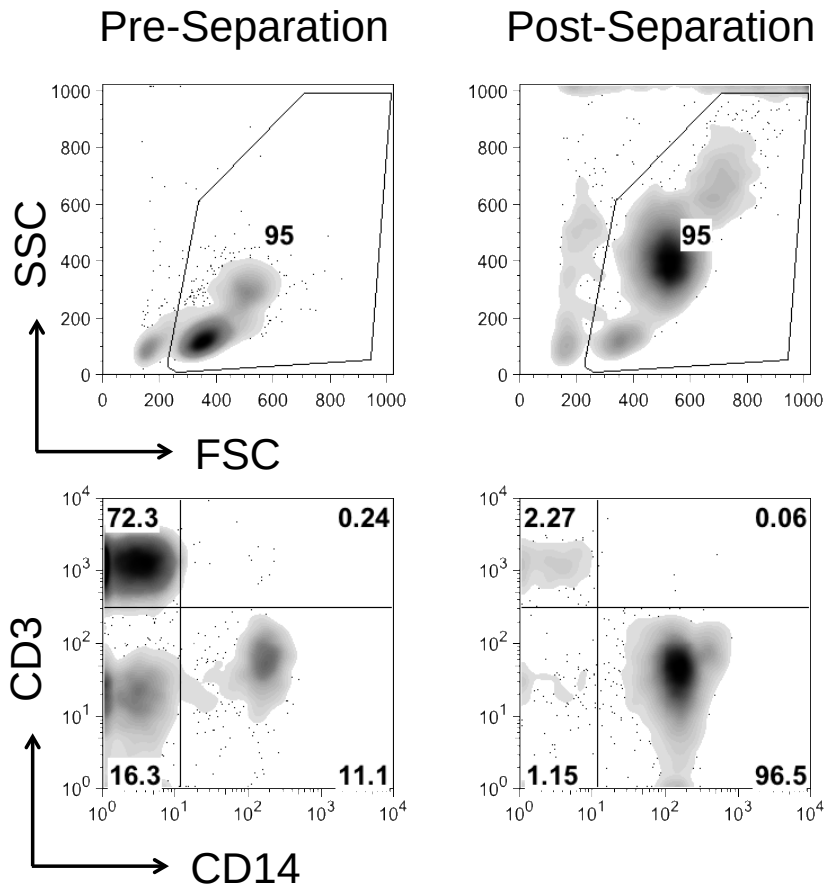

Figure 2-3 ***Monocytes can be isolated from peripheral blood and enriched to a high purity.*** **Samples of cells (~1 x 10^5) were taken pre- and post-MACS separation with magnetic CD14 positive-selection beads. Cells were stained with CD3 and CD14 antibodies in order to assess purity by flow cytometry.** ***Top:*** **FSC-SSC gating profiles used to assess monocyte and lymphocyte populations. Gates were set on live cells.** ***Bottom:*** **CD3-CD14 quadrants to show percentages of lymphocytes and monocytes both pre- and post-MACS separation. Quadrants were determined by isotype controls and unstained controls. Data are representative of two experiments.**

The CD14-depleted PBMC fraction was then centrifuged at 500 x *g* for 10 mins, and the pellet was re-suspended in cryopreservation media at 1 x 10^7 cells / mL, and stored at -80°C in cryovials.

2.2.3.2 Generation of DC from blood $CD14^+$ monocytes

Isolated human monocytes were initially checked for purity and phenotype by flow cytometry. For this, a sample of cells was taken before and after MACS separation, and these were assessed for the monocyte marker CD14 by flow cytometry. Purity ($CD14^+$ cells) typically ranged from 96-98%. A representative purity assessment is shown in Figure 2-3. As can be seen, MACS separation induced $CD14^+$ enrichment from 11.1% to 96.5%, with only minimal contamination from $CD3^+$ lymphocytes.

To differentiate into DC, purified monocytes were counted and re-cultured in fresh complete media containing 100 ng/mL recombinant GM-CSF and 50 ng/mL recombinant IL-4. Monocytes were seeded at 2.5 x 10^5/mL in 6-well plates and cultured for 5-7 days at 37°C in 5% CO_2.

As can be seen in Figure 2-4, monocytes acquired a DC-like phenotype over the duration of the differentiation in IL-4 and GM-CSF. This was measured by flow cytometric analysis of the acquisition of the DC marker and lipid antigen presentation molecule CD1a, and also down-regulation of the monocyte marker CD14. By day 5, cells were $CD14^{lo}$ and $CD1a^+$, which indicated a typical DC phenotype. Therefore, DC were harvested and used between days 5-7 for all experiments.

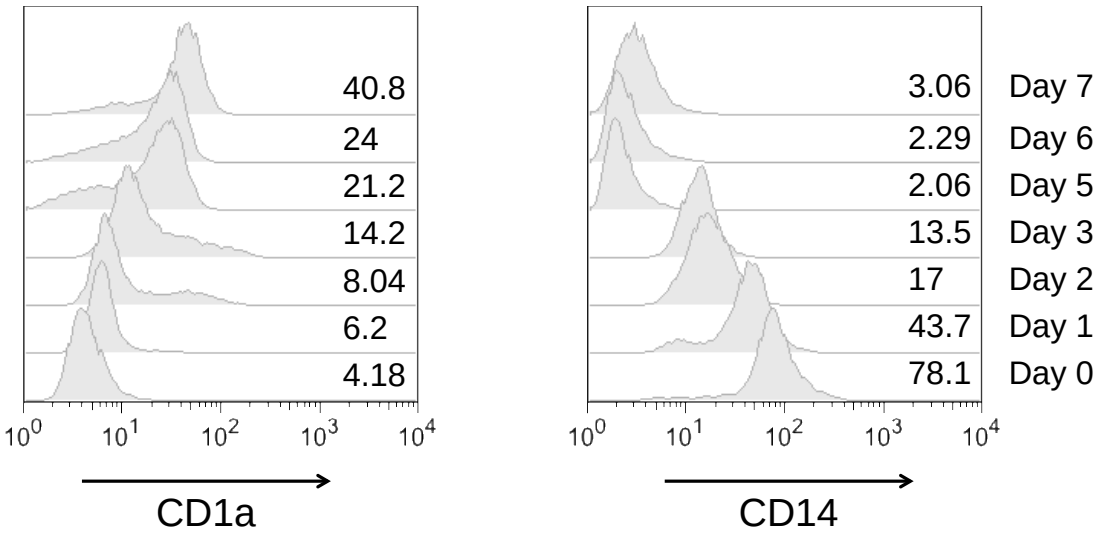

Figure 2-4 ***Monocytes cultured in the presence of IL-4 and GM-CSF are able to differentiate into DC.*** **Monocytes were cultured in 50 ng/mL IL-4 and 100 ng/mL GM-CSF for up to 7 days as described in the text of the Materials and Methods. At various times (as indicated on the histograms), cell samples (1 x 10^5) were aspirated from the culture and assessed for surface expression of CD1a (a DC marker) and CD14 (a monocyte marker) by flow cytometry. MFI values are displayed on the histogram.**

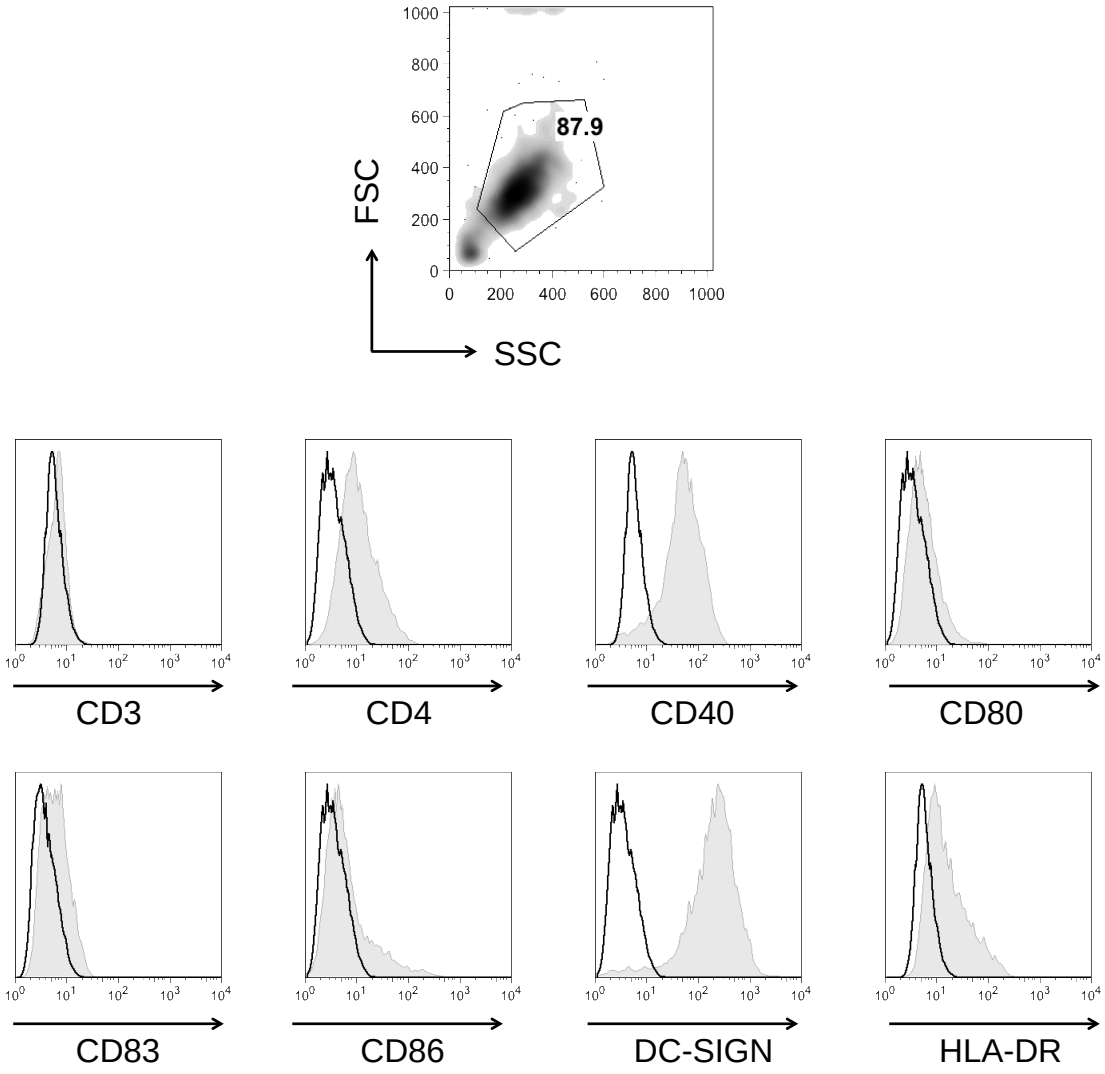

Figure 2-5 *DC differentiated from monocytes show a classic immature DC phenotype.* Monocytes were differentiated in 50 ng/mL IL-4 and 100 ng/mL GM-CSF for 7 days as described in the Materials and Methods, and on the final day of culture an aspirated sample of cells was assessed for characteristic DC markers. CD3 (to measure lymphocyte contamination), CD4, CD40, CD80, CD83, CD86, DC-SIGN and HLA-DR (markers of DC activation and differentiation) were then measured by flow cytometry. *Top*: DC live gate. *Bottom*: Isotype controls are shown by solid lines, and specific antigens are shown in grey filled histograms. Data are representative of three experiments.

On day 7, DC were further analysed for surface phenotype (Figure 2-5), and were found to be $CD3^{-}CD4^{+}CD40^{+}CD83^{lo}CD80^{lo}CD86^{lo}$DC-$SIGN^{+}HLA\text{-}DR^{+}$, which is consistent with previous reports and findings (Mallon et al., 1999).

2.2.3.3 Isolation of $CD4^{+}$ T-cells from PBMCs

T-cells were isolated from frozen CD14-depleted PBMC stocks. For T-cell isolation, frozen PBMC were gently thawed at 37°C and immediately transferred to 30 mL pre-warmed (37°C) FCS. Cells were then centrifuged at 500 x *g* for 10 mins and the supernatant was discarded. PBMC were then re-suspended in 30 mL complete media and centrifuged again at 500 x *g* for 10 mins, after which the supernatant was discarded. Cells were adjusted to a total cell count of less than 1 x 10^{7} PBMC, and 40 µL ice-cold MACS buffer was then added to the cell pellet. Next, 20 µL Miltenyi Pan $CD4^{+}$ or Naive $CD4^{+}$ T-cell Antibody Cocktail was added to the cell suspension, and the cells were incubated at 4°C for 30 mins. After the first incubation, another 40 µL MACS buffer and 40 µL T-cell Microbead Cocktail was added to the cell suspension, and the cells were incubated for a further 30 mins at 4°C. Next, an LS column was attached to a Miltenyi Magnet system and primed with 3 mL MACS buffer. Once the 3 mL had run through, the PBMC suspension was aliquoted onto the MS column, and allowed to pass through by gravity flow.

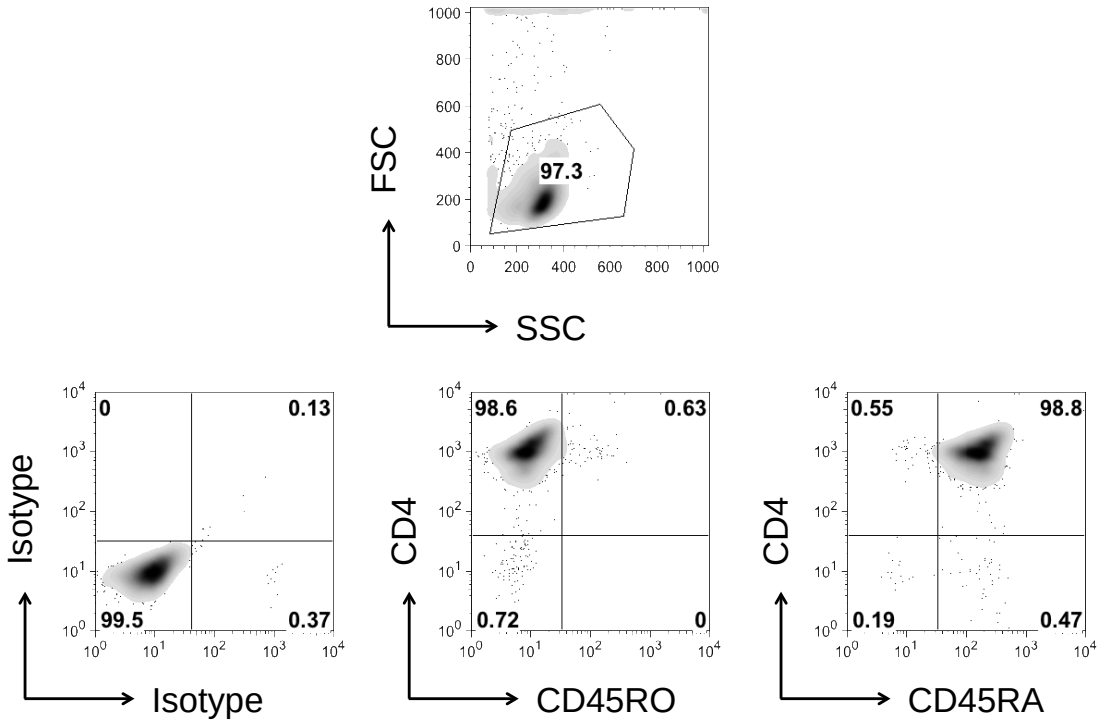

Figure 2-6 *CD4$^+$ naive T-cells can be enriched from CD14-depleted PBMCs by magnetic cell separation.* **Frozen CD14-depleted PBMC stocks underwent naive CD4$^+$ T-cell enrichment using the Miltenyi CD4$^+$ Naive T-cell Isolation Kit, as described in the Materials and Methods section. Samples of cells (1 x 10^5) were taken post-separation, and assessed for the T-cell markers CD4, CD45RA and CD45RO by flow cytometry. *Top:* FSC-SSC gating profiles used to determine T-cell purity. Gates were set on live cells. *Bottom:* Percentage of naive and memory cells were measured, using a gate determined by an isotype control and unstained control. Data are representative of two experiments.**

The flow-through was collected in a fresh container below the column. Immediately after the suspension had flowed through the column, it was washed with 3 x 3 mL MACS buffer, and then the entire flow-through was collected and centrifuged at 500 x *g* for 10 mins. The cell pellet represented the enriched T-cell fraction, which was used for the experiments.

Flow cytometry was used to determine the purity of the T-cells. An aliquot of the cells was taken post-MACS separation, and assessed for CD4, CD45RA and CD45RO expression. T-cell enrichment typically generated $CD4^+$ purity higher than 98%. As can be seen in Figure 2-6, naive $CD45RA^+$ T-cells could be generated to high purity using MACS separation, with minimal CD45RO contamination. Similar results were obtained for the Miltenyi Biotech Pan $CD4^+$ T-cell Isolation Kit (data not shown).

2.2.4 General DC stimulation

On day 5-7 of differentiation DC were harvested and washed twice (500 x *g* for 10 mins) in RPMI containing 10% FCS in order to wash off any remaining antibiotics. DC were re-counted by hemocytometer, re-suspended in fresh RPMI with 10% FCS and seeded in polypropylene tubes at 2.5 x 10^5/mL in 500 µL total volume. DC were then stimulated in individual tubes with the relevant stimuli as indicated in figure legends and incubated at 37°C in 5% CO_2 in a humidified incubator for indicated times. Some DC were also co-treated with inhibitors, vehicle controls or co-stimuli. DC were then used for gene, protein or cellular analysis, or used in a further assay, as described in the relevant section.

2.2.5 Bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BM-DC) were kindly provided by Dr Holly Stephenson and Dr Nayani Pramanik (UCL Institute of Child Health, UK). These cells were prepared from the femurs of wild-type C57BL/6 mice aged 6-12 weeks as previously described (Stephenson et al., 2014). Once differentiated, BMDC were cultured and stimulated identically to human DC.

2.2.6 DC migration assay

To assess chemotaxis/migration, DC were stimulated as indicated in the figure legends, and then washed in complete media twice (500 x *g* for 10 mins) and re-counted on a hemocytometer. Meanwhile, increasing concentrations of CCL19 diluted in complete media were placed in the wells of a Corning transwell-adapted 24-well plate at 600 µL per well. An 8 µm pore transwell was then inserted into the well, and 200 µL of DC suspension containing 2.5 x 10^5 cells was added to the upper compartment. The plate was then incubated at 37°C in 5% CO_2 in a humidified incubator for 5 h, and then the transwells were carefully lifted out of the plate and discarded. The liquid in the bottom chamber was then mixed thoroughly, and an aliquot was taken for counting the migrated DC by hemocytometer. Cell counting was performed in triplicate and an average was taken. Visual inspection of the wells was also used to approximately confirm the readings on the hemocytometer.

2.2.7 T-cell polarisation assay

To measure T-cell polarisation, T-cells were first isolated using the Naive CD4+ Isolation Kit. Meanwhile, stimulated or control DC were seeded in complete media into round-bottom 96-well plates at 5 x 10^4 cells per well in 100 µL volume. SEB (10 ng/mL) was added to each well. 2.5 x 10^5 naive CD4+ T-cells were then added to each well, and allowed to divide for 5 days in 37°C and 5% CO_2. On day 5, cells from the 96-well plate were aspirated and aliquoted into 2 mL fresh complete media containing 2 U/mL IL-2, in a 48-well plate. T-cells were then re-incubated and allowed to expand for a further 7-9 days. At the end of the culture, cells were treated with a cocktail of brefeldin A (1 µg/mL), PMA (100 ng/mL) and ionomycin (1 µg/mL) for 3 h. Cells were then harvested into polystyrene flow cytometry tubes and processed for flow cytometry according to the methodology described elsewhere.

2.2.8 T-cell proliferation assay

To measure T-cell proliferation, T-cells were first isolated using the Pan $CD4^+$ T-Cell Isolation Kit. T-cells were then washed twice in PBS to remove protein (500 x *g* for 10 mins) and then re-suspended in 1 mL PBS. To the cell suspension, 1 µM of CFSE was added, and the cells were gently mixed and then incubated at 37°C in 5% CO_2 for 8 mins, while protected from light. Then, 2 mL FCS was added to the tube in order to stop the CFSE binding, and cells were re-incubated for a further 2 mins. After 2 mins, cells were washed twice

in complete media and then re-suspended to the desired concentration and volume.

Meanwhile, stimulated or control DC were seeded in complete media into round-bottom 96-well plates at 5 x 10^4 cells per well in 100 µL volume. To some wells, SEB (10 ng/mL) or soluble anti-CD3 (clone OKT3, 1 µg/mL) was added. Then, T-cells were added at the concentration indicated in the relevant figure legends (e.g. for a 1:1 ratio, 5 x 10^4 $CD4^+$ T-cells were added), and the total volume of each well was adjusted with complete media to make a final volume of 200 µL. On day 5, cells were transferred to polystyrene flow cytometry tubes and then processed in the usual way for flow cytometric analysis.

2.2.9 Flow cytometry

All flow cytometry experiments were performed by first transferring 1-2.5 x 10^5 cells to Falcon round-bottom polystyrene flow cytometry tubes. 3 mL ice-cold flow cytometry buffer was then added to each tube, and cells were centrifuged at 500 x *g* for 10 mins, and the supernatant was discarded. For experiments involving surface staining, to ensure equal staining between samples, a master mix (containing the appropriate antibodies diluted in flow cytometry buffer) was made, and then 100 µL of master mix was added to the relevant tube. Cells were then gently mixed by pipetting, and then incubated at 4°C for 30 mins while protected from light. After 30 mins, 3 mL flow cytometry buffer was added to the tubes and these were centrifuged at 500 x *g* for 5 mins, and supernatants were discarded. This step was then repeated to ensure removal

of any excess staining antibody. Finally, cells were then fixed in 100-200 µL BD CellFix and briefly vortexed. Samples were run and analysed on either a BD FACSCalibur or BD LSR II flow cytometer within 24h of fixation. FlowJo version 8.8.7 software was used to analyse flow cytometry data and generate figures.

To determine positive and negative populations (e.g. $CD45RA^+$ and $CD45RA^-$ T-cells), isotype controls conjugated to the appropriate fluorochrome were sometimes used at equivalent concentration as the target antibody, as stated in the figure legends. Moreover, isotype controls were also used when first establishing the DC phenotype. However, isotype controls are often problematic, and it has been reported that they can give erroneous results (Keeney et al., 1998). Therefore, isotype controls were only used when needed in order to distinguish positive and negative populations. To determine the antibody concentrations to use in experiments, titration experiments were performed to determine the saturating concentration (data not shown).

2.2.10 Intracellular flow cytometry

To detect intracellular epitopes, cells were harvested and washed once (500 x *g* for 5 mins) and a surface stain (as described in section 2.2.8) was performed if needed. After the optional surface stain, cells were then fixed in 300 µL BD CellFix for 30 mins at 4°C. Cells were then washed twice (500 x *g* for 5 mins): first with 3 mL flow cytometry buffer and then second in permeabilisation buffer. The supernatant was discarded. Cells were re-

suspended in 100 µL of master mix (required antibodies diluted in permeabilisation buffer) and then incubated for a further 30 mins at 4°C shielded from light. Cells were then washed twice (500 x *g* for 5 mins; once with permeabilisation buffer and once with flow cytometry buffer) and re-suspended in 100 µL BD CellFix ready for analysis on the flow cytometer.

2.2.10.1 Intracellular PhosFlow

For the detection of phosphorylated intracellular proteins by flow cytometry, DC were stimulated for the indicated time-points at 37°C before fixation with pre-warmed BD Fix Buffer at a 1:1 ratio with the cell media. Cells were fixed for 10 mins at 37°C and 5% CO_2. Cells were washed once with PBS containing 2.5% FCS (500 x *g* for 10 mins) before a permeablisation step with 1 mL BD Perm Buffer III. Cells were then incubated on ice for 30 mins, before washing three times in PBS containing 2.5% FCS. Cells were then stained with the following antibodies: AlexaFluor647-conjugated p-STAT1-Y701 or p-STAT3-Y705 or PE-conjugated p-STAT1-S727 for 45 mins at 4°C while protected from light, before washing (500 x *g* for 5 mins), re-suepension in 200 µL PBS and immediate analysis on a BD FACSCalibur or BD LSRII instrument.

2.2.11 Lentivirus quantification

All lentiviruses in this study were kind gifts from Dr Emma Chan (UCL Institute of Child Health, UK) and Dr Holly Stephenson (UCL Institute of Child Health, UK/ Max Plank Institute, Germany). Lentiviruses were HIV-1-based vectors

with self-inactivating (SIN) elements, and were generated as previously described (Chan et al., 2012).

For determination of virus concentration, 293T cells (seeded by Dr Emma Chan) were grown in complete media at 37°C and 5% CO_2 until sub-confluent. Then, 1 x 10^5 cells per well were infected with serial dilutions of GFP-reporter (SEW) lentivirus (GFP-LV) stock and re-incubated for a further 72h. At 72h, cells were harvested and then assessed for lentivirus transduction by flow cytometry. GFP^+ cells were measured to distinguish those that had been effectively transfected with virus.

To calculate virus titre, the amount of virus that gave 0-10% was selected, as this was unlikely to represent cells with multiple copies of virus (shown in Figure 2-7). Then, the background from the untransfected cells was subtracted (i.e. 0.35%) from the total, to give 7.69% GFP^+ cells. Given that 0.016 µL GFP-LV stock caused 7.69 x 10^3 HEK 293T cells to be transduced, it was assumed that 0.016 µL of GFP-LV stock contained 7.69 x 10^3 infectious particles. Therefore, the concentration of the GFP-LV virus stock was determined to be 4.8 x 10^8 IU/mL. This methodology was used to determine multiplicity of infection (MOI) for DC transfection experiments.

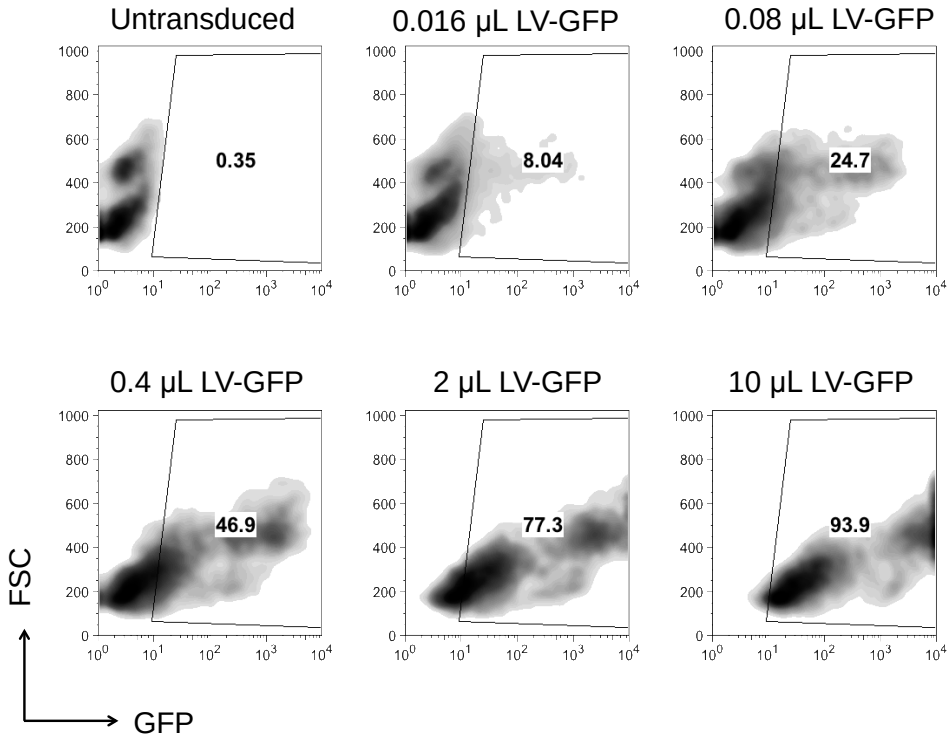

Figure 2-7 ***Titration of GFP-expressing lentiviral transfection of HEK-293T cells can be used to calculate virus titre.*** **HEK-293T cells were grown in complete media until sub-confluent and infected with increasing doses (0.016 to 10 µL) of a lentivirus containing a GFP construct (LV-GFP). Cells were then re-incubated for 72h and subsequently assessed for GFP expression by flow cytometry. Gating percentage of GFP+ cells was determined by an untransduced control.**

2.2.11.1 Transfection of DC with lentivirus

Transfection of DC with lentivirus was performed according to a previously published protocol (Breckpot et al., 2007), with some minor modifications. After monocyte isolation, DC were cultured in transfection media according to the standard protocol. However, on day 3 of differentiation, DC were harvested and then washed in X-VIVO 15 media twice by centrifugation at 500 x *g* for 10 mins. 1 x 10^6 DC were re-suspended in a 100 µL droplet of 'transduction cocktail' (X-VIVO 15 media containing 5 MOI lentivirus, 10 µg/mL protamine sulfate) within a 6-well plate for 2h and incubated at 37°C in 5% CO_2 in a humidifed incubator. After 2h, 4 mL transfection media was added to each droplet, and DC were cultured as normal for a further 3 days. Transfection efficiency was assessed by flow cytometry by measuring GFP^+ DC, and was further optimised in Chapter 4.

2.2.11.2 Lentivirus NF-κB assay

DC were transfected with a lentivirus containing a NF-κB reporter plasmid (from Dr Holly Stephenson, UCL Institute of Child Health, UK) (Stephenson et al., 2014) as previously described above. DC were then stimulated in technical duplicates for 4h as described in the relevant figure legends, and then 100 µL BrightGlo reagent was added for 2 mins before measuring luciferase activity on a luminometer.

2.2.12 Western Blot

DC were stimulated at 2.5 x 10^5 cells/condition as described in the relevant figure legends. Cell lysates were made by adding 3 mL ice-cold PBS to DC and centrifuging samples for 10 mins at 500 *x* g. Supernatants were discarded, and 100 µL lysis buffer was added to the DC, and the cell suspension was vigorously pipetted more than 20 times, and incubated at 4°C for 30 mins. Cell lysates were frozen at -80°C until further use.

Upon thawing, cell lysates were centrufiged at maximum speed for 10 mins, and the supernatant was extracted from the lysates in order to remove debris. Lysates were diluted in a 1:1 ratio with x2 Laemmli buffer and heated to 95°C on a heat-block for 10 mins. Samples were then loaded onto 12% acrylamide gels (purchased from Thermo Scientific or made in-house) in a Mini-Protean Tetra-Cell (Bio-Rad) system containing running buffer with an electrical current of 120mV applied for ~1.5 h. The acrylamide gel was then placed in a Mini-Protean Transfer System (Bio-Rad) with a PVDF membrane and transfer buffer, and an electrical current of 200mA was applied for 1h. The PVDF membrane was then blocked for 0.5-2h in 0.1% Tween-PBS containing 0.1-5% powdered milk at room temperature. The primary antibody of interest was then added at a dilution of 1/1000 in a solution of 0.1% Tween-PBS containing 1-5% powdered milk, and incubated in a 50 mL Falcon tube at 4°C for 16h under constant movement. The membrane was then washed in 0.1% Tween-PBS three times in 10-minute intervals and incubated with a secondary HRP-linked antibody in 0.1% Tween-PBS containing 0.1% powdered milk for 1h at room temperature. After washing three times for 5 mins in 0.1% Tween-PBS,

the membrane was then placed on a Kodak Biomax Cassette (Sigma) and exposed to reconstituted ECL solution for a few mins before exposure to X-ray film and processing in a dark room by a developer.

To ensure similar levels of protein loading, some PVDF membranes were bathed in stripping buffer for 30 mins and then washed extensively with 0.1% Tween-PBS. Membranes were then re-blocked and incubated with a different primary antibody. The same protocol as previously described was then used for the second blot after the initial strip and wash.

2.2.13 Enzyme-Linked Immunosorbent Assay

ELISAs for IL-1β, IL-6 and TNF-α were performed using the eBioscience Ready-SET-Go ELISA kits. For chemokines, Peprotech ELISA Development Kits were used. Supernatants used for these assays were aspirated from DC cultures and then passed through a 0.2 μm PVDF membrane filter in order to remove any cells or bacteria.

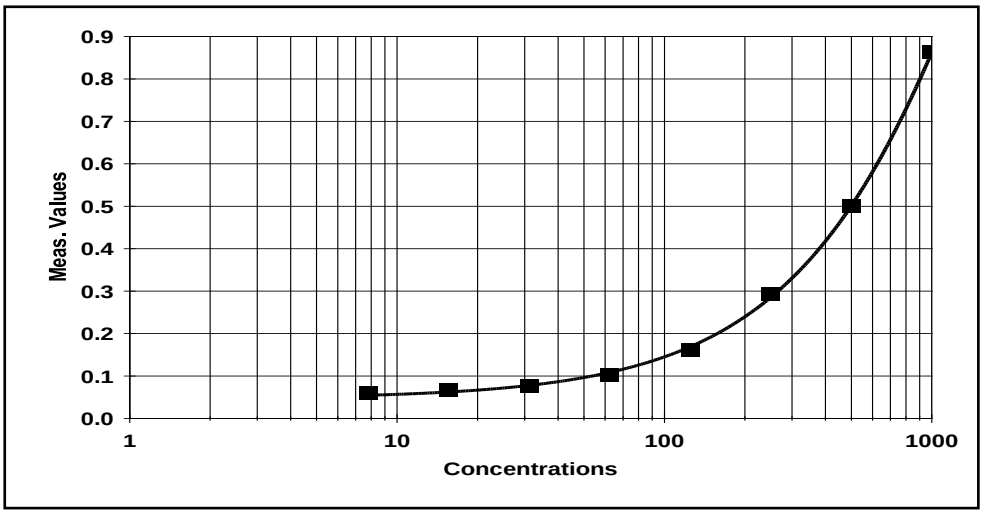

Figure 2-8 ***A standard curve used to calculate cytokine concentrations by ELISA.*** **Shown is a representative standard curve calculated by using serial dilutions of recombinant human IL-1β (7.8 - 1000 pg/mL). Measured spectrophotometry values (O.D. 450 nm) are depicted by ■ (x-axis) and plotted against IL-1β concentration in pg/mL (y-axis), and a predicted line-of-best-fit was plotted using a quadratic polynomial fit ($y = a+b*x+c*x^2$) on Revelation software. The correlation coefficient (R^2) was >0.99, indicating a good quality standard curve.**

First, an ELISA plate was coated with 50 µL of coating buffer, containing the diluted capture antibody (at 1 µg/mL) required for the assay. The plate was then incubated overnight at 4°C. After incubation, the plate was washed vigorously three times with saturating amounts of ELISA wash buffer. Then, the plate was blocked at room temperature for 1h with assay diluent at 100 µL per well. After blocking, the plate was again washed three times with ELISA wash buffer. Serial dilutions of the relevant recombinant protein were added in duplicate at 100 µL final volume with a typical range of 7.8-1,000 pg/mL. 100 µL volume of samples were also added in duplicate to the plate. Samples were diluted 1:10-1:500 in assay diluent, using previously published cytokine quantities in response to meningococcal antigens as an approximate guideline (Jones et al., 2007; Kolb-Maurer et al., 2001). After the addition of samples, the plate was incubated overnight at 4°C, and then washed more than three times in ELISA wash buffer. Next, the plates were incubated with 50 µL of detection antibody diluted in assay diluent. After 2h, plates were washed vigorously more than three times with ELISA wash buffer. Then, 50 µL streptavidin-HRP conjugated antibody diluted in assay diluent was added to each well, and the plate was incubated at room temperature for 10 mins. After 10 mins, 50 µL stop solution (1M H_2SO_4) was added to each well in order to stop the reaction. The plate was read immediately on a Dynatech microplate reader at 450 nm wavelength, and sample values were calculated by a standard curve (see Figure 2-8), by using Revelation ELISA software.

2.2.14 Real-time PCR and analysis of gene expression

2.2.14.1 RNA extraction

For analysis of gene expression, 2.5 x 10^5 DC were lysed in 350 µL RNA Later buffer (RLT plus buffer, Qiagen) containing 1% 2-mercaptoethanol. Lysates were homogenised by passing through a QIAshredder (Qiagen) spin column by centrifugation at 15,000 x *g* for 2 mins and the flow-through was frozen at -80°C until RNA extraction. RNA was extracted using the Qiagen RNeasy Mini Plus Kit according to manufacturer's instructions. In detail, 350 µL of cell lysate was passed through a genomic DNA elimination column by centrifugation at 8,000 x *g* for 30 seconds before mixing with 350 µL buffer containing 70% ethanol. The sample (700 µL) was transferred to an RNeasy spin column and centrifuged at 8000 x *g* for 15 seconds to bind the RNA and flow-through was discarded. RNA attached to the column was then washed with 700 µL of Buffer RW1 followed by centrifugation at 8000 x *g* for 15 seconds. The column was then washed twice with 500 µL of buffer RPE, before transfer of the RNeasy spin column to a new collection tube and centrifugation at 15,000 x *g* for 1 minute in order to remove residual buffer. 30 µL of RNase-free water was added to the spin column membrane and the column was then placed in a 1.5mL Eppendorf tube and centrifuged at 8000 x *g* to elute the RNA. RNA was frozen at -80°C until use. RNA concentration and purity was assessed by Nanodrop spectrophotometry and was routinely found to have a 260/280 ratio above 1.8, indicative of high purity RNA. See Figure 2-9 for a typical plot and yield.

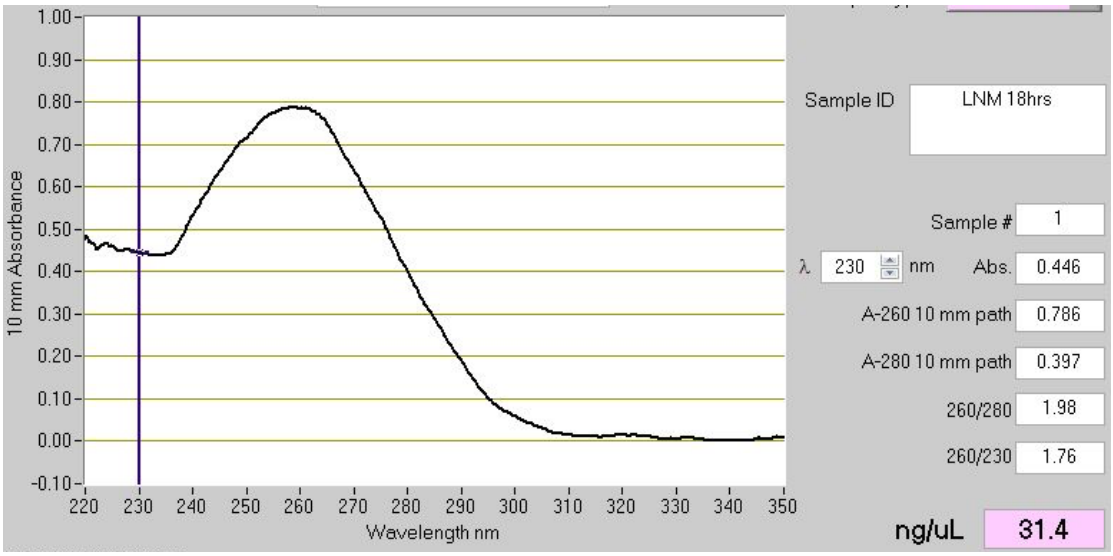

Figure 2-9 ***Representative emission spectrum of extracted DC RNA.*** **RNA was extracted from DC as described in the Materials and Methods. A small sample of RNA diluted in RNAse-free H_20 (1 µL) was then measured for purity and concentration by NanoDrop (Thermo Scientific) at 230 nm, after blanking the instrument with the appropriate solvent. RNA emits fluorescence at 260 nm, and 260/280 ratio was calculated to be 1.98, which indicated high-purity RNA with minimal contamination. The concentration of RNA in this sample was calculated to be 31.4 ng/µL.**

2.2.14.2 First strand cDNA synthesis

RNA served as a template for cDNA synthesis using first strand cDNA synthesis by using the Superscript II Reverse Transcriptase kit (Life technologies) and random hexamer primers. First, 9 µL of purified RNA (max 1 µg) was mixed with 2.5 µL of random hexamers (100 ng/µL) and incubated at 70°C for 10 mins. The following reaction mixture was used for cDNA synthesis: 4.5 µL 5x First Strand Buffer, 2 µL 0.1M DTT, 1 µL 10 mM dNTPs, 1 µL RNAse Out and 0.5 µL Superscript II Reverse Transcript.

8.5 µL of the cDNA synthesis cocktail was added to the 11.5 µL RNA/hexamer mix and incubated at room temperature for 10 mins before incubation at 42°C for 1h. cDNA synthesis was then terminated by incubation at 72°C for 10 mins, and 80 µL of RNase-free water added to the 20 µL cDNA reaction to give 100 µL of cDNA template, which was frozen at -20°C until use.

2.2.14.3 Primers and real-time PCR

Primers were designed using Primer Blast software (NCBI) and validated for specificity through the analysis of melt curves generated following PCR amplification using SYBR Green Mastermix on the Rotor-gene 6000 Corbert thermocycler (Qiagen). Each primer generated a single peak size was confirmed by agarose gel electrophoreisis. The reaction mix consisted of: 10 µL x2 SYBR Green Mastermix, 6 µL DNase-free water, 1 µL forward primer at 500 nM, 1 µL reverse primer at 500 nM, 2ul of cDNA template.

The following primer sequences were used:

CD86 F: AGCACAGACACACGGATGAG, R: GGAAGGCCATCACAAAGAGA; *IL6* F: GTGCCTCTTTGCTGCTTTCAC, R: GGTACATCCTCGACGGCATCT; *IL1B* F: GTAGTGGTGGTCGGAGATTCG, R: CTAAACAGATGAAGTGCTCCT; *IL8* F: AATCTGGCAACCCTAGTCTGCTA, R: AAACCAAGGCACAGTGGAACA; *IDO1* F: GAGAAGTTAAACATGCTCAGC, R: GACACAGTCTGCATAAACCAA; *IFNB* F: AGCTGCAGCAGTTCCAGAAG, R: AGTCTCATTCCAGCCAGTGC; *IFNG* F: TGACCAGAGCATCCAAAAGA, R: CTCTTCGACCTCGAAACAGC; *CISH* F: GTCCAGCCGAGTCCCCACTCC, R: TGCTCACCCCTGAACGCAGAG; *SOCS1* F: GAGAACCTGGCTCGCATC, R: AACACGGCATCCCAGTTAAT; *SOCS3* F: GTCACCCACAGCAAGTTTCC, R: TCACTGCGCTCCAGTAGAAG; *TNFA* and the house keeping gene *GAPDH* primers were from Qiagen (TNFA: QT01079561; GAPDH: QT01192646).

For analysis of gene expression, the cDNA from samples was amplified using SYBR green technology (Bio-Rad) on a rotor-gene 6000 Corbert thermocycler (Qiagen) using the following conditions: 95°C 5 mins, then 40 cycles of 95°C 30 seconds, 60°C 30 seconds and 72°C 30 seconds. Melt curve and SYBR green emission data were collected. Samples were run in technical duplicates and the average Cycle thresholds (Cts) of the duplicates determined for the gene of interest as well as the housekeeping gene GAPDH. A representative melt-curve showing a single PCR product is shown in Figure 2-10.

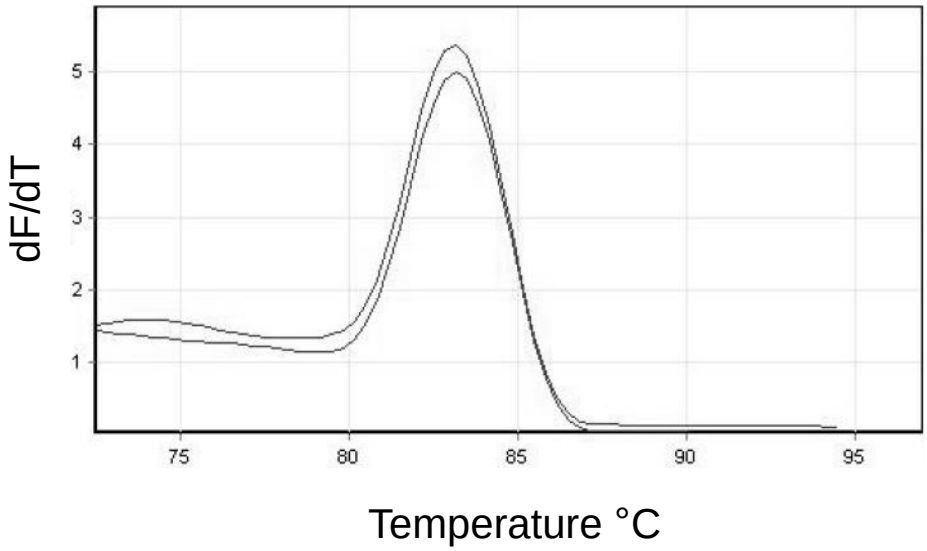

Figure 2-10 ***A representative melt-curve showing a single product from RT-PCR.*** **RNA was extracted from DC and converted to cDNA, and then copies of *CD86* mRNA were quantified by RT-PCR as described in the Materials and Methods. dF/dT (derivative fluorescence/derivative temperature) plotted against temperature (°C) showed a single peak, indicative of a single PCR product.**

Expression of the gene of interest was first normalised to the housekeeping gene *GAPDH* using the deltaCt method, before standardising to T=0 DC expression using the -deltadeltaCT (-ddCT) method (Livak and Schmittgen, 2001) and the following formulae:

Treated DC gene **dCT**= (Treated DC *gene of interest* Ct) - (Treated DC *GAPDH* Ct)

T=0 DC gene **dCT** = (T=0 DC *gene of interest* Ct) - (T=0 *GAPDH* Ct)

-ddCT=(Treated DC gene **Ct**)-(T=0 DC gene **CT**)

Fold change in expression = 2^{-ddCT} =$2^{-(\text{Treated DC gene Ct})-(\text{T=0 DC gene CT})}$

2.3 Statistics and Software

All statistical analysis was done using GraphPad Prism (version 5.0) or Microsoft Excel (version 2010) software. Molecular modelling of proteins was performed using the SWISS model online software (http://swissmodel.expasy.org). All one- and two-way ANOVA statistical tests (as indicated in figure legends) were followed by a Bonferroni post-test in order to correct for multiple testing.

3 Chapter 3: Viable *N. meningitidis* Subverts Dendritic Cell Maturation

3.1 Introduction

Human DC express a multitude of TLRs, such as TLR2, TLR3, TLR4, TLR6 and TLR9, in order to respond to microbial antigens (Muzio et al., 2000). Upon TLR stimulation, DC undergo a maturation program that involves the up-regulation of maturation markers and the production of pro-inflammatory cytokines. However, many bacteria have developed sophisticated strategies to subvert this process. This is unsurprising, considering that DC maturation is critical for the effective clearance of many bacterial infections *in vivo* (Macedo et al., 2008; Schreiber et al., 2010), and many obligate pathogens depend on the human host for their own survival.

There are two major ways by which pathogens can exploit dendritic maturation to divert immune responses to a favourable outcome. The first method is modulation of DC cytokine production. Cytokines are important for both priming non-immune cells to induce an anti-viral or anti-bacterial state (Miyajima et al., 1992), and also in polarising T-cell differentiation lineages (Chen and Flies, 2013). Furthermore, cytokines can recruit other immune cells to the site of infection (Vieira et al., 2009). *Campylobacter jejuni* can use flagellin to target DC immunoreceptors and induce IL-10 production that may suppress the host immune system and lead to bacterial persistence (Stephenson et al., 2014). *Helicobacter pylori* can similarly induce TGF-β

secretion in DC to effect a tolerogenic microenvironment dominated by T_{REG} differentiation (Kao et al., 2010). Within the Neisseria genus, *N. gonorrhoeae* can skew T-cell differentiation from a protective T_H1/T_H2 balance to a pathogenic and inflammatory T_H17 response by inducing co-production of IL-6 and TGF-β in myeloid DC (Liu and Russell, 2011; Liu et al., 2012).

The second technique pathogens employ to evade the immune system via DC is modulation of expression of maturation markers. As previously discussed, maturation markers are involved in antigen presentation and the co-stimulation of T-cells. Arguably, the expression of DC maturation markers ('signal 1' and 'signal 2') is more important for the initiation of adaptive immune responses than cytokine production ('signal 3'). Mature DC co-cultured with naive T-cells in the presence of neutralising antibodies against IL-12 can still drive strong T_H1 responses (Rissoan et al., 1999), and pharmacologically modified DC expressing low levels of CD86 yet producing high levels of IL-12 are still biased towards inducing a T_H2 response, which is reversed upon CD86 up-regulation (Turnquist et al., 2010). Indeed, the expression of certain DC maturation markers alone is sufficient to generate T_H1 responses independently of IL-12 (Soares et al., 2007). Patients with IL-12 deficiency can still generate protective immunity to intracellular pathogens (Fieschi et al., 2003), and IL-12-KO mice develop protective T_H1 responses against many bacteria (Jankovic et al., 2002). Therefore, the surface phenotype of mature DC may be sufficient for the initiation of effective cellular immune responses, but the cytokine signal is still an important enhancement factor towards cellular polarisation.

Pathogens can suppress the up-regulation of maturation markers on DC by a variety of mechanisms (Khan et al., 2012). *Mycobacterium tuberculosis* cell wall components target DC-SIGN to suppress DC maturation by active interference with the TLR pathway; thus, infected DC have low levels of CD40, CD80 and CD86 (Geijtenbeek et al., 2003). Similarly, *Enterobacter sakazakii* targets DC-SIGN via surface-expressed OmpA protein to inhibit MAPK activation and thus prevent DC maturation (Mittal et al., 2009). For many bacteria, the mechanism behind the inhibition of DC maturation is mechanistically unresolved, such as the interaction between *Haemophilus ducreyi* and human myeloid DC (Banks et al., 2007).

Generally, viable bacteria have a higher availability of native antigens, and are therefore better able to mature DC. For example, viable *Salmonella enterica* activates DC very rapidly, but prolonged exposure to heat-killed bacteria is required for the same level of maturation (Kalupahana et al., 2005). Similarly, viable *E. coli* is slightly better than heat-killed bacteria at inducing DC maturation *in vivo* (Trez et al., 2005). However, for those bacteria that possess immune evasion mechanisms, the inhibition of DC maturation may or may not depend on bacterial viability. For instance, the LPS of *Coxiella burnetti* blocks TLR signalling and therefore does not induce DC maturation. This occurs whether the bacterium is viable or inactivated through heat-killing (Shannon et al., 2005). By contrast, viability is essential for the suppression of DC maturation by *Yersinia enterocolitica*, which depends on active transcription of genes within its virulence plasmid to inhibit maturation marker up-regulation within the host cell (Erfurth et al., 2004).

N. meningitidis and its virulence factors are potent inducers of human DC maturation. Meningococcal PorA protein alone can induce up-regulation of HLA-DR, CD40, CD80 and CD86. Furthermore, PorA alone can also induce IL-8 and modest IL-1β production (Al-Bader et al., 2004b). Meningococcal LOS is sufficient for the up-regulation of DC maturation markers and the secretion of pro-inflammatory cytokines (Kolb-Maurer et al., 2001), and similar results have been found in macrophages by using LOS-deficient mutant strains (Pridmore et al., 2001). Notably, the presence of LOS in the meningococcal cell membrane leads to higher levels of phagocytosis (Dixon et al., 2001) which elicits optimal IL-12 production in human DC (Uronen-Hansson et al., 2004a). Consistent with these observations, capsule-deficient mutants of *N. meningitidis* provoke higher amounts of inflammatory cytokine production due to enhanced levels of phagocytosis (Kolb-Maurer et al., 2001). Given the robust maturation of DC by meningococci and its constitutive components, DC exposed to *N. meningitidis* and co-cultured with naive $CD4^+$ T-cells leads to strong T_H1 and T_H2 immune responses (Steeghs et al., 2006). Moreover, *N. meningitidis* can drive DC-mediated T_H17 polarisation in memory T-cells through potent production of IL-23 via intracellular NOD2 (van Beelen et al., 2007).

While these studies have been conducted entirely on inactivated bacteria, it has been since shown that viable and killed *N. meningitidis* serogroup B (NmB) are differentially recognised by human DC (Jones et al., 2007). DC exposed to viable NmB had dampened levels of maturation marker up-regulation compared to those exposed to killed bacteria. This was found to be

a contact-dependent mechanism and was independent of the method of bacterial inactivation. Importantly, the effect of live NmB was dominant over killed bacteria, underscoring the importance of this interaction for human immunity. Since large quantities of viable and killed bacteria can be recovered from the bodily fluids of patients with meningococcal septicaemia (Øvstebø et al., 2004), this observation is particularly pertinent. This could therefore represent a novel mechanism of immune evasion by the meningococcus, i.e. an evolutionary strategy employed by the bacterium to suppress effective immunity in the mucosal tissue. Since NmB is an obligate coloniser of the human host, with no non-human animal or environmental reservoir, immune evasion is an essential requirement for carriage in the nasopharynx (Lo et al., 2009). The aim of this thesis therefore was to further characterise this interaction between live NmB and DC, starting with an investigation into the basic interaction between these cells.

3.2 Aims and Objectives

The aim of the experiments in this chapter was to elucidate the basic interaction between NmB and DC. Based upon previous work in the laboratory, it was hypothesised that viable and killed NmB would exert differential effects on DC maturation. To investigate this, DC were stimulated under various conditions and tested for multiple aspects of activation by assessing all of the signals required to induce effective T-cell responses.

3.3 Results

3.3.1 DC are activated upon stimulation with LPS

It was first determined if DC differentiated from peripheral blood monocytes could respond to LPS. DC express high levels of TLR4 (Uronen-Hansson et al., 2004b), and it has been demonstrated that LPS can activate DC *in vitro* in numerous studies. Importantly, this would establish a useful positive control for DC maturation for the rest of the study. TLR4 contains TRIF and MyD88 adaptors, and therefore a TLR4 agonist is an ideal control for full DC activation. LPS is a heterogeneous class of molecules, and some forms of LPS are much more immunogenic than others (Attridge et al., 2009). With this consideration, LPS derived from *E. coli* strain 0111:B4 was chosen for its immunogenic potency.

DC were stimulated for 24h with tenfold incremental concentrations of LPS to test the threshold of activation. Flow cytometry was then used to measure CD40 and CD86 up-regulation, which are markers of DC maturation[1]. As Figure 3-1 shows, it was found that even low doses of LPS (0.01 µg/mL) were able to induce up-regulation of CD40 (unstimulated MFI: 73.1, LPS stimulated MFI: 131) and CD86 (unstimulated MFI: 118, LPS stimulated MFI: 818). Similar results were obtained after stimulation with up to 10 µg/mL LPS.

[1] The colour key *grey: unstimulated, green: LPS*, *blue: killed NmB* and *red: live NmB* is used throughout the entire study.

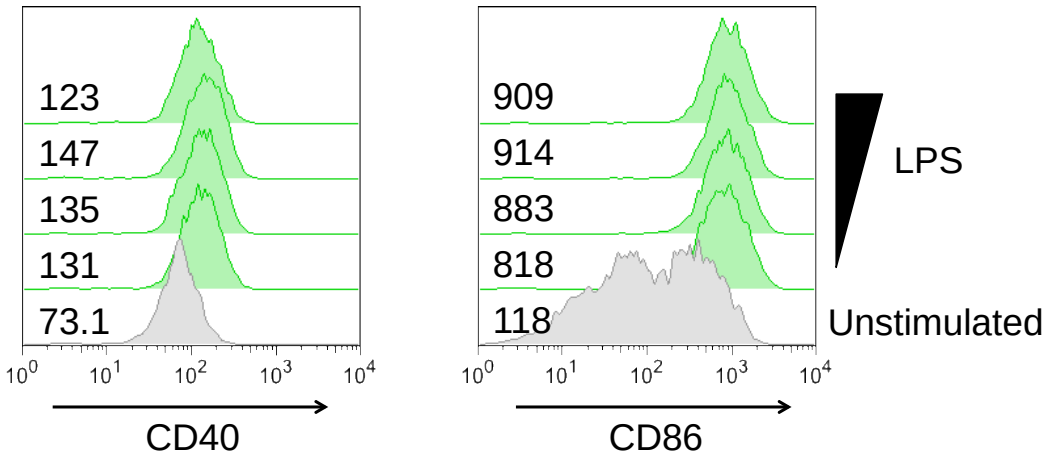

Figure 3-1 *DC are matured by culture with the TLR4 agonist LPS.* DC were stimulated for 24h with increasing concentrations of LPS derived from *E. coli* (from lowest to highest: 0.01, 0.1, 1 and 10 µg/mL), as described in the Materials and Methods. Stimulated DC are shown in green histograms, and unstimulated DC are in grey histograms. CD40 and CD86 expression were then measured by flow cytometry, gating on viable cells determined by FSC-SSC. MFI values are displayed on the histogram. Data are representative of two experiments.

To test pro-inflammatory cytokine production in response to LPS, DC were stimulated for 24h and IL-1β was measured by intracellular flow cytometry, as described in the Materials and Methods. As Figure 3-2 shows, like maturation marker expression, even small doses of LPS (0.01 μg/mL) induced cytokine production (2.55 %), and this reached near maximum (7.76 %) at 0.1 μg/mL. Interestingly, IL-1β production was reduced upon very high LPS stimulation, likely due to homeostatic negative regulation. Hence, DC easily reached maximum or near-maximum phenotypic maturation even with low doses of LPS. Based on these data and cost effectiveness, it was decided that 100 ng/mL (0.1 μg/mL) LPS would be sufficient to induce DC maturation, and this was used as a positive control for all of the following experiments in the study.

3.3.2 DC are activated by killed NmB but not live NmB

As previously discussed, several studies have shown that killed NmB can induce DC maturation (Al-Bader et al., 2004b; Dixon et al., 2001; Kolb-Maurer et al., 2001; Steeghs et al., 2006; Uronen-Hansson et al., 2004a). Therefore, to reproduce these findings, and also to test cell responsiveness to meningococci in the present system, DC were stimulated with a range of doses (MOI 1, MOI 10 and MOI 100) of PFA-fixed bacteria, as described in the Materials and Methods. As Figure 3-3 shows, DC up-regulated co-stimulatory molecules (CD40, CD86) and antigen presenting molecules (HLA Class I, HLA-DR) in response to even low doses (MOI 1) of killed NmB. And so similarly to LPS, killed NmB effectively induced DC maturation.

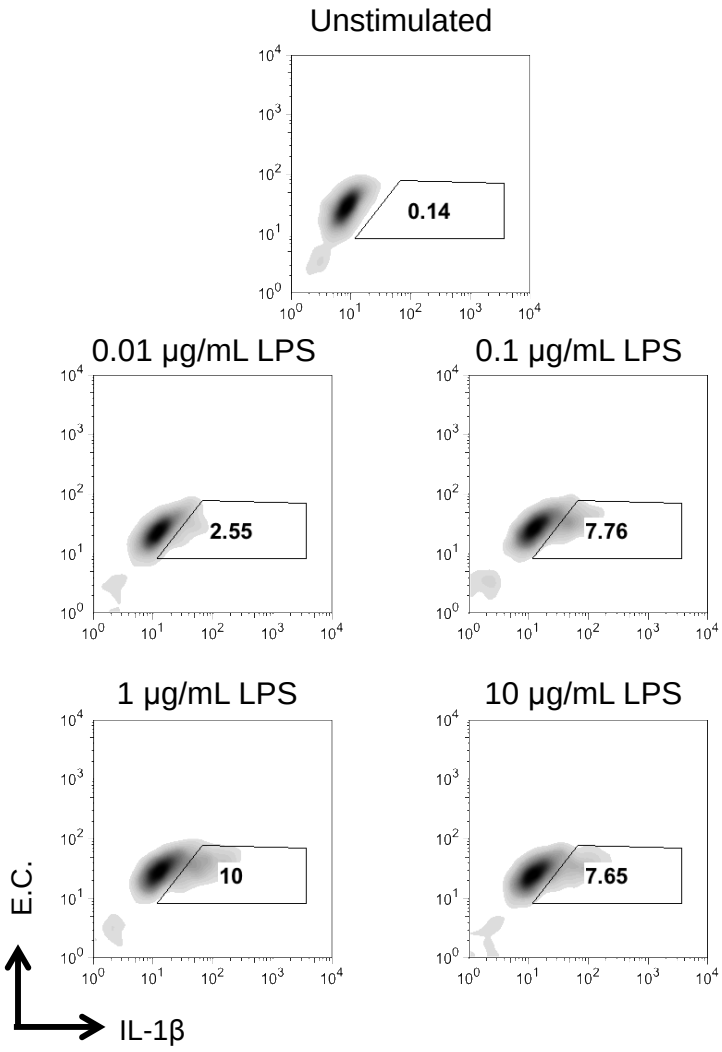

Figure 3-2 ***DC produce inflammatory cytokine IL-1β in response to LPS stimulation.*** **DC were stimulated for 24h with increasing concentrations of** ***E. coli*** **LPS (0.01, 0.1, 1 and 10 µg/mL). DC were then fixed and peameabilised and intracellular IL-1β was measured by intracellular flow cytometry, as described in the Materials and Methods. Gating was determined by an unstained control. E.C. = empty fluorescence channel. Data are representative of two experiments.**

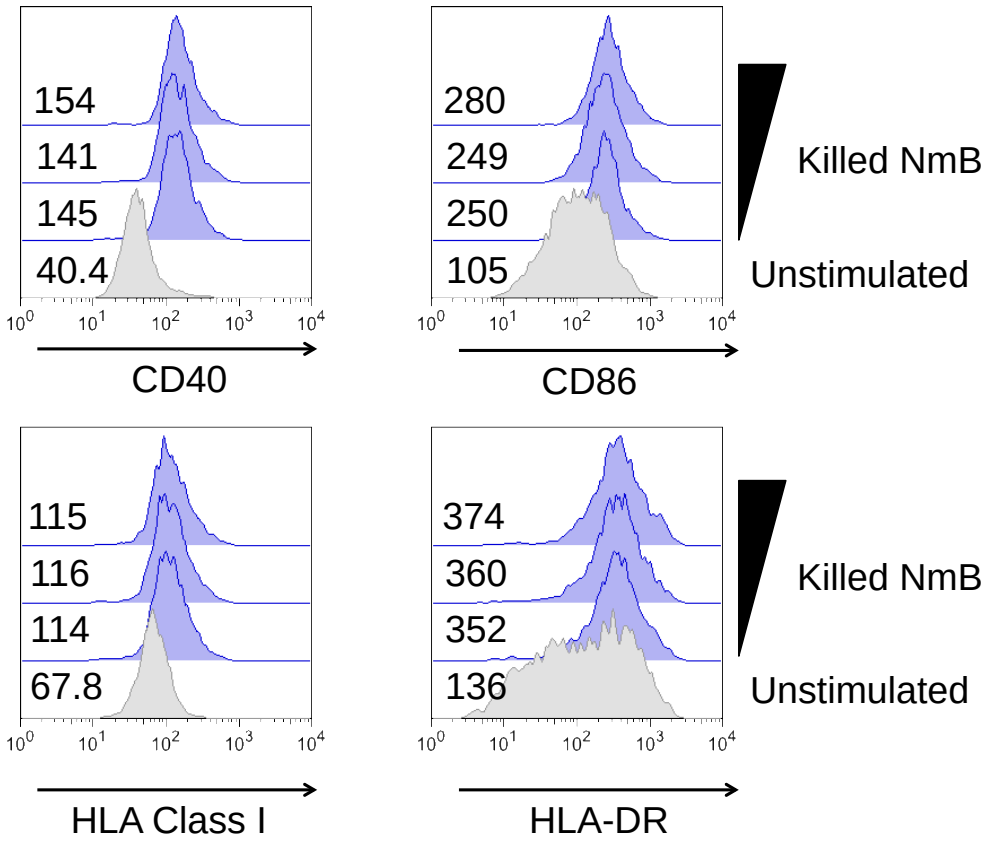

Figure 3-3 *Killed meningococci mature DC independetly of MOI*. DC were stimulated for 24h with an MOI of 1, 10 or 100 (lowest to highest) PFA-fixed NmB, and DC surface expression of CD40, CD86, HLA Class I and HLA-DR (DC maturation markers) were measured by flow cytometry. MFI values are displayed on the histogram. Data are representative of three experiments.

Next, DC were tested for their ability to mature after infection with live NmB. A previous study by Jones et al. has shown that DC fail to mature in response to viable forms of the bacteria (Jones et al., 2007). As Figure 3-4 shows, unlike killed NmB, live bacteria failed to induce DC maturation at a range of MOIs. Even at MOI 100, there was no up-regulation of surface maturation markers (e.g. unstimulated DC CD40 MFI: 40.4, live NmB-infected DC CD40 MFI: 39.4). Furthermore, as Figure 3-5 shows, a time-course analysis of HLA Class I, CD40 and CD86 expression showed a persistent lack of maturation marker up-regulation, which also excluded differential kinetics as a reason for phenotype induced by live NmB. Since MOI 10 seemed to give the best activation of some maturation markers, all further experiments were performed with this dose unless otherwise stated.

Using CD40 and CD86 as indicators of maturation marker up-regulation, the ability of DC to respond to LPS, killed NmB and live NmB was tested in multiple donors (Figure 3-6), and there was a significant difference between killed NmB and live NmB in the up-regulation of both CD40 and CD86 (p = <0.001). Killed NmB induced a mean average 4.8-fold increase in CD40 MFI, whereas live NmB induced only a 1.8-fold increase in CD40. Notably, in some donors there was a trend of minor up-regulation of maturation markers in response to live NmB. Importantly, control markers such as DC-SIGN were unaffected, meaning that the suppression of DC maturation was unlikely to be a general disruption of the DC cell membrane (data not shown). Therefore, live NmB consistently failed to induce maturation of DC.

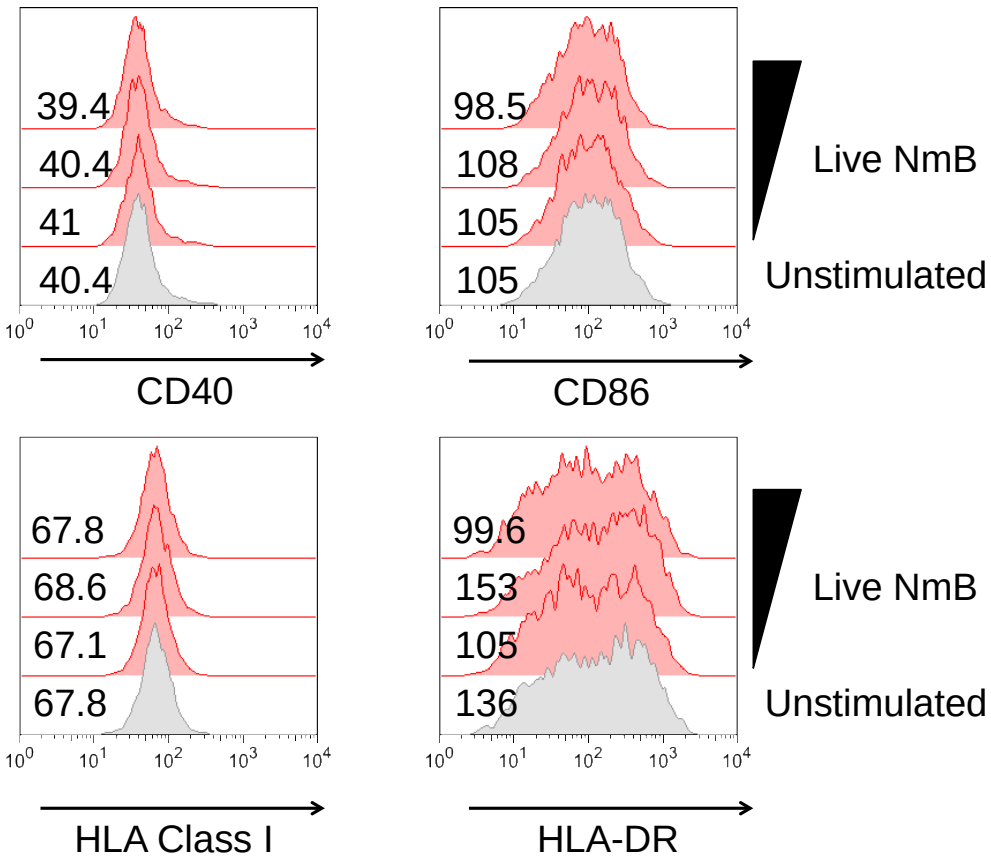

Figure 3-4 *Live meningococci fail to mature DC independently of MOI*. DC were stimulated for 24h with an MOI of 1, 10 or 100 (lowest to highest) live NmB, and surface expression of CD40, CD86, HLA Class I and HLA-DR were measured by flow cytometry. MFI values are displayed on the histogram. Data are representative of three experiments.

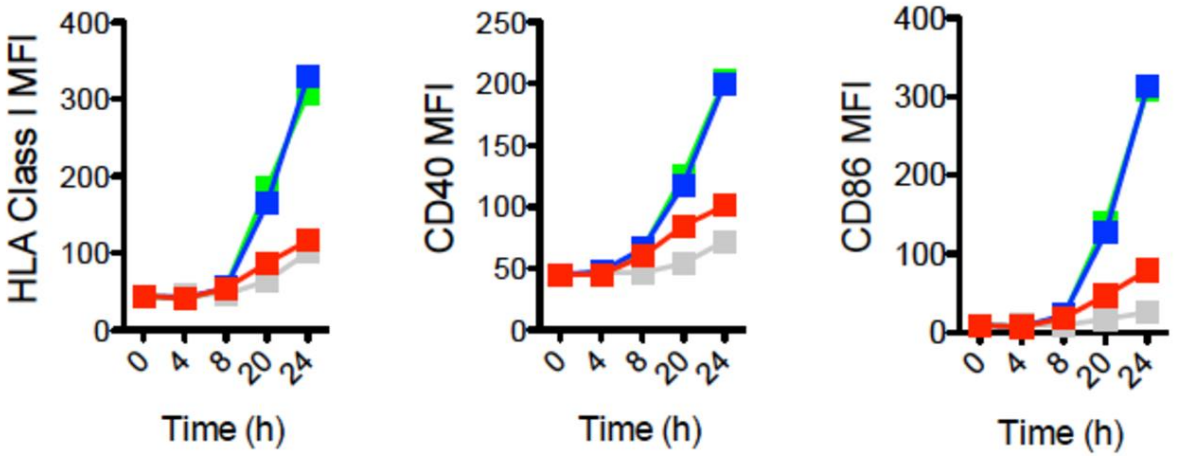

Figure 3-5 Figure 3-6 ***The failure of live NmB to induce DC maturation is independent of kinetics*****. DC were stimulated for the indicated times (0-24h) with 100 ng/mL LPS (green), 10 MOI killed (blue) or 10 MOI live (red) NmB. Some DC were left unstimulated (grey). Surface expression of HLA Class I, CD40 and CD86 was then assessed by flow cytometry. Data are representative of two experiments.**

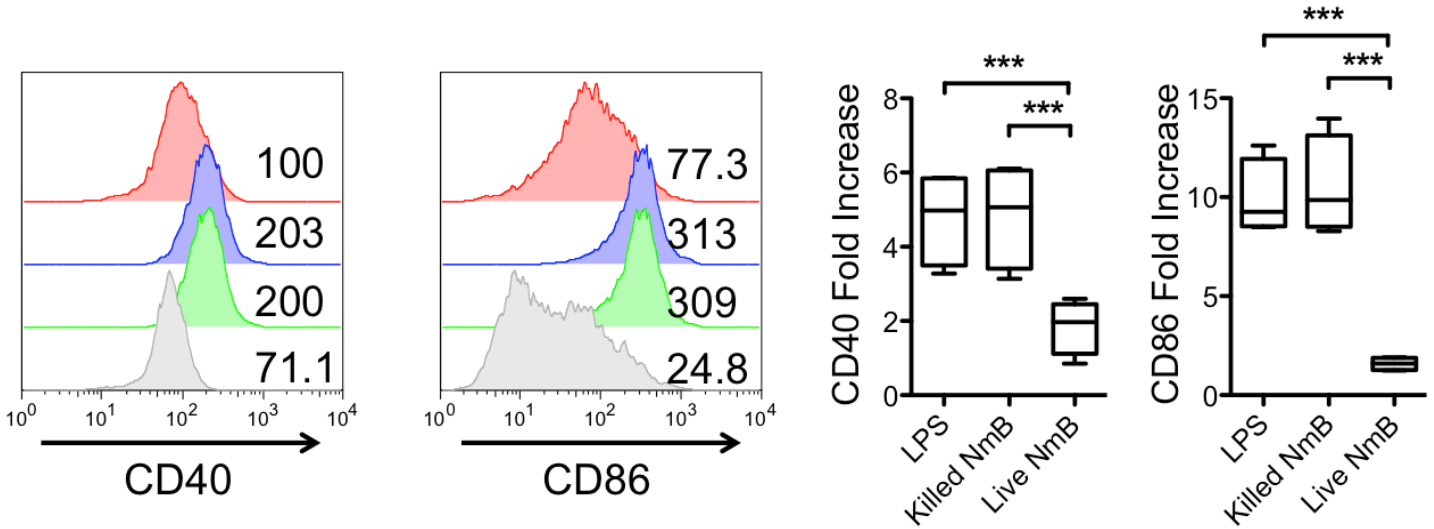

Figure 3-6 *Live NmB fails to induce DC maturation in multiple donors*. DC were stimulated for 24h with 100 ng/mL LPS, 10 MOI killed or 10 MOI live NmB for 24h and then assessed for surface expression of CD40 and CD86 by flow cytometry. *Left:* representative histogram showing CD40 and CD86 expression (Grey: unstimulated, green: LPS, blue: killed NmB, red: live NmB). MFI values are displayed on the histogram. *Right*: Pooled data from four individual donors showing the fold change in MFI compared to the unstimulated control. Box-plots depict medians and interquartile ranges. Significance was tested by one-way ANOVA with a Bonferroni post-test. **P*<0.05, ***P*<0.01, ****P*<0.001

3.3.3 Differential effects of live NmB on mouse and human DC

Mouse and human APC possess significant differences, such as the expression of different immunological receptors (Mestas and Hughes, 2004). For example, murine B-cells express TLR4 and will mitogenically respond to LPS, while B-cells do not express TLR4 and are largely inert when exposed to even high concentrations. Furthermore, mouse and human TLR4 differentially recognise meningococcal LOS (Steeghs et al., 2008), and mouse DC lack critical human pathogen receptors such as DC-SIGN. Given these differences, it was questioned whether murine bone marrow derived myeloid DC would display the same phenotype when exposed to viable meningococci. Mouse DC were cultured with killed and viable NmB for 24h at a range of MOIs, and surface expression of CD40, CD80, CD86 and MHC Class II were assessed by flow cytometry (Figures 3-7 and 3-8). Interestingly, for most surface maturation markers, viable NmB induced greater up-regulation of these molecules than killed bacteria (10 MOI killed NmB CD86 MFI: 14.7 vs 10 MOI live NmB CD86 MFI: 25.8). There was a slightly lower amount of CD40 up-regulation in DC stimulated with live NmB, however this was only marginal, and there was approximately 4-fold up-regulation of CD40 MFI induced by all MOIs of live NmB compared to the control. These data emphasize the fundamental differences in the ability of murine and human DC to respond to pathogens adapted to the human mucosa — and therefore human DC were subsequently used for the rest of the study.

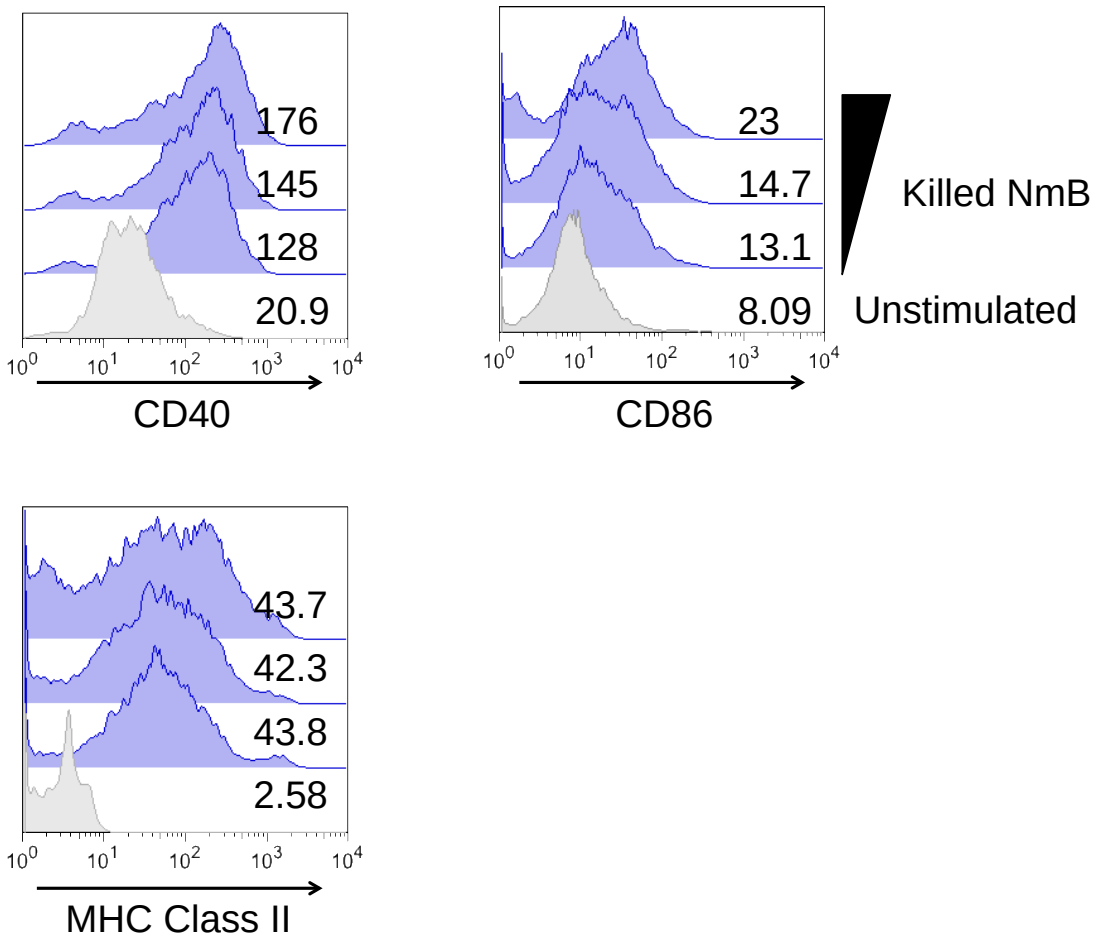

Figure 3-7 *Killed meningococci mature murine DC.* Murine bone marrow derived DC (BM-DC) were stimulated for 24h with an MOI of 1, 10 or 100 (lowest to highest) PFA-fixed killed NmB (blue histograms), and surface expression of CD40, CD86 and MHC Class II were measured by flow cytometry. Unstimulated control DC are shown in grey histograms. MFI values are displayed on the histogram. Data are representative of three experiments.

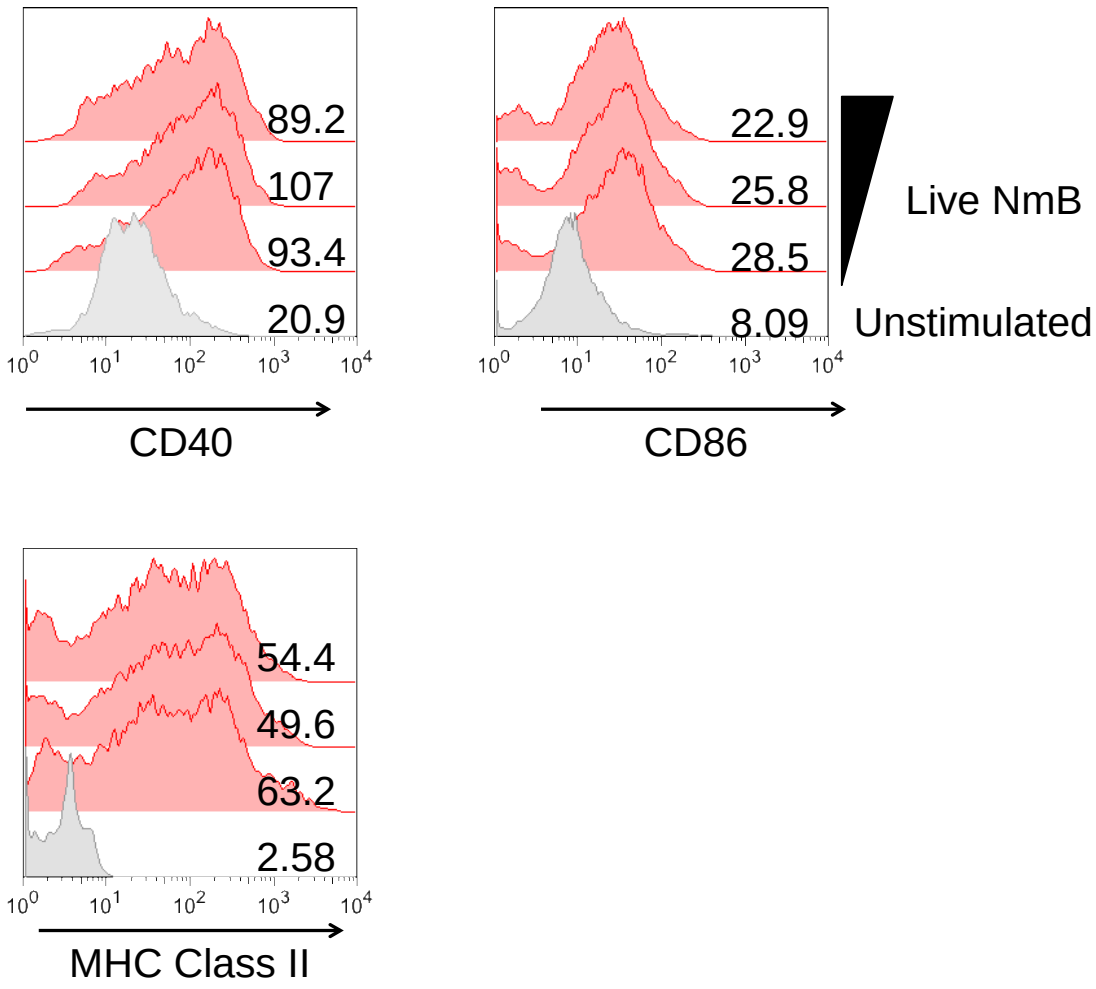

Figure 3-8 *Live meningococci mature murine DC.* Murine bone marrow derived DC (BM-DC) were stimulated for 24h with an MOI of 1, 10 or 100 (lowest to highest) viable NmB (red histograms), and surface expression of CD40, CD86 and MHC Class II were measured by flow cytometry. Unstimulated control DC are shown in grey histograms. MFI values are displayed on the histogram. Data are representative of three experiments.

3.3.4 DC are activated by other viable bacterial pathogens

The possibility that other viable pathogenic or commensal bacteria would activate DC was investigated by using a Gram-positive pathogen (*S. pneumoniae*), Gram-negative pathogen (*C. jejuni*) or Gram-positive commensal (*Lactobacilli* reuteri) control. *C. jejuni* purportedly induces DC maturation whether viable or killed (Hu et al., 2006), and live *S. pneumoniae* (Noske et al., 2009) also induces DC maturation. Similarly, the commensal *L.* reuteri induces DC activation whether heat-killed or viable (Mohamadzadeh et al., 2005). The testing of these bacteria in the same infection protocol used within this study would therefore validate the results to a greater degree.

DC were stimulated with LPS, killed bacteria or live bacteria and then surface expression of CD40 and CD86 was measured by flow cytometry. Unlike NmB, viable *C. jejuni*, *S. pneumoniae* and *L.* reuteri all induced up-regulation of CD40 and CD86 comparable to killed bacteria. For instance, *C. jejuni* induced 270 CD86 MFI when killed but 322 CD86 MFI when viable, whereas *L.* reuteri induced 106 CD86 MFI when killed but 188 CD86 MFI when viable. *S. pneumoniae* was generally weaker at inducing DC maturation, likely due to the lack of endotoxin. These results suggested that the inhibition of maturation marker up-regulation by live NmB was unlikely to be an epiphenomenon of live bacterial infection *per se*—since these results resound with previously published work by other laboratories—but was rather a specific effect of live NmB.

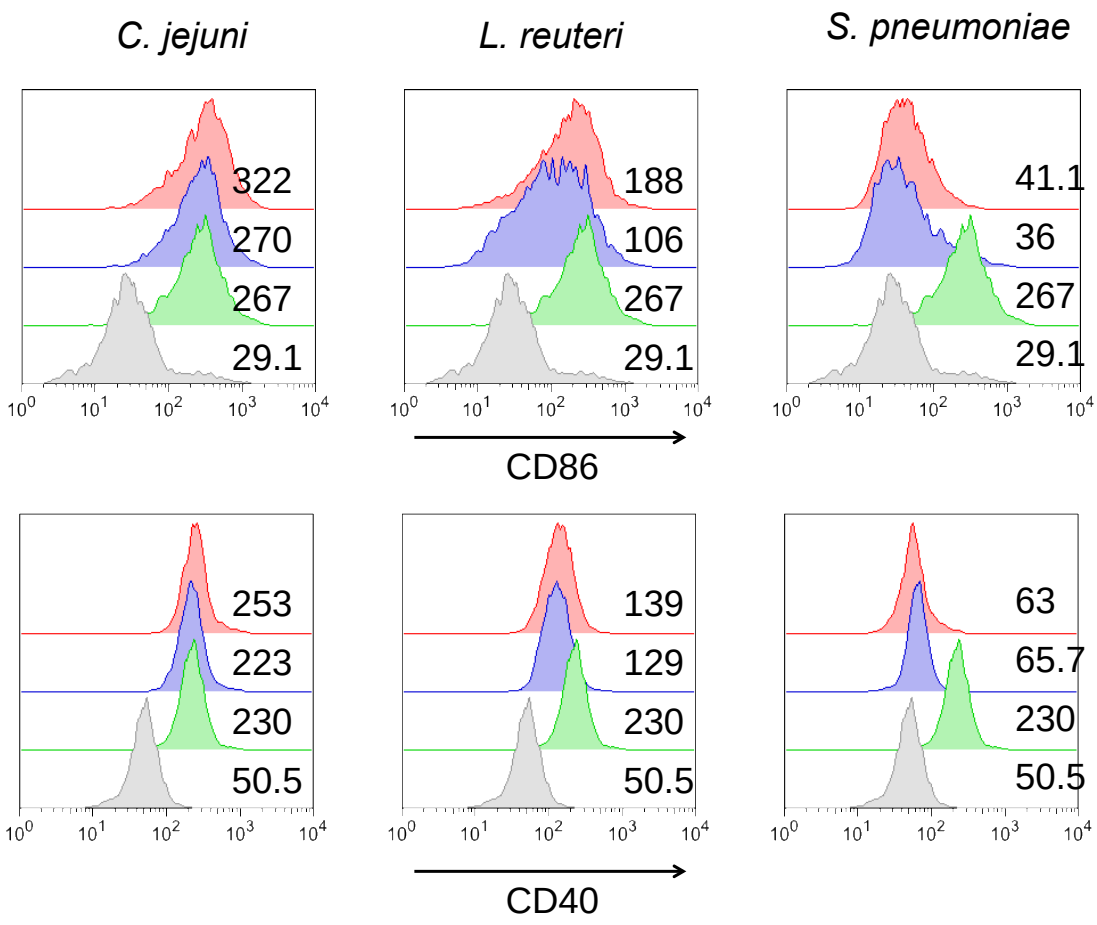

Figure 3-9 *DC are matured by many viable bacterial species*. DC were stimulated with 100 ng/mL LPS (green), 10 MOI live (red) bacteria, 10 MOI killed (blue) bacteria or left unstimulated (grey) for 24h. Bacterial strains used were *C. jejuni* strain 11168H, *L. reuteri* strain DSM12246 and *S. pneumoniae* strain ATCC 11733. Bacteria were killed by the same method as NmB (see Materials and Methods). Surface expression of CD40 and CD86 was assessed by flow cytometry. MFI values are displayed on the histograms. Data are representative of two experiments.

3.3.5 Suppression of DC maturation by live NmB is dominant over exogenous stimuli

The data reported so far were unclear as to whether the failure of live NmB infection to mature DC was due to a lack of signal or an active suppression. Hence, DC were stimulated for 24h with the TLR ligands peptidoglycan (PGN) (TL2/6), poly(I:C) (TLR3) and LPS (TLR4). This was to determine whether the effect of live NmB would be dominant over bacterial or viral ligands. As Figure 3-10 shows, when DC were cultured alone with these stimuli, each induced strong up-regulation of CD86 (peptidoglycan CD86 MFI: 124; LPS CD86 MFI: 365; poly(I:C) MFI: 365). However, when these ligands were used in conjunction with live NmB infection, the up-regulation of this marker was lost (e.g. live NmB + LPS CD86 MFI: 59). It can be concluded from these results that live NmB actively suppressed DC phenotypic maturation in the context of different TLR signals.

3.3.6 Viable NmB selectively up-regulates the T-cell inhibitory molecule PD-L1

DC also up-regulate negative T-cell co-stimulatory molecules in response to certain microbes. Some pathogens, such as HIV-1, *S. typhimurium* and *M. tuberculosis* can induce the expression of these molecules on phagocytes (Newland et al., 2011; Rodríguez-García et al., 2011; Schreiber et al., 2010).

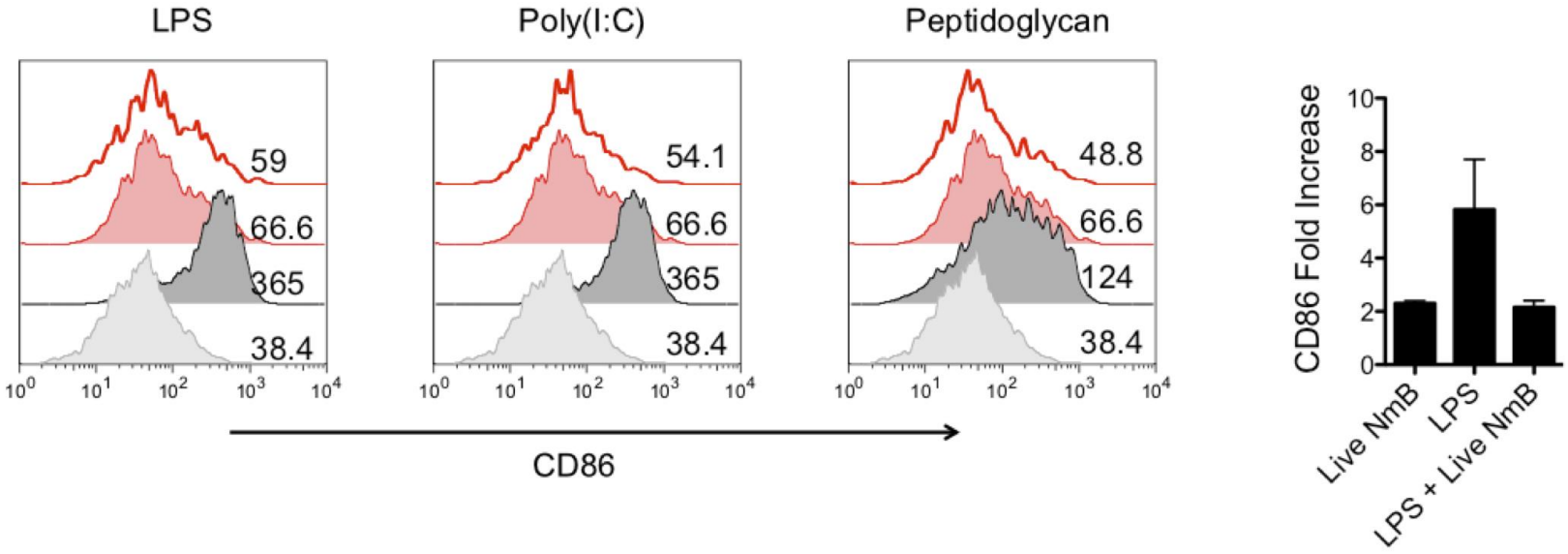

Figure 3-10 L*ive NmB blocks the immunostimulatory activity of TLR ligands*. *Left:* DC were stimulated for 24h in with 100 ng/mL LPS, Poly(I:C) (10 µg/mL) or peptidoglycan (1 µg/mL) and surface expression of CD86 was determined by flow cytometry. Grey: unstimulated cells, black: TLR ligand alone, red filled: live NmB, red open: live NmB + TLR ligand. MFI values are displayed on the histogram. Data are representative of three experiments. *Right:* Pooled data from 3 donors showing 24h post-stimulation CD86 expression in response to various stimuli. Graphs depict mean averages ± SEM.

However, this has not been shown for *N. meningitidis*. PD-L1 and PD-L2 can bind to T-cell expressed PD-1 and induce cellular anergy. Therefore, It was questioned whether NmB could induce the inhibitory co-stimulatory molecules PD-L1 and PD-L2. DC were stimulated for 24h with LPS, killed or live NmB and then surface expression of PD-L1 and PD-L2 was measured by flow cytometry (Figure 3-11). Strikingly, in contrast to CD40 and CD86 expression, PD-L1 was fully up-regulated by live NmB (LPS MFI fold up-regulation: 7.0 vs live NmB MFI fold up-regulation: 7.3). There was no significant difference between PD-L1 expression between DC stimulated with LPS, killed or live NmB. In contrast to PD-L1, PD-L2 failed to fully up-regulate after DC infection with live NmB. Indeed, there was a significant difference between PD-L2 expression induced by LPS, killed NmB and live NmB (p = <0.05). A ratio comparison of CD86:PD-L1 MFIs revealed that live NmB selectively up-regulated PD-L1 (p = <0.01), when compared to killed NmB.

3.3.7 DC infected with live NmB have decoupled cytokine responses

The up-regulation of DC maturation markers—antigen presenting molecules and co-stimulatory molecules—represents two ('Signal 1' and 'Signal 2') facets of full DC activation. To be considered *bona fide* mature cells, DC must also secrete cytokines (Signal '3'). Therefore, DC were tested for their ability to secrete the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. Previous reports claim that killed NmB induces high amounts of pro-inflammatory cytokine release from human DC (Kolb-Maurer et al., 2001).

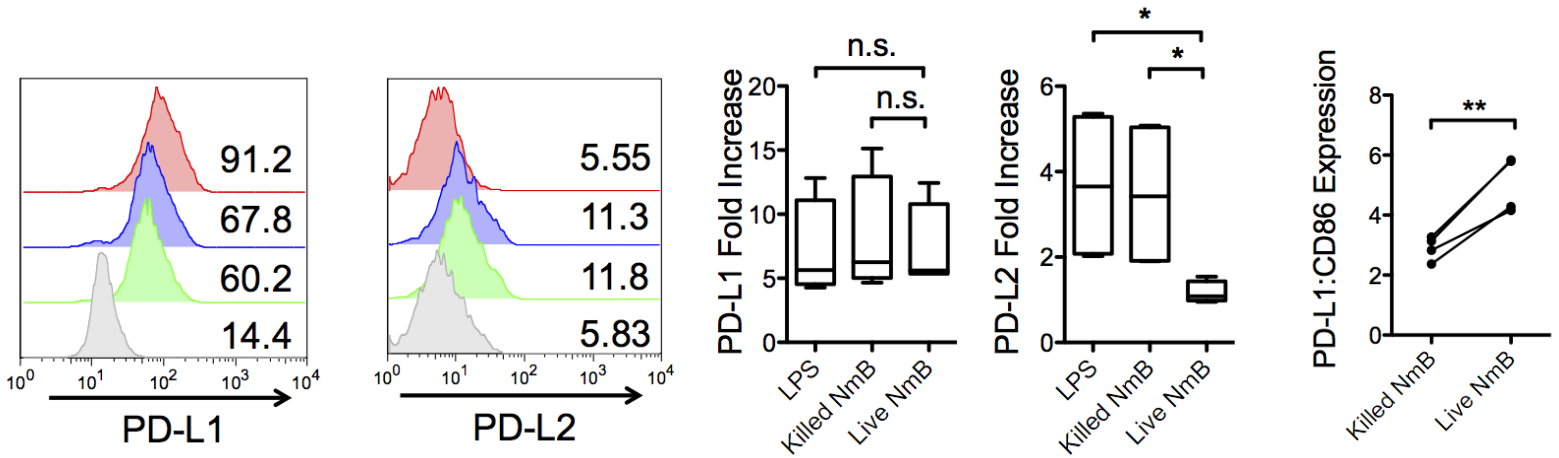

Figure 3-11 ***Live NmB induces the selective up-regulation of PD-L1.*** **DC were stimulated for 24h by 100 ng/mL LPS (green), 10 MOI killed (blue) or 10 MOI live (red) NmB, or left unstimulated (grey).** ***Left:*** **Representative donor showing surface expression of PD-L1 and PD-L2. MFI values are displayed on the histograms.** ***Middle:*** **Pooled data from 4 individual donors. Box-plots depict medians and interquartile ranges. Significance was tested by one-way ANOVA with a Bonferroni post-test.** ***Right:*** **Calculation of PD-L1:CD86 MFI ratio by the formula (PD-L1 MFI Fold Change/CD86 MFI Fold Change) in four individual, matched donors as indicated by lines. Significance was tested by a paired t-test. *P <0.05, **P <0.01, ***P <0.001 ***P <0.001.**

To interrogate this immunological function, DC were stimulated for 24h with either LPS, killed or live NmB and then secreted cytokine was measured in the cell culture supernatants by ELISA (Figure 3-13). In contrast to the surface phenotype data, live NmB was able to induce higher levels of pro-inflammatory cytokine secretion than both LPS and killed NmB (p = <0.01). This was most true for IL-1β (killed NmB: mean 575.4 pg/mL; live NmB: mean 20,535 pg/mL) and TNF-α (killed NmB: mean 73.9 ng/mL; live NmB: mean 299.9 ng/mL). Consequently, live NmB decoupled maturation marker expression and cytokine production.

3.3.8 Live NmB induces low chemokine secretion

DC also secrete chemokines in response to pathogens. These allow the DC to recruit other cells, such as neutrophils and monocytes, to the site of infection. Since infection with live NmB resulted in high-level production of cytokines, it was expected that this would also result in a similar trend with regards to chemokine production. DC were stimulated for 24h with LPS, killed or live NmB and supernatants were assessed for levels of MCP-1 and MIP-3α (Figure 3-13). Surprisingly, it was found that live NmB induced significantly less (p = <0.05) MCP-1 than the positive control LPS, and a similar trend compared to killed NmB. Similarly, there was a non-significant trend of lower MCP-1 levels in supernatants from infection with live infection compared to killed NmB, although these were similar between LPS and live NmB. Therefore, in contrast to pro-inflammatory cytokines, chemokine secretion was not enhanced by live NmB.

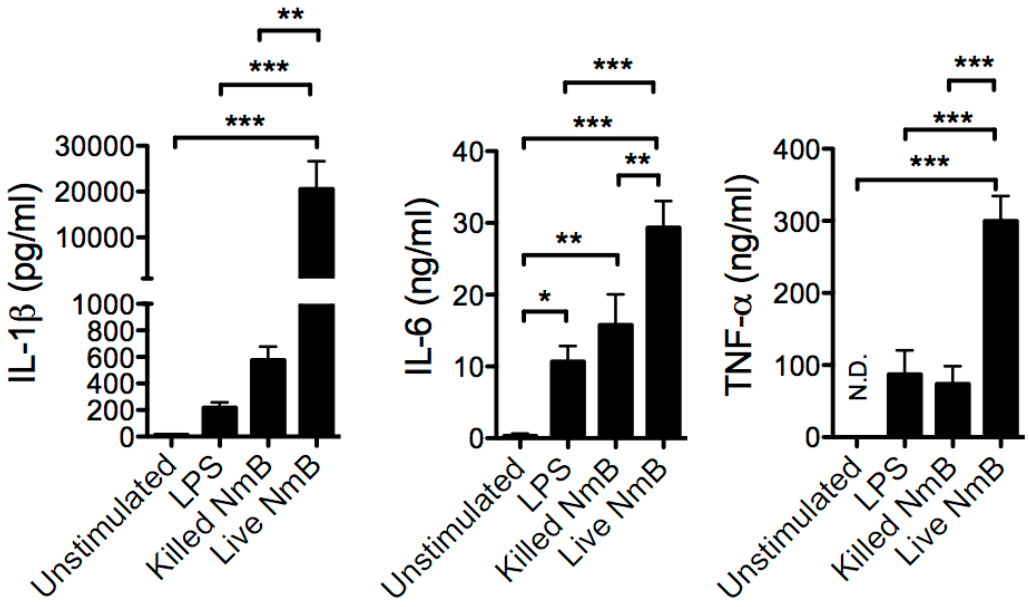

Figure 3-12 ***Live NmB induces enhanced pro-inflammatory cytokine release from DC compared to LPS and killed NmB*****. DC from 4-7 donors were for 24h with 100 ng/mL LPS, 10 MOI kiled NmB or 10 MOI live NmB, and some were left unstimulated. Sterile (0.2 µm filtered) supernatants were collected and analysed for IL-1β , IL-6 and TNF-α protein levels by ELISA. Graphs depict mean averages ± SEM. Significance was tested by one-way ANOVA with a Bonferroni post-test (N.D. = none detected.) *P <0.05, **P <0.01, ***P <0.001**

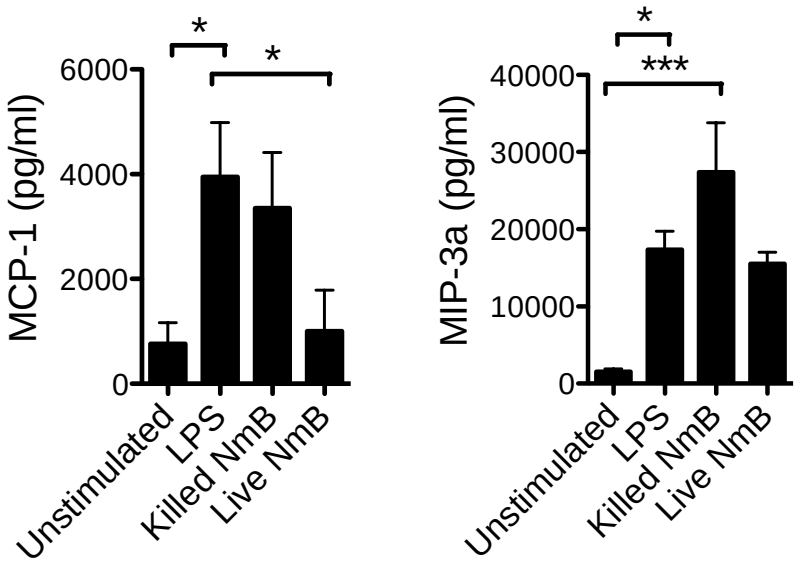

Figure 3-13 *Reduced production of chemokines in DC infected with live NmB*. DC from 3-6 donors were for 24h with 100 ng/mL LPS, 10 MOI kiled NmB or 10 MOI live NmB, and some were left unstimulated. Sterile (0.2 µm filtered) supernatants were collected and analysed for MCP-1 and MIP-3α protein levels by ELISA. Graphs depict mean averages ± SEM. Significance was tested by one-way ANOVA with a Bonferroni post-test. *P <0.05, **P <0.01, ***P <0.001

3.3.9 Dysregulation of DC maturation is transcriptionally controlled

The up-regulation of maturation markers is subject to transcriptional and post-translational regulation. TLR signalling induces transcriptional changes in the DC due to activation of transcription factors such as NF-κB, AP-1 and STAT1. This results in an up-regulation of the transcription of genes such as *CD40*, *CD86*, *IL1B*, *IDO1, IL6* and *TNFA*. Once co-stimulatory and antigen presentation molecules are transcribed and translated, they are also subject to post-translational modifications such as ubiquitination, which crucially determine their fate within the cell. The master regulator of this process is membrane-associated RING-CH1 (MARCH1), which can ubiquitinate molecules such as CD86 and label them for internalisation from the cell surface, which ultimately leads to proteasomal degradation (Corcoran et al., 2011; Tze et al., 2011). Upon DC maturation, *MARCH1* is down-regulated, thus stabilising the CD86 molecule and allowing it to persist at the cell surface. Similar mechanisms involving MARCH1 negative regulation have been found for HLA Class II and other DC maturation markers (De Gassart et al., 2008). Bacteria such as *Francisella tularensis* can subvert this process to prevent the induction of effective immune responses in macrophages (Wilson et al., 2009), and thus it seemed to be a plausible hypothesis that live NmB might be utilising a similar strategy.

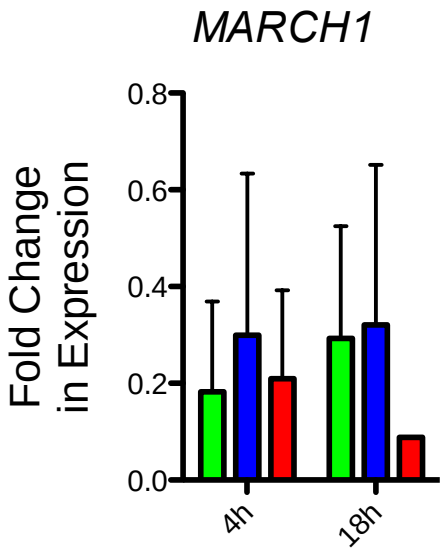

Figure 3-14 ***The post-translational controller of DC maturation*** **MARCH1** ***is down-regulated by live NmB*****. DC were stimulated for 4 or 18h with either 100 ng/mL LPS (green), 10 MOI killed (blue) or 10 MOI live NmB (red). RNA was extracted and transcripts of** ***MARCH1*** **mRNA were measured by RT-PCR relative to a time 0 (T_0) control, as described in the Materials and Methods. Bar graphs depict mean averages ± SEM. Data are from two individual donors.**

To interrogate these processes, DC were stimulated for 4h and 18h with LPS, killed and live NmB and then RNA was extracted as described in the Materials and Methods. Transcripts of the *MARCH1* gene at these time-points were then quantified by RT-PCR (Figure 3-14). As the data show, *MARCH1* was down-regulated by LPS, killed NmB and live NmB at 4h and 18h (fold change in expression = < 0.6 for all conditions), implying normal post-translational control of CD86 and other molecules.

To exclude the possibility of CD86 remaining in intracellular reservoirs, DC were assessed for surface vs. intracellular (intracellular and surface) levels of CD86, using a saponin-based cell permeabilisation method as described in the Materials and Methods (Figure 3-15). The data clearly show that intracellular CD86 was also depleted upon live NmB infection when compared to LPS and killed NmB (intracellular CD86 MFI killed NmB: 23; intracellular CD86 MFI live NmB: 9.67), which corresponded to the surface phenotype. There was an overall slight reduction in CD86 MFI across all conditions in the intracellular stain compared to the surface stain, however this was likely due to the fixation method interfering with the CD86 antibody epitope. Together, these data implicated transcriptional defects as the driver of DC dysregulation.

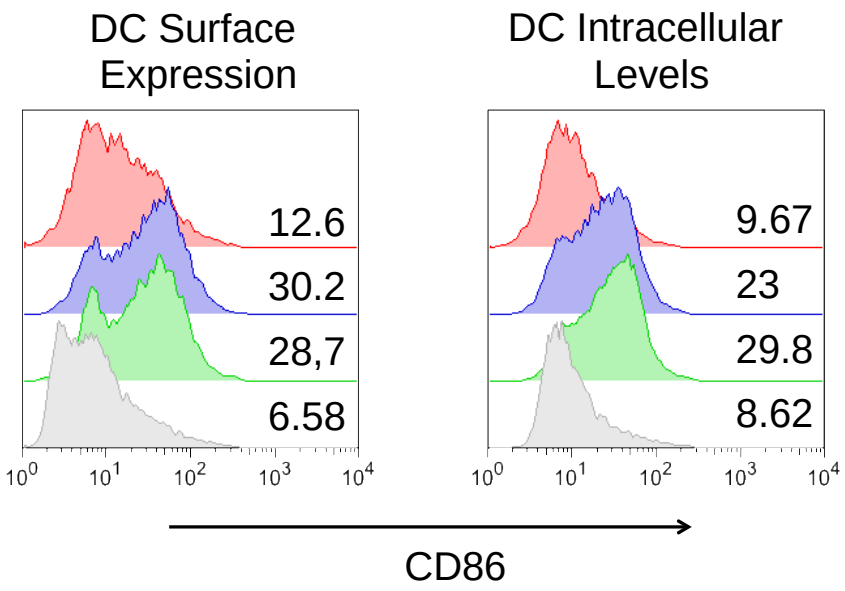

Figure 3-15 ***Upon infection with live NmB, CD86 is not stored in internal reservoirs.*** **DC were stimulated for 24h with either 100 ng/mL LPS (green), 10 MOI killed (blue), 10 MOI live (red) NmB or left unstimulated (grey). CD86 was then measured on the cell surface (left) or within the saponin-permeabilised cell (right) via flow cytometry. MFI values are displayed on the histogram. Data are representative of two experiments.**

Next, *CD86, IL1B, IL6* and *TNFA* transcription were measured 4 or 18h post-stimulation with LPS, killed and live NmB (Figure 3-16). Consistent with the flow cytometry and ELISA data, *CD86* transcription was down-regulated by live NmB at 18h (mRNA fold change <1), whereas pro-inflammatory cytokine transcription was sustained. This was found to be a significant difference between killed and live NmB (p = <0.05), with a non-significant trend for *TNFA*. Taken together, these data strongly supported the theory that live NmB was suppressing DC maturation by a transcriptional defect that was selective to the maturation markers, while cytokine production was retained or enhanced during late-phase maturation.

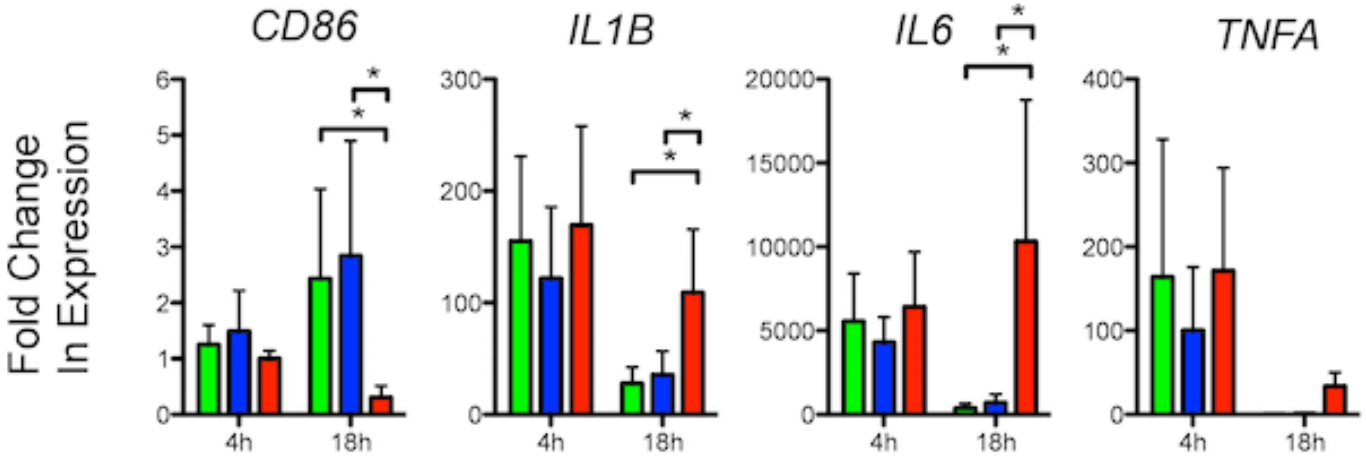

Figure 3-16 ***Live NmB sustains the transcription of pro-inflammatory cytokines while suppressing the transcription of CD86*****. DC were stimulated for 4 or 18h with either 100 ng/mL LPS (green), 10 MOI killed (blue) or 10 MOI live NmB (red). RNA was extracted and transcripts of** ***CD86, IL1B, IL6 and TNFA*** **mRNA were measured by RT-PCR relative to a T_0 control. Bar graphs depict mean averages ± SEM. Data are from three individual donors. Singificance was tested by two-way ANOVA with a Bonferroni post-test. *P <0.05, **P <0.01, ***P <0.001**

3.4 Discussion

In this chapter, the data have shown that live NmB failed to induce full DC maturation. This was indicated by the lack of maturation marker up-regulation upon infection with live bacteria, whereas positive controls induced vigorous DC activation. These results confirm earlier findings in the literature (Dixon et al., 2001; Jones et al., 2007). Additionally, this appeared to be an active suppression of the DC maturation process since the immuno-stimulatory activity of potent TLR ligands was completely blocked by live bacteria. It was also observed that mouse DC were able to respond effectively to viable NmB, as indicated by the up-regulation of antigen presentation and co-stimulatory molecules. This is perhaps unsurprising, given the major differences in human and mouse immunology. Nevertheless, these data do underscore the importance of the unique adaptations of an obligate human pathogen.

Remarkably, DC were still able to produce vast quantities of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. Therefore, it would be more accurate to describe DC maturation in response to live NmB as dysregulated, as opposed to globally inhibited. Furthermore, this is a particularly unusual phenotype, since maturation marker expression and cytokine production are two tightly controlled and co-regulated aspects of DC maturation. Though many bacteria can manipulate DC maturation, the result is usually a complete inhibition of DC functions. For instance, *E. sakazakii* prevents DC maturation via MAPK deactivation and thus the cells cannot up-regulate CD40, CD86 or produce TNF-α (Mittal et al., 2009). Analogously, *Mycobacterium leprae* also suppresses both DC HLA-DR up-regulation and TNF-α production (Murray et

al., 2007). To the author's knowledge, the only pathogen that exerts a similar phenotype to the observations described herein is *H. ducreyi*, and the mechanism behind this interaction is completely unknown (Banks et al., 2007).

The enhancement of pro-inflammatory cytokine secretion would suggest that certain DC signalling pathways remained intact. What remained unclear from these data was how live NmB was able to induce higher amounts of pro-inflammatory cytokine than killed NmB. One possible explanation was that viable bacteria were able to divide in culture, and therefore the higher quantities of cytokine in the supernatant may have merely reflected the higher amounts of bacterial PAMPs. If this were the case, it would still be an intriguing finding: that DC produce any cytokine at all in response to an infectious agent while remaining persistently $CD86^{lo}$ is a rare and unprecedented phenomenon. However, this was an unlikely interpretation for multiple reasons. As the LPS titration experiment showed, DC readily matured with even low doses of antigen, but reached a maximal plateau of activation, which was reflected in the 'capping' of CD86 up-regulation and IL-1β production. Similar findings have been reported elsewhere (Kolb et al., 2014), i.e. that high doses of LPS saturate the DC maturation response, and after a certain threshold, increasing doses fail to induce higher levels of *CD80*, *CD86* or *IL6* transcription. Furthermore, chemokine secretion did not follow the same trend as pro-inflammatory cytokines, as the amounts secreted in response to live NmB were either lower or similar to killed NmB. Hence, these data suggested that the increased cytokine secretion induced by live NmB was not

due to bacterial growth or availability of PAMPs *per se*, but was rather a specific manipulation of DC maturation.

These findings also suggest that the suppression of up-regulation of maturation markers was transcriptionally—as opposed to translationally—controlled. The master CD86 protein regulator MARCH1 was equally down-regulated by live NmB and positive controls. This would probably exclude ubiquitination, degradation and receptor recycling as the cause of low maturation marker expression. Additionally, a time-course experiment showed that CD86 and other markers had persistently low expression. CD86 can accumulate in intracellular reservoirs (Smyth et al., 1998), which would not be detectable by DC surface staining. To exclude the possibility that DC were transcribing *CD86* but that it was not being transported to the cell surface, an intracellular stain was performed, revealing lower amounts of intracellular CD86 in DC infected with live NmB, which was directly comparable to cell surface quantities. In further support of this, an analysis of *CD86, IL1B, IL6* and *TNFA* mRNA transcripts indicated that live NmB suppressed the up-regulation of maturation markers, while failing to down-regulate cytokine production. Collectively, these data implied that the dysregulation of DC maturation was transcriptionally controlled, and was unlikely to be a result of post-translational modifications to the maturation markers.

Not all DC co-stimulatory molecules were suppressed by infection with viable bacteria. PD-L1, but not PD-L2, was up-regulated by live NmB to the same extent as positive controls. This was particularly interesting as PD-L2 is usually co-regulated with CD86 via the IFN-β-STAT1 pathway (Wiesemann et

al., 2008), whereas PD-L1 was found to be MAPK dependent in monocytes (Wölfle et al., 2011). In addition, the chemokines MCP-1 and MIP-3α have STAT1 binding sites in their promoters, and IFN-β or IFN-γ treatment induces transcription of these chemokines (Dai et al., 2008; Li et al., 2010; Pattison et al., 2013; Staab et al., 2013).

There are compelling data in the literature that suggest mouse TRIF-/- DC may be able to mature solely via TLR2-mediated NF-κB activation (Weatherill et al., 2005); and intriguingly, the induction of pro-inflammatory cytokines by live NmB implicated this pathway as fully functional in human DC. Hence, these data supported the hypothesis that a specific NF-κB-independent pathway—likely a component of the IFN-STAT1 signalling axis—was being targeted by live NmB to disrupt human DC maturation. It is this possibility that is explored in the following chapter.

4 Chapter 4: Dendritic Cell Signalling Pathways Induced by *N. meningitidis*

4.1 Introduction

Dendritic cell maturation requires cascades of intracellular signals to produce a fully activated cell (Türeci et al., 2003b). Indeed, the most recent gene arrays have revealed that over 2,000 genes are differentially regulated during this process (Jin et al., 2010). The kinetics of DC maturation are important for assessing maturation, as the various aspects of cellular activation are subject to strict temporal control. For example, transcription of pro-inflammatory genes (e.g. *il12p40*) is a rapid event that can occur only mins after DC stimulation (Langenkamp et al., 2000). On the other hand, morphological and phenotypic changes can continue to occur over 24 hours post-stimulation (Verdijk et al., 2004). Therefore, it is important that DC maturation is assessed at both early and late stages, as it is a relatively extended process.

As previously discussed, DC maturation requires two major signals: early MyD88-driven NF-κB signalling, which primarily induces cytokine transcription, and TRIF-dependent STAT1 signalling that results in an up-regulation of the expression of surface maturation markers (Kolb et al., 2014). There is significant cross talk between these two signalling axes. TLR activation can induce early yet transient STAT1 signalling (Sikorski et al., 2012), and the autocrine IFN axis can also amplify NF-κB activity via STAT1-independent mechanisms (Gil et al., 2001). Discrete activation of unilateral

signalling pathways is therefore unlikely to occur to any stimulus; rather, models involving waves of signal amplification and repression are more biologically representative.

STAT1 can be activated by type I and type II IFNs. IFN-α and IFN-β signal via IFNAR1/IFNAR2, leading to activation of the kinases JAK1 and Tyk2. This leads to phosphorylation of STAT1 and STAT2, which form a STAT1-STAT-2 heterodimer in association with IRF9, and a STAT1-STAT1 homodimer, and then bind to ISRE and GAS (interferon-gamma -activated sites) sites, respectively. This results in the induction of STAT1-dependent genes, such as DC maturation markers. IFN-γ signals via the IFN gamma receptor (IFNγR) by activating JAK1 and JAK2, which induce the phosphorylation of a STAT1 homodimer that activates GAS-dependent transcription (Platanias, 2005). Both IFN-β and IFN-γ autocrine loops have been described for DC maturation; but the ablation of STAT1 is sufficient in itself to prevent up-regulation of DC maturation markers (Johnson and Scott, 2007).

No data exist in the literature concerning the signalling induced by *N. meningitidis* in DC, and surprisingly little is known about the intracellular signalling events induced by the meningococcus in other phagocytes and non-immune cells. It is known that LOS from the bacterium can activate both the MyD88- and TRIF-dependent axes of the TLR4 pathway in monocytes, and that this results in the activation of NF-κB and the production of IFN-β (Liu et al., 2010). This has also been shown in HEK cells, whereby infection with *N. meningitidis* activates NF-κB and also IRF3-mediated IFN-β production (Mogensen et al., 2006a). Similarly, infection of epithelial cells results in

prolonged NF-κB signalling and the release of pro-inflammatory cytokines (Deghmane et al., 2011). The meningococcus is able to activate the MAPKs ERK, p38 and JNK in endothelial cells, leading to enhanced invasion and inflammatory cytokine release (Sokolova et al., 2004). In macrophages, *N. meningitidis* can modulate nitric oxide production (Stevanin et al., 2005), and this can lead to an inhibition of apoptotic signalling pathways (Tunbridge et al., 2006).

Differences in the signalling induced between viable and inactivated *Neisseria* spp. have been noted in several studies. In a HEK cell line, viable and killed *N. meningitidis* can activate transfected TLRs to equal extents, although viable bacteria can induce more TLR9 signalling (Mogensen et al., 2006b). However, more prominent differences in the immune response to live and killed have been observed. Viable *N. lactamica* can modulate peroxisome proliferator-activated receptor gamma (PPAR-γ) to suppress TLR2-NF-κB signalling, while killed bacteria cannot (Tezera et al., 2011). Moreover, viable—but not killed—*N. meningitidis* can suppress the induction of certain immunity genes in epithelial cell lines (Wong et al., 2011). In macrophages, infection with live *N. gonorrhoeae* results in higher cytokine production than killed bacteria, but this is independent of bacterial growth (Zughaier et al., 2014). In summary, species-specific differences in *Neisseria* spp.-induced cell signalling exist, but none of the available data explain the phenotype exerted on DC by live bacteria.

4.2 Aims and Objectives

The aim of the investigations in this chapter was to investigate the hypothesis that viable NmB was targeting a major pathway involved in DC maturation. Data from the previous chapter supported the concept of the bacteria manipulating a signalling pathway to decouple cytokine production and surface maturation, and so the key signalling molecules of the MyD88 and TRIF signalling axes, and other canonical pathways, were examined.

4.3 Results

4.3.1 Lentivirus transfection does not mature DC

Lentiviruses are useful biotechnological tools with which to study intracellular signalling events. Unlike adenoviral vectors, lentiviruses can be transfected without eliciting strong immune responses in the host cell (Morelli et al., 2000). Despite being less immunogenic, DC express virus-recognising TLRs such as TLR3 and TLR7 that can detect high titres of lentivirus and therefore induce maturation *in vitro* (Breckpot et al., 2010). For this reason, careful optimisation of the transfection protocol is necessary in order to achieve a desirable balance of transfection rates and cellular activation.

DC were first transfected with a lentivirus containing a constitutively active GFP reporter element (LV-GFP) (a kind gift from Dr Emma Chan, UCL) in order assess transfection rates and DC immuno-activation. Two different protocols were used: (1) using 1% heat-inactivated autologous human serum, as previously described (Breckpot et al., 2007); and (2) using heat-inactivated FCS. In contrast to previous reports, it was found that lentivirus transfection at MOI 5 performed in the presence of human serum was potently immunogenic, resulting in marked up-regulation of CD86 expression and low transfection rates (28%) (Figure 4-1). However, transfection in the presence of FCS did not induce DC maturation, and also had higher levels of transfection (50.1%). Therefore, FCS was used for all future transfections.

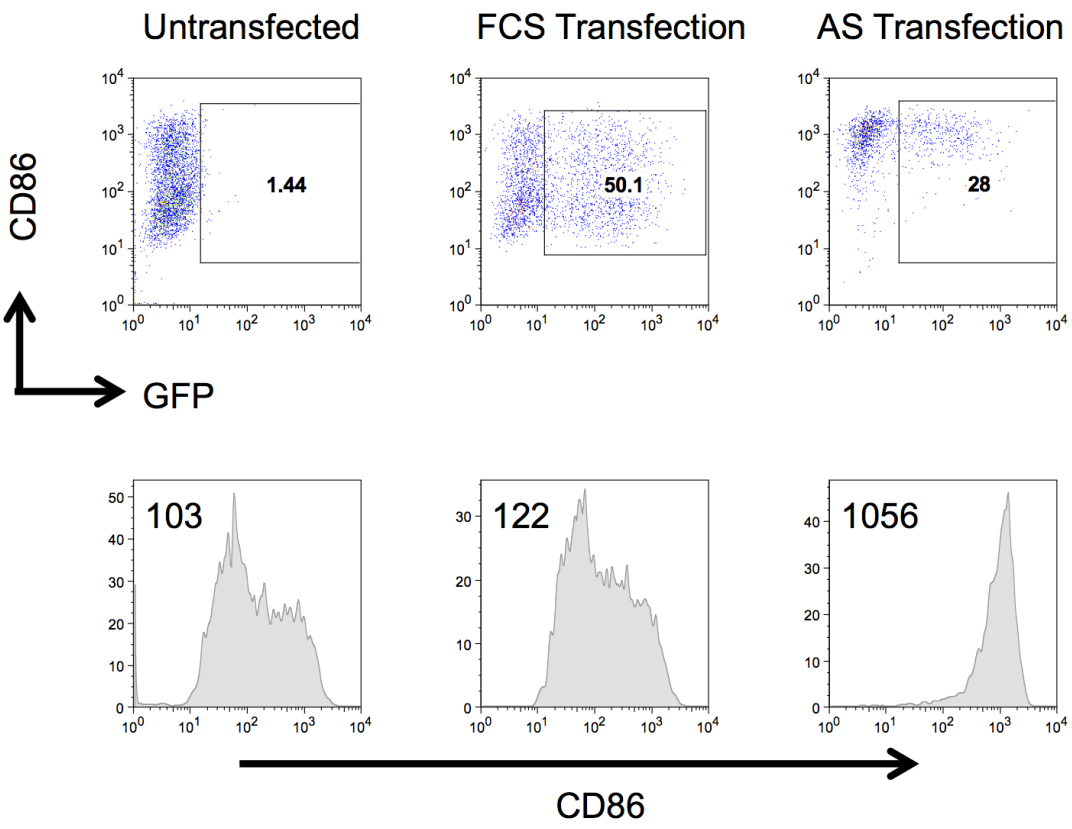

Figure 4-1 ***DC can be efficiently transfected with lentivirus without resulting in phenotypic maturation.*** **DC were transfected with MOI 5 of a GFP-expressing lentiviral vector (LV-GFP) on day 3 of differentiation, and after 72h they were assessed for transfection rates (GFP^+) and cellular activation (CD86 expression). Frequency of GFP^+ cells (top) and CD86 MFI (bottom) are displayed on the graphs. FCS transfection: DC transfected in the presence of 10% FCS; AS transfection: DC transfected in the presence of 1% heat-inactivated autologous serum. Data are representative of two experiments.**

4.3.2 Inhibition of DC maturation by live NmB is NF-κB-independent

The NF-κB pathway is targeted by many bacteria to disrupt host cell immune responses (Rahman and McFadden, 2011). NF-κB activation is a direct result of early MyD88-dependent TLR signalling, and so it was hypothesised that NmB might be blocking this pathway in order to disrupt DC maturation. To interrogate this pathway, DC were transfected with a lentivirus containing an NF-κB reporter plasmid. Thus, any NF-κB transcriptional activity would be directly quantifiable by luciferase activity. DC were then stimulated for 4h and luciferase activity was quantified by luminometer (Figure 4-2). Unexpectedly, it was found that LPS, killed NmB and live NmB activated early NF-κB activity to similar extents, with all three conditions showing over 3-fold NF-κB transcriptional activity above the unstimulated control.

To confirm this finding, IκB-α degradation was probed by Western blot. IκB-α is an inverse surrogate marker for NF-κB nuclear translocation, as it ordinarily captures the transcription factor in the cytoplasm to prevent spontaneous immune responses. IκB-α is ubiquitinated and subsequently degraded by the proteasome upon TLR signalling. In agreement with the previous data, it was found that IκB-α degradation occurred with similar kinetics in DC stimulated with killed or live bacteria. For both conditions (Figure 4-3), IκB-α degradation occurred between 30 mins and 3 h post-infection, with a recovery at 4h.

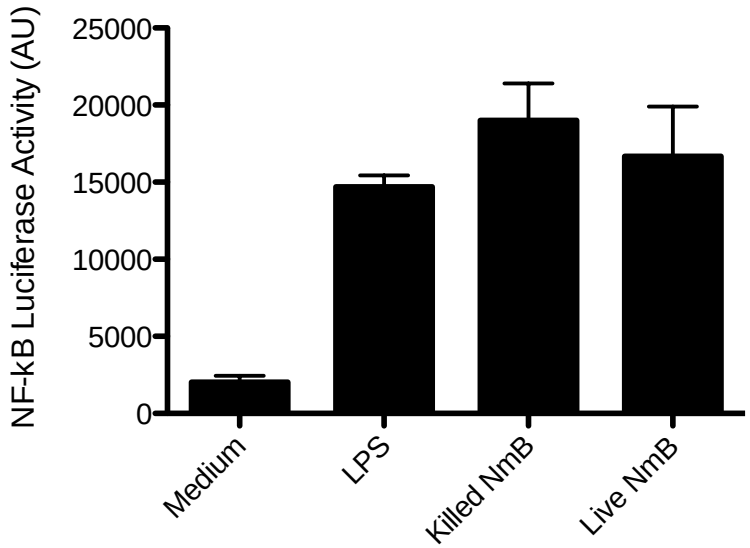

Figure 4-2 Live *NmB induces normal transcriptional NF-κB activity*. DC were transfected with a lentivirus containing an NF-κB luciferase reporter construct on day 3 of differentation. After 72h, DC were then stimulated with LPS (100 ng/mL), 10 MOI killed NmB or 10 MOI live NmB for 4h and NF-κB transcriptional activity was determined by luciferase activity by the addition of BrightGlo and measuring luminescence on a luminometer. Data shown are from two individual donors. Bars depict mean averages ± SEM.

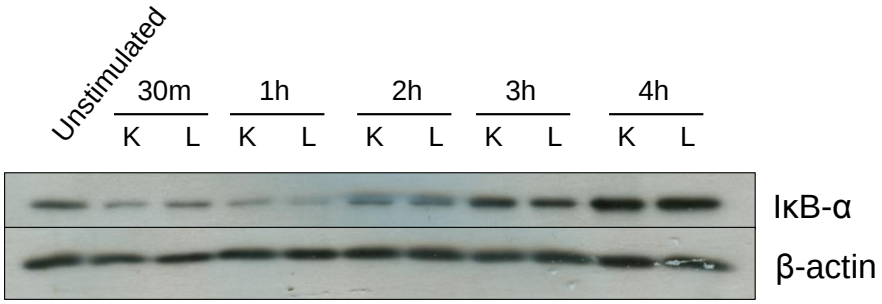

Figure 4-3 ***DC stimulated with live or killed NmB induce rapid IκB-α degradation.*** **DC were stimulated with either 10 MOI killed or 10 MOI live NmB (K = killed NmB, L = live NmB) for the indicated times and then cellular lysates were probed by Western blot for the presence of IκB-α, a surrogate marker of NF-κB activation. Some DC were left unstimulated and lysates were collected at 4h. β-actin was used as a loading control. Data are representative of two independent experiments.**

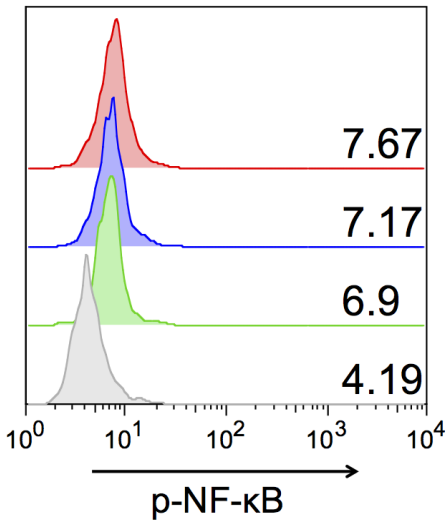

Figure 4-4 *NmB induces late-phase NF-κB phosphorylation.* DC were stimulated with 100 ng/mL LPS (green), 10 MOI killed (blue) or 10 MOI live NmB (red) for 18h and p-NF-κB (p-p65) was measured by intracellular PhosFlow cytometry. MFI values are displayed on the histogram. Data are representative of one experiment.

Late-phase NF-κB activity was further validated by intracellular flow cytometry. At 18h post-stimulation, DC were assessed for NF-κB phosphorylation after stimulation with LPS, killed NmB or live NmB (Figure 4-4). In agreement with the previous data, LPS, killed and live NmB displayed similar levels of NF-κB phosphorylation (LPS p-NF-κB MFI: 6.9; killed NmB p p-NF-κB MFI 7.17; live NmB p-NF-κB MFI: 7.97).

4.3.3 Activation of mitogen activated protein kinases by NmB

DC were next assessed for MAPK activity in response to viable and killed NmB. MAPKs are important in mediating central aspects of DC maturation, and pathogens may inhibit their activation to modulate cytokine production and up-regulation of maturation markers (Mittal et al., 2009). Therefore, inhibition of MAPKs can prevent effective T-cell responses. To test MAPK activation, DC were stimulated with killed NmB or live NmB for various times (as indicated in the figure legends) and then lysates were probed for MAPK phosphorylation by Western blot. As can be seen in Figure 4-5, it was found that both live and killed bacteria activated induced phosphorylation—and therefore activation—of the MAPKs ERK, p38 and JNK to similar extents at 2 and 4h post-stimulation.

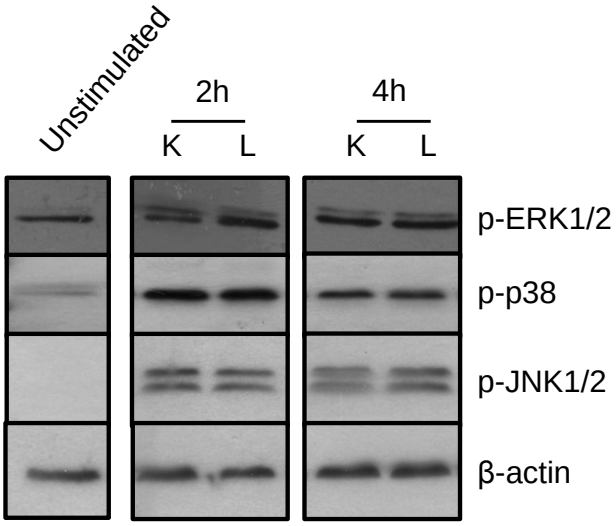

Figure 4-5 *NmB induces the activation of multiple MAPK kinases.* DC were stimulated with either 10 MOI killed or 10 MOI live NmB (K = killed NmB, L = live NmB) for the indicated times and then cellular lysates were probed by Western blot for the presence of ERK1/2 (T202/Y204), p38 (T180/Y182) and JNK1/2 (T183/Y185) phosphorylation. Some DC were left unstimulated and lysed at 4h. β-actin was used as a loading control. Black boxes indicate cropped blots to depict relevant lanes. Data are representative of three independent experiments.

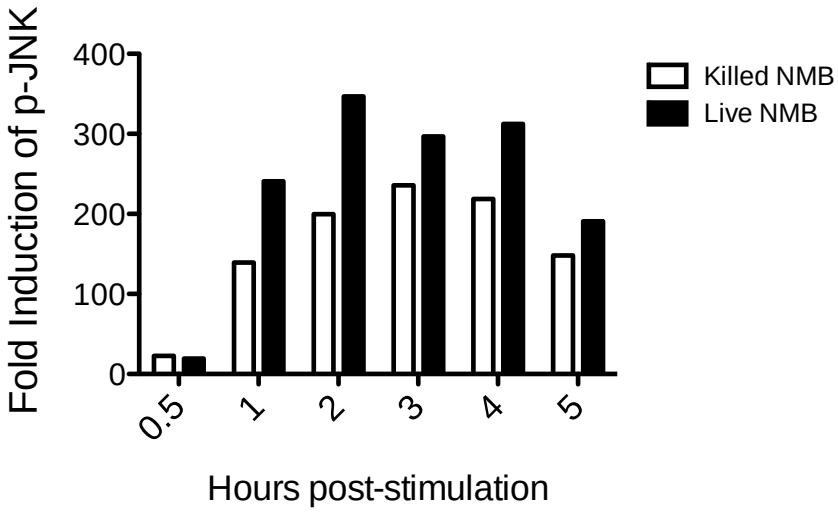

Figure 4-6 *Kinetics of JNK activation by NmB*. DC were stimulated with either 10 MOI killed or 10 MOI live NmB for up to 5 h, and cellular lysates were taken at the indicated time-points. Lysates were then probed for p-JNK and total JNK by Western blot. Fold induction (induced p-JNK - background p-JNK) was calculated relative to total JNK protein by densitometry using ImageJ software. Data are from one donor and representative of two experiments.

Since excessive JNK signalling can negatively regulate DC maturation (Handley et al., 2005), temporal dynamics of p-JNK was further quantified by densitometry, using total JNK protein as a control. As can be seen in Figure 4-6, both killed and live bacteria induced a strong early signal (0.5 h) that then showed sustained activity up to 5 h post-stimulation.

4.3.4 NmB induces normal activation of standard DC maturation pathways

Several pathways of DC maturation have been reported to decouple cytokine production and up-regulation of maturation markers. These include GSK-3, which is constitutively active in immature DC and is deactivated upon maturation (Rodionova et al., 2007); Src family proteins, which can dysregulate aspects of DC maturation via modulation of ITAM-ITIM signalling (Napolitani et al., 2003); and Akt signalling, which can be also be modulated for the same purpose (Turnquist et al., 2010). To interrogate these pathways, DC were stimulated with killed or live NmB for 4h or 18h, and phospho-epitopes were probed by Western blot (Figure 4-7).

Similarly to NF-κB and MAPK signalling, it was found that killed and live NmB activated/deactivated these signalling pathways to similar extents during both early (4h) and late (18h) maturation, thus implicating a different signalling pathway being targeted by live NmB to prevent full DC maturation.

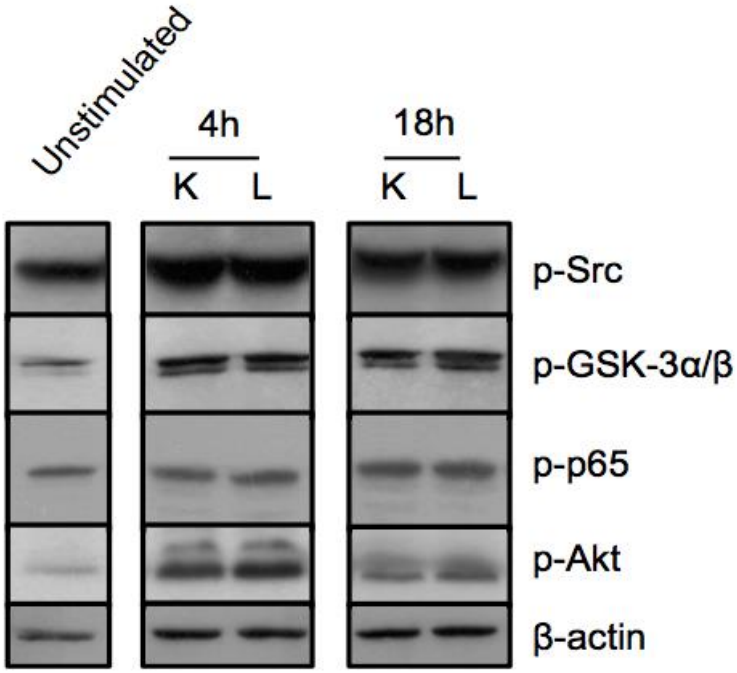

Figure 4-7 *Live and killed NmB display similar cellular signalling patterns.* DC were stimulated with either 10 MOI killed or 10 MOI live NmB (K = killed NmB, L = live NmB) for 4 or 18h and then cellular lysates were probed by Western blot for the indicated proteins: p-Src family kinases (Y416), p-GSK-3 (S21), p-p65 (S536) and p-Akt (S473). Some DC were left unstimulated and lysed at 18h. β-actin was used as a loading control. Black boxes indicate cropped blots to depict relevant lanes. Data are representative of two (p-Src, p-65, p-Akt) and four (p-GSK3) independent experiments.

4.3.5 Live NmB infection results in selective tyrosine dephosphorylation of STAT1

Up-regulation of DC maturation markers depends on autocrine IFN signalling that activates STAT1. It was therefore hypothesised that live NmB might be modulating this pathway to prevent the up-regulation of CD86 and other maturation markers. STAT1 binding sites exist in the promoters of the maturation markers (Weatherill et al., 2005), and STAT1-KO DC are therefore poorly immunogenic in T-cell proliferation assays (Longhi et al., 2009). STAT1 requires two major post-translational modifications in order to gain canonical signalling functions: (1) tyrosine phosphorylation of the Y701 residue, which allows it to dimerise and translocate to the nucleus, and (2) serine phosphorylation of the S727 residue, which potentiates and primes its full activation.

DC STAT1-Y701 and -S727 phosphorylation was measured by Western blot at early (4h) and late (18h) maturation after stimulation with killed and live NmB (Figure 4-8). Remarkably, DC infected with live NmB showed virtually complete dephosphorylation of STAT1-Y701 at 18h, compared to phosphorylation at 4h. DC stimulated with killed NmB retained STAT1-Y701 phosphorylation at both early and late maturation. By contrast, live NmB infection induced strong STAT1-S727 signalling at early (4h) and late (18h) maturation. Killed NmB followed the same trend.

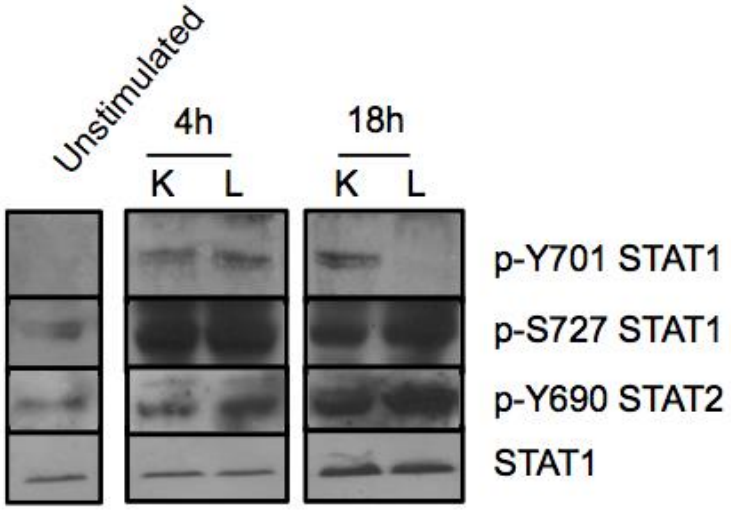

Figure 4-8 *Live NmB induces tyrosine dephosphorylation of STAT1*. DC were stimulated for indicated times with 10 MOI killed NmB or 10 MOI live NmB (K = killed NmB, L = live NmB), and cell lysates were analysed for levels of the designated proteins by Western blot. Some DC were left unsitmulated and lysates were collected at 18h. STAT1 was used as a loading control. Black boxes indicate cropped blots to depict relevant lanes. Data are representative of four (STAT1-Y701) or three (STAT1-S727 and STAT2-Y690) individual experiments.

Moreover, in juxtaposition to STAT1 activation, the activatory tyrosine 690 (Y690) phosphorylation of STAT2 remained intact after DC exposure to both stimuli (Figure 4-8). DC were also tested for STAT3 activation, and STAT3 tyrosine phosphorylation (Y705) was measured by intracellular PhosFlow after stimulation with various stimuli (Figure 4-9). LPS, killed and live NmB all induced similar levels of STAT3 phosphorylation (and therefore activation).

Since Western blots are only semi-quantitative (Gassmann et al., 2009), it was decided that intracellular flow cytometry would be utilised in order to better quantify STAT1 activity. DC were stimulated with LPS, killed or live NmB for 18h and levels of STAT1-Y701 and STAT1-S727 phosphorylation were measured intracellularly using a PhosFlow protocol. Consistent with the Western blot data (Figure 4-10), LPS and killed NmB induced 1.44- and 1.45-fold STAT1 p-Y701 induction above an unstimulated control, while STAT1 p-Y701 induction by live NmB was virtually absent (1.0-fold). This was found to be a significant difference (p = <0.001) between live NmB and LPS and killed NmB.

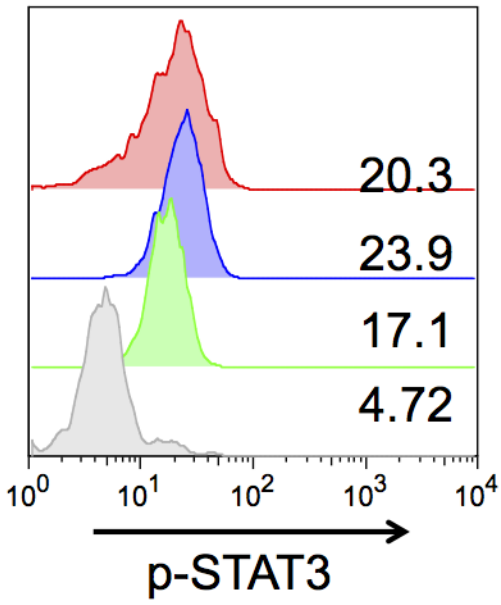

Figure 4-9 *DC infected with live NmB retain STAT3 activation*. DC were stimulated for 18h with 100 ng/mL LPS (green histogram), 10 MOI killed NmB (blue histogram) or 10 MOI live NmB (red histogram) and assessed for STAT1 Y701 phosphorylation by intracellular PhosFlow flow cytometry. Some DC were left unstimulated (grey histogram). MFI values are displayed on the histograms. Data are representative of three experiments.

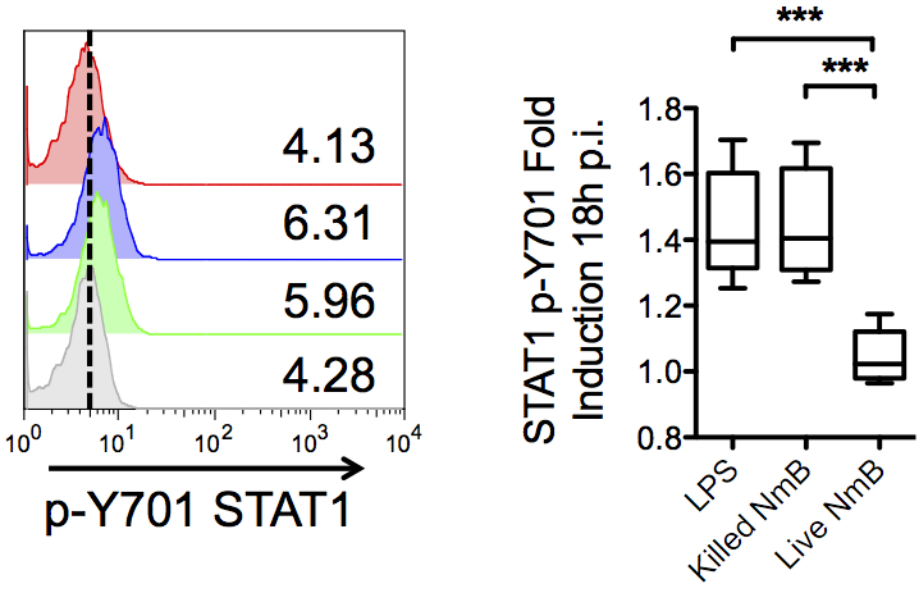

Figure 4-10 *Live NmB suppresses STAT1 tyrosine phosphorylation in DC*. DC were stimulated for 18h with 100 ng/mL LPS (green histogram), 10 MOI killed NmB (blue histogram) or 10 MOI live NmB (red histogram) and assessed for STAT1-Y701 phosphorylation by intracellular PhosFlow flow cytometry. Some DC were left unstimulated (grey histogram). *Left:* Data from a representative donor with MFI values displayed on the histogram. *Right:* Combined data from six individual donors showing fold-change in p-Y701 STAT1 MFI above the unstimulated control. Box-plots depict medians and interquartile ranges. Significance was tested by one-way ANOVA with a Bonferroni post-test. **P*<0.05, ***P*<0.01, ****P*<0.001.

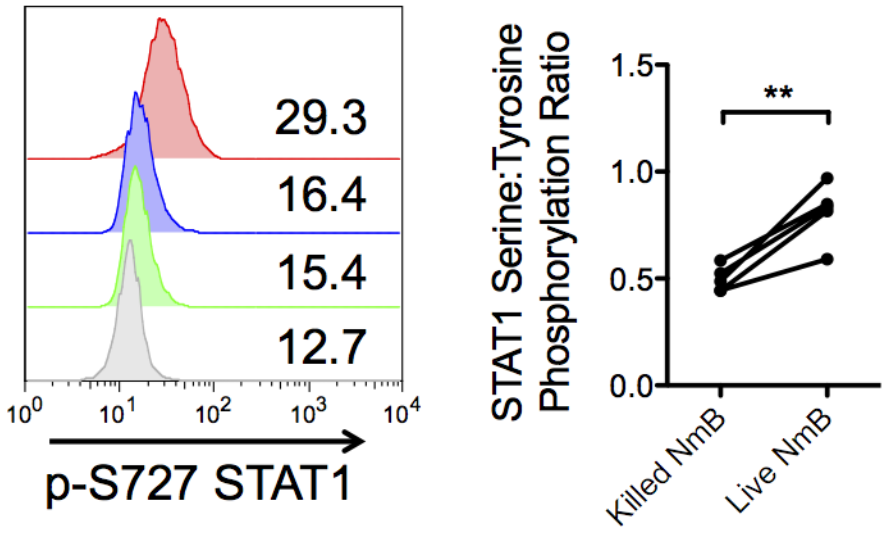

Figure 4-11 *STAT1 serine phosphorylation is sustained in DC infected with viable NmB*. DC were stimulated for 18h with 100 ng/mL LPS (green histogram), 10 MOI killed NmB (blue histogram) or 10 MOI live NmB (red histogram) and assessed for STAT1-S727 phosphorylation by intracellular PhosFlow flow cytometry. Some DC were left unstimulated (grey histogram). *Left*: Analysis of STAT1-S727 phosphorylation at 18h post-stimulation in a representative donor with MFI values displayed on the histogram. *Right:* Calculation of STAT1 Serine:Tyrosine phosphorylation ratio at 18h by the formula (STAT1 p-S272 MFI Fold Change/STAT1 p-Y701 MFI Fold Change) in 5 individual matched donors as indicated by lines. Significance was tested by paired t-test *P<0.05, **P<0.01, ***P<0.001.

STAT1 p-S727 was also measured by intracellular flow cytometry. DC were stimulated with killed or live NmB for 18h and this epitope was quantified. Intriguingly, live NmB induced stronger STAT1 p-S727 phosphorylation (MFI: 29.3) than LPS (MFI: 15.4) or killed NmB (MFI:16.4) (Figure 4-11). A comparison of tyrosine:serine STAT1 phosphorylation between killed and live NmB also showed that live NmB induced significantly more STAT1-S727 phosphorylation compared to killed bacteria (p = <0.01).

STAT1-Y701 phosphorylation is essential for STAT1 dimerisation and translocation into the nucleus, and this results in the transcription of STAT1-dependent genes (Shuai et al., 1993). To confirm that the dephosphorylated STAT1 was not inducing target genes, the transcriptional activity of the well-characterised STAT1-dependent reporter gene *IDO1* (Chon et al., 1996) was measured by RT-PCR. As Figure 4-12 shows, DC infected with live NmB displayed significantly reduced (p = <0.05) levels of *IDO1* compared to the positive controls LPS and killed NmB. The NF-κB-dependent gene *IL8* was used as a control, and this showed both early and late *IL8* transcription induced by live NmB. Notably, live NmB drove significantly stronger (p = <0.01) *IL8* gene expression than killed NmB and LPS. Therefore, the data suggested that STAT1-dependent genes were being transcriptionally suppressed by live bacteria.

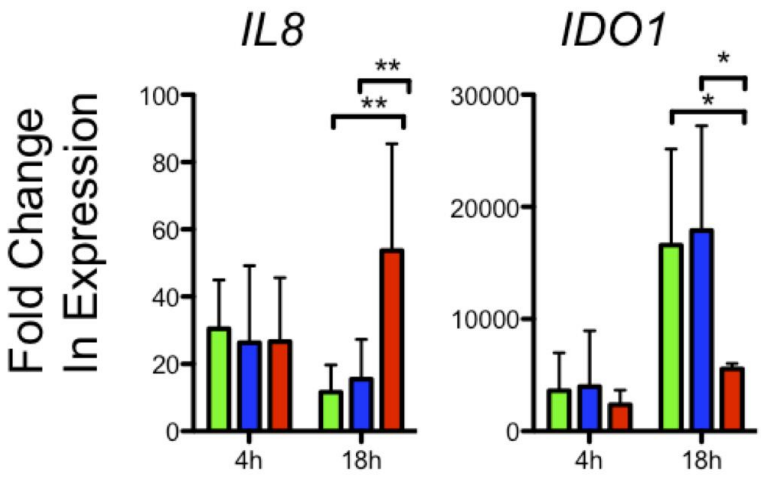

Figure 4-12 *Live NmB can suppress STAT1 reporter genes while sustaining NF-κB reporter genes*. DC were stimulated for the indicated times with 100 ng/mL LPS (green), 10 MOI killed NmB (blue) or 10 MOI live NmB (red), and then RNA was extracted to quantitatively assess *IL8* (NF-κB-dependent gene) and *IDO1* (STAT1-dependent gene) mRNA expression relative to a time 0 (T_0) control. Bar graphs depict mean averages ± SEM. Data are from four individual donors. Singificance was tested by two-way ANOVA with a Bonferroni post-test. **P*<0.05, ***P*<0.01, ****P*<0.001.

4.3.6 STAT1 activation correlates with CD86 expression and DC maturation

Interestingly, flow cytometric analysis of $CD86^{lo}$ and $CD86^{hi}$ populations after DC were stimulated with live NmB for 18h revealed more STAT1-Y701 phosphorylation (MFI: 29.8) in the few $CD86^{hi}$ DC (Figure 4-13). This was not dependent on which stimulus the DC had received (data not shown). Moreover, a Spearman correlation test of raw flow cytometry data (CD86 fluorescence vs. STAT1-Y701 fluorescence) using FlowJo data analysis functions showed a strong correlation (r = 0.56) between STAT1-Y701 phosphorylation and CD86 expression in individual cells.

4.3.7 DC infected by live NmB still transcribe interferon

It was hypothesised that viable NmB may have been preventing normal STAT1 activation by disrupting the autocrine IFN loop that DC require for maturation. To test this, DC were stimulated for 4 and 18h with LPS, killed and live NmB and then *IFNB* and *IFNG* mRNA transcripts were measured by RT-PCR (Figure 4-14). Remarkably, DC infected with live NmB were actively producing much more IFN transcripts than either LPS or killed NmB. At 4h, live NmB induced >2000-fold induction of *IFNB* compared to the unstimulated T_0 control, and significantly more than LPS (p = < 0.001) and killed NmB (p = < 0.01) at the same time-point.

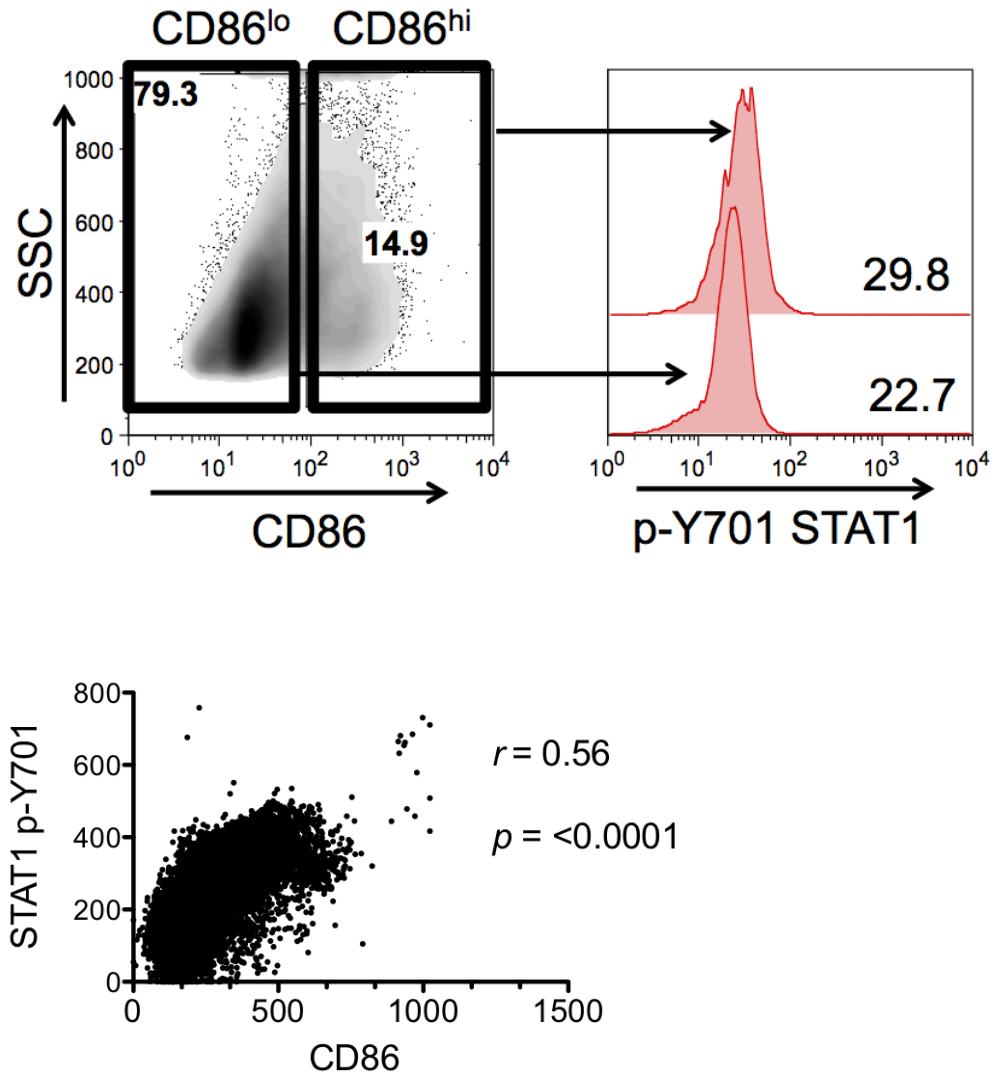

Figure 4-13 ***DC CD86 expression correlates with STAT1 Y701 phosphorylation*****.** ***Top:*** **DC were gated on CD86^{lo} and CD86^{hi} populations and assessed for STAT1-Y701 phosphorylation by intracellular PhosFlow flow cytometry after 18h infection with 10 MOI live NmB. MFI values are displayed on the histogram.** ***Bottom*****: >59,000 DC infected with 10 MOI live NmB for 18h from a representative donor of six were analysed by PhosFlow flow cytometry for raw CD86 and STAT1-pY701 flouresence values (Y and X axes, respectively). Correlation (*** ***r)*** **and significance of pairing (*** ***p*****) was measured using a Spearman's rank test.**

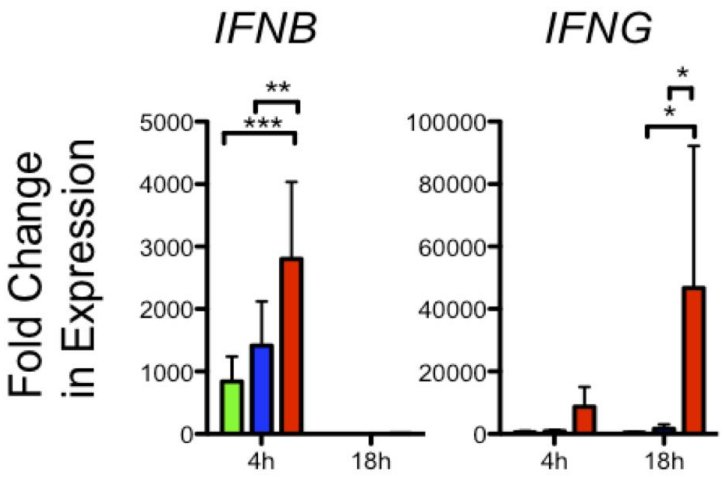

Figure 4-14 *DC infected with live NmB still transcribe type I and type II IFNs.* DC were stimulated for the indicated times with 100 ng/mL LPS (green), 10 MOI killed NmB (blue) or 10 MOI live NmB (red), and then RNA was extracted to quantitatively assess *IFNB* and *IFNG* mRNA expression relative to a time 0 (T_0) control. Bar graphs depict mean averages ± SEM. Data are from four individual donors. Singificance was tested by two-way ANOVA with a Bonferroni post-test. **P*<0.05, ***P*<0.01, ****P*<0.001.

These data were very surprising, since these two cytokines are extremely potent inducers of STAT1 signalling, and late-phase Y701 dephosphorylation was observed at the same time-point of excess IFN production. This suggested a very potent inhibitory signal being delivered to STAT1, in order to over-ride these positive cytokine signals.

To exclude the possibility that IFN was being transcribed but not secreted, DC were also primed for 2h with IFN-β and IFN-γ before infection with live bacteria. Despite priming with both cytokines (Figure 4-15), DC infected with live NmB were still unable to up-regulate CD86 in response to live bacteria, while uninfected DC were activated by the IFN priming, thus suggesting an active blockade of STAT1 activation rather than a lack of signal *per se*.

4.3.8 Live NmB induces IFN hyporesponsiveness in DC

DC were next tested for active hyporesponsiveness to brief IFN pulsing during the late maturation phase. At 18h post-stimulation, DC were pulsed with a high dose of IFN-γ, which is a potent STAT1 activator (Figure 4-16). Unstimulated, LPS- and killed NmB-treated DC all showed a strong induction of STAT1-Y701 phosphorylation after 15 mins of stimulation (51.2%, 86.5% and 83.6% respectively), while DC infected with live NmB showed blunted tyrosine phosphorylation (27.6%). This was found to be a significant trend between multiple donors (p = <0.01 killed NmB vs live NmB).

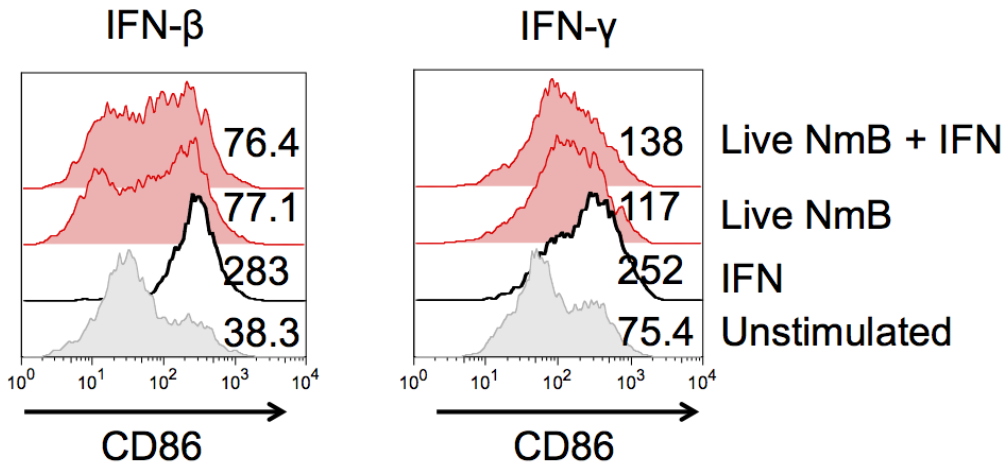

Figure 4-15 ***IFN priming is insufficient to restore DC maturation upon infection with live NmB*****. DC were primed for 2h with either 20ng/mL IFN-β or 20 ng/mL IFN-γ and then some DC were stimulated for 24h with 10 MOI live NmB (red), alone or in combination with exogeneous IFN, or left unstimulated (grey). Surface CD86 expression was assessed by flow cytometry. MFI values are shown on the histograms. Data are representative of three experiments; two different donors are shown for each cytokine.**

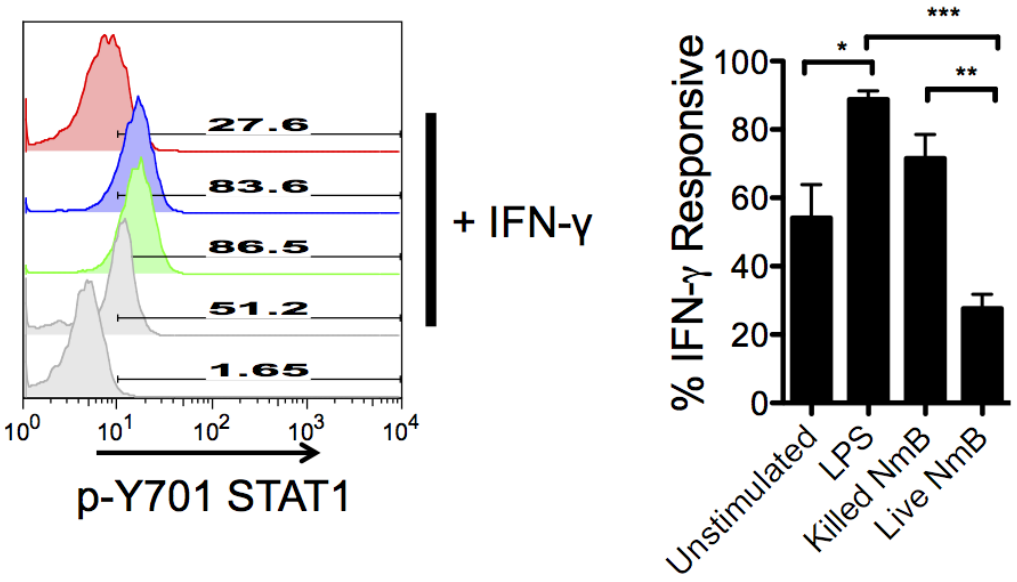

Figure 4-16 *DC are hyporesponsive to IFN pulsing after* infection with *NmB.* DC were stimulated with 100 ng/mL LPS (green), 10 MOI killed NmB (blue) or 10 MOI live NmB (red) for 18h and then pulsed with 10 ng/mL IFN-γ for 15m. Some DC were left unstimulated (grey). Cells were then fixed and permeabilised and STAT1-Y701 phosphorylation was measured by intracellular PhosFlow flow cytometry. *Left:* Data from one representative donor, with gates showing percentage of 'STAT1 responders' as set by the unstimulated control. *Right:* Combined data from three donors showing percent responsive DC to IFN-γ pulsing. Bars depict mean averages ± SEM. Significance was tested by one-way ANOVA with a Bonferroni post-test. **P*<0.05, ***P*<0.01, ****P*<0.001.

4.3.9 JAK kinase signalling is intact in DC infected with live NmB

In order to induce STAT1 phosphorylation, IFN must bind to its cognate IFN receptor and cause JAK kinase activation. These JAK kinases (JAK1, JAK2 and Tyk2) are bound to the IFN receptor and directly phosphorylate STAT proteins. Therefore, their activation (i.e. phosphorylation) was examined by Western blot. This would inform as to whether the IFN receptor was fully functional, as these kinases are the immediate downstream effectors of the cytokine-IFN receptor interaction.

DC were stimulated for 18h with either killed NmB or live NmB, and cell lysates were probed for phosphorylation of JAK1, JAK2 or Tyk2 by Western blot (Figure 4-17). Interestingly, there were similar levels of phosphorylation induced by killed an live bacteria for p-JAK1, while there appeared to be more phosphorylation of p-JAK2 induced by live NmB. There were multiple bands detected for p-JAK2, which may reflect both the antibody polyclonality (e.g. binding multiple phosphate groups) and that JAK proteins undergo heavy post-translational modification (e.g., polyubiquitination) in response to cytokine signalling (Ungureanu et al., 2002). Tyk2 was also similarly activated by killed and live bacteria, and in some donors there was a trend for live NmB to induce stronger phosphorylation of this protein, which is consistent with the enhanced levels of IFN production induced by live bacteria.

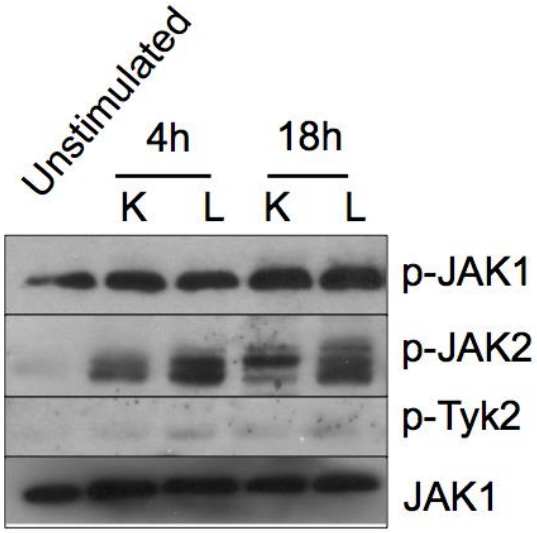

Figure 4-17 *DC infected with live NmB retain JAK kinase activation.* DC were stimulated for indicated times with 10 MOI killed NmB or 10 MOI live NmB (K = killed NmB, L = live NmB), and cell lysates were analysed for levels of the designated proteins by Western blot (p-JAK1 Y1022/Y1023, p-JAK2 Y1007/Y1008, p-Tyk2 Y1054/Y1055). Some DC were left unsitmulated and lysates were collected at 18h. JAK1 was used as a loading control. Black boxes indicate cropped blots to depict relevant lanes. Data are representative of three experiments.

4.3.10 Live NmB infection leads to suppression of SOCS protein expression

SOCS proteins are homeostatic molecules that negatively regulate the IFN and TLR pathways. *SOCS1* and *CISH1* (also known as CIS) are transcribed in response to STAT1 signalling (Lejeune et al., 2001), while SOCS3 can be induced by NF-κB or STAT3 signalling pathways (Pauli et al., 2008). These proteins can prevent the phosphorylation of STAT proteins by occluding the JAK-IFN receptor interaction or the JAK-STAT interaction.

It was hypothesised that live NmB may be inducing these proteins to block STAT1 signalling and prevent DC maturation. To test this, DC were stimulated for 4h and 18h with LPS, killed or live NmB and *SOCS1, SOCS3 and CISH* mRNA was measured by RT-PCR (Figure 4-18). There was an observed trend of *SOCS1* down-regulation by live NmB at 18h compared to positive controls, while there was a significant suppression of *CISH1* gene expression at 18h (p = <0.05 killed NmB vs. live NmB). *SOCS3* was similar amongst all conditions tested. SOCS1 protein levels were further validated by Western blot, and there was a clear reduction in SOCS1 protein levels induced by live NmB infection. Therefore, SOCS proteins were in fact reduced by live NmB, excluding these molecules as the drivers of STAT1 dysregulation.

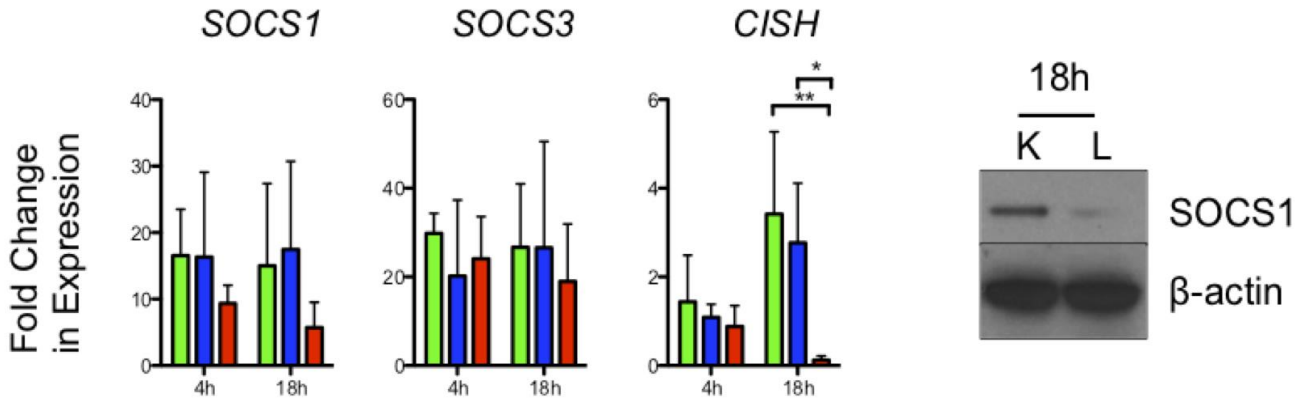

Figure 4-18 Live NmB suppresses up-regulation of STAT1-dependent SOCS gene transcription. *Left:* DC were stimulated for the indicated times and then RNA was extracted to quantitatively assess *SOCS1, SOCS3 and CISH* gene expression relative to a time 0 (T_0) control. Green: 100 ng/mL LPS, blue: 10 MOI killed NmB, red: 10 MOI live NmB. Graphs depict mean averages ± SEM. Data from three individual donors are shown. Significance was tested by two-way ANOVA with a Bonferroni post-test. **P*<0.05, ***P*<0.01, ****P*<0.001. *Right*: Levels of DC SOCS1 protein expression determined by Western blot of DC lysates 18h post-stimulation with 10 MOI killed (K) or 10 MOI live (L) NmB. Data are representative of two independent experiments

4.3.11 JAK-STAT signalling is required for DC maturation in response to meningococcal antigens

A recent vaccinology study reported that the meningococcal vaccine Menomune, which consists of bacterial antigens (i.e. polysaccharides), activates that JAK-STAT pathway in human DC and induces a robust maturation response (Banchereau et al., 2014). Since live NmB was interfering with this pathway, It was questioned whether this signalling axis was important for the up-regulation of maturation markers in response to NmB. To test this, DC were stimulated with killed NmB for 18h in the presence of the JAK inhibitor tofacitinib or the NF-κB inhibitor BAY-11-7085 and CD40 and CD86 up-regulation were measured. As Figure 4-19 shows, tofacitinib exerted a strong negative effect on up-regulation of maturation markers, and this effect was dose-dependent, with a maximal suppression at 500 μM. In contrast, BAY-11-7085 had only a minimal effect on maturation marker expression, highlighting the requirement for the IFN signalling axis for full DC maturation in response to NmB.

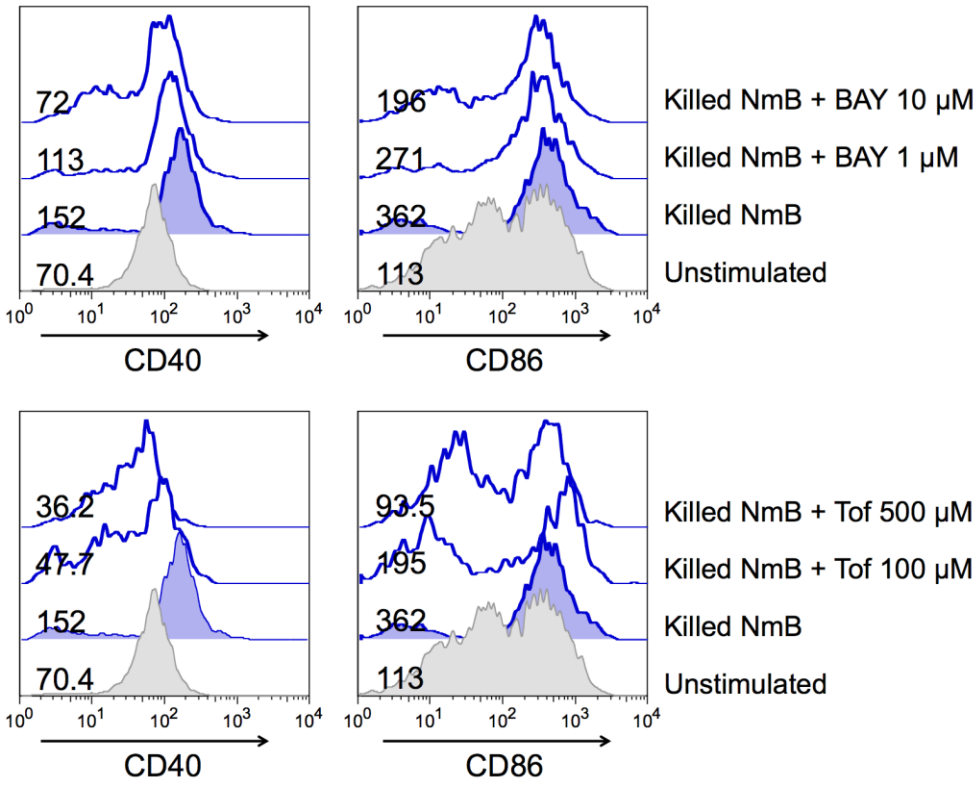

Figure 4-19 ***The IFN pathway is an important determinant of DC maturation responses to meningococcal antigens*****. DC were stimulated with 10 MOI killed NmB (blue shaded histogram) for 24h in the presence of the NF-κB inhibitor BAY11-7085, the JAK inhibitor tofacitinib or a vehicle control (0.5% DMSO). Some DC were left unstimulated (grey histogram). DC were then assessed for expression of CD40 and CD86 by flow cytometry. MFI values are displayed on the histogram. Data are representative of two experiments.**

4.4 Discussion

The data in this chapter support the concept of live NmB modulating a critical signalling path in DC maturation. While nearly all examined pathways had equivalent activation, there was differential induction of STAT1 signalling, likely mediated by the Y701 dephosphorylation. This led to IFN hyporesponsiveness in the DC that was linked to inhibition of maturation.

Unlike previous reports, it was found that FCS induced higher lentivirus transfection rates and was less immunogenic than human serum. There are several reasons why these results may differ from those previously published. Human serum can contain varying amounts of contaminants such as platelets (Tammen et al., 2005), which can secrete bioactive chemokines and cytokines and also express CD40L (Henn et al., 1998), which are all potent inducers of DC maturation. It is known that gravitational forces (i.e. centrifugation) can activate immune cell signalling pathways (Verhaar et al., 2014). It is possible that the harsh centrifugation of blood using an 'in-house' method may have activated contaminating platelets and resulted in the maturation of DC. Moreover, activated DC are much more resistant to lentiviral transfection. This is because activated DC up-regulate virus resistance factors upon sensing viral PAMPs (Cella et al., 1999). It is therefore plausible that this is why DC matured by autologous serum had lower rates of viral transfection.

Using a lentivirus NF-κB reporter and IκB-α degradation assays, it was demonstrated that killed and live bacteria activated this pathway to similar

extents. Given that DC stimulated with killed and live NmB both induce a potent inflammatory response (i.e. TNF-α, IL-1β and IL-6 secretion), it is logical that NF-κB was found to be activated by both of these stimuli. However, while NF-κB is important for DC maturation, the extent to which it contributes towards a fully mature phenotype remains complex. The use of NF-κB inhibitors and MyD88-deficient mutant cells suggests that the early NF-κB response predominantly induces cytokine signalling. However, some studies on CD40 transcriptional regulation have revealed a need for NF-κB 'priming' at the enhancer elements of the promoter region, followed by late-phase STAT1 association induced by autocrine IFN (Qin et al., 2005). Similarly, the CD86 promoter contains both NF-κB and STAT1 binding elements (Weatherill et al., 2005), despite TRIF signalling being reported the main driver of co-stimulatory molecule expression (Kolb et al., 2014). The extent to which certain DC genes depend on MyD88 or TRIF signalling, however, may ultimately depend on the TLR stimulus used, and there is undoubtedly synergism involving both pathways.

NF-κB signalling in response to a stimulus such as LPS is usually biphasic, consisting of an early and late-phase response — the latter being primarily due to autocrine effectors (Kawai and Akira, 2007). The data do not completely eliminate the possibility that live NmB could be interfering with the late-phase NF-κB pathway. Indeed, the NF-κB assays mainly focused on the early response as a read-out for TLR engagement. However, this possibility is unlikely for three reasons. Firstly, p65 showed similar phosphorylation between killed NmB and live NmB at the 18th time-point. Secondly, flow

cytometric analysis of late maturation also confirmed this finding. And thirdly, *IL8* transcription (which is NF-κB dependent) was robustly induced by live bacteria during late maturation. Therefore, it can be inferred from the data that the NF-κB pathway was normally induced by live bacteria, excluding this as a mechanism of DC modulation.

MAPK activation was also sustained by live NmB, with equivalent kinetics in JNK activation in particular. MAPKs are required for diverse aspects of DC maturation — inhibition of any of the three can result in an aberrant phenotype (Nakahara et al., 2006). The observation that *N. meningitidis* can activate MAPKs in DC is consistent with reports in the literature of the bacterium activating these pathways in brain endothelial cells, leading to pro-inflammatory cytokine release (Sokolova et al., 2004). Notably, some background activation of ERK was detected in the unstimulated control. This was likely due to the presence of growth factors (e.g. EGF) in the DC growth media resulting in baseline MAPK activation, since this has previously been reported in other cell types (Nakao et al., 2002). Background levels of p65 phosphorylation and NF-κB transcription were also observed. Interestingly, a study by Kanada et al. (2011) found that there was a constitutive level of NF-κB bound to the CD86 promoter in unstimulated DC (Kanada et al., 2011). It may be surmised that there is a basal level of constitutive NF-κB activity in cultured DC, which may be required for low-level transcription of certain molecules, and that these data are therefore consistent with previous findings. Even in an immature state, DC contain relatively high levels of CD86 and other co-stimulatory molecules compared to other immune cells.

DC also had normal activation of Akt and Src proteins upon infection with live bacteria, which are important for DC maturation. In addition, the inhibitory serine/threonine kinase GSK-3 was phosphorylated and therefore deactivated by positive controls and live NmB. GSK-3 plays a central role in integrating metabolic signalling pathways and coupling cytokine production with up-regulation of maturation markers in DC (Alessandrini et al., 2011; Rodionova et al., 2007; Turnquist et al., 2010). Therefore, it may be considered a central checkpoint of DC maturation, connecting the PI3K-Akt, Wnt and TLR signalling axes. The observation that GSK-3 was undergoing similar repression by all stimuli tested would suggest that live NmB was targeting a very specific pathway downstream of most effector kinases.

Strikingly, analysis of the STAT proteins revealed a stark difference in STAT1 Y701 phosphorylation induced by live and killed bacteria. STAT1-Y701 was virtually dephopshorylated by live NmB at 18h, while there was still strong phosphorylation induced by stimulation with killed NmB. This effect was specific to the tyrosine residue, as STAT1-S727 was strongly phosphorylated by live bacteria. This activation state of STAT1 (pY701-off/p-S727-on) has been described in the literature as 'alternative activation' (Schroder et al., 2007), and to the author's knowledge, has not been described in relation to a specific pathogen before. STAT1-Y701 is essential for STAT1 dimersation and nuclear translocation (Shuai et al., 1993b), and in agreement with this, it was found that classic STAT1 reporter genes such as *IDO1* were down-regulated in live NmB-infected DC. STAT2 and STAT3 were also unaffected

in these cells, showing equivalent phosphorylation patterns between killed and live bacteria.

Paradoxically, the apparatus of the IFN pathway was fully functional as far as the data indicated. DC transcribed even higher amounts of *IFNB* and *IFNG* upon infection with live NmB compared to positive controls, and the JAK kinases were also fully activated. The STAT1-dependent SOCS genes *SOCS1* and *CISH* were not fully up-regulated by live NmB compared to killed NmB, which excluded these as the drivers of the IFN signalling blockade, but failed to explain why STAT1 was not fully phosphorylated upon IFN-γ pulsing. SOCS3, which is dependent on STAT3 and NF-κB signalling (Lejeune et al., 2001; Pauli et al., 2008), showed no significant difference between killed and live NmB. Thus, STAT1 was being precisely and selectively targeted by live NmB to a single residue, resulting in dephosphorylation despite relatively higher amounts of autocrine IFN and lower amounts of negatively regulating SOCS proteins. What remains unresolved from these data is how DC infected with live NmB transcribed higher levels of IFN genes and also pro-inflammatory cytokines. However, this may be explained by data in the literature demonstrating that SOCS proteins (and in particular SOCS1) can negatively regulate both the IFN and TLR pathways (Baetz et al., 2004). In human DC, RNA silencing of *SOCS1* alone causes massively enhanced cytokine production (Hong et al., 2009), and similar results have been found *in vivo* (Shen et al., 2004). Therefore, failure to up-regulate SOCS proteins by live NmB—presumably because of STAT1 tyrosine dephosphorylation—is likely to cause dysregulated pro-inflammatory cytokine production in DC.

STAT1 is a major target of viruses, given its pivotal role in anti-viral immunity (Goodbourn et al., 2000). However, few bacteria have been shown to manipulate this pathway. Currently, no Neisserial species has been demonstrated to target this signalling axis, and so these data are the first description of such a bacterium modulating host immunity in this particular way. STAT1 is essential for DC functions, but most of this work has been performed using murine cells. In mice, IFNAR1-/- (Longhi et al., 2009) or STAT1-/- (Johnson and Scott, 2007) DC fail to up-regulate CD86 or other maturation markers upon TLR stimulation. Consistent with these findings, the data herein indicating that IFN-β and IFN-γ alone could induce CD86 up-regulation, combined with the data showing the correlation between STAT1-pY701 and CD86 expression, would strongly support the conclusion that both human and mouse DC require an IFN-derived STAT1 signal to up-regulate maturation markers.

In further support of this, the pan-JAK inhibitor tofacitinib was used to block autocrine IFN signalling in response to killed NmB. Tofacitinib is a well-characterised inhibitor that has previously been shown to potently suppress STAT1-Y701 phosphorylation and therefore reduce DC maturation marker expression (Kubo et al., 2013). In the present study, it was found that tofacitinib potently reduced CD40 and CD86 up-regulation in response to inactivated NmB, while the NF-κB inhibitor BAY 11-7085 had only a modest effect in reducing the expression of these molecules.

Tofacitinib was selected to inhibit the JAK-STAT pathway for a number of important reasons. While many excellent inhibitors exist for STAT3 due to its

role in oncogenesis, STAT1 is traditionally been considered a less attractive target for inhibition, since its role in cancer is pro-apoptotic (Avalle et al., 2012). Fludarabine has been suggested as a selective inhibitor of STAT1 (Frank et al., 1999), however the data are controversial. Some studies suggest that fludarabine is ineffective in preventing STAT1 activation (Hanafi et al., 2014; Martinez et al., 2006), while others determine that fludarabine may in fact enhance STAT1-Y701 phosphorylation (Youlyouz-Marfak et al., 2007).

From these data, it may be suggested that the TRIF-dependent autocrine IFN axis may be more important than NF-κB signalling for up-regulation of maturation markers in response to meningococcal antigens. An important caveat to consider is that these inhibitors have varying potencies and toxicities, and so an exact comparison between the two pathways would be difficult to achieve in human cells.

Another limitation with these data is the lack of information about maturation markers besides CD86 for certain assays. However, CD86 was chosen as a good candidate molecule for DC maturation in these assays for two reasons: (1) CD86 has a large MFI increase upon maturation (see Chapter 3), thus making it a sensitive marker of DC activation, and (2) at the time of performing the experiments, few antibodies for DC maturation markers had been experimentally validated for intracellular PhosFlow. The harsh methanol fixation required for measuring intracellular STAT1 phosphorylation destroys many protein epitopes, and thus CD40 staining while assessing STAT1 phosphorylation would not have been possible with commercially available

antibodies. However, CD86, CD40 and HLA Class I & II are tightly co-regulated, and are all up-regulated in response to IFN (data not shown). Therefore, it can be assumed that CD86 expression is representative of general maturation marker regulation.

Collectively, these data suggest a mechanism by which NmB can elegantly manipulate DC signalling pathways by a precise modification of a single transcription factor. The findings in this chapter strongly indicate that live meningococci induce a highly specific tyrosine dephosphorylation of STAT1, blocking IFN signal transduction, and resulting in selective inhibition of maturation marker up-regulation. By contrast, NF-κB signalling was normal, and this may explain the earlier finding of sustained cytokine production. These data therefore provide a mechanism for the de-coupling of cytokine production and the up-regulation of DC maturation markers by the meningococcus. And lastly, these data support the paradigm of evidence in the literature that NF-κB is necessary, but not sufficient, for DC maturation.

5 Chapter 5: *N. meningitidis* impairs DC immunological functions

5.1 Introduction

A common misconception in the field of DC immunobiology is that phenotypic maturation (either the expression of maturation markers, or the production of pro-inflammatory cytokines) will result in effective immune responses (Reis e Sousa, 2006). Indeed, mature DC expressing high levels of co-stimulatory molecules and antigen presentation molecules can paradoxically induce tolerogenic T-cell responses *in vivo* in some circumstances (Albert et al., 2001). The ability of DC to induce T-cell proliferation and differentiation depends on a number of competing positive (e.g. CD80/CD86) and negative (e.g. PD-L1) signals. Some of these DC-T-cell receptor molecules have poorly defined function. For example, there are conflicting reports regarding the role of PD-L2 in T-cell stimulation, specifically as to whether it has a positive (Gao et al., 2013) or negative (Pfistershammer et al., 2006) role in cellular immunity. It is therefore necessary to test the ability of immature or mature DC to induce T-cell responses, as immunogenicity cannot always be inferred from phenotypic data.

DC are the most potent APC at driving T-cell responses, and are able to robustly polarise naive $CD4^+$ T-cells into various subsets. The surface expression of DC maturation markers is the most critical requirement for this

process to occur, as T-cell proliferation and polarisation can still be induced independently of cytokine production (Jankovic et al., 2002). The exact repertoire of positive and negative co-stimulatory molecules determines whether DC-mediated T-cell stimulation occurs. For example, high levels of PD-L1 and low levels of B7 co-stimulatory molecules on DC are associated with T-cell anergy. This ratio has been noted in chronic viral infections (Benedict et al., 2008; Zheng et al., 2014), and is posited as a method by which pathogens may induce immunological 'exhaustion', thus allowing microbial persistence in the host. Moreover, *M. tuberculosis* can also induce PD-L1^{hi}CD86^{lo} DC in mice, and these are associated with cellular immune function impairment at the site of chronic bacterial infection (Schreiber et al., 2010). *S. typhimurium* can also limit T-cell responses in murine models of autoimmunity by enhancing the expression of PD-L1 on DC (Newland et al., 2011). Notably, this is independent of T_{REG} induction and appears to be a direct immunosuppressive effect of the DC on immune cells.

Another important aspect of DC maturation is the ability to elicit active immunological tolerance. DC are capable of inducing T_{REG} expansion by secretion of anti-inflammatory cytokines, thus causing T-cells to express FOXP3. Probiotic bacteria can modulate DC to express high levels of IL-10, TGF-β and IDO, thus causing an expansion of FOXP3^{+} T_{REG} and an amelioration of mucosal inflammatory responses (Kwon et al., 2010). Therefore, there are diverse mechanisms by which microbes can program DC to either evade or manipulate cellular immunity.

T-cell responses (i.e. cellular immunity) to *N. meningitidis* have received much less research attention than B-cell responses, as the presence of serum bactericidal antibody is essential for protection against *N. meningitidis*. . In fact, the 'gold standard' test for assessing protective immune responses to the meningococcus is the serum bactericidal assay (SBA) (Borrow et al., 2005). However, it has been postulated that this assay may in fact underestimate protective immunity to *N. meningitidis* (Welsch and Granoff, 2007). While B-cells are responsible for antibody production, many B-cell functions, such as immunoglobulin class switching, require T-cell help. T-cell dependent antigens (such as outer membrane proteins) are not able to directly stimulate B-cells, and therefore require a B-cell-T-cell interaction.

T-cells are important for protection against mucosal pathogens because they secrete cytokines that prime the epithelium to resist microbial threats (Pappu et al., 2012), and also because they recruit front-line phagocytes such as neutrophils to the site of infection (Appelberg, 1992). Furthermore, T-cells can induce B-cell IgA secretion as a general protective mechanism in the mucosal tissue (Bemark et al., 2012). It has been noted that T-cell deficiency in cases of primary immunodeficiency or secondary infection has resulted in an increased risk of meningococcal disease (Cohen et al., 2010; Foster et al., 2010). Therefore, cellular immunity may play an important protective role in host resistance to the meningococcus.

A second important DC function is the ability to undergo chemotaxis and migrate. DC maturation is accompanied by an up-regulation of the surface marker chemokine receptor 7 (CCR7) (Hansson et al., 2006). CCR7 is a

major chemokine receptor that binds to chemokine C-C motif chemokine 19 (CCL19), which is a constitutively expressed chemokine present in the lymphatic system (Gunn et al., 1999). Ergo, DC that are fully activated can respond to this messenger cue and migrate to lymph nodes to present antigen to T-cells.

5.2 Aims and Objectives

The aim of this chapter was to define and characterise DC function after infection with NmB. To do this, *in vitro* immunological assays were used. These assays measured chemotaxis, $CD4^+$ T-cell proliferation and $CD4^+$ T-cell differentiation. The hypothesis was that live NmB would prevent effective T-cell proliferative functions in DC.

5.3 Results

5.3.1 Live NmB suppresses the up-regulation of CCR7

CCR7 is an important receptor for the migration of DC to lymphatic tissue. DC were tested for CCR7 surface expression in response to various conditions. DC were stimulated with LPS, killed NmB and live NmB for 24h and surface expression of CCR7 was measured by flow cytometry. As Figure 5-1 shows, it was found that both LPS and killed NmB induced an increase in the percentage of $CCR7^+$ DC (39.8% and 35.2%, respectively); this was also reflected in an increase in MFI (7.03 MFI and 6.55 MFI, respectively). Live NmB inhibited CCR7 expression (23.2%), and there was also an inhibition of MFI up-regulation (4.22 MFI) compared to LPS and killed NmB. Therefore, the expression of CCR7 followed a similar pattern to other maturation markers, such as CD40 and CD86.

5.3.2 DC infected with live NmB have impaired chemotactic responses to CCL19

Given the suppression of CCR7 expression induced by live NmB, it was questioned whether this would result in DC that were unable to migrate in response to the CCR7 ligand, CCL19. To test this, an *in vitro* DC migration assay was used, based on a previously published methodology with some modifications (Bouma et al., 2007).

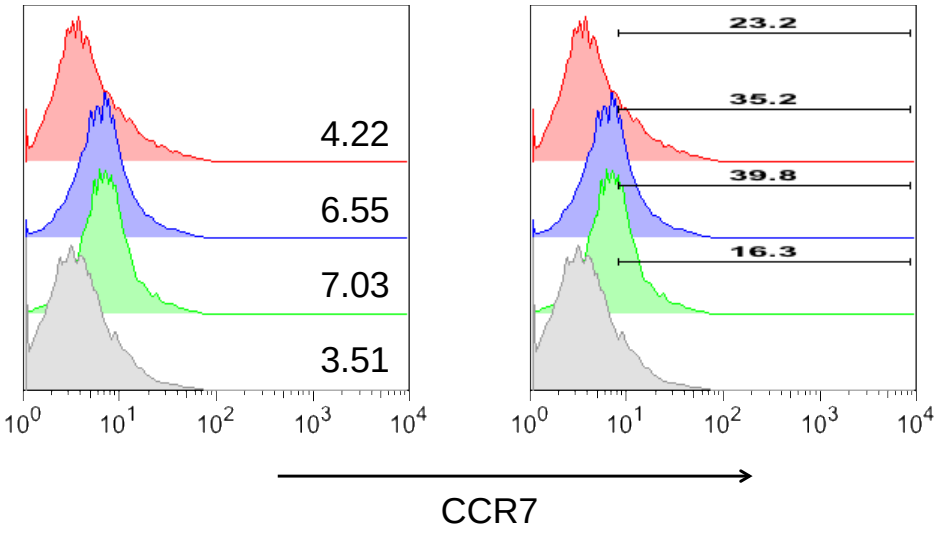

Figure 5-1 *Live NmB induces reduced expression of CCR7 on DC compared to killed NmB or LPS.* DC were stimulated with 100 ng/mL LPS (green), 10 MOI killed NmB (blue) or 10 MOI live NmB (red) for 24h. Some DC were left unstimulated (grey). Surface expression of CCR7 was then measured by flow cytometry. *Left*: Representative donor showing CCR7 MFI displayed on the histogram. *Right:* Representative donor showing percentage CCR7$^+$ DC; gating was determined by an unstained control. Data are representative of three experiments

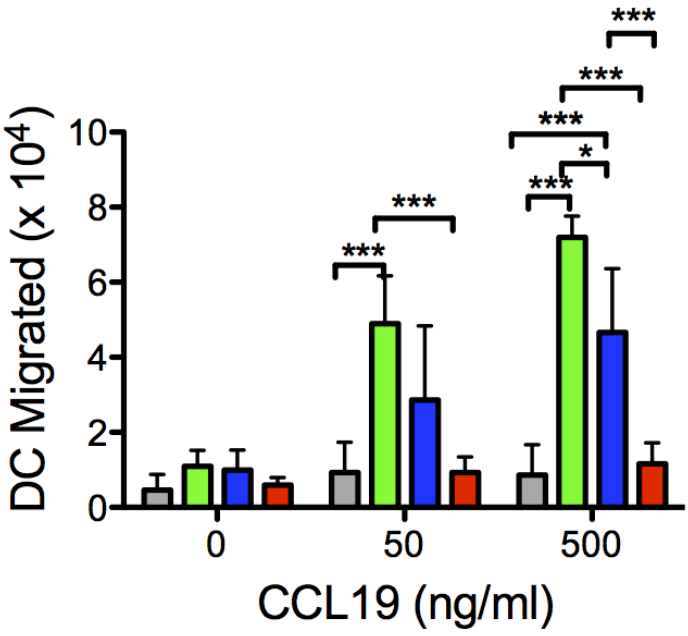

Figure 5-2 *DC infected with live NmB are hyporesponsive to CCL19 in an* in vitro *chemotaxis assay*. DC were stimulated for 24h with 100 ng/mL LPS (green), 10 MOI killed NmB (green) or 10 MOI live NmB (red). Some DC were left unstimulated (grey). DC were then washed with complete media containing 100 U/mL penicillin/streptomycin and 2 x 10^5 DC were loaded into the upper compartment of a 8 µm pore transwell. In the lower compartment were various concentrations of CCL19. After 5h, migratory DC in the lower compartment were then counted by haemocytometer in duplicate. Data are from three experiments. Significance was tested by two-way ANOVA with a Bonferroni post-test. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

DC were first stimulated with LPS, killed NmB or live NmB for 24h. Then, DC were washed in antibiotic-containing media, counted and re-seeded in the upper chamber of an 8 µm pore transwell. In the bottom chamber, increasing concentrations of CCL19 were used, alongside a negative control with no CCL19. DC were then allowed to migrate for 5h, and the migrated DC in the lower chamber were quantified by haemocytometer.

As expected, it was found that DC stimulated with LPS or killed NmB were able to migrate the most at 500 ng/mL CCL19 (mean average 6.9×10^4 and 4.7×10^4 DC, respectively). This was a significant difference (p = <0.001) compared to the unstimulated control for both conditions. DC infected with live NmB had no significant difference compared to the unstimulated control at any concentration of CCL19. Therefore, good correlation was shown for CCR7 expression and DC migration to CCL19.

5.3.3 LPS can Induce T-cell Proliferation via DC

There are numerous protocols to assess the ability of DC to stimulate T-cell proliferation. *In vitro* DC-mediated T-cell responses are subject to a high number of variables, such as ratio, co-stimuli and kinetics (Langenkamp et al., 2000), which can critically influence the potency and phenotype of the T-cell response. Therefore, to investigate the potential of DC to induce T-cell responses after infection with live NmB, an optimisation experiment using bacterial antigen (LPS) was first performed to determine the best conditions with which to base further proliferation experiments.

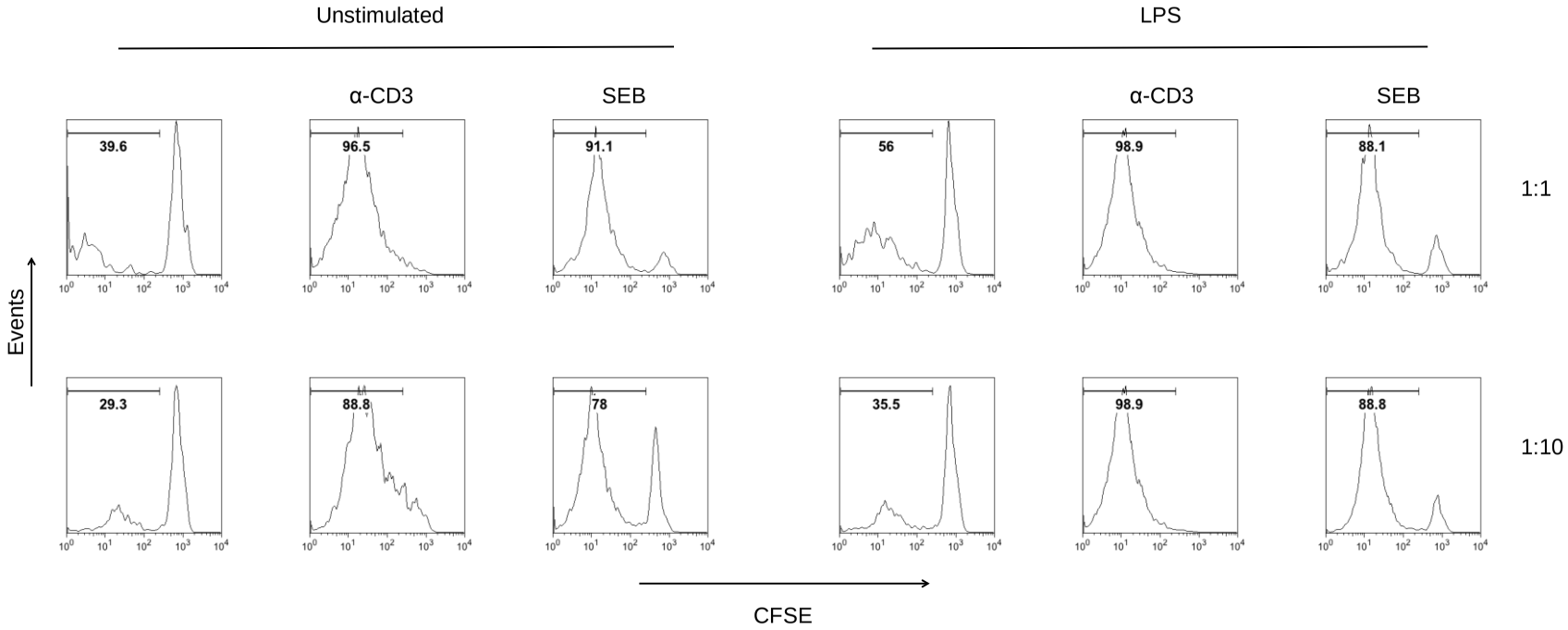

Figure 5-3 ***DC stimulated with LPS can induce robust T-cell proliferative responses.*** **DC were stimulated for 24h with 100 ng/mL LPS for 24h (right), or left unstimulated (left). DC were then washed and re-counted, and seeded at 5 x 10^4 in a 96-well plate with 1:1 or 1:10 allogeneic CFSE labelled purified $CD4^+$ T-cells for five days. For some conditions, α-CD3 (1 µg/mL soluble) or SEB (10 ng/mL) was added to the co-culture. T-cell division was then measured on day 5 by flow cytometry, using $CFSE_{dim}$ cells as an indicator or proliferation. Data are representative of two experiments.**

$CD4^+$ T-cells can respond to anti-CD3 antibodies (which mimic an MHC-peptide signal (Verma et al., 2010)), super-antigens (which bind the TCR β chain and MHC Class II (Herman et al., 1991)) and allogeneic MHC (due to the high affinity of the TCR for MHC molecules generally (Felix and Allen, 2007)). These co-stimuli were tested in an *in vitro* DC-T-cell co-culture model under various conditions. First, DC were stimulated with LPS for 24h and then washed and re-counted. DC were then co-cultured with CFSE-labelled allogeneic highly-purified (>96%) $CD4^+$ T-cells at different ratios for 5 days, and T-cell proliferation was measured by flow cytometric analysis.

The data show that for all conditions, a higher ratio of DC:CD4 T-cell was able to induce stronger proliferative responses. Also for all conditions, the use of SEB superantigen or soluble α-CD3 antibody was able to induce much stronger T-cell responses than the control without an added co-stimulus. For example, at a 1:1 ratio, unstimulated DC were able to induce 39.6% proliferation without a co-stimulus, but were able to induce 96.5% and 91.1% proliferation when the α-CD3 or superantigen was added to the co-culture. DC stimulated with LPS were also able to generally induce more T-cell proliferation than unstimulated DC. At a 1:1 ratio, DC stimulated with LPS before co-culture could induce 16.4% more proliferation than the unstimulated control.

Based on these data, it was decided that allogeneic co-cultures without the use of co-stimuli would be used for all further proliferation experiments, as the

inclusion of α-CD3 or SEB induced near-maximum T-cell division even with unstimulated DC.

5.3.4 DC infected with live NmB show reduced allostimulatory capacity

It was hypothesised that DC infected with live NmB would not induce strong T-cell proliferative responses, since DC maturation markers are critical for delivering co-stimulatory signals. To investigate this, DC were stimulated for 24h with LPS, killed NmB or live NmB and then washed in antibiotic-containing media, re-counted and co-cultured with allogeneic CFSE-labelled $CD4^+$ T-cells at a range of ratios. Some DC were cultured alone and checked for cell death for 4h after the initial antibiotic wash, and there was found to be no apoptosis during this period for any condition (data not shown). On day 5 of the co-culture, $CD4^+$ T-cells were assessed for proliferation using flow cytometry, by gating on the $CFSE_{dim}$ population. The percentage of cell division relative to the unstimulated control was then calculated.

Interestingly, at 1:2, 1:5 and 1:10 DC:$CD4^+$ T-cell ratios, DC infected with live NmB showed significantly reduced levels of induced T-cell proliferation compared to LPS and killed NmB (p = <0.01). At the 1:2 DC:$CD4^+$ T-cell ratio, LPS and killed NmB induced 70% and 76% more proliferation than the unstimulated control, respectively. By contrast, live NmB induced -16% proliferation compared to the unstimulated control. Therefore, live NmB was able to actively suppress T-cell responses at a range of ratios, whereas the positive controls LPS and killed NmB enhanced T-cell proliferation.

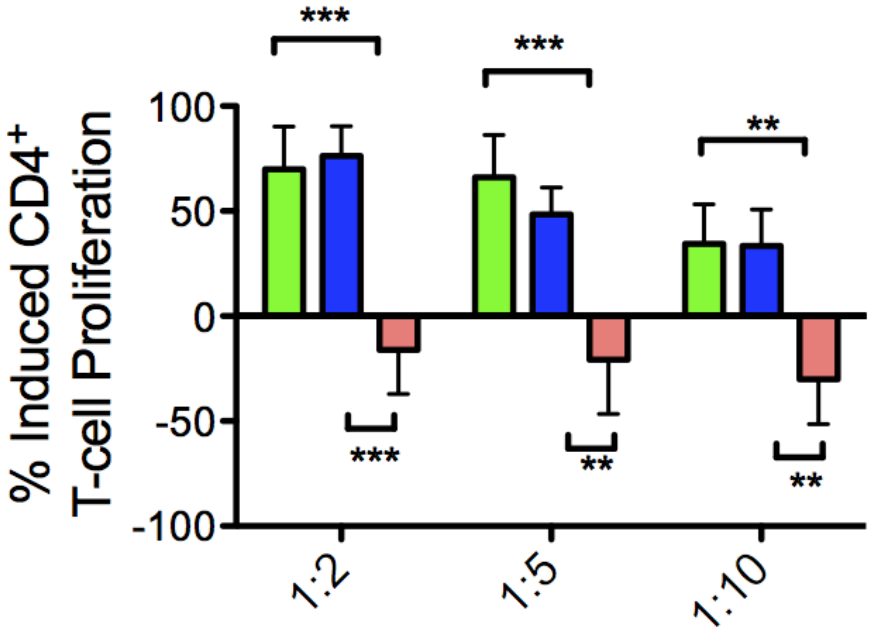

Figure 5-4 ***DC infected with live NmB induce poor CD4+ T-cell proliferative responses at a variety of ratios.*** **DC were stimulated for 24h with 100 ng/mL LPS (green), 10 MOI killed NmB (blue) or 10 MOI live NmB (red); some DC were left unstimulated. DC were then washed in complete media containing 100 U/mL penicillin/streptomycin and and re-counted, and seeded at 5 x 10^4 in a 96-well plate with 1:2, 1:5 or 1:10 allogeneic CFSE labelled purified $CD4^+$ T-cells for five days. T-cell division was then measured on day 5 by flow cytometry, using $CFSE_{dim}$ cells as an indicator of proliferation. Data are shown as percentage induction relative to the unstimulated control using the formula [(%$CFSE_{dim}$ stimulus - %$CFSE_{dim}$ unstimulated control) / %$CFSE_{dim}$ unstimulated control x 100] Data are from four individual donors. Significance was tested by two-way ANOVA with a Bonferroni post-test. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$**

5.3.5 PD-L1 up-regulation by live NmB can be suppressed by inhibiting the MAPKs

Since live NmB seemed to be actively suppressing the induction of T-cell proliferation, it was speculated that the selective up-regulation of PD-L1 (see Chapter 3) might be actively inhibiting T-cell division. Therefore, it was first seen whether PD-L1 up-regulation by live NmB could be prevented, in order to test this hypothesis.

It has been reported that PD-L1 up-regulation on APCs is driven by MAPK signalling (Wölfle et al., 2011), therefore this signal transduction pathway was interrogated. ERK (via MEK), p38 or JNK were inhibited with the following inhibitors: PD 325901, SB 203580 or SP 600125. The remaining DC were cultured in a vehicle control (0.5% DMSO). DC were then infected with live NmB. As can be seen in Figure 5-5, the inhibition of all three MAPKs suppressed PD-L1 up-regulation in response to live NmB infection to various extents. With MEK inhibition, PD-L1 was lowered from 1225 MFI to 703 MFI; with p38 inhibition 1225 MFI to 492 MFI, and with JNK inhibition 1225 MFI to 328 MFI. Therefore, JNK was the most potent at inducing PD-L1 expression. When tested in multiple donors, there was a significant trend for JNK inhibition to greatly reduce PD-L1 expression induced by live NmB (p = < 0.01). From these data, it can be concluded that live NmB utilised MAPKs to induce PD-L1 expression.

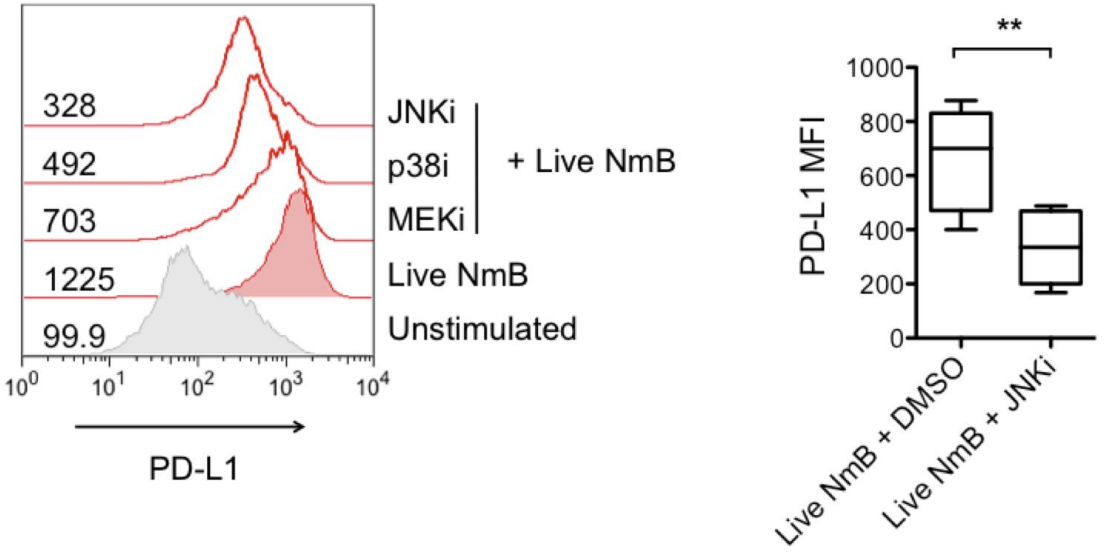

Figure 5-5 ***The up-regulation of PD-L1 by live NmB depends on MAPK signalling*****. DC were stimulated for 24h with 10 MOI live NmB in the presence of a vehicle control (0.5% DMSO). For some conditions, DC were infected in the presence of 10 μM MEK inhibitor (MEKi) (PD 325901), 10 μM p38 inhibitor (p38i) (SB 203580) or 10 μM JNK inhibitor (JNKi) (SP 600125). Some DC were left unstimulated. Surface expression of PD-L1 was then assessed by flow cytometry. *Left:* Histogram showing unstimulated DC (grey), live NmB-infected DC (red), and live NmB-infected DC in the presence of various inhibitors (red unshaded). MFI values are displayed on the histogram. Data are from a representative donor. *Right:* Pooled data from six individual donors showing PD-L1 MFI in response to live NmB, either in the presence of a vehicle control (0.5% DMSO) or 10 μM JNKi. Box-plots depict medians and interquartile ranges. Significance was tested by paired t-test. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$**

5.3.6 PD-L1 inhibition can enhance T-cell proliferation induced by DC infected with live NmB

The extent to which selective PD-L1 up-regulation contributed towards the suppression of $CD4^+$ T-cell proliferation was next investigated. It was hypothesised that inhibition or blockade of PD-L1 might enhance the T-cell responses induced by DC infected with live NmB.

DC were stimulated for 24h with LPS, killed NmB or live NmB and then washed in antibiotic-containing media, re-counted and co-cultured with allogeneic CFSE-labelled $CD4^+$ T-cells at a range of ratios. For some conditions, DC were also cultured with a JNK inhibitor (SP 600125) during infection. For other conditions, a PD-L1 blocking antibody was used during the entirety of the co-culture. As can be seen in Figure 5-6, live NmB induced poor T-cell proliferative responses compared to LPS and killed NmB (9% vs 68.1% and 67.3%, respectively). However, the use of the JNK inhibitor greatly enhanced T-cell proliferation (41.5%), and so did the use of the PD-L1 blocking antibody (29.7%). These levels almost reached the level induced by the unstimulated control (54%). These data suggest PD-L1 is essential for the active suppression of $CD4^+$ T-cell proliferation by DC infected with live NmB.

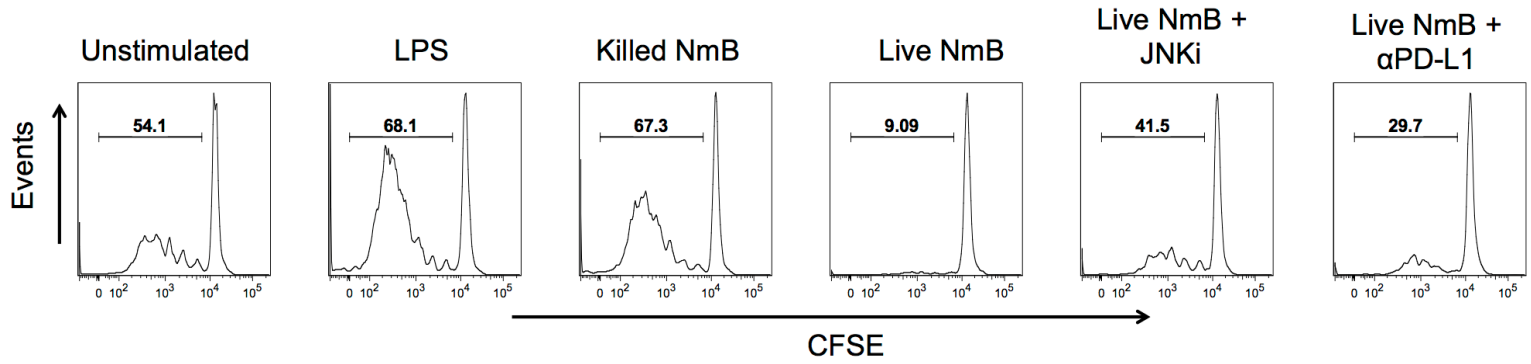

Figure 5-6 ***Inhibition of JNK or the blockade of PD-L1 enhances the T-cell proliferative responses in DC infected with live NmB*****. DC were stimulated for 24h with 100 ng/mL LPS, 10 MOI killed NmB or 10 MOI live NmB; some DC were left unstimulated. All were cultured in the presence of a 0.5% DMSO vehicle control. For some conditions, DC were also cultured with 10µM JNKi (SP 600125). DC were then washed in complete media containing 100 U/mL penicillin/streptomycin and and re-counted, and seeded at 5 x 10^4 in a 96-well plate with 1:5 allogeneic CFSE labelled purified $CD4^+$ T-cells for five days. For some conditions, a polyclonal PD-L1 blocking antibody (50 µg/mL) was added to the culture. T-cell division was then measured on day 5 by flow cytometry, using $CFSE_{dim}$ cells as an indicator or proliferation. Data are representative of two experiments.**

5.3.7 DC infected with live NmB show reduced T_H1-like responses

DC are able to polarise naive $CD4^+$ T-cells into T_H1 or T_H2 effectors, which can be defined by the ability to produce IFN-γ and IL-4. To test the ability of live NmB to induce these T-cell cytokine signatures, T-cells were analysed for intracellular cytokine production.

DC were stimulated with either killed NmB or live NmB and then co-cultured with allogeneic high purity (>98%) naive ($CD45RA^+$) $CD4^+$ T-cells for 5 days. T-cells were then stimulated with PMA and ionomycin in the presence of brefeldin A for 3h, and analysed for IFN-γ and IL-4 by intracellular flow cytometry. It was found that T-cells only produced negligible amounts of cytokine (Figure 5-7), with DC stimulated with killed NmB inducing 4.03% IFN-γ^+ T-cells and live NmB inducing only 1.53% IFN-γ^+ T-cells. However, there was a trend for killed NmB inducing more T_H1-type responses and live NmB inducing more T_H2-type responses, as live NmB induced 5.82% IL-4^+ T-cells while killed NmB only induced 2.95% IL-4^+ T-cells.

It was therefore decided that a protocol utilising the superantigen SEB and the use of IL-2 expansion as previously described (van Beelen et al., 2007) would be used to achieve higher levels of cytokine production. As controls for T_H1 and T_H2 polarisation, poly(I:C) (T_H1) and cholera toxin (CT) (T_H2) were used, as these have been previously reported to induce such responses (de Jong et al., 2002).

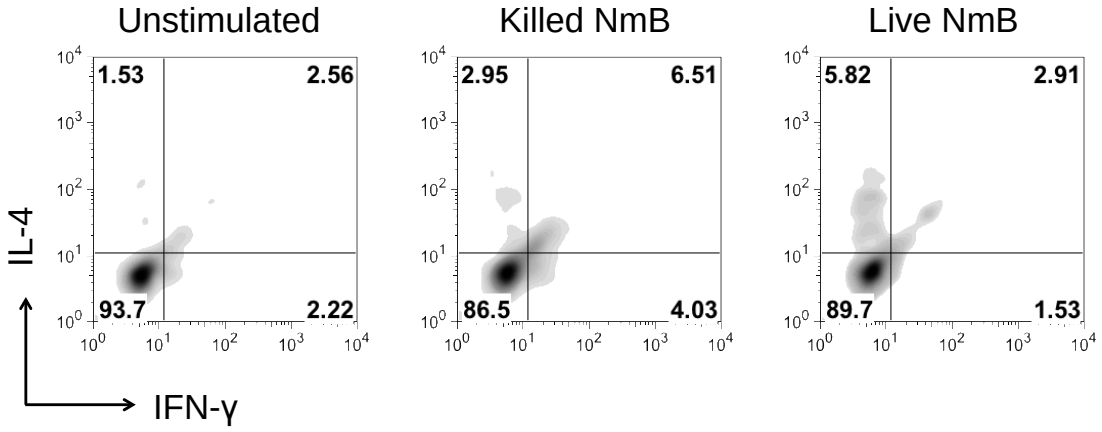

Figure 5-7 ***Allogeneic co-cultures in the absence of co-stimuli induce minimal cytokine production in naive CD4$^+$ T-cells.*** **DC were stimulated with 10 MOI killed NmB, 10 MOI live NmB or left unstimulated for 24h. DC were then washed in complete media containing 100 U/mL penicillin/streptomycin and and re-counted, and seeded at 5 x 10^4 in a 96-well plate with 1:5 allogeneic purified naive CD45RA$^+$ CD4$^+$ T-cells for five days. On day 5, T-cells were stimulated with PMA (100 ng/mL) and ionomycin (1 µg/mL) for 3h in the presence of brefeldin A (1 µg/mL) for 3 h. Intracellular levels of IL-4 and IFN-γ were then determined by intracellular flow cytometry, using a permeabilisation protocol. Gates were set by an isotype control on live T-cells. Data are representative of three experiments.**

DC were stimulated for 24h with LPS, killed NmB, live NmB, poly(I:C) or CT. DC were then washed in antibiotic-containing media, re-counted and co-cultured with allogeneic naive $CD4^+$ T-cells in the presence of SEB. On day 5, T-cells were expanded with IL-2. On day 12-14, T-cells were then stimulated with PMA and ionomycin for 3h in the presence of brefeldin A, and production of IFN-γ and IL-4 were measured by intracellular flow cytometry.

As Figure 5-8 shows, it was found that both poly(I:C) and CT were able to induce IFN-γ (unstimulated: 34.6% IFN-γ^+; poly(I:C): 42.7% IFN-γ^+) and IL-4 (unstimulated: 8.43%; CT: 11.8%) T-cell cytokine production above that of the unstimulated control. Therefore, DC were able to effectively polarise naive T-cells to desired phenotypes. With regards to NmB, killed NmB induced 57.1% IFN-γ^+ and 9.47% IL-4^+ T-cells, while live NmB induced 49.3% IFN-γ^+ and 15% IL-4^+ T-cells. The reduction in T_H1-type cells was found to be significantly reduced across multiple donors by live NmB in comparison to killed NmB (p = < 0.01), while there was a non-significant trend in most donors for live NmB to induce stronger T_H2-type responses (Figure 5-9).

Taken together, these data demonstrate that live NmB can suppress T-cell proliferation via PD-L1, and also impair the generation of T_H1-type responses in naive $CD4^+$ T-cells.

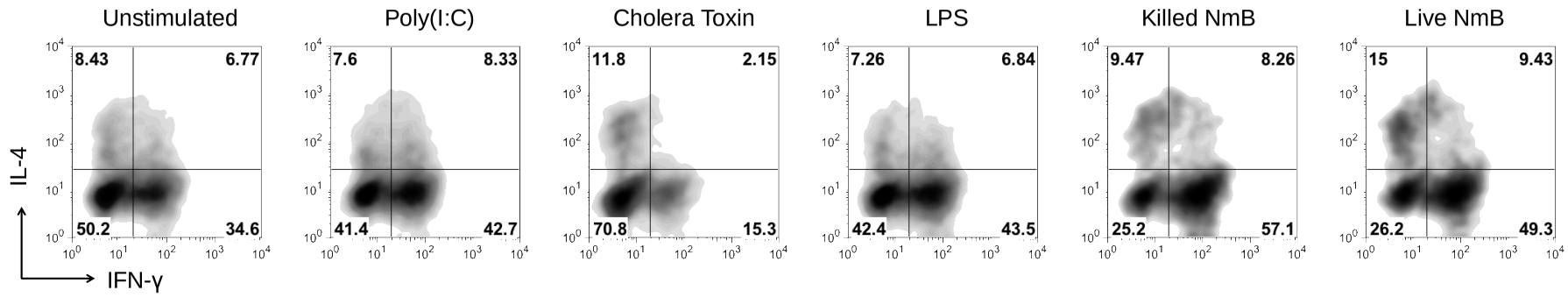

Figure 5-8 ***Killed- and live NmB-stimulated DC induce differential CD4$^+$ T-cell cytokine responses*****. DC were stimulated with 10 µg/mL poly(I:C), 1µg/mL cholera toxin, 100 ng/mL LPS, 10 MOI killed NmB, 10 MOI live NmB or left unstimulated for 24h. DC were then washed in complete media containing 100 U/mL penicillin/streptomycin and and re-counted, and seeded at 5 x 10^4 in a 96-well plate with 1:5 allogeneic purified naive CD45RA$^+$ CD4$^+$ T-cells for five days. On day 5, T-cells were expanded in fresh media with 10 U/mL rhIL-2 for a further 7-9 days. Cells were then stimulated with PMA (100 ng/mL) and ionomycin (1 µg/mL) for 3h in the presence of brefeldin A (1 µg/mL) for 3 h. Intracellular levels of IL-4 and IFN-γ were then determined by intracellular flow cytometry, using a permeabilisation protocol. Gates were set by an isotype control. Data are representative of five experiments.**

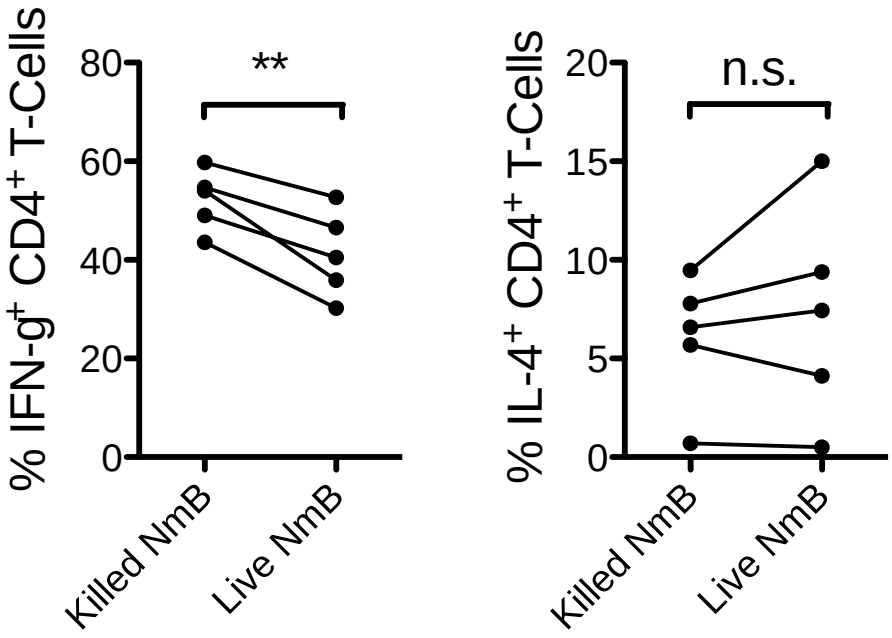

Figure 5-9 *Live NmB suppresses the induction of T_H1-type responses by DC.* DC were stimulated with 10 MOI killed NmB, 10 MOI live NmB or left unstimulated for 24h. DC were then washed in complete media containing 100 U/mL penicillin/streptomycin and re-counted, and seeded at 5 x 10^4 in a 96-well plate with 1:5 allogeneic purified naive $CD45RA^+$ $CD4^+$ T-cells for five days. On day 5, T-cells were expanded in fresh media with 10 U/mL rhIL-2 for a further 7-9 days. Cells were then stimulated with PMA (100 ng/mL) and ionomycin (1 µg/mL) for 3h in the presence of brefeldin A (1 µg/mL) for 3 h. Intracellular levels of IL-4 and IFN-γ were then determined by intracellular flow cytometry, using a permeabilisation protocol. Data are from five individual donors. Significance was tested by paired t-test. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

5.4 Discussion

DC migration and T-cell stimulation are the critical immunological 'outputs' of the maturation process. Herein, it was found that live NmB modulated both of these processes, resulting in functionally paralysed DC. This is a logical finding given the previous data, considering the phenotype induced by the viable bacterium that was explored in the Chapter 3.

DC migration was impaired by live NmB in a CCL19 chemotaxis assay, showing minimal chemotaxis compared to the unstimulated control. This correlated with CCR7 expression, since live NmB also failed to up-regulate/induce CCR7 on DC. It should be noted that there was a significant trend for LPS to induce more chemotaxis than killed NmB at 500 ng/mL CCL19, and a non-significant trend at 50 ng/mL. This may be partially explained by the modest increase in expression of CCR7 induced by LPS. Alternatively, the phagocytosis of whole bacteria may have led to DC being unable to migrate as effectively through the transwell pores.

In vitro CCL19 migration assays correlate well to *in vivo* DC migration, as has been previously published. For example, the parasite *Toxoplasma gondii* can induce the up-regulation and acquisition of DC CCR7 *in vitro*, which renders DC hypermigratory to CCL19. The parasite can induce the same phenotype *in vivo*, which then allows it to hijack migratory DC and gain access to the lymphatic tissue of the brain in mice (Fuks et al., 2012). Similarly, Wiskott-Aldrich syndrome patients have impaired DC chemotaxis in transwell CCL19

assays *in vitro*, and this is reflected in Wiskott-Aldrich syndrome protein (WASP) mutant murine DC (Bouma et al., 2007).

An important limitation to these data is that CCL19-mediated chemotaxis is just one aspect of DC motility and migration. General DC motility in response to activatory stimuli involves more widespread changes in cytoskeletal structure and actin filamentation besides CCR7 up-regulation (Verdijk et al., 2004). If time had permitted, a DC-endothelial cell assay in order to investigate the effect of live NmB on DC endothelial transmigration would have been carried out. Moreover, although DC can migrate to the lymphatic tissue and polarise naive T-cell differentiation, T-cells can patrol lymphatic tissue and home to mucosal sites of inflammation and infection (Sallusto et al., 1999). Therefore, these data describing the inhibited migratory functions of DC in response to live meningococci must be considered in the wider context of more general impaired immunological functions.

It was also found that live NmB induced the selective up-regulation of PD-L1 via the MAPKs ERK, p38 and JNK, although JNK inhibition was the most potent at reducing its expression. All of these signalling molecules were shown to be activated by live NmB in the previous chapter. This corresponds to other findings in the literature; a MAPK-STAT3 axis was found to induce PD-L1 expression in human monocytes (Wölfle et al., 2011), and a study on melanoma cells found that the JNK-induced c-Jun transcription factor binds to the PD-L1 promoter to induce up-regulation and immunotolerance (Jiang et al., 2013). It can therefore be concluded that live NmB uses these pathways to induce PD-L1 up-regulation in the absence of other maturation marker

expression, as PD-L1 expression does not appear to be dependent on STAT1.

Live NmB was able to suppress the induction of DC-mediated CD4+ T-cell proliferation at all ratios tested. In many donors, this was below the level of proliferation induced by the unstimulated control. This may have been due to the selective up-regulation of the potent T-cell inhibitory receptor PD-L1, as the use of a JNK inhibitor or a PD-L1 blocking antibody on DC infected with live NmB was able to enhance the ability of these DC to induce T-cell proliferation. Notably, these two treatments were not able to induce T-cell proliferation resembling the positive controls killed NmB and LPS, presumably because DC infected with live NmB remained $CD86^{lo}$.

There was a general trend for lower levels of proliferation at higher ratios of T-cells. This is unsurprising, as fewer available DC results in a reduction in available positive signals per T-cell. Interestingly, there was always a notable background of proliferation in the unstimulated control. This may be explained by the fact that DC are not truly negative for any of the co-stimulatory molecules, such as CD40 and CD86. Indeed, DC maturation is an up-regulation of these markers that are acquired upon differentiation from monocytic precusors. DC are phenotypically defined by the presence of these molecules, which are expressed at higher levels compared to other cell types even in an immature state; therefore it can be expected that even immature DC are capable of inducing a moderate level of proliferation.

It is possible that apoptosis may account for the suppression of DC infected with live NmB to induce T-cell proliferation. However, this is unlikely to be the main driver of suppressed T-cell proliferation for several reasons. Although live NmB induces higher levels of apoptosis in DC (Jones et al., 2007), DC viability stabilised upon the re-culturing of DC in fresh antibiotic-containing media, presumably because of the removal and killing of bacteria (data not shown). Additionally, the use of a blocking PD-L1 antibody markedly boosted the T-cell proliferation induced by DC infected with live NmB, and it is unlikely that a blocking antibody would affect DC viability. Together, these data enforce the importance of the DC surface marker repertoire in determining the resulting T-cell responses.

A secondary effect was that T-cells co-cultured with DC that had been infected with live NmB were less able to produce IFN-γ in response to PMA and ionomycin stimulation. Killed NmB was found to effectively induce these T_H1-type cytokines in T-cells, as has been previously published in a study investigating a panel of Gram-negative bacteria and their ability to induce T_H1/T_H17 responses (van Beelen et al., 2007). T_H1-type responses have been shown to correlate with CD40 expression and the general maturation state of the DC. Hence, immature DC are more likely to induce a T_H2-type response, while mature DC are more biased towards T_H1-type responses (de Heusch et al., 2004; Langenkamp et al., 2000). There was also a non-significant trend between the ability of killed NmB and live NmB stimulated DC to induce IL-4 production, as live NmB induced more of this cytokine in most (3 out of 5) of the co-culture assays. While interesting, these observations are most

probably less important than the overall suppression of T-cell responses, since the cytokine assays depended on expansion of the T-cells with exogenous IL-2 and supernantigen.

A major limitation of these data is that the T-cell experiments were performed in an antigen-independent system. SEB and allogeneic MHC were used to induce T-cell responses, but these are non-specific and will always activate a certain percentage of T-cells. It would be interesting to replicate these experiments by using a specific recall antigen in vaccinated volunteers, such as tetanus toxoid (TT), as has been described elsewhere (Le Bert et al., 2011).

In summary, these data show that live NmB is able to disable two core DC functions: chemotaxis and T-cell stimulation. $CD4^+$ T-cells are critical for the protection of mucosal surfaces, and so these data may represent a method of immune evasion by NmB via DC dysregulation. However, while these data suggest that the selective up-regulation of PD-L1 on DC may induce active repression of T-cell proliferation, they still do not uncover the mechanism of STAT1-Y701 dephosphorylation that is required for full maturation. The mechanism behind this dephosphorylation is explored in the following chapter.

6 Chapter 6: Reversing the immunomodulation of DC by *N. meningitidis*

6.1 Introduction

The data in the previous chapters shows that live NmB suppressed DC maturation by selective tyrosine dephosphoryation of STAT1. This correlated with reduced levels of maturation markers, reduced levels of anti-inflammatory SOCS genes *SOCS1* and *CISH*, and enhanced cytokine production. Furthermore, these DC were poorly immunogenic in allogeneic co-culture models. Given the possibility that a host or bacterial effector might be interfering with this pathway, the mechanism(s) behind the deactivation of STAT1 were investigated.

The manipulation of host cell signalling by pathogens is a relatively common occurrence. Many pathogens encode enzymes specifically for this purpose, and have no other known role in bacterial cell physiology. Bacteria usually require a secretion system to inject effectors into the host cell. A notable example is the type 3 secretion system (T3SS) of *Yersinia* spp., which can secrete immuno-modulatory proteins into human phagocytes. For example, *Yersinia* outer protein P (YopP) can prevent DC from functionally maturing by inhibiting the MAPK signal transduction cascade (Autenrieth et al., 2007). This requires direct contact between the human cell and the viable bacterium.

Many pathogens, particularly viruses, have been shown to modulate the STAT1 pathway specifically (Goodbourn et al., 2000). For example, vaccinia

virus (VV) encodes a protein called VH1, which is a dual-activity tyrosine and serine phosphatase (Guan et al., 1991). This phosphatase can dephosphorylate STAT1, resulting in impaired signalling in cell lines and interference with IFN responses (Najarro et al., 2001). Furthermore, VV infection *in vivo* interferes with APC MHC Class II expression via STAT1 deactivation, and thus limits effective T-cell responses (Li et al., 2005). Coincidentally, VV can also block human DC maturation by an unknown mechanism, and these two findings have not yet been linked by the authors of the studies (Engelmayer et al., 1999). Another prominent example is the measles virus, which encodes a protein known as 'Measles P Protein', which can prevent STAT1 binding effectively to the IFNR-JAK complex, thus preventing phosphorylation and signal transduction (Devaux et al., 2013).

Pathogens can also prevent STAT1 signalling via host enzymes. Cytomegalovirus (CMV) can induce the activation of the host tyrosine/serine phosphatase SHP-2, which dephosphorylates STAT1 and thereby prevents the up-regulation of HLA-DR (Baron and Davignon, 2008). It is worth noting that CMV can also suppress DC maturation through an unknown mechanism (Moutaftsi et al., 2002) while inducing selective up-regulation of PD-L1 (Benedict et al., 2008); although again, these phenomena have not been connected within the literature. Influenza A virus, too, can inhibit STAT1 signal transduction by hyper-activation of NF-κB (Pauli et al., 2008). Over-triggering of NF-κB can elevate SOCS3 protein levels, resulting in blockade of IFN signal transduction.

Modulation of STAT1 signal transduction is less well characterized for bacteria, however there are a few notable examples in the literature. Viable—but not inactivated—enterohaemorraghic *E. coli* (EHEC) can down-regulate STAT1 phosphorylation by an unidentified, heat-sensitive secretory product (Ceponis et al., 2003). However, it is unknown what implications this finding has regarding immune responses to the bacterium. Similarly to Influenza A, *L. monocytogenes* can induce up-regulation of *SOCS3* and prevent STAT1 phosphorylation in macrophages (Stoiber et al., 2001). Using a systems biology approach, one study found that *Brucella abortus* down-modulates the IFN pathway in DC, correlating with less STAT1 transcriptional activity and a less mature phenotype (Gorvel et al., 2014). However, this is likely to be through TLR recognition, rather than a specific targeting of the IFN pathway.

Currently, no data exist in the literature regarding the role of STAT1 in meningococcal infection. While STAT1 is required for DC to induce effective T-cell responses *in vivo* (Longhi et al., 2009), it has a complex and ambiguous role in bacterial sepsis and immunity. STAT1 signalling in DC is crucial for the protection of mice against lethal *L. monocytogenes* infection (Kernbauer et al., 2012). The same study also found that STAT1-deficient mice have enhanced levels of inflammatory cytokines, consistent with the proposed model herein of meningococcal modulation of DC. Moreover, STAT1-dependent negative regulators of cytokine production, such as SOCS1, have been shown to restrict the inflammatory response in murine models of sepsis, implicating STAT1 in a protective role (Kinjyo et al., 2002; Nakagawa et al., 2002; Tanimoto et al., 2005). However, STAT1-deficient mice are noted for being

more resistant to the onset of septicaemia after endotoxin challenge (Kamezaki et al., 2004), correlating with reduced levels of pro-inflammatory cytokine in the peripheral blood. This discrepancy may be explained by the different stimuli used in these assays, such as the use of whole organisms versus specific components. Alternatively, STAT1-deficient mice may have more highly inflammatory DC (due to lowered SOCS protein expression), but the overall effect could be anti-inflammatory in certain circumstances due to the lack of DC-initiated, $CD4^+$ T-cell-derived cytokines.

In this chapter, the mechanism of STAT1 tyrosine dephosphorylation was explored further. Experiments were carried out to reverse STAT1 signal transduction modulation via pharmacological inhibitors, to determine if this would result in reversion of DC to conventional phenotype and function. Finally, possible bacterial factors responsible for the immunomodulation of DC were investigated.

6.2 Aims and Objectives

The aim of this chapter was to reverse the phenotype exerted on DC by live NmB. To do this, various inhibitors were used to uncover the mechanism behind STAT1 tyrosine dephosphorylation. The hypothesis was that reversal of STAT1-Y701 dephosphoryation would revert all aspects of DC phenotype that were modulated by live NmB.

6.3 Results

6.3.1 Dysregulation of DC maturation by live NmB is independent of apoptosis

The role of apoptosis in the modulation of DC maturation was first investigated. Pathogens can induce DC apoptosis as a method of escaping cellular immune responses (Kushwah and Hu, 2010). It has previously been reported that live NmB can induce more apoptosis in DC than killed bacteria, but is likely to be independent to the inhibition of phenotypic maturation (Jones et al., 2007).

Importantly, 'alternative activation' of STAT1 (serine phosphorylated, tyrosine dephosphorylated) has been described as having pro-apoptotic activity in immune and non-immune cells. The serine phosphorylation is critical for the induction of STAT1-mediated apoptosis (Kim and Lee, 2005; Stephanou et al., 2001). Biochemical analysis has revealed that this is via a caspase-dependent mechanism.

It was questioned whether the selective serine phosphorylation of STAT1 could explain the earlier finding of increased apoptosis of DC in response to viable NmB infection. To test this hypothesis, an annexin-V assay, based on binding of annexin-V to 'flipped' phosphatidylserine only present on apoptotic cells, was used as a indicator of DC apoptosis.

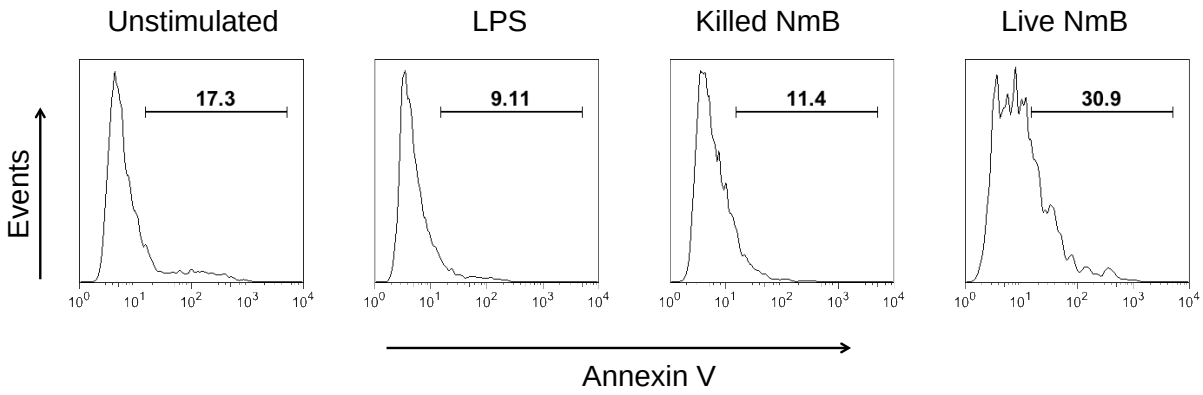

Figure 6-1 *Live NmB induces more apoptosis in DC than killed NmB*. DC were stimulated for 24h with 100 ng/mL LPS, 10 MOI killed NmB or 10 MOI live NmB. Apoptosis was then measured by flow cytometry, using annexin-V$^+$ DC as an indicator of apoptosis. Gates were set by an unstained control. Data are representative of three experiments.

To confirm previous findings, DC were stimulated for 24h with LPS, killed NmB or live NmB and stained for phosphostidylserine expression via FITC-labelled annexin-V. Consistent with previous work (Jones et al., 2007), live NmB was found to induce more apoptosis than killed NmB and LPS (Figure 6-1), as indicated by approximately three-fold more annexin-V^+ DC (live NmB: 30.9%, LPS: 9.11%, killed NmB: 11.4%).

Since p38 has been reported to phosphorylate STAT1 on the S727 residue (Goh et al., 1999), and it was also found that live NmB induced p38 activation (as shown in Chapter 4), a well-characterised inhibitor (SB 203580) was used to reverse S727 phosphorylation. DC were infected with live NmB for 24h either in the presence of the p38 inhibitor or a vehicle control. Levels of STAT1-S727 phosphorylation were then determined by intracellular PhosFlow cytometry (Figure 6-2). As expected, the use of a p38 inhibitor was sufficient to reduce the levels of STAT1-S727 phosphorylation induced by live NmB.

Next, to see if p38 inhibition could reverse the pro-apoptotic effects of live bacteria, DC were stimulated with LPS, killed NmB or live NmB for 24h and apoptosis was gauged by live FSC-SSC gating (as previously described (Handley et al., 2005)) and annexin-V staining. For some conditions, DC were stimulated in the presence of a p38 inhibitor.

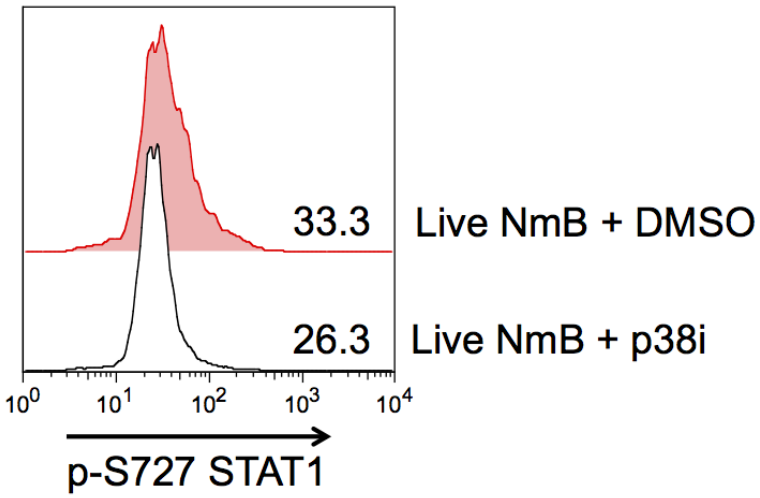

Figure 6-2 *STAT1-S727 phosphorylation induced by live NmB is dependent on p38*. DC were stimulated for 18h with 10 MOI live NmB in the presence of the p38 inhibitor SB 203580 (1 µg/mL) (white histogram) or equivalent vehicle control (0.1 % DMSO) (red histogram). STAT1-S727 phosphorylation was then measured by intracellular PhosFlow cytometry. MFI values are displayed on the histograms. Data are representative of two experiments.

As Figure 6-3 shows, live NmB-infected DC cultured in the presence of a p38 inhibitor showed a higher percentage of viable cells (p = <0.01) compared to the vehicle control, as determined by percentage of live gated DC. Furthermore, as shown in Figure 6-4, DC stimulated in the presence of a p38 inhibitor showed markedly reduced apoptosis. This was more prominent in DC stimulated with live NmB, where p38 inhibition almost completely reverted the levels of apoptosis to that of the unstimulated control (live NmB DC: 28.6% annexin-V^+ vs. live NmB + p38i DC: 10.8% annexin-V^+). By contrast, p38 inhibition had only a minor reduction on the apoptosis induced by killed NmB (7.03% annevin-V^+ vs. 5.98% annevin-V^+, respectively).

To determine the specificity of p38 inhibition controlling apoptosis, a control JNK inhibitor (SP 600125) was also tested. This was used to determine whether apoptosis was a general property of MAPK activation, or specific to p38 alone. DC were infected with live NmB with either a vehicle control or JNK inhibitor for 24h, and annexin-V staining was determined by flow cytometry. Unlike p38 inhibition, JNK inhibition did not cause a reduction in annexin-V staining (Figure 6-5). Moreover, there was a slight increase in apoptosis with JNK inhibition (29.7% vs. 40.7%, respectively).

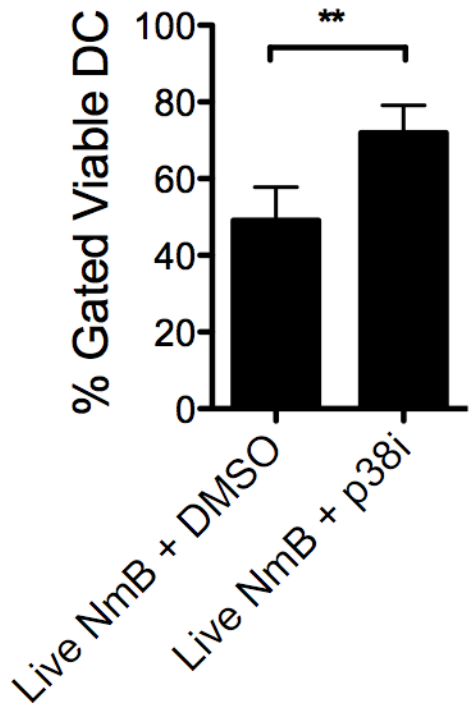

Figure 6-3 *Inhibition of p38 leads to an increase in viability in DC infected with live NmB.* DC were infected with 10 MOI live NmB for 24h in the presence of p38 inhibitor SB 203580 (1 µg/mL) or vehicle control (0.5% DMSO). Viability was then determined by flow cytometry, by measuring percentage of DC within the FSC-SSC live DC gate. Data are from give donors. Significance was tested by paired t-test. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Importantly, p38 inhibition was not able to restore DC CD86 expression after 24h infection with live NmB in these assays, highlighting the distinctness of maturation and apoptosis in this system (Figure 6-6). These data strongly suggested that p38 activation by live NmB induced selective STAT1-S727 phosphorylation, which was the main driver of apoptosis in this infection model. However, this pro-apoptotic effect was distinct from the inhibition of DC maturation marker up-regulation.

6.3.2 Live capsule, PilE, RmpM deficient NmB induce similar DC maturation profiles to live WT NmB

N. meningitidis possesses several virulence factors that determine the pathogenic potential of the organism and also influence the interaction of the bacterium with the human immune system. Capsule- or LOS-deficient NmB is differentially recognised by human DC, eliciting a dissimilar phenotype to wild-type organisms. It was hypothesised that these virulence factors might be responsible for the observed phenotype. To test this, DC were exposed to live and killed variants of several NmB mutants for 24h: *SiaD*, a capsule-deficient mutant, *PilE*, a pilus-deficient mutant and *RmpM*, a mutant deficient for an OmpA homologue, chosen for its similarity to a common protein across many bacterial species found to inhibit DC maturation (Mittal et al., 2009, 2010). Surface expression of CD40 was then measured by flow cytometry.

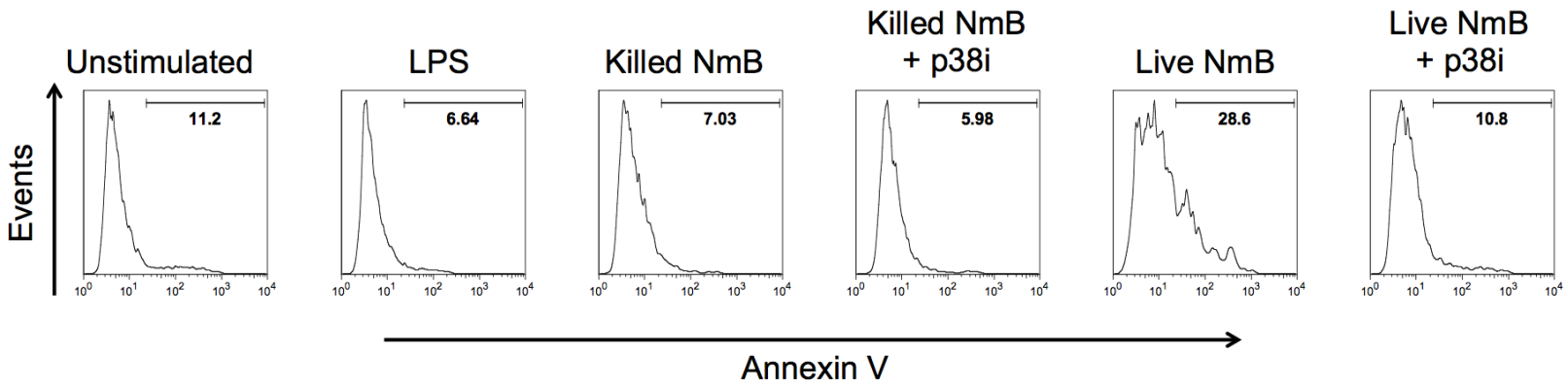

Figure 6-4 ***Inhibition of p38 rescues DC infected with live NmB from apoptosis.*** **DC were stimulated for 24h with 100 ng/mL LPS, 10 MOI killed NmB or 10 MOI live NmB, with either a vehicle control (0.5% DMSO) or the p38 inhibitor SB 203580 (1 µg/mL). Apoptosis was then measured by flow cytometry, using annexin V^+ DC as an indicator of apoptosis. Gates were set by an unstained control. Data are representative of three experiments.**

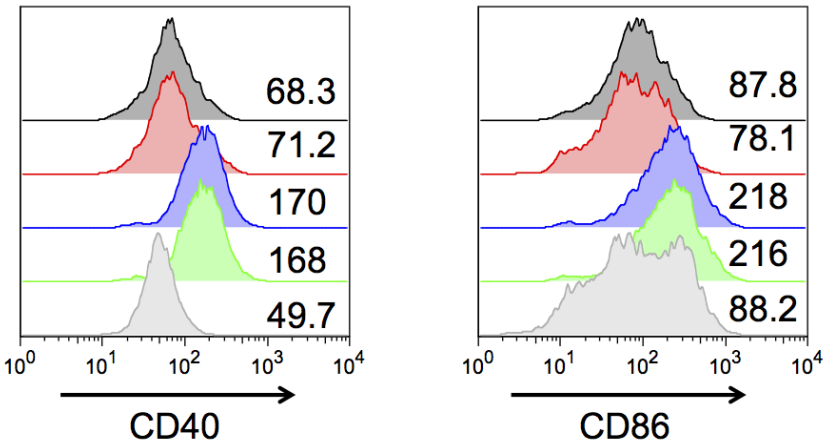

Figure 6-5 ***Inhibition of p38 does not restore DC maturation after infection with live NmB.*** **DC were stimulated with 100 ng/mL LPS (green), 10 MOI killed NmB (blue), 10 MOI live NmB (red) or left unstimulated (grey) in the presence of vehicle control (0.5 % DMSO) or live NmB + p38 inhibitor SB 203580 (1 µg/mL) (black). Surface expression of CD40 and CD86 was then measured by flow cytometry. MFI are displayed on the histograms. Data are representative of two experiments.**

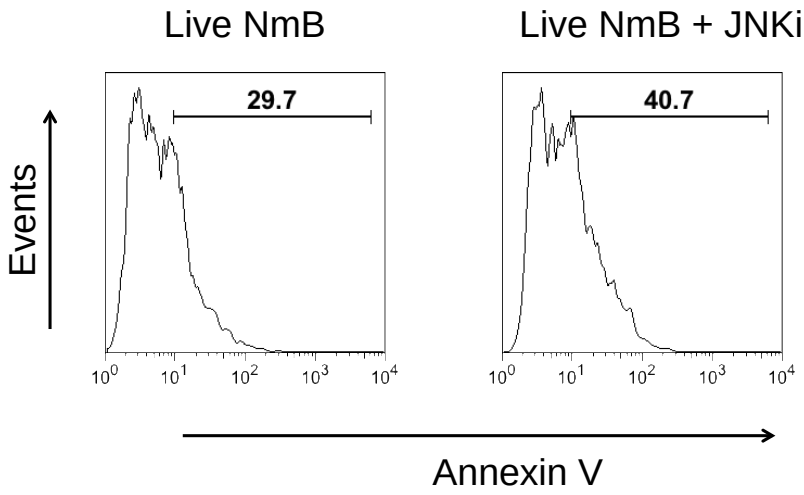

Figure 6-6 *JNK inhibition does not down-modulate apoptosis during infection with live NmB.* DC were stimulated for 24h with 10 MOI live NmB in the presence of a vehicle control (0.5 % DMSO) or JNK inhibitor SP 600125 (1 µg/mL). Apoptosis was then measured by flow cytometry, using annexin V^+ DC as an indicator of apoptosis. Gates were set by an unstained control. Data are representative of one experiment.

As Figure 6-7 displays, all viable mutants failed to induce the up-regulation of the maturation marker CD40 comparable to their isogenic killed counterparts. Therefore, it was concluded that the inhibition of DC maturation by live NmB was independent of these known virulence factors.

6.3.3 Suppression of DC surface phenotype maturation by live NmB is independent of CEACAM1 and SHP-2

Several lines of evidence have supported the notion that *N. meningitidis* can bind to CEACAM receptors and modulate intracellular signalling. As previously mentioned, *Neisseria* spp. express Opa proteins that can bind to CEACAM and induce host tyrosine phosphatase (SHP-2) activity (Boulton and Gray-Owen, 2002). A recent paper has found that gonococcal Opa binding to human CEACAM1 can down-regulate CD83 expression in DC, which is a marker of maturation (Yu et al., 2013). This was an attractive hypothesis to explain the dysregulation of DC maturation, since SHP-2 can selectively dephosphorylate STAT1 in the absence of STAT3 dephosphorylation (Liang et al., 2008).

To investigate this possibility, a pan-CEACAM blocking antibody was first used to determine if live NmB was using this interaction to dysregulate DC maturation. DC were stimulated for 24h with either killed NmB or live NmB in the presence of a blocking CEACAM antibody. However, as shown in Figure 6-8, a pan-CEACAM blocking antibody had only a minor effect on CD40 and CD86 expression during live NmB infection, which was dose-independent.

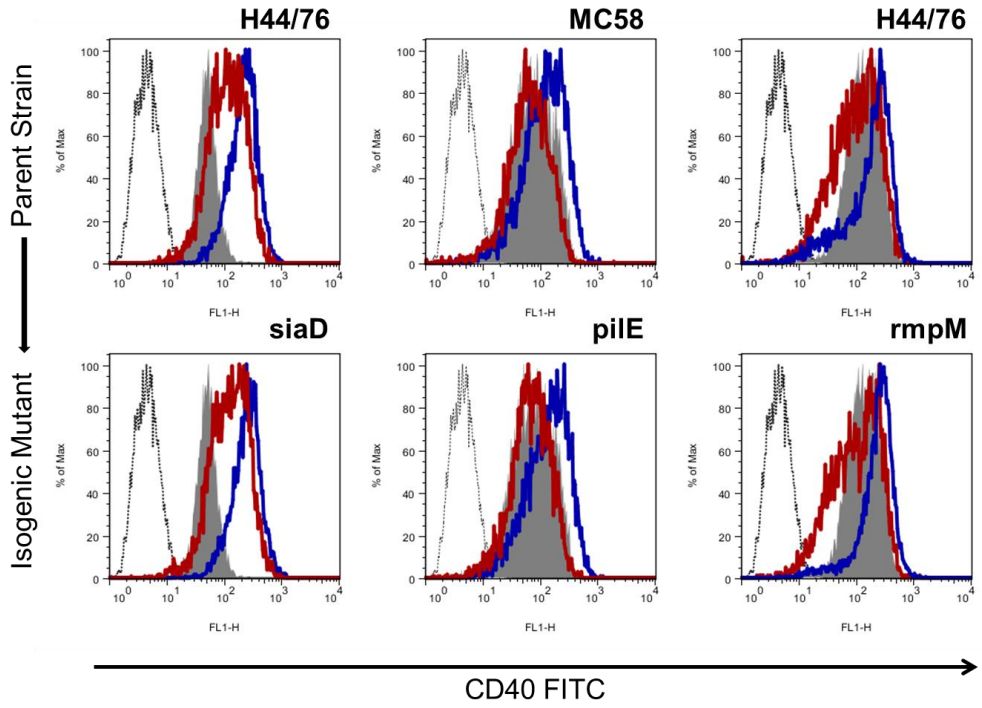

Figure 6-7 *Conventional mutant strains of NmB fail to restore DC maturation when viable*. DC were stimulated with 10 MOI killed (blue) NmB, 10 MOI live (red) NmB or left unstimulated (grey). Isotype controls are shown in the unshaded histogram. The wild-type (WT) parent strain (WT H44/76 or WT MC58) is shown on the top layer, and the mutant knock-out strain is shown on the bottom (siaD = capsule deficient, pilE = pili deficient, rmpM = rmpM protein deficient). After 24h, CD40 expression was measured by flow cytometry. Data are representative of two experiments.

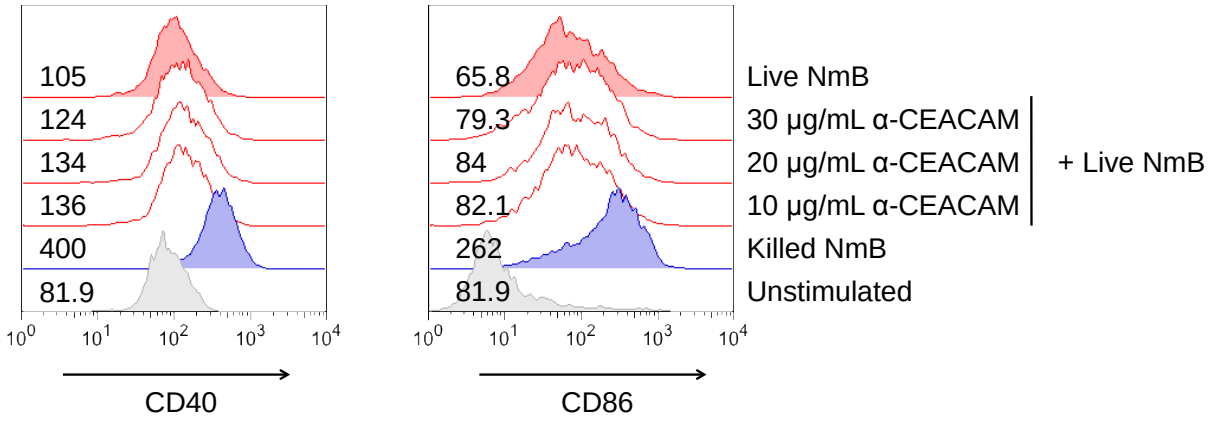

Figure 6-8 ***The dysregulation of DC maturation by live NmB is not reversed by CEACAM blocking.*** **DC were stimulated for 24h with 10 MOI killed NmB (blue), 10 MOI live NmB (red) or left unstimulated (grey). For some conditions, DC were cultured in the presence of 50 µg/mL CEACAM blocking antibody (D14HD11) during infection with live NmB (red unshaded). Surface expression of CD40 and CD86 was measured by flow cytometry. MFI are displayed on the histograms. Data are representative of two experiments.**

This small effect may have been solely due to dissociation of the bacterium from the DC. CEACAM blocking antibody had no effect on the induction of CD40 and CD86 by killed NmB (data not shown).

Furthermore, SHP-2 activity was then measured by flow cytometry. DC were stimulated for 24h with LPS, killed NmB and live NmB. SHP-2 phosphorylation was then measured by intracellular PhosFlow flow cytometry. As Figure 6-9 shows, it was found that LPS and killed NmB induced a mean 1.1-fold up-regulation in SHP-2 phosphorylation, while live NmB did not appear to induce SHP-2 phosphorylation beyond the unstimulated control. Finally, the use of a SHP-2 inhibitor (NSC 87877) had no effect on the suppression of CD40 and CD86 up-regulation by live NmB, since DC cultured in the presence of this inhibitor and then infected for 24h by live bacteria had the same expression of these markers. Therefore, these data excluded the CEACAM-SHP2 signalling axis as a probable cause of DC dysregulation by viable NmB.

6.3.4 Dysregulation of DC maturation requires active bacterial protein synthesis

Bacteriostatic concentration of chloramphenicol was used to arrest bacterial protein synthesis. Chloramphenicol binds to the bacterial ribosome and inhibits peptidyl transferase activity, thus preventing any new protein synthesis. This is specific to prokaryotic cells, which means DC protein synthesis is unaffected in this system.

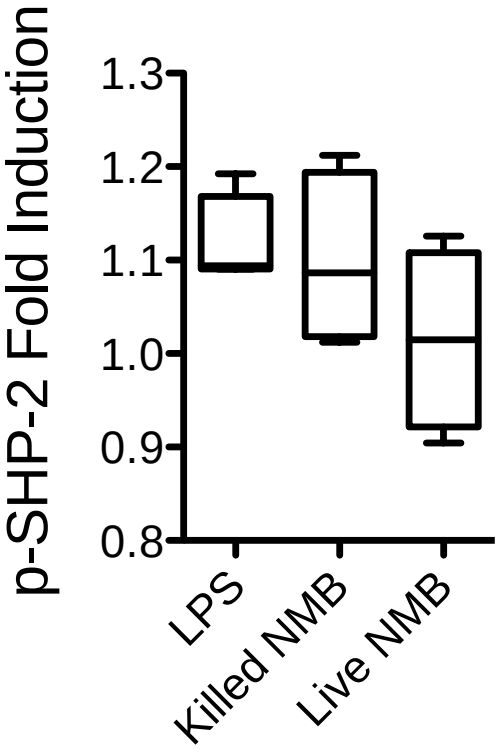

Figure 6-9 ***Live NmB does not induce enhanced SHP-2 activity compared to killed NmB.*** **DC were stimulated for 18h with 100 ng/mL LPS, 10 MOI killed NmB or 10 MOI live NmB and SHP-2 phosphorylation (p-Y452 MFI) was measured relative to an unstimulated control. SHP-2 phosphorylation was determined by intracellular PhosFlow cytometry. Data are from four individual donors. Box plots depict medians and interquartile ranges.**

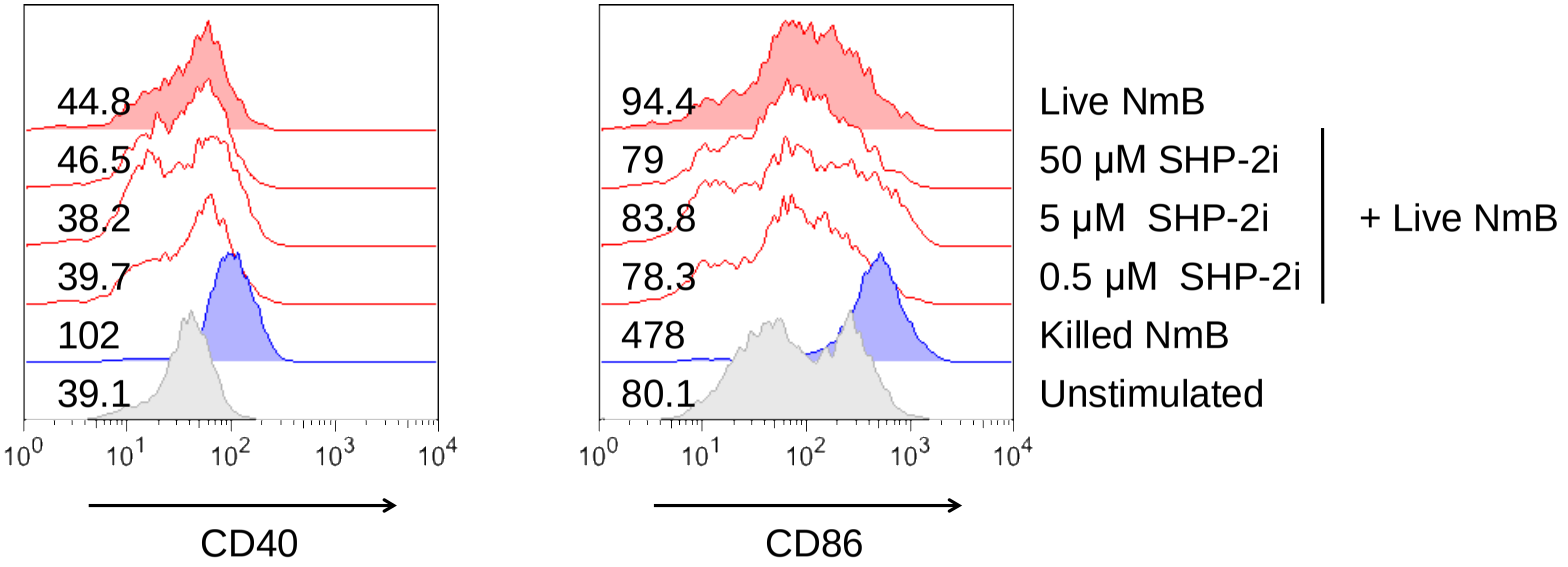

Figure 6-10 *SHP-2 inhibition does not restore DC maturation responses*. DC were stimulated with 10 MOI killed NmB (blue), 10 MOI live NmB (red) or left unstimulated (grey) with a vehicle control (0.5% DMSO). Some DC were stimulated with live NmB in the presence of 50 μM SHP-2 inhibitor NSC 87877 (red unshaded). Surface expression of CD40 and CD86 expression was then measured by flow cytometry. MFI values are displayed on the histograms. Data are representative of two experiments.

Many bacteria up-regulate the transcription of virulence factors in response to cellular and physiological stresses, or by recognition of host immune cells and antimicrobial measures. To test if the suppression of DC phenotypic maturation was dependent on active bacterial protein synthesis, DC were treated with bacteriostatic levels of chloramphenicol (10ug/ml) at various time-points after infection with live NmB, and CD40 and CD86 expression was measured by flow cytometry. Bacterial viability was confirmed by plating after washing and removal of chloramphenicol. As can be seen in Figure 6-10, chloramphenicol treatment before 4h duration of infection with live NmB led to a normal maturation response. Chloramphenicol had no effect on killed NmB or untreated DC (data not shown). These data suggested that the suppression of surface maturation marker expression requires bacterial protein synthesis.

6.3.5 Sodium orthovanadate restores STAT1 phosphorylation in DC infected with live NmB

Many bacteria actively secrete tyrosine phosphatases into the host cell. These proteins often have roles in bacterial cell physiology—such as capsule production—but some are encoded solely for the purpose of host cell modulation. The most well-characterised of these proteins may be the *Yersinia* spp. phosphatases, which can modulate host cell actin formation. These are actively secreted into the host cell via a T3SS complex.

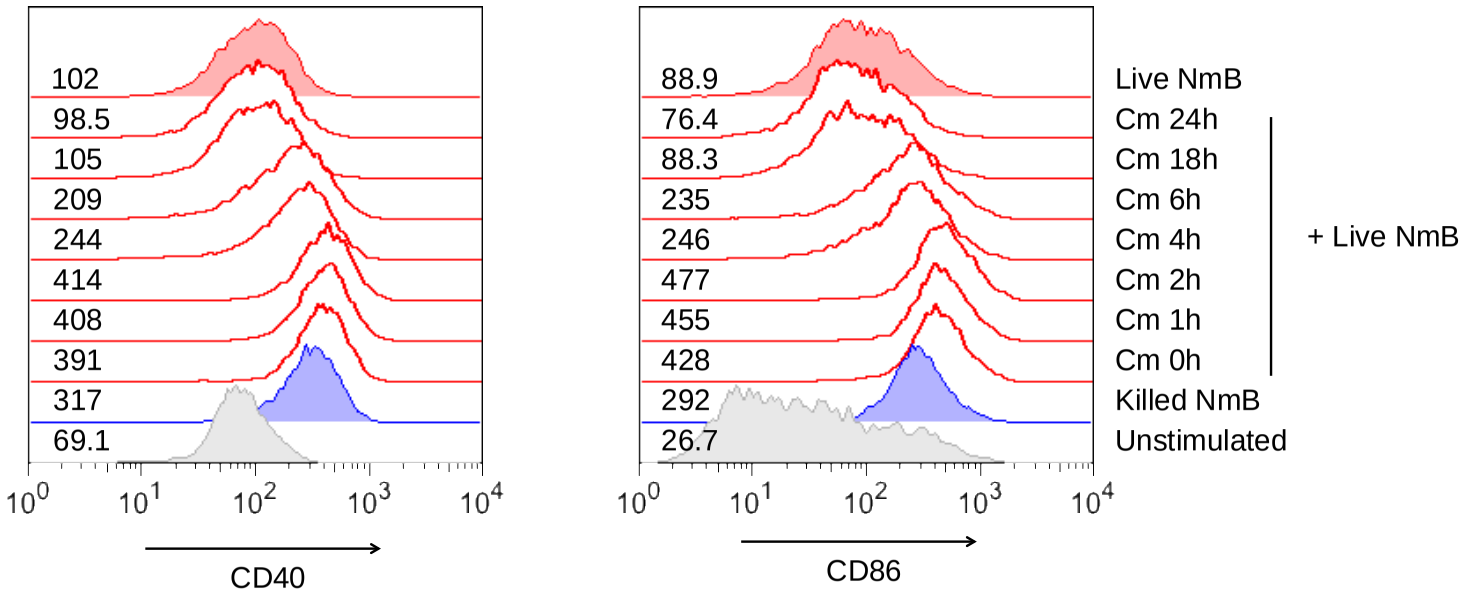

Figure 6-11 ***Early chloramphenicol treatment restores DC maturation in response to live NmB infection.*** **DC were stimulated for 24h with 10 MOI killed NmB (blue), 10 MOI live NmB (red) or were left unstimulated (grey) in the presence of a vehicle control (0.1% ethanol). For some conditions, DC were treated with bacteriostatic levels of chloramphenicol (10 µg/mL) at the designated times in combination with live NmB infection (red unshaded). Surface expression of CD40 and CD86 was measured by flow cytometry. MFIs are displayed on the histograms. Data are representative of two experiments.**

While *N. meningitidis* does not possess a secretion system, it does have autotransporters and two-partner secretion systems that may enable the secretion of effectors into the host cell (Khalid and Sansom, 2006).

It was speculated that NmB was secreting a bacterially encoded tyrosine phosphatase. This could explain the following observations: 1) the selective tyrosine dephosphorylation of STAT1, 2) the requirement for bacterial active bacterial protein synthesis, 3) the requirement for DC and NmB contact-dependence as previously found by transwell experiments (Jones et al., 2007), and 4) the marked hyporesponsiveness to IFN. To test this, sodium orthovanadate (vanadate) was used to inhibit any potential tyrosine phosphatases. Vanadate has been used in many infection models (Ceponis et al., 2003; Pauli et al., 2008), and pathogenic tyrosine phosphatases are exquisitely sensitive to this inhibitor (Guan et al., 1991).

First, it was determined that vanadate did not affect bacterial viability — although in some bacteria it has been shown that they use vanadate as a metabolite for respiration (Antipov et al., 2000). NmB was grown in various concentrations of vanadate for several h and then bacterial growth was measured by spectrophotometry (Figure 6-11). High doses (500 μM) of vanadate were shown not to effect bacterial growth.

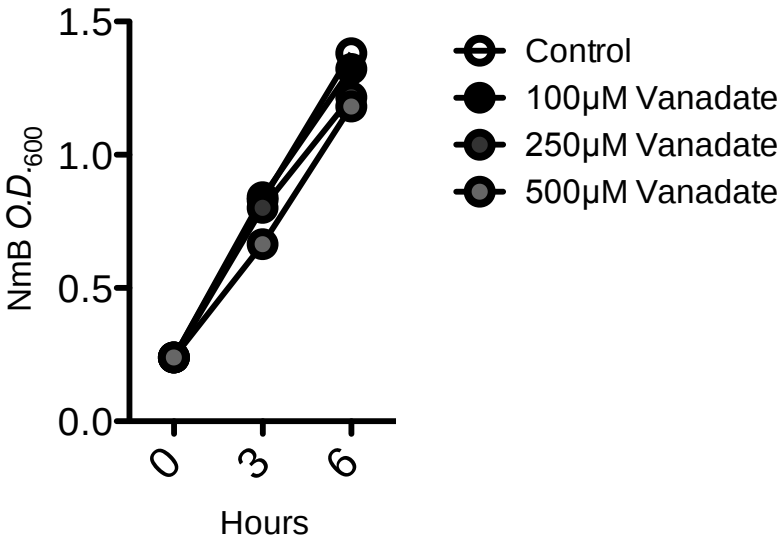

Figure 6-12 ***Vanadate does not inhibit NmB growth.*** **NmB was adjusted to OD_{600} 0.25 in RPMI without phenol red and grown at 37°C for up to 6h. For some conditions, NmB was treated with 100-500 μM vanadate. OD_{600} measurements were then taken at 3 and 6 h of growth. Data shown are from two independent experiments. Bars depict mean averages ± SEM.**

To restore STAT1-Y701 phosphorylation, some DC were pre-treated with vanadate for 2h before infection with live NmB. At 18h post-stimulation, cellular lysates were assessed for the presence of phosphorylated STAT1-Y701 by Western blot. As Figure 6-12 shows, vanadate treatment restored STAT1-Y701 phosphorylation in a dose-dependent manner.

Next, DC were pre-treated with vanadate for 2h and stimulated with LPS, killed NmB and live NmB for 24h. DC maturation was then assessed by surface expression of CD86. Consistent with the restoration of STAT1-Y701 phosphorylation, the use of vanadate greatly enhanced the expression of CD86, which was found to be significant amongst all donors (p = <0.01). By contrast, vanadate modestly lowered CD86 expression in DC stimulated with killed NmB, likely due to slight inhibitor toxicity (Figure 6-13).

6.3.6 Vanadate restores SOCS1 and normalises cytokine production

It was questioned whether vanadate would also enhance SOCS1 expression. SOCS1 is a STAT1-dependent regulator of DC cytokine production, and it was hypothesised that the enhanced levels of STAT1-Y701 phosphorylation would result in increased levels of SOCS1 protein. To test this, DC were infected with live NmB for 18h in the presence or absence of vanadate, and cellular lysates were probed for total SOCS1 protein by Western blot. In line with STAT1-Y701 phosphorylation, vanadate treatment greatly enhanced the levels of SOCS1 protein induced by live NmB (Figure 6-14).

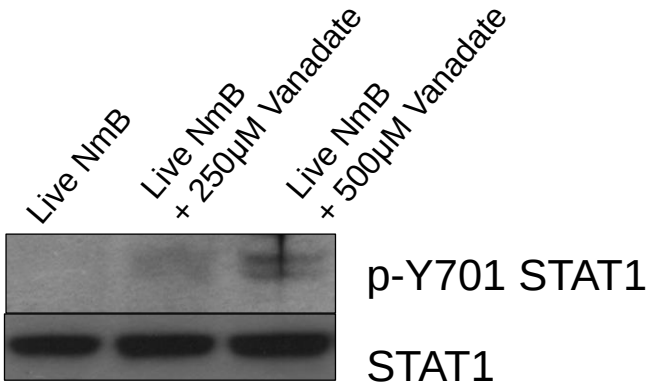

Figure 6-13 ***Vanadate treatment restores STAT1-Y701 phosphorylation in response to live NmB infection*****. DC were infected for 18h with 10 MOI live NmB; for some conditions, DC were treated with 250-500 μM vanadate during the infection. Lysates were collected at 18h and probed for the presence of p-Y701 phosphorylation by Western blot. Total STAT1 protein was used as a loading control. Data are representative of three experiments.**

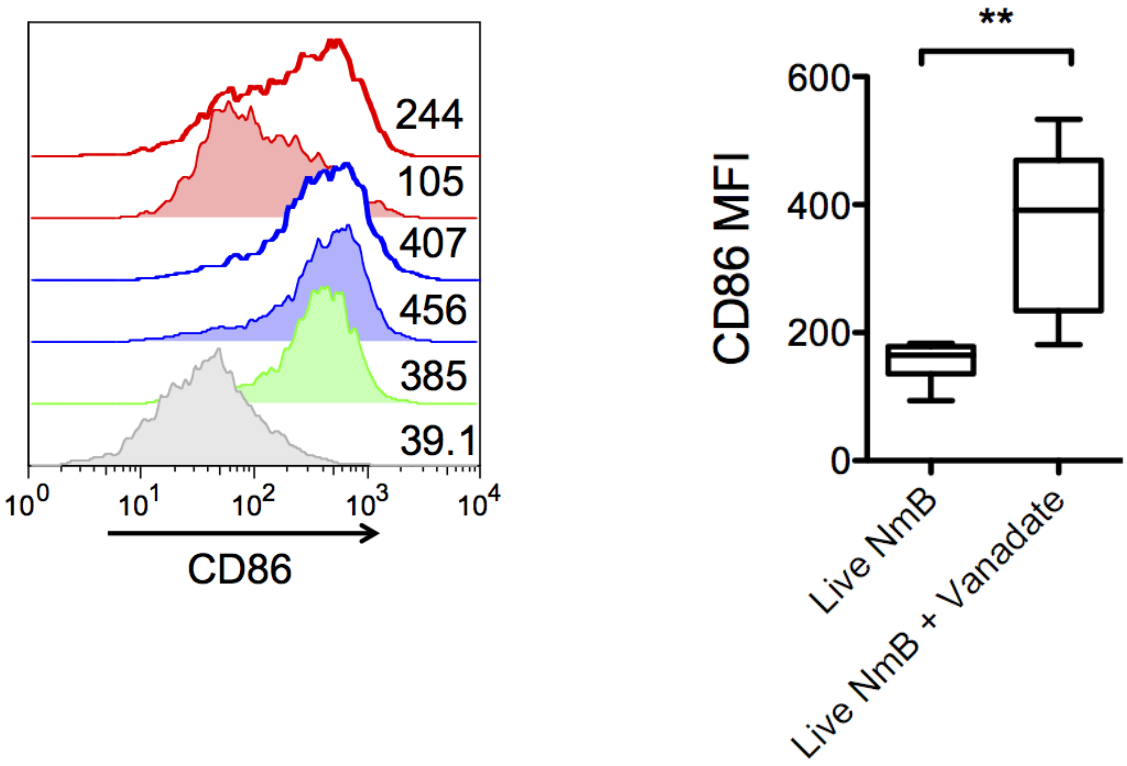

Figure 6-14 ***Vanadate treatment restores CD86 up-regulation in response to infection with live NmB. Left:*** **DC were stimulated for 24h with 100 ng/mL LPS (green), 10 MOI killed NmB (blue), 10 MOI live NmB (red) or left unstimulated (grey). For some conditions, DC were also treated with 500 µM vanadate during stimulation (red unshaded: live NmB; blue unshaded: killed NmB). CD86 expression was measured by flow cytometry. Data are from a representative donor. MFI values are displayed on the histograms. Right: Pooled CD86 MFI data from eight individual donors comparing 100-500 µM vanadate treated and untreated DC stimulated with live NmB. Box-plots depict medians and interquartile ranges. Significance was tested by paired t-test. *P<0.05, **P<0.01, ***P<0.001.**

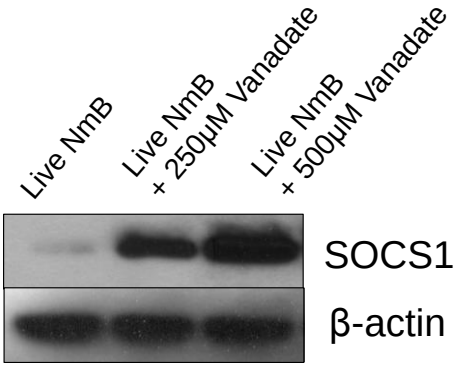

Figure 6-15 ***Vanadate treatment enhances levels of SOCS1 protein induced by live NmB infection*****. DC were infected for 18h with 10 MOI live NmB; for some conditions, DC infected in the presence of 250-500 μM vanadate. Lysates were collected at 18h and probed for the presence of SOCS1 protein by Western blot. Total β-actin protein was used as a loading control. Data are representative of three experiments**

Since SOCS1 is a master regulator of DC cytokine production, levels of pro-inflammatory cytokines were measured after vanadate treatment. Some DC were pre-treated with vanadate for 2h and then stimulated with LPS, killed NmB or live NmB for 24h. Levels of intracellular IL-1β were then measured by flow cytometry, using a saponin-based cell permeabilisation protocol. It was found that vanadate treatment significantly lowered the levels of IL-1β production in DC (p = <0.01), correlating with the restoration of SOCS1 (Figure 6-15). By contrast, vanadate had no real effect on the levels of IL-1β induced by killed NmB. As shown in Figure 6-16, similar results were found for secreted levels of IL-6 after stimulation with live NmB: vanadate effectively reduced the levels of IL-6 secretion by live NmB (mean 19,000 pg/mL vs. mean 11,250 pg/mL).

6.3.7 Vanadate treatment enhances T-cell responses induced by live NmB

Since vanadate restored STAT1-Y701 phosphorylation and increased maturation marker expression induced by live NmB, the functional consequences on T-cell proliferative responses were investigated. To test this, DC were pre-treated with vanadate for 2h and then stimulated with LPS, killed NmB and live NmB for 24h. DC were then washed, re-counted and cultured with CFSE-labelled allogeneic $CD4^+$ T-cells for 5 days. T-cell proliferation was then measured by flow cytometry, as determined by the $CFSE_{dim}$ population frequency.

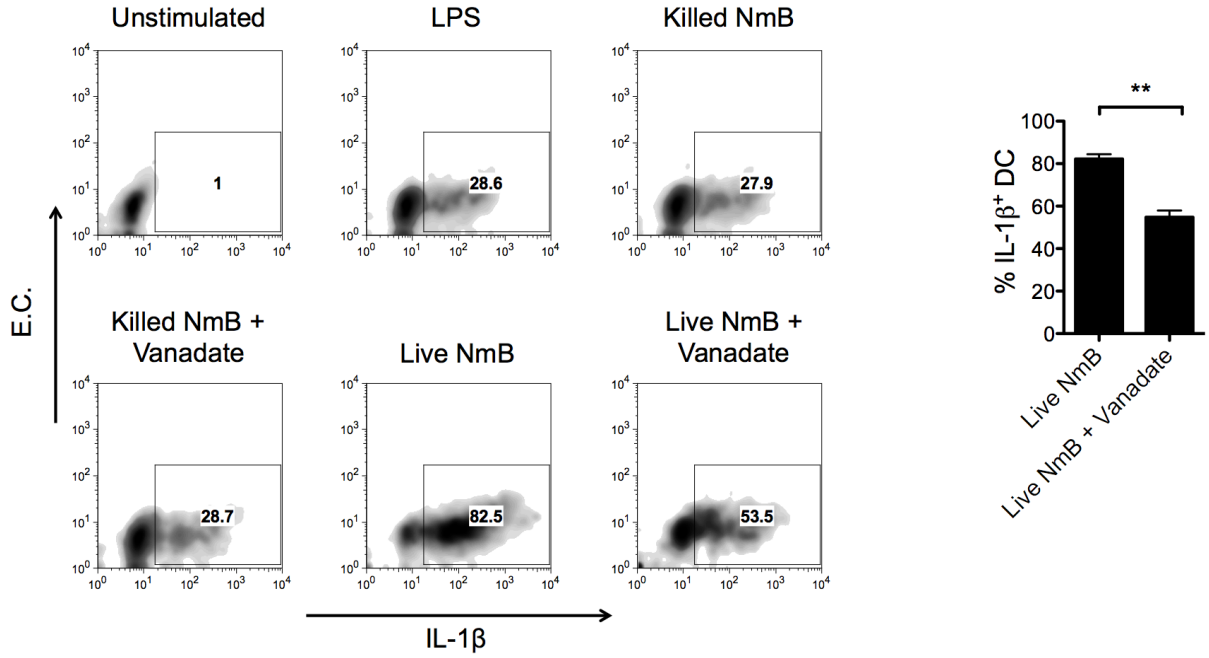

Figure 6-16 ***Vanadate treatment reduces the production of IL-1 β induced by infection with live NmB. Left:*** **DC were stimulated with 100 ng/mL LPS, 10 MOI killed NmB or 10 MOI live NmB for 24h. Brefeldin A (1 µg/mL) was added during the final 4h. For some conditions, DC were cultured in the presence of 500 µM vanadate. Intracellular IL-1β was measured by intracellular flow cytometry. E.C. = empty channel. Gate was set by an unstained control. Data show a representative donor.** ***Right:*** **Pooled data from three donors showing the frequency of IL-1β$^+$ DC. Bars depict mean ± SEM. Significance was tested by paired t-test. *P<0.05, **P<0.01, ***P<0.001.**

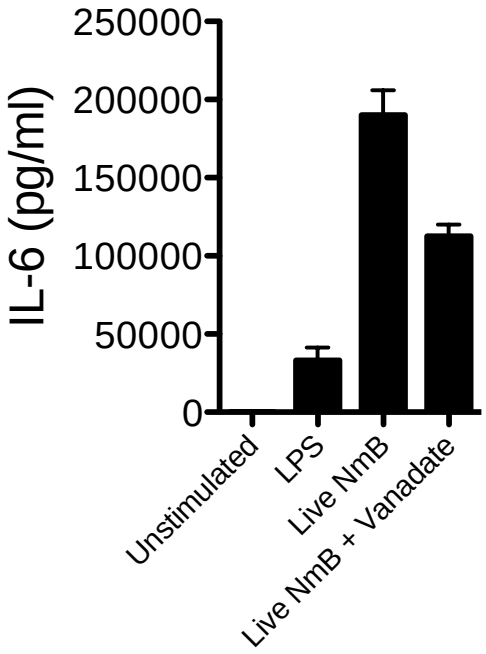

Figure 6-17 ***Vanadate reduces the amount of secreted IL-6 induced by live NmB infection.*** **DC were stimulated for 24h with 100 ng/mL LPS, 10 MOI live NmB or 10 MOI live NmB in the presence of 500 µM vanadate. Filtered supernatants were collected and assessed for total IL-6 protein by ELISA. Data are from two individual donors. Bars depict mean ± SEM.**

As Figure 6-17 shows, in accordance with CD86 expression, the use of vanadate restored the allostimulatory capacity of DC infected with live NmB. Collectively, this set of experiments showed that the restoration of STAT1-Y701 phosphorylation by vanadate could revert core aspects of conventional DC activation.

6.3.8 A candidate virulence factor

(*With Dr Craig Ross, St George's University, London*)

The experiments thus far suggested that a tyrosine phosphatase—likely bacterially encoded—was responsible for the dysregulation of the DC maturation response. The use of the tyrosine phosphatase inhibitor, vanadate, successfully restored DC to conventional activation as evidenced by up-regulation of CD86 expression, normalisation of the inflammatory cytokine profile, and reversion of allostimulatory capacity.

Given that the most likely host tyrosine phosphatase SHP-2 had normal activity, and that inhibition of bacterial protein synthesis reverted the phenotype, it was hypothesised that a bacterial tyrosine phosphatase was mediating the tyrosine dephosphorylation of STAT1.

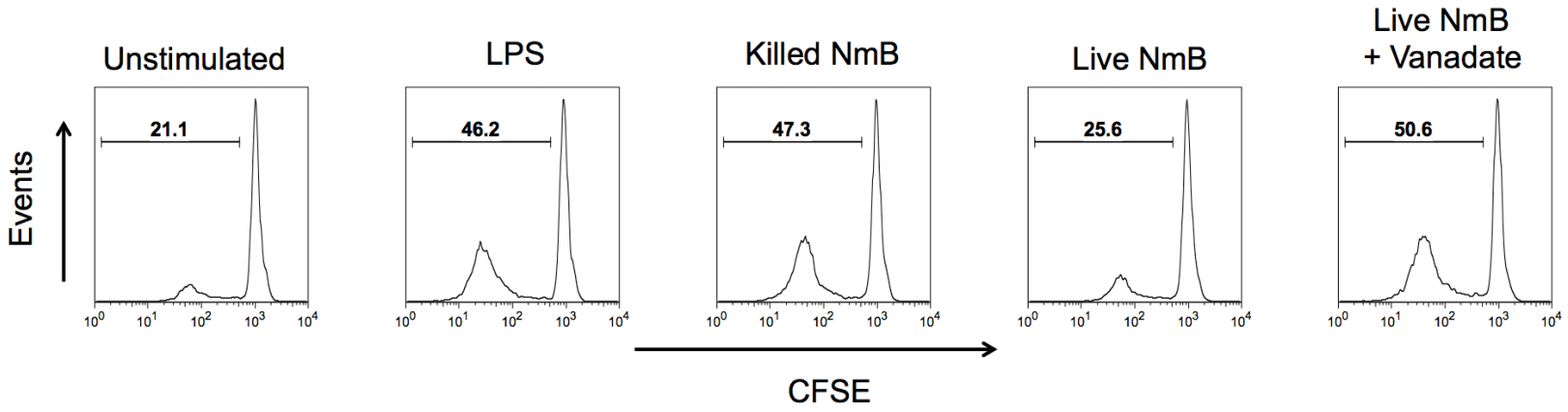

Figure 6-18 ***Vanadate restores the allostimulatory capacity of DC infected with live NmB.*** **DC were stimulated for 24h with 100 ng/mL LPS, 10 MOI killed NmB or 10 MOI live NmB. For some conditions, DC were cultured in the presence of 500 µM vanadate. DC were then washed and cultured in a 1:5 ratio with purified, CFSE-labelled allogeneic $CD4^+$ T-cells. After 5 days, T-cell proliferation was measured by flow cytometry, using the $CFSE_{dim}$ population as an indicator of cell division. Data are repsentative of two experiments.**

A search of the literature and the NmB genome revealed two putative tyrosine phosphatases of unknown function. The first was discovered by a group in 2007 and the molecular structure was solved by X-ray crystallography (Krishna et al., 2007). This revealed an active site with homology to the eukaryotic tyrosine phosphatase PTP1B, which has been reported to dephosphorylate STAT1. However, the enzyme appeared to have a shallow active site domain, and also lacked the P-loop, critical for enzymatic activity. As yet, no ligand has been identified for this protein. Though initially identified in *N. meningitidis* serogroup A, a homologue was found in the NmB genome, identified as NMB_0495.

A second candidate phosphatase was found by a search of the NmB strain H44/76 genome (Budroni et al., 2011). This was a 151 amino acid protein, designated NMB_0945, with putative tyrosine phosphatase activity based on the low molecular weight tyrosine phosphatase (LMW-PTP) motif. A molecular model of NMB_0945 was predicted using SWISS-MODEL online software (Biasini et al., 2014), which calculated good sequence homology to the *Bacillus subtilis* tyrosine phosphatase yfkJ, and this served as a model for structure prediction. Interestingly, these LMW-PTPs in *B. subtilis* are often expressed at very low levels, but are up-regulated during events of bacterial stress and cell cycle growth (Musumeci et al., 2005). As can be seen in Figure 6-19, the predicted NMB_0945 model contained the classic 'P-loop' structure, which is required by PTPs to bind phosphate. Therefore, it was hypothesised that this protein was a more likely candidate than NMB_0495.

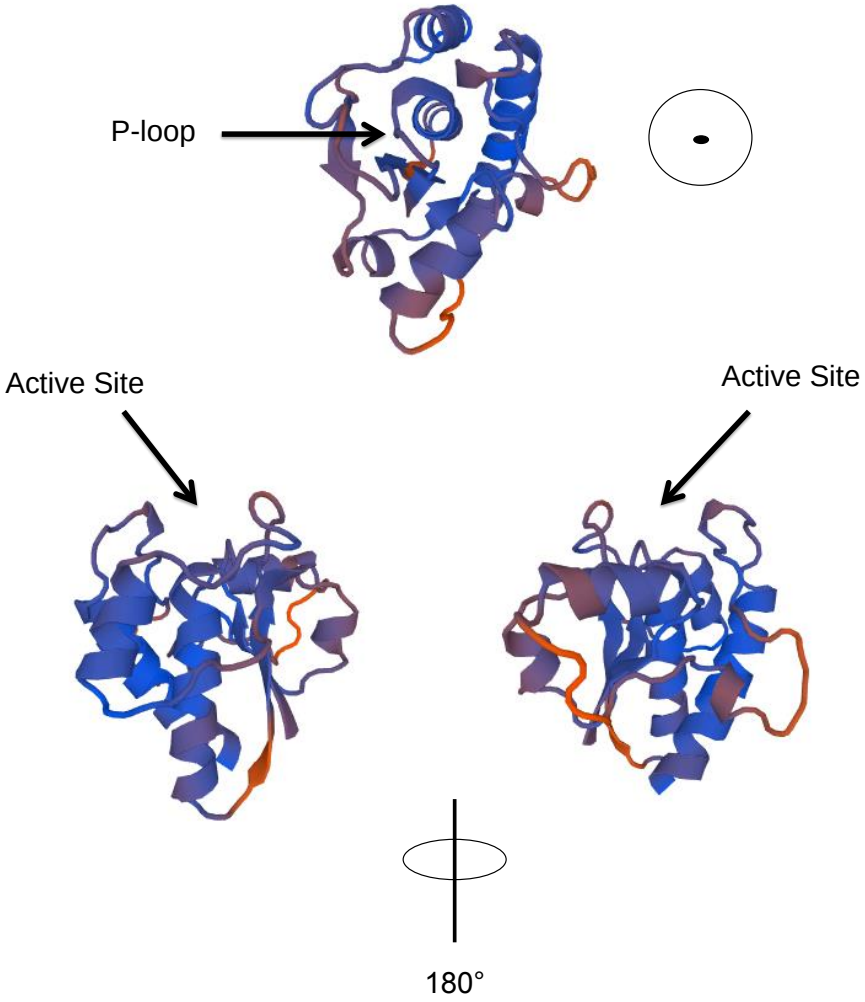

Figure 6-19 *An* in silico *model of NMB_0945 predicted secondary and tertiary structure.* The amino acid sequence of NMB_0945 was used to predict secondary and tertiary protein structure. Using SWISS-MODEL software, 44% homology was found to the *B. subtilis* phosphatase yfkJ and this was used to generate a prediction of protein structure. *Top:* A top-down view of the protein showing the P-loop determinant that allows phosphotyrosine binding and catalytic activity. *Bottom:* Two perspectives of the NMB_0945 active site, showing the cleft containing the P-loop.

To test the activity of these two candidate proteins in their ability to block STAT1 signalling, an *in vitro* IFN assay was used as a proof-of-principle pilot experiment. First, genomic DNA was extracted from NmB and tested for purity by spectroscopy. Genomic DNA was then sent to Dr Craig Ross (St George's University, London) who tested these genes in a well-characterised IFN signal transduction assay which had previously been used in their laboratory (Childs et al., 2012). Briefly, NMB_0495 and NMB_0945 were cloned into a eukaryotic vector and then transfected into Vero cells containing an ISRE reporter. Thus, any IFN-STAT1 signalling was measurable by β-galactosidase activity. Three positive control proteins were used: PIV5:V, FPV012 and FPV014, which are viral proteins that have all been shown to block IFN signal transduction (Childs et al., 2012; Laidlaw et al., 2013; Precious et al., 2007). Once transfected, Vero cells were then pulsed with IFN-β and ISRE activity was measured. As Figure 6-20 shows, PIV:5 caused a near-complete blockade of IFN signalling relative to the vector control, while FPV012 and FPV014 inhibited IFN signalling by 44% and 48% respectively. The two meningococcal proteins, NMB_0495 and NMB_0945, had differential effects on IFN signalling: the former enhanced IFN signalling by 141%, while the latter inhibited IFN signalling by 37%, comparable to the positive controls FPV014 and FPV016. These data described the first proof-of-principle example of a meningococcal protein disrupting a host signal transduction pathway, and also a promising candidate virulence factor that may be responsible for undermining cellular immunity to the meningococcus.

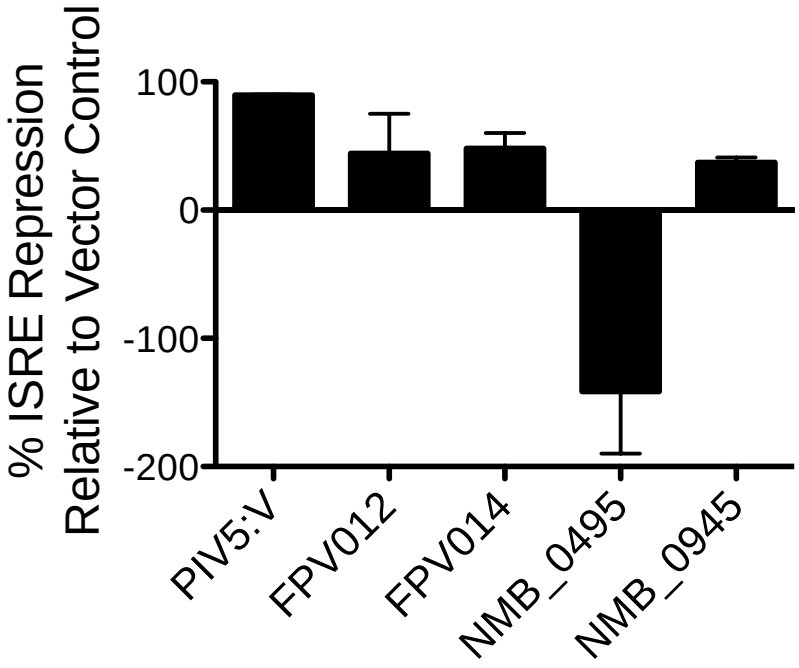

Figure 6-20 *NMB_0945 expression represses IFN-β transcriptional activity*. ISRE-reporter Vero cells were transfected with proteins derived from *Poxviridae* viruses that were used as positive controls: PIV5:V, FPV012, FPV014. Cells were also transfected with the NmB proteins NMB_0495 and NMB_0945. Cells were then pulsed with 10 ng/mL IFN-β and β-galactosidase activity was measured as an indicator of ISRE activity. Values were calculated relative to the IFN-β-induced ISRE activity in the negative control (empty vector transfection). Data are from two experiments. Bars depict mean ± SEM.

6.4 Discussion

It was found in this chapter that 'alternatively activated' STAT1 had a pro-apoptotic effect in DC. The increase in apoptosis in response to live NmB has been observed previously (Jones et al., 2007), although the mechanism remained unknown. Notably, NmB can be anti-apoptotic or pro-apoptotic in various cell types. In B-cells, NmB can inhibit apoptosis by a PorB-dependent mechanism (Massari et al., 2000); while in epithelial cells, NmB can be pro-apoptotic by a TNF-α dependent mechanism (Deghmane et al., 2009). In DC, maturation and survival are governed by separate (though overlapping) signalling nodes (Rescigno et al., 1998). Importantly, even bacterial stimuli that induce apoptosis can cause CD86 up-regulation (Lehner et al., 2001).

The STAT1-S727 phosphorylation induced by live NmB was found to be dependent on p38, which was shown to be activated by live bacteria in a previous chapter. Inhibition of p38 dampened STAT1-S727 phosphorylation, and in turn reduced the amount of apoptotic DC. Since selectively serine phosphorylated STAT1 is pro-apoptotic, this is consistent with numerous reports in the literature (Stephanou et al., 2001). Importantly, this also excluded apoptosis as the driver of inhibited DC maturation.

The S727 phosphorylation has recently been shown to be pro-inflammatory through an undefined, SOCS-independent mechanism (Luu et al., 2014). Therefore, the enhanced levels of pro-inflammatory cytokines may be caused by both direct and indirect mechanisms, i.e. not solely by SOCS protein

reduction. It is unknown if p38 inhibition would therefore lower cytokine production via a direct effect on STAT1, since p38 is also necessary for epigenetic modifications that allow transcriptional access to the promoters of these genes (Carter et al., 1999). In such an experiment, it would be difficult to infer if any observed cytokine reduction was due to either of these two effects. Interestingly, since alternatively activated STAT1 induces pro-inflammatory cytokines, it is also unclear whether this resulted in direct or indirect apoptosis. Although serine phosphorylated STAT1 can activate caspases, the autocrine effect of excess TNF-α and IL-1β could also synergise in enhancing cell death.

The data in this chapter also elucidate that STAT-Y701 dephosphorylation is not due to conventional virulence factors or likely host effectors. PilE, RmpM and SiaD mutants were all able to suppress DC maturation marker up-regulation when viable. In further support of this, various OMVs (which contain many of these proteins) are reportedly able to fully mature DC (Jones et al., 2014). However, chloramphenicol was able to reverse this effect if used early enough during infection, indicating that active bacterial protein synthesis was required for DC dysregulation. This observation is consistent with many virulence models in the literature. Numerous bacteria require direct cell contact in order to secrete effectors into the cytoplasm of the host cell. For example, *Pseudomonas aeruginosa* requires contact with the host cell in order to switch-on a regulon controlling the transcription of secreted virulence factors; this is dependent on direct contact with the host cell and also active protein synthesis (Cisz et al., 2008; Vallis et al., 1999).

The use of vanadate was also able to revert all tested aspects of DC activation. Treatment of DC with vanadate during infection with live NmB was sufficient to restore STAT1-Y701 phosphorylation, correlating with an increase in CD86 expression; vanadate also enhanced SOCS1 protein levels, while correlating with a reduction in pro-inflammatory cytokines. Although vanadate can enhance basal levels of STAT1 activation (Pauli et al., 2008), it is also a potent inhibitor of tyrosine phosphatases, and even pathogenic phosphatases. YopH from *Yersinia* spp. (Andersson et al., 1996), SptP from *S. typhimurium* (Kaniga et al., 1996) and VH1 from VV H1 (Guan et al., 1991) are all inhibited by the presence of vanadate.

Endogenous PTPs such as TC-PTP, CD45, SHP-1, SHP-2 and PTP1B can dephosphorylate STAT1 under various conditions. However, these phosphatases are usually associated with a reduction in cytokine production. To the author's knowledge, only SHP-2 is capable of enhancing cytokine production while simultaneously dephosphorylating STAT1. This is due to its role in enhancing NF-κB activity and subsequently elevating IL-1 and IL-6 production (You et al., 2001). Since CEACAM is a well-characterised ligand of the Neisserial protein Opa, which can in turn recruit SHP-2, it seemed a plausible hypothesis that live NmB might be ligating CEACAM and inducing SHP-2 activity to dephosphorylate STAT1 and arrest DC maturation. However, both a blocking antibody to CEACAM1 and various SHP-2 assays indicated that this was not likely to be the case. Furthermore, endogenous PTPs usually have activity against JAK family kinases (Shuai and Liu, 2003), which were shown to be activated by live NmB in a previous chapter. Given

these data, it can be concluded that the PTP responsible for STAT1-Y701 dephosphorylation was likely to be bacterial in origin.

One such bacterial candidate protein was NMB_0945, and this was able to inhibit IFN signalling in a eukaryotic cell line containing an ISRE binding domain, which is capable of binding a STAT1-STAT2 heterodimer in response to type I IFN. Though this was a modest reduction, it was still comparable to some of the positive controls. It is worth noting that while STAT1-Y701 was virtually completely dephosphorylated by live NmB in previous assays, NMB_0945 may still be an attractive candidate effector molecule for this phenomenon despite its moderate activity. Bacterial proteins are often subject to numerous post-translational modifications that enhance their function. Therefore, NMB_0945 may have needed further modifications—encoded by accessory bacterial proteins—in order to teach its full activity. Bacteria can methylate, lipidate, acetylate and phosphorylate their own enzymes (Cain et al., 2014). In order to reach optimal enzymatic activity reflective of complete STAT1-Y701 dephosphorylation, NMB_0945 may have needed to be expressed as part of a larger regulon.

Another possibility is that NMB_0945 required a low pH in order to reach optimal enzymatic activity. An early paper on Neisserial protein phosphatases found that the activity of these enzymes increased in acidic conditions (Leahy et al., 1940). This is an intriguing observation that may hold the key to NmB-mediated DC dysfunction, as there are notable examples of other pH-dependent virulence factors in the literature. *S. typhimurium* requires a TLR-triggered pH lowering in order to activate pathogenic tyrosine phosphatases

and transcribe various virulence factors. TLR signalling in DC induces an acidic extracellular and intracellular environment, such as the production of acid lactate and endosome acidification (Everts et al., 2014; Krawczyk et al., 2010). For *S. typhimurium*, this acidification process takes approximately 4-5 h after initial infection (Alpuche Aranda et al., 1992), which is the same 'window' within which chloramphenicol can be added to prevent suppression of DC maturation by live NmB. It is tempting to speculate that this initial TLR-mediated immune recognition, resulting in a pH drop, may be needed for NMB_0945 to reach optimal activity. Therefore, the dephosphorylation of STAT1 by NMB_0945 (or indeed another PTP) could hypothetically be considered to be a 'self-propagating inflammatory cycle', since dysregulated TLR signalling (via SOCS protein down-regulation) would likely encourage more lactate production and further acidification.

In summary, these data showed that the serine phosphorylation of STAT1 was pro-apoptotic, but that reversion of STAT1 tyrosine dephosphorylation could revert DC to conventional maturation. But more importantly, they implicated a putative bacterial tyrosine phosphatase— NMB_0945—in the specific targeting of a host transcription factor. These are novel findings for the pathophysiology of the meningococcus, and may have important ramifications for the understanding of natural immunity to the bacterium.

7 Chapter 7: Final Discussion and Future Work

In recent decades there was no cross-protective vaccine against NmB. Cross-protective vaccines had been developed for other meningococcal serogroups (such as group C) based on the polysaccharide antigen coupled to a protein carrier. These vaccines induced robust immune responses in the form of protective antibodies. However, an NmB vaccine was problematic due to the fact that the capsule was homogenic to human tissue. Therefore, a 'reverse vaccinology' approach was undertaken, which involved mining the NmB genome for conserved proteins that could serve as good vaccine antigens. This research produced the vaccine 4CMenB (also branded as Bexsero®). Clinical trials showed that it is effective in producing cross-protection against multiple strains of NmB, although there is significant scope for improvement (Shea, 2013). Given the relative rarity of disease, natural immunity arguably functions adequately in the protection of the host against meningococcal disease. Therefore, to develop optimum vaccine protection, it may also be important to understand the natural immune response to the bacterium.

The immunological relationship between *N. meningitidis* and the human host is complex and not fully understood. The absence of good animal models has been a prohibitive factor in the understanding of immune responses to the meningococcus. Recently, a CEACAM1 transgenic murine model was

developed and this was shown to induce high levels of bacterial colonisation (Johswich et al., 2013). Such a model could allow a deeper understanding of natural immunity and pathogenesis in meningococcal disease. However, many of the key cellular receptors that determine the virulence potential of *N. meningitidis* are fundamentally different or entirely absent in the mouse. It has been shown that murine and human TLR4 respond differently to a lipid A pre-cursor, which is the toxic component of LPS (Akashi et al., 2001). Correspondingly, penta-acylated meningococcal LOS activates murine but not human TLR4 (Steeghs et al., 2008). The phagocyte receptor DC-SIGN, which is a critical molecule for the recognition of Gram-negative bacteria (Zhang et al., 2006), is not expressed on murine cells. Terminal sugar moieties within meningococcal LOS allow the bacterium to bind to DC-SIGN, which results in uptake and phagocytosis (Steeghs et al., 2006). Mouse and human B-cells also respond differently to *N. meningitidis*. Previous reports have found that the bacterium can inhibit murine B-cell function (Melancon-Kaplan et al., 1986), while human studies have found that meningococcal antigens are able to directly activate B-cells (Wetzler et al., 1996).

Given these major differences in human and mouse immune responses to the *N. meningitidis*, even a transgenic mouse model may not be a good model with which to investigate immune responses to the bacterium. On the other hand, *in vitro* activation of human DC (particularly the up-regulation of surface maturation markers) by vaccine antigens corresponds extremely well to levels of protective antibodies in humans. The same study also found that there was no correlation between murine DC activation and levels of antibody protection

(Li et al., 2013). The use of human cells for understanding the immune response to *N. meningitidis* may therefore represent a rare example of *'in vitro veritas'*.

It is thought that immune evasion mechanisms may allow the bacterium to persist in the human host (Lo et al., 2009). These mechanisms are necessary because the meningococcus is an obligate coloniser of the human nasopharynx; a non-human animal or environmental reservoir has not been found. The exquisite adaptations that allow the bacterium to colonise the human mucosa are also key determinants of pathogenesis. A pertinent example of this is the bacterial capsule: while it is rich in sialic acid and can therefore disable complement deposition (which is abundant in the mucosal tissue), it also allows the bacterium to survive in the bloodstream during instances of invasive disease. A dominant paradigm in the literature is that *N. meningitidis* is an "accidental pathogen" (Stollenwerk and Jansen, 2003), and that it has evolved to rarely cause invasive disease. Indeed, symptomatic invasive disease represents an evolutionary 'dead-end' for the bacterium, as the death of the host prevents further infections.

In this study, live NmB was shown to dysregulate DC maturation by selective tyrosine dephosphorylation of STAT1 — a model for this is proposed in Figure 7-1. STAT1 is essential for the up-regulation of DC maturation markers—such as CD40 and CD86—and therefore DC infected with live NmB were phenotypically immature. But strikingly, DC were able to induce very high levels of inflammatory cytokines such as IL-1β, however normal NF-κB and MAPK activation was observed. Therefore, NmB was able to decouple two

fundamental aspects of DC maturation. Since pro-inflammatory cytokine production is an early-phase aspect of DC maturation, and up-regulation of maturation markers is a late-phase event, the effect of live NmB could be described as an 'arrest' of DC maturation in an early, hyper-inflammatory phase.

DC are often described as the 'sentinels' of the immune system, due to their ability to control and dictate adaptive immune responses (Stockwin et al., 2000). As well as being the most important APC for stimulating naive $CD4^+$ T-cells, DC are also essential for thymus-dependent antibody responses via germinal B-cells (Inaba et al., 1983). DC can also generate robust antibody responses via $CD4^+$ T-cell-B cell interactions when targeted by vaccine adjuvants (Shortman et al., 2009). The suppression of the up-regulation of DC co-stimulatory and antigen presenting molecules could be an evolutionary strategy by which to persist in the human host. As previously mentioned, *M. tuberculosis* disables local T-cell responses *in vivo* by inducing a similar $CD86^{lo}PD\text{-}L1^{hi}$ phenotype to the one reported herein; restoration of DC function enables bacterial clearance (Schreiber et al., 2010). Hence, the dysregulation of DC maturation by live NmB may have important physiological relevance.

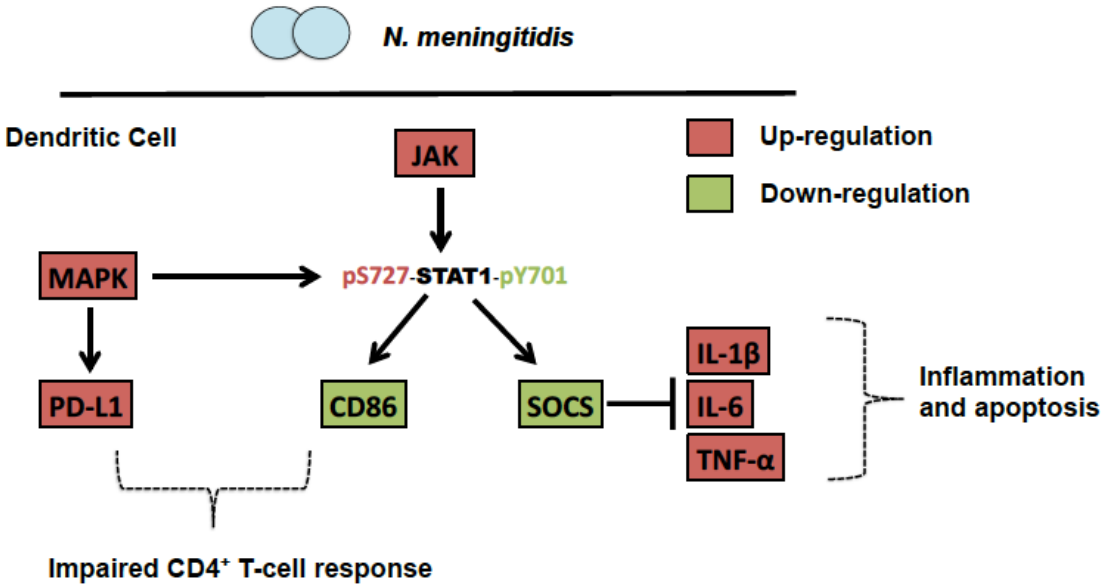

Figure 7-1 ***A proposed model of the dysregulation of DC by live NmB*****. Shown is a schematic of live NmB up-regulated/activated (red) and down-regulated/deactivated (green) cellular targets and signalling axes, and the result of these modulations on DC phenotype. The disruption of STAT1-Y701 phosphorylation arrests DC in a hyper-inflammatory, apoptotic state which correlates with low levels of SOCS genes *SOCS1* and *CISH*. Moreover, DC are CD86^{lo}, but MAPK-dependent PD-L1^{hi}, resulting in impaired T-cell responses.**

It was found that DC infected with live NmB were poor inducers of $CD4^+$ T-cell proliferation, even compared to unstimulated DC. Part of this effect was due to the selective up-regulation of PD-L1, which was dependent on MAPK activity. Commensal bacteria can cause PD-L1 up-regulation to induce a tolerogenic environment in the airway mucosal tissue (Gollwitzer et al., 2014), but this is the first time that such an effect has been described with NmB. During the course of these experiments, a study was published that showed *N. gonorrhoeae* was able to restrict $CD4^+$ T-cell responses via PD-L1 expression on DC (Zhu et al., 2012). The authors showed that PD-L1 blockade enhanced (but did not completely restore) T-cell proliferation. This effect of T-cell suppression could reflect a more general property of the *Neisseria* genus.

However, there are important drawbacks to the study by Zhu et al. that are worth consideration. *N. gonorrhoeae* appeared to induce a normal maturation profile ($CD86^{hi}PD\text{-}L1^{hi}$), comparable to many bacterial stimuli, and so the blockade of PD-L1 leading to enhanced T-cell proliferation may have been an artefact based on the removal of a standard negative signal, rather than a reversal of the specific cause of gonococcal-mediated T-cell suppression. Due to a lack of positive controls in their study (e.g. LPS), it is difficult to ascertain whether *N. gonorrhoeae* induced quantitatively more PD-L1 up-regulation than would be normal. It is unlikely that the mechanism of gonococcal suppression of T-cell responses is similar to the one presented herein.

Though it was found that live NmB dysregulated DC maturation via STAT1-Y701 dephosphorylation and prevented T-cell proliferation, natural immunity

to *N. meningitidis* has been observed in humans. Serological analyses have revealed the presence of naturally occurring humoral immunity to NmB in adults and children. These antibodies are predominantly of the IgG1 and IgG3 sub-class (Pollard et al., 1999a). Many of these antibodies are directed against the PorA surface protein, which is subject to intense phase-variation, yet appears to be a dominant immuno-stimulatory epitope.

Cellular immunity to the meningococcus has received much less research attention than humoral immune responses. However, a small number of studies have addressed this topic. Early studies looking at cellular immunity to *N. meningitidis* used crude methods to assess this matter, such as stimulation of whole blood PBMCs with meningococcal OMV and measurement of cytokine profiles by ELISA. Though the authors of such a study concluded that older children induced a more T_H2-type response (Pollard et al., 1999b), it is worth noting that many mononuclear cells can produce T_H2-like cytokines. Monocytes can secrete vast quantities of IL-10 in response to bacterial antigens (Malefyt et al., 1991), which was the same read-out that the authors used as confirmation of a T_H2 response. Hence, only extremely limited conclusions can be drawn from such reports.

More advanced and relevant studies have used a different approach to investigate cellular immunity to the meningococcus: the stimulation of cells from excised palatine tonsils with meningococal OMV or bacterial antigens. These studies found a pronounced T_H1-type naive and memory T-cell response after stimulation with OMV from NmB, and also the strong induction of T-cell proliferation (Davenport et al., 2003, 2007). This reflects another

prominent investigation, which also found the polarisation of a robust T_H1-type profile after *in vitro* stimulation of DC with killed NmB (van Beelen et al., 2007). It is worth noting that these previous studies have two important limitations: (1) the use of detergent-extracted OMV, which do not contain the full repertoire of outer membrane proteins (Koeberling et al., 2008), and (2) palatine tonsils are removed during cases of inflammation and infection, which may produce biased or inconsistent results. Regardless of these limitations, these studies raise an important question: if DC are disabled by live NmB as data in the present study suggest, why are there memory T_H1 cells to meningococcal antigens in the tonsils?

There are several evidence-based explanations that may answer this question. The first is that the T_H1 memory cells identified in the studies by Davenport et al. may not in fact represent *bona fide* memory T-cells specific to the meningococcus. To justify their conclusions, the authors used the surface marker CD45RO in their experiments as an indicator of a T-cell memory phenotype. However, memory $CD8^+CD45RO^+$ T-cells express a variety of PRRs that can respond non-selectively to antigens such as LPS, inducing strong proliferative and cytokine responses (Komai-Koma et al., 2009). Similar results have been found with $CD4^+CD45RO^+$ memory T-cells, which can also respond to a variety of TLR ligands and subsequently produce high amounts of IFN-γ (Caron et al., 2005). This phenomenon has also been found *in vivo*: infection of mice with *S. typhimurium* induces non-specific naive and memory $CD4^+$ T-cell IFN-γ production and proliferation, even in the absence of MHC Class II (Srinivasan et al., 2007). Since OMV contain high amounts of LOS

and other bacterial TLR ligands, this could explain the strong induction of IFN-γ and proliferation in tonsillar memory T-cells, i.e. a non-specific response to general TLR stimulation.

A second explanation is that commensal species of *Neisseria*, such as *N. lactamica*, may provide cross-protection against *N. meningitidis*. *N. lactamica* and *N. meningitidis* share many cross-reactive antigens (Troncoso et al., 2000), and it has been found that colonisation of humans with *N. lactamica* can generate protective antibodies against *N. meningitidis* (Evans et al., 2011). This has been explored as a promising strategy with which to offer protection against meningococcal disease. Beyond *N. lactamica*, it has also been found that even influenza vaccination can generate T-cell memory responses to bacterial antigens (Su et al., 2013). Indeed, the same study found that HIV-unexposed adults possessed endogenous T-cells with a memory phenotype to the virus. Hence, the scope of cross-protection by shared motifs in the human TCR repertoire may be larger than previously thought. Since the tonsils are rich in bacterial and viral antigens, other bacterial—or even viral—organisms may provide cross-protection due to similarity with meningococcal antigens. It may be speculated that the dysregulation of DC maturation by live NmB may necessitate another organism providing cross-protective immunity to the bacterium, which may be somewhat less vigorous than that of a direct meningococcal antigen, and therefore advantageous for colonisation.

Finally, a third explanation is that the inhibition of DC maturation by live NmB may necessitate a different APC generating T-cell immunity. The immune

system has sometimes been described as being comparable to a football team (Sompayrac, 2012): when one player is removed from the game, other members of the team can step-in to fulfill the function of the missing player. Indeed, primary human DC deficiencies have been more well-described in the last ten years (Collin et al., 2011). While these patients are subject to a higher rate of bacterial infections, these are less severe and associated with lower rates of morbidity than would be expected, considering the importance of DC in orchestrating the immune system. Importantly, an increased incidence of meningococcal disease in these patients has not been found.

In support of the hypothesis that a different APC might be generating T-cell immune responses to the meningococcus during natural infection, a prominent study found that patients with B-cell deficiency had impaired cellular immunity to *N. meningitidis,* despite these patients having relatively normal DC function in response to LPS (Morales-Aza et al., 2009). It may be that the dysregulation of DC maturation by live NmB may necessitate B-cells acting in a role of a 'compensatory APC' in generating and maintaining cellular immunity to the meningococcus. In further support of this hypothesis, the up-regulation of co-stimulatory and antigen presenting molecules on human B-cells may be more dependent on NF-κB signalling than in DC, as CD80 transcription can be induced by NF-κB signalling alone in germinal center B-cells (Niu et al., 2003). Thus, the tyrosine dephosphorylation of STAT1 induced by live NmB may not have the same effect on these cells. Human tonsil mucosal tissue is rich in memory B-cells that can rapidly up-regulate CD80 and CD86, and subsequently stimulate T-cell responses (Liu et al.,

1995). The less-specialised B-cell, as opposed to the professional DC, may thus be the prime inductor of T-cell immunity to the meningococcus, which could provide an advantage to the bacterium in terms of the strength and scope of the immune response. Indeed, it has been found that tonsillar T-cell immunity does not correlate to bactericidal antibodies (Davenport et al., 2003), suggesting sub-par natural cellular immunity.

Preliminary experiments found that live NmB appeared to induce a higher percentage of $CD86^+$ B-cells than killed NmB (data not shown). It would be intriguing to investigate this further, by exposing human primary naive $CD19^+$ B-cells to live and killed NmB, and then testing the ability of these B-cells to induce T-cell proliferation. Though an *in vitro* assay, this would elucidate whether B-cells are better able to respond to NmB, providing further support for the hypothesis that B-cells generate natural cellular immunity to the bacterium *in vivo*.

Further, live NmB was found to significantly reduce the amount of T_H1-type $CD4^+$ T-cells compared to killed NmB via infection of DC. There was a non-significant trend of enhanced IL-4^+ T_H2-type cells, which is consistent with lower expression of co-stimulatory molecules induced by live bacteria. T_H1 cells can produce IFN-γ, which can prime macrophages and other front-line leukocytes. Considering this, a reduction in T_H1-type cells may be theoretically important for the survival of NmB in the mucosa. Further work could explore the ability of live NmB to induce T_H17 and T_{REG} cells via DC, as these are also important in maintaining the balance between inflammation and tolerance. IL-17 can up-regulate the production of defensins in human

epithelial cells, which are important for broad protection against bacterial infection (Kao et al., 2004). It may be that live NmB suppresses T_H17 responses in a similar way to T_H1 responses.

Another aspect of the model presented here is that live NmB was able to induce higher amounts of pro-inflammatory cytokine production. This correlated with a reduction in SOCS1 and CISH transcripts and protein. SOCS1 is a master controller of human DC cytokine production, and silencing of this gene can alone results in uncontrolled pro-inflammatory cytokine production (Hong et al., 2009). The use of vanadate restored STAT1-Y701 phosphorylation, enhanced SOCS1 expression and also lowered the production of pro-inflammatory cytokines. Though SOCS1 is critical for controlling DC cytokine production, activated STAT1 also has a direct role in suppressing the inflammasome and restricting IL-1β production (Guarda et al., 2011). Thus, it is unclear to what extent the restoration of STAT1-Y701 phosphorylation directly and indirectly contributed to the reduction of pro-inflammatory cytokine production. It would be interesting to test how much SOCS1 contributes to the elevated levels of pro-inflammatory cytokines produced in response to live NmB infection. This could be investigated by lentiviral transfection of a high-copy SOCS1 construct into DC infected with live NmB, which could then be assessed for cytokine production. It is likely that both STAT1 itself and STAT1-induced SOCS1 may contribute towards the reduction in IL-1β after vanadate treatment.

These data are interesting because LOS is classically regarded as the central driver of inflammation in meningococcal disease. However, repeated LPS

stimulation (such as might occur during sepsis) has been noted as being tolerogenic (Fan and Cook, 2004). Critically, the down-regulation of SOCS1 could provide a mechanism that allows this inflammation to go unchecked and unregulated in the context of invasive disease, thus providing an important additional element to the pathophysiology of sepsis in meningococcal disease. *SOCS1* silencing in mice exacerbates the cytokine storm after challenge with bacteria or endotoxin (Chung et al., 2007; Kinjyo et al., 2002; Nakagawa et al., 2002). Yet despite its importance in this role, SOCS1 has not been studied in the context of meningococcal infection. A recent paper by Wang and colleagues has established that SOCS1 induction is likely to be a general property of Gram-negative bacteria (Zhang et al., 2014); in contrast, the data herein uniquely show that viable NmB can inhibit SOCS1 up-regulation, which may represent a novel mode of pathogenesis. These data thus expand the paradigm of understanding the pathophysiology of meningococcal disease.

While it is known that DC with low expression of co-stimulatory molecules are poor at clearing infection, the induction of pro-inflammatory cytokines is not a clear immune evasion strategy. However, an intriguing study has demonstrated that NmB can internalise human cytokines and use these as DNA-binding proteins to up-regulate complement resistance genes (Mahdavi et al., 2013). The induction of these pro-inflammatory cytokines may therefore be of benefit to colonisation, and these data could provide mechanistic precedent for these observations. Furthermore, early TLR signalling and cytokine production are now known to induce a rapid immuno-metabolic shift

towards lactate secretion by DC (Everts et al., 2014; Krawczyk et al., 2010); correspondingly, research by Tang and colleagues has previously found that lactate is essential for NmB colonisation (Exley et al., 2005). Thus, amplification of cytokine production by SOCS1/TLR dysregulation may aid the bacterium in both complement resistance and nutrient acquisition, but with the fitness cost of increased risk of dissemination in the host. In support of this hypothesis, a transgenic mouse model of NmB infection has shown that colonisation induces a rapid burst of pro-inflammatory cytokines, but that this does not correspond to immediate bacterial clearance, even several days post-infection (Johswich et al., 2013). Compelling new evidence has revealed the presence of meningococcal RNA thermosensors that induce up-regulation of immune evasion factors (e.g. capsule) during elevated temperatures (e.g. influenza infection) (Loh et al., 2013). This positions NmB as being uniquely capable of coping with the physiological penalties of enhanced pro-inflammatory cytokine secretion, which may not result in bacterial clearance. But more importantly, these data suggest that NmB may exist in an equilibrium of low-level inflammation and non-productive adaptive immunity, which may be exacerbated by co-infection or a disruption of the host-pathogen interface, leading to pathogenic behaviour.

The question remains of whether live NmB would induce higher pro-inflammatory cytokine secretion generally across many cell types, or if this observation is limited to DC. A previous report has found that live NmB induces higher levels of IL-6 in epithelial cells compared to killed NmB (Tezera et al., 2011); hence, it may be that this effect of enhanced cytokine

production is more widespread, and possibly mediated via STAT1 tyrosine dephosphorylation, as was found in the present study. Many analyses of the bacterial microbiome of the upper respiratory tract have determined that colonisation with *N. meningitidis* is associated with asthma and other conditions of chronic respiratory inflammation (Bisgaard et al., 2007; Hilty et al., 2010). Although these associative studies did not posit causation, it is tempting to venture that the excessive inflammation induced by live NmB across many cell types via a STAT1-dependent mechanism at the mucosal barrier may lead to low-level chronic inflammation, resulting in occasional airway inflammatory disease.

Lastly, the present study found some evidence that a bacterial tyrosine phosphatase was targeting STAT1-Y701. Canonical STAT1 activation is essential for DC activation and the induction of T-cell responses, and is typically induced by an autocrine IFN loop that is fundamental for late-phase maturation. Emerging evidence suggests that this signalling axis may in fact supersede PRR engagement in terms of importance for DC functions (Pantel et al., 2014). Both type I (Kubo et al., 2013; Pollara et al., 2006) and type II (Fricke et al., 2006; Pan et al., 2004) IFNs have been noted in this process, although the type I IFN axis is sufficient for human DC. Studies on CD40 regulation in murine phagocytes have shown that an early NF-κB signal is required to bind to the promoter, followed by a IFN-derived STAT1 signal, in order to achieve full up-regulation (Qin et al., 2005). In DC, blockade of either NF-κB (O'Sullivan and Thomas, 2002) or the autocrine IFN axis (Kubo et al., 2013) can suppress the up-regulation of maturation markers.

The use of the PTP inhibitor vanadate to restore STAT1 tyrosine phosphorylation and DC maturation would support the notion of a bacterial tyrosine phosphatase targeting STAT1. Indeed, many viable bacteria actively translocate tyrosine phosphatases into host cells to modify function (Whitmore and Lamont, 2012), and many pathogenic tyrosine phosphatases (e.g. YopH from Yersinia spp. (Andersson et al., 1996), SptP from *S. typhimurium* (Kaniga et al., 1996) and VH1 from vaccinia virus H1 (Guan et al., 1991)) are exquisitely sensitive to vanadate. Furthermore, it was shown that bacteriostatic levels of chloramphenicol (which disrupts *de novo* bacterial protein synthesis) rendered DC resistant to the modulatory effects of live NmB.

Tyrosine dephosphorylation of STAT1 was likely to be a direct targeting of the transcription factor for several reasons. Firstly, it was shown that the effect was dominant over the addition of exogenous stimuli (TLR ligands or IFN), therefore it is unlikely to be a lack of a signal *per se* but rather an active inhibition. Secondly, it was shown that the upstream IFN pathway (IFN secretion and JAK activation) was fully functional. And lastly, at saturating levels of stimulation, STAT1 can be tyrosine phosphorylated independently of the IFN-JAK axis. The tyrosine kinase Src, which was also activated by live NmB, can fulfill this function (Rawlings et al., 2004). LPS alone can activate Src (Napolitani et al., 2003), and given a high enough level of LPS stimulation, DC can still up-regulate CD86 even with blocked IFN receptors (Pollara et al., 2006). Given this redundancy, it is therefore logical that a highly active bacterial phosphatase was targeting STAT1 directly.

Analysis of the meningococcal genome found two candidate proteins that had potential tyrosine phosphatase activity. Cloning of the proteins into a eukaryotic ISRE reporter cell line found that NMB_0945 alone caused a reduction in IFN-β signalling that was comparable to viral protein positive controls. Since meningococcal phosphatases possess optimum activity at a low pH (Leahy et al., 1940), it may be that the acidification that accompanies immune cell activation (Azuma et al., 1996) is required for optimal enzymatic activity, and therefore optimal dephosphorylation of STAT1-Y701. These data provide an exciting framework with which to explore the bacterial determinant of DC dysregulation.

Homologues of this gene appear across the Neisseria genus, and are also present in *N. lactamica*, *N. gonorrhoeae* and other serogroups of *N. meningitidis*. Indeed, preliminary data have shown that DC respond similarly to *N. lactamica* (i.e. inhibited maturation marker up-regulation and enhanced cytokine secretion), and experiments within this study showed that both WT MC58 and WT H44/76 exerted a similar phenotype. Therefore, this could represent an important virulence factor across the *Neisseria* genus that endows these bacteria with pro-inflammatory properties.

Important further work could involve knocking out the NMB_0945 gene in NmB with an antibiotic resistance cassette and then infecting DC with the viable mutant strain. STAT1-Y701 phosphorylation could then be measured, and the overall maturation response could be deduced. If this mutant strain were to restore DC maturation, it would be extremely compelling evidence that this could be considered an entirely novel inflammatory virulence factor

(and immune evasion factor) for the meningococcus. Also, any future vaccine would probably benefit from excluding this protein from the preparation, given its immunosuppressive properties.

In conclusion, this study has shown that live NmB can dysregulate DC maturation by the precise targeting of a single tyrosine residue on the transcription factor STAT1. This had wide-ranging implications for the immunological functions of these cells, such as the impairment of adequate T-cell responses. However, the reversal of this effect was sufficient to restore DC functions. This was linked to a possible protein encoded by the bacterium. These data could help to further our understanding of human immunity to the meningococcus, but they also link this to a novel mechanism of pathogenesis and immune evasion. Importantly, the proposed model of DC dysregulation by NmB links pathogenesis and immune evasion into a single mechanism, which is a wide-ranging motif in meningococcal research. Therefore, these data expand the paradigm of host-pathogen interactions for the meningococcus.

8 References

Abt, M.C., Osborne, L.C., Monticelli, L.A., Doering, T.A., Alenghat, T., Sonnenberg, G.F., Paley, M.A., Antenus, M., Williams, K.L., Erikson, J., et al. (2012). Commensal Bacteria Calibrate the Activation Threshold of Innate Antiviral Immunity. Immunity *37*, 158–170.

Achouak, W., Heulin, T., and Pagès, J.-M. (2001). Multiple facets of bacterial porins. FEMS Microbiol. Lett. *199*, 1–7.

Lai A Fat, R.F.M., Suurmond, D., and Van Furth, R. (1973). In vitro synthesis of immunoglobulins, secretory component and complement in normal and pathological skin and the adjacent mucous membranes. Clin. Exp. Immunol. *14*, 377–395.

Akashi, S., Nagai, Y., Ogata, H., Oikawa, M., Fukase, K., Kusumoto, S., Kawasaki, K., Nishijima, M., Hayashi, S., Kimoto, M., et al. (2001). Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition. Int. Immunol. *13*, 1595–1599.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat. Rev. Immunol. *4*, 499–511.

Ala'Aldeen, D. a. A., Neal, K.R., Ait-Tahar, K., Nguyen-Van-Tam, J.S., English, A., Falla, T.J., Hawkey, P.M., and Slack, R.C.B. (2000). Dynamics of Meningococcal Long-Term Carriage among University Students and Their Implications for Mass Vaccination. J. Clin. Microbiol. *38*, 2311–2316.

Albert, M.L., Jegathesan, M., and Darnell, R.B. (2001). Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. Nat. Immunol. *2*, 1010–1017.

Albiger, B., Johansson, L., and Jonsson, A.-B. (2003). Lipooligosaccharide-Deficient Neisseria meningitidis Shows Altered Pilus-Associated Characteristics. Infect. Immun. *71*, 155–162.

Alessandrini, A., Haseth, S.D., Fray, M., Miyajima, M., Colvin, R.B., Williams, W.W., Benedict Cosimi, A., and Benichou, G. (2011). Dendritic cell maturation occurs through the inhibition of GSK-3β. Cell. Immunol. *270*, 114–125.

Alpuche Aranda, C.M., Swanson, J.A., Loomis, W.P., and Miller, S.I. (1992). Salmonella typhimurium activates virulence gene transcription within acidified macrophage phagosomes. Proc. Natl. Acad. Sci. U. S. A. *89*, 10079–10083.

Andersson, K., Carballeira, N., Magnusson, K.E., Persson, C., Stendahl, O., Wolf-Watz, H., and Fällman, M. (1996). YopH of Yersinia pseudotuberculosis

interrupts early phosphotyrosine signalling associated with phagocytosis. Mol. Microbiol. *20*, 1057–1069.

Anonychuk, A., Woo, G., Vyse, A., Demarteau, N., and Tricco, A.C. (2013). The cost and public health burden of invasive meningococcal disease outbreaks: a systematic review. PharmacoEconomics *31*, 563–576.

Antipov, A.N., Lyalikova, N.N., and L'vov, N.P. (2000). Vanadium-binding protein excreted by vanadate-reducing bacteria. IUBMB Life *49*, 137–141.

Ap, L., Qa, T.-T., A, M., C, B., and Rp, S. (2005). Serum amyloid A, properdin, complement 3, and toll-like receptors are expressed locally in human sinonasal tissue. Am. J. Rhinol. *20*, 117–123.

Appelberg, R. (1992). CD4+ T cells are required for antigen-specific recruitment of neutrophils by BCG-immune spleen cells. Immunology *75*, 414–419.

Attridge, S.R., Wallerström, G., Li, B.-L., Morona, R., and Holmgren, J. (2009). Differential immunogenicity of Vibrio cholerae O139 variants expressing different combinations of naturally occurring and atypical forms of the serogroup polysaccharide. Vaccine *27*, 1055–1061.

Autenrieth, S.E., Soldanova, I., Rösemann, R., Gunst, D., Zahir, N., Kracht, M., Ruckdeschel, K., Wagner, H., Borgmann, S., and Autenrieth, I.B. (2007). Yersinia enterocolitica YopP inhibits MAP kinase-mediated antigen uptake in dendritic cells. Cell. Microbiol. *9*, 425–437.

Avalle, L., Pensa, S., Regis, G., Novelli, F., and Poli, V. (2012). STAT1 and STAT3 in tumorigenesis. JAK-STAT *1*, 65–72.

Azuma, E.K., Yuo, A., Matsushima, K., Kasahara, T., Mizoguchi, H., Saito, M., Takaku, F., and Kitagawa, S. (1996). Activation and priming of human monocytes by monocyte chemotactic and activating factor: Cooperation with other inflammatory cytokines and close association between an increase in cytoplasmic free Ca2+ and intracellular acidification. Exp. Hematol. *24*, 169–175.

Al-Bader, T., Jolley, K.A., Humphries, H.E., Holloway, J., Heckels, J.E., Semper, A.E., Friedmann, P.S., and Christodoulides, M. (2004a). Activation of human dendritic cells by the PorA protein of Neisseria meningitidis. Cell. Microbiol. *6*, 651–662.

Al-Bader, T., Jolley, K.A., Humphries, H.E., Holloway, J., Heckels, J.E., Semper, A.E., Friedmann, P.S., and Christodoulides, M. (2004b). Activation of human dendritic cells by the PorA protein of Neisseria meningitidis. Cell. Microbiol. *6*, 651–662.

Baetz, A., Frey, M., Heeg, K., and Dalpke, A.H. (2004). Suppressor of Cytokine Signaling (SOCS) Proteins Indirectly Regulate Toll-like Receptor Signaling in Innate Immune Cells. J. Biol. Chem. *279*, 54708–54715.

Baker, M., McNicholas, A., Garrett, N., Jones, N., Stewart, J., Koberstein, V., and Lennon, D. (2000). Household crowding a major risk factor for epidemic meningococcal disease in Auckland children. Pediatr. Infect. Dis. J. *19*, 983–990.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.-J., Pulendran, B., and Palucka, K. (2000). Immunobiology of Dendritic Cells. Annu. Rev. Immunol. *18*, 767–811.

Banchereau, R., Baldwin, N., Cepika, A.-M., Athale, S., Xue, Y., Yu, C.I., Metang, P., Cheruku, A., Berthier, I., Gayet, I., et al. (2014). Transcriptional specialization of human dendritic cell subsets in response to microbial vaccines. Nat. Commun. *5*, 5283.

Banks, K.E., Humphreys, T.L., Li, W., Katz, B.P., Wilkes, D.S., and Spinola, S.M. (2007). Haemophilus ducreyi partially activates human myeloid dendritic cells. Infect. Immun. *75*, 5678–5685.

Baron, M., and Davignon, J.-L. (2008). Inhibition of IFN-γ-Induced STAT1 Tyrosine Phosphorylation by Human CMV Is Mediated by SHP2. J. Immunol. *181*, 5530–5536.

Van Beelen, A.J., Zelinkova, Z., Taanman-Kueter, E.W., Muller, F.J., Hommes, D.W., Zaat, S.A.J., Kapsenberg, M.L., and de Jong, E.C. (2007). Stimulation of the Intracellular Bacterial Sensor NOD2 Programs Dendritic Cells to Promote Interleukin-17 Production in Human Memory T Cells. Immunity *27*, 660–669.

Bemark, M., Boysen, P., and Lycke, N.Y. (2012). Induction of gut IgA production through T cell-dependent and T cell-independent pathways. Ann. N. Y. Acad. Sci. *1247*, 97–116.

Benedict, C.A., Loewendorf, A., Garcia, Z., Blazar, B.R., and Janssen, E.M. (2008). Dendritic cell programming by cytomegalovirus stunts naive T cell responses via the PD-L1/PD-1 pathway. J. Immunol. Baltim. Md 1950 *180*, 4836–4847.

Bernard, S.C., Simpson, N., Join-Lambert, O., Federici, C., Laran-Chich, M.-P., Maïssa, N., Bouzinba-Ségard, H., Morand, P.C., Chretien, F., Taouji, S., et al. (2014). Pathogenic Neisseria meningitidis utilizes CD147 for vascular colonization. Nat. Med. *20*, 725–731.

Le Bert, N., Chain, B.M., Rook, G., and Noursadeghi, M. (2011). DC Priming by M. vaccae Inhibits Th2 Responses in Contrast to Specific TLR2 Priming and Is Associated with Selective Activation of the CREB Pathway. PLoS ONE *6*, e18346.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, T.G., Bertoni, M., Bordoli, L., et al. (2014). SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res. *42*, W252–W258.

Bisgaard, H., Hermansen, M.N., Buchvald, F., Loland, L., Halkjaer, L.B., Bønnelykke, K., Brasholt, M., Heltberg, A., Vissing, N.H., Thorsen, S.V., et al. (2007). Childhood Asthma after Bacterial Colonization of the Airway in Neonates. N. Engl. J. Med. *357*, 1487–1495.

Bjerknes, R., Guttormsen, H.K., Solberg, C.O., and Wetzler, L.M. (1995). Neisserial porins inhibit human neutrophil actin polymerization, degranulation, opsonin receptor expression, and phagocytosis but prime the neutrophils to increase their oxidative burst. Infect. Immun. *63*, 160–167.

Borrow, R., Balmer, P., and Miller, E. (2005). Meningococcal surrogates of protection—serum bactericidal antibody activity. Vaccine *23*, 2222–2227.

Bos, M.P., Hogan, D., and Belland, R.J. (1997). Selection of Opa+ Neisseria gonorrhoeae by limited availability of normal human serum. Infect. Immun. *65*, 645–650.

Botos, I., Segal, D.M., and Davies, D.R. (2011). The Structural Biology of Toll-like Receptors. Structure *19*, 447–459.

Boulton, I.C., and Gray-Owen, S.D. (2002). Neisserial binding to CEACAM1 arrests the activation and proliferation of CD4+ T lymphocytes. Nat. Immunol. *3*, 229–236.

Bouma, G., Burns, S., and Thrasher, A.J. (2007). Impaired T-cell priming in vivo resulting from dysfunction of WASp-deficient dendritic cells. Blood *110*, 4278–4284.

Bozza, F.A., Salluh, J.I., Japiassu, A.M., Soares, M., Assis, E.F., Gomes, R.N., Bozza, M.T., Castro-Faria-Neto, H.C., and Bozza, P.T. (2007). Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. Crit. Care *11*, R49.

Brandtzaeg, P. (2011). Immune functions of nasopharyngeal lymphoid tissue. Adv. Otorhinolaryngol. *72*, 20–24.

Brandtzaeg, P., and Pabst, R. (2004). Let's go mucosal: communication on slippery ground. Trends Immunol. *25*, 570–577.

Brandtzaeg, P., Bjerre, A., Øvstebø, R., Brusletto, B., Joø, G.B., and Kierulf, P. (2001). Invited review: Neisseria meningitidis lipopolysaccharides in human pathology. J. Endotoxin Res. *7*, 401–420.

Breckpot, K., Emeagi, P., Dullaers, M., Michiels, A., Heirman, C., and Thielemans, K. (2007). Activation of Immature Monocyte-Derived Dendritic

Cells After Transduction with High Doses of Lentiviral Vectors. Hum. Gene Ther. *18*, 536–546.

Breckpot, K., Escors, D., Arce, F., Lopes, L., Karwacz, K., Van Lint, S., Keyaerts, M., and Collins, M. (2010). HIV-1 Lentiviral Vector Immunogenicity Is Mediated by Toll-Like Receptor 3 (TLR3) and TLR7. J. Virol. *84*, 5627–5636.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil Extracellular Traps Kill Bacteria. Science *303*, 1532–1535.

Brown, E.J., Hosea, S.W., and Frank, M.M. (1983). The role of antibody and complement in the reticuloendothelial clearance of pneumococci from the bloodstream. Rev. Infect. Dis. *5 Suppl 4*, S797–S805.

Brownlie, R.J., and Zamoyska, R. (2013). T cell receptor signalling networks: branched, diversified and bounded. Nat. Rev. Immunol. *13*, 257–269.

Budroni, S., Siena, E., Hotopp, J.C.D., Seib, K.L., Serruto, D., Nofroni, C., Comanducci, M., Riley, D.R., Daugherty, S.C., Angiuoli, S.V., et al. (2011). Neisseria meningitidis is structured in clades associated with restriction modification systems that modulate homologous recombination. Proc. Natl. Acad. Sci. *108*, 4494–4499.

Cain, J.A., Solis, N., and Cordwell, S.J. (2014). Beyond gene expression: the impact of protein post-translational modifications in bacteria. J. Proteomics *97*, 265–286.

Callaghan, M.J., Jolley, K.A., and Maiden, M.C.J. (2006). Opacity-associated adhesin repertoire in hyperinvasive Neisseria meningitidis. Infect. Immun. *74*, 5085–5094.

Cameron, P.U., and Stent, G. (2001). Isolation of human tonsillar dendritic cells. Methods Mol. Med. *64*, 121–136.

Caron, G., Duluc, D., Frémaux, I., Jeannin, P., David, C., Gascan, H., and Delneste, Y. (2005). Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells. J. Immunol. Baltim. Md 1950 *175*, 1551–1557.

Carter, A.B., Knudtson, K.L., Monick, M.M., and Hunninghake, G.W. (1999). The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP). J. Biol. Chem. *274*, 30858–30863.

Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I., and Lanzavecchia, A. (1999). Maturation, Activation, and Protection of Dendritic Cells Induced by Double-stranded RNA. J. Exp. Med. *189*, 821–829.

Ceponis, P.J.M., McKay, D.M., Ching, J.C.Y., Pereira, P., and Sherman, P.M. (2003). Enterohemorrhagic Escherichia coli O157:H7 Disrupts Stat1-Mediated Gamma Interferon Signal Transduction in Epithelial Cells. Infect. Immun. *71*, 1396–1404.

Cerutti, A. (2008). The regulation of IgA class switching. Nat. Rev. Immunol. *8*, 421–434.

Chan, E., Schaller, T., Eddaoudi, A., Zhan, H., Tan, C.P., Jacobsen, M., Thrasher, A.J., Towers, G.J., and Qasim, W. (2012). Lentiviral Gene Therapy Against Human Immunodeficiency Virus Type 1, Using a Novel Human TRIM21-Cyclophilin A Restriction Factor. Hum. Gene Ther. *23*, 1176–1185.

Chandran, A., Herbert, H., Misurski, D., and Santosham, M. (2011). Long-term Sequelae of Childhood Bacterial Meningitis: An Underappreciated Problem. Pediatr. Infect. Dis. J. *30*, 3–6.

Chen, L., and Flies, D.B. (2013). Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat. Rev. Immunol. *13*, 227–242.

Chen, G., Shaw, M.H., Kim, Y.-G., and Nuñez, G. (2009). NOD-Like Receptors: Role in Innate Immunity and Inflammatory Disease. Annu. Rev. Pathol. Mech. Dis. *4*, 365–398.

Childs, K., Randall, R., and Goodbourn, S. (2012). Paramyxovirus V Proteins Interact with the RNA Helicase LGP2 To Inhibit RIG-I-Dependent Interferon Induction. J. Virol. *86*, 3411–3421.

Chirumbolo, S. (2012). State-of-the-art review about basophil research in immunology and allergy: is the time right to treat these cells with the respect they deserve? Blood Transfus. *10*, 148–168.

Chon, S.Y., Hassanain, H.H., and Gupta, S.L. (1996). Cooperative role of interferon regulatory factor 1 and p91 (STAT1) response elements in interferon-gamma-inducible expression of human indoleamine 2,3-dioxygenase gene. J. Biol. Chem. *271*, 17247–17252.

Chu, C.-C., Di Meglio, P., and Nestle, F.O. (2011). Harnessing dendritic cells in inflammatory skin diseases. Semin. Immunol. *23*, 28–41.

Chung, C.-S., Chen, Y., Grutkoski, P.S., Doughty, L., and Ayala, A. (2007). SOCS-1 is a central mediator of steroid-increased thymocyte apoptosis and decreased survival following sepsis. Apoptosis Int. J. Program. Cell Death *12*, 1143–1153.

Cisz, M., Lee, P.-C., and Rietsch, A. (2008). ExoS Controls the Cell Contact-Mediated Switch to Effector Secretion in Pseudomonas aeruginosa. J. Bacteriol. *190*, 2726–2738.

Coen, P.G., Cartwright, K., and Stuart, J. (2000). Mathematical modelling of infection and disease due to Neisseria meningitidis and Neisseria lactamica. Int. J. Epidemiol. *29*, 180–188.

Cohen, C., Singh, E., Wu, H.M., Martin, S., de Gouveia, L., Klugman, K.P., Meiring, S., Govender, N., and von Gottberg, A. (2010). Increased incidence of meningococcal disease in HIV-infected individuals associated with higher case-fatality ratios in South Africa: AIDS *24*, 1351–1360.

Collin, M., Bigley, V., Haniffa, M., and Hambleton, S. (2011). Human dendritic cell deficiency: the missing ID? Nat. Rev. Immunol. *11*, 575–583.

Collin, M., McGovern, N., and Haniffa, M. (2013). Human dendritic cell subsets. Immunology *140*, 22–30.

Conley, M.E., Dobbs, A.K., Farmer, D.M., Kilic, S., Paris, K., Grigoriadou, S., Coustan-Smith, E., Howard, V., and Campana, D. (2009). Primary B Cell Immunodeficiencies: Comparisons and Contrasts. Annu. Rev. Immunol. *27*, 199–227.

Conly, J.M., and Stein, K. (1992). The production of menaquinones (vitamin K2) by intestinal bacteria and their role in maintaining coagulation homeostasis. Prog. Food Nutr. Sci. *16*, 307–343.

Corcoran, K., Jabbour, M., Bhagwandin, C., Deymier, M.J., Theisen, D.L., and Lybarger, L. (2011). Ubiquitin-mediated regulation of CD86 protein expression by the ubiquitin ligase membrane-associated RING-CH-1 (MARCH1). J. Biol. Chem. *286*, 37168–37180.

Costerton, J.W., Irvin, R.T., and Cheng, K.J. (1981). The Bacterial Glycocalyx in Nature and Disease. Annu. Rev. Microbiol. *35*, 299–324.

Creagh, E.M., and O'Neill, L.A.J. (2006). TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends Immunol. *27*, 352–357.

Dai, X., Sayama, K., Tohyama, M., Shirakata, Y., Yang, L., Hirakawa, S., Tokumaru, S., and Hashimoto, K. (2008). The NF-kappaB, p38 MAPK and STAT1 pathways differentially regulate the dsRNA-mediated innate immune responses of epidermal keratinocytes. Int. Immunol. *20*, 901–909.

Davenport, V., Guthrie, T., Findlow, J., Borrow, R., Williams, N.A., and Heyderman, R.S. (2003). Evidence for naturally acquired T cell-mediated mucosal immunity to Neisseria meningitidis. J. Immunol. Baltim. Md 1950 *171*, 4263–4270.

Davenport, V., Groves, E., Hobbs, C.G., Williams, N.A., and Heyderman, R.S. (2007). Regulation of Th-1 T cell-dominated immunity to Neisseria meningitidis within the human mucosa. Cell. Microbiol. *9*, 1050–1061.

Davila, S., Wright, V.J., Khor, C.C., Sim, K.S., Binder, A., Breunis, W.B., Inwald, D., Nadel, S., Betts, H., Carrol, E.D., et al. (2010). Genome-wide association study identifies variants in the CFH region associated with host susceptibility to meningococcal disease. Nat. Genet. *42*, 772–776.

Deghmane, A.-E., Veckerlé, C., Giorgini, D., Hong, E., Ruckly, C., and Taha, M.-K. (2009). Differential Modulation of TNF-α–Induced Apoptosis by Neisseria meningitidis. PLoS Pathog *5*, e1000405.

Deghmane, A.-E., El Kafsi, H., Giorgini, D., Abaza, A., and Taha, M.-K. (2011). Late Repression of NF-κB Activity by Invasive but Not Non-Invasive Meningococcal Isolates Is Required to Display Apoptosis of Epithelial Cells. PLoS Pathog *7*, e1002403.

Dehio, C., Gray-Owen, S.D., and Meyer, T.F. (1998). The role of neisserial Opa proteins in interactions with host cells. Trends Microbiol. *6*, 489–495.

Van Deuren, M., Brandtzaeg, P., and van der Meer, J.W.M. (2000). Update on Meningococcal Disease with Emphasis on Pathogenesis and Clinical Management. Clin. Microbiol. Rev. *13*, 144–166.

Deutch, S., Labouriau, R., Schønheyeder, H.C., Ostergaard, L., Nørgård, B., and Sørensen, H.T. (2004). Crowding as a risk factor of meningococcal disease in Danish preschool children: a nationwide population-based case-control study. Scand. J. Infect. Dis. *36*, 20–23.

Devaux, P., Priniski, L., and Cattaneo, R. (2013). The measles virus phosphoprotein interacts with the linker domain of STAT1. Virology *444*, 250–256.

Dinauer, M.C. (2007). Disorders of neutrophil function: an overview. Methods Mol. Biol. Clifton NJ *412*, 489–504.

Dixon, G.L.J., Newton, P.J., Chain, B.M., Katz, D., Andersen, S.R., Wong, S., Ley, P. van der, Klein, N., and Callard, R.E. (2001). Dendritic Cell Activation and Cytokine Production Induced by Group B Neisseria meningitidis: Interleukin-12 Production Depends on Lipopolysaccharide Expression in Intact Bacteria. Infect. Immun. *69*, 4351–4357.

Drickamer, K. (1999). C-type lectin-like domains. Curr. Opin. Struct. Biol. *9*, 585–590.

Duffin, R., Leitch, A.E., Fox, S., Haslett, C., and Rossi, A.G. (2010). Targeting granulocyte apoptosis: mechanisms, models, and therapies. Immunol. Rev. *236*, 28–40.

Dunn, K.L.R., Virji, M., and Moxon, E.R. (1995). Investigations into the molecular basis of meningococcal toxicity for human endothelial and epithelial cells: the synergistic effect of LPS and pili. Microb. Pathog. *18*, 81–96.

Engelmayer, J., Larsson, M., Subklewe, M., Chahroudi, A., Cox, W.I., Steinman, R.M., and Bhardwaj, N. (1999). Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. J. Immunol. Baltim. Md 1950 *163*, 6762–6768.

Erfurth, S.E., Grobner, S., Kramer, U., Gunst, D.S.J., Soldanova, I., Schaller, M., Autenrieth, I.B., and Borgmann, S. (2004). Yersinia enterocolitica Induces Apoptosis and Inhibits Surface Molecule Expression and Cytokine Production in Murine Dendritic Cells. Infect. Immun. *72*, 7045–7054.

Escors, D., Liechtenstein, T., Perez-Janices, N., Schwarze, J., Dufait, I., Goyvaerts, C., Lanna, A., Arce, F., Blanco-Luquin, I., Kochan, G., et al. (2013). Assessing T-cell responses in anticancer immunotherapy. Oncoimmunology *2*.

Evans, C.M., Pratt, C.B., Matheson, M., Vaughan, T.E., Findlow, J., Borrow, R., Gorringe, A.R., and Read, R.C. (2011). Nasopharyngeal Colonization by Neisseria lactamica and Induction of Protective Immunity against Neisseria meningitidis. Clin. Infect. Dis. *52*, 70–77.

Everts, B., Amiel, E., Huang, S.C.-C., Smith, A.M., Chang, C.-H., Lam, W.Y., Redmann, V., Freitas, T.C., Blagih, J., van der Windt, G.J.W., et al. (2014). TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. Nat. Immunol. *15*, 323–332.

Exley, R.M., Goodwin, L., Mowe, E., Shaw, J., Smith, H., Read, R.C., and Tang, C.M. (2005). Neisseria meningitidis Lactate Permease Is Required for Nasopharyngeal Colonization. Infect. Immun. *73*, 5762–5766.

Fan, H., and Cook, J.A. (2004). Molecular mechanisms of endotoxin tolerance. J. Endotoxin Res. *10*, 71–84.

Felix, N.J., and Allen, P.M. (2007). Specificity of T-cell alloreactivity. Nat. Rev. Immunol. *7*, 942–953.

Fieschi, C., Dupuis, S., Catherinot, E., Feinberg, J., Bustamante, J., Breiman, A., Altare, F., Baretto, R., Deist, F.L., Kayal, S., et al. (2003). Low Penetrance, Broad Resistance, and Favorable Outcome of Interleukin 12 Receptor β1 Deficiency Medical and Immunological Implications. J. Exp. Med. *197*, 527–535.

Fife, B.T., Pauken, K.E., Eagar, T.N., Obu, T., Wu, J., Tang, Q., Azuma, M., Krummel, M.F., and Bluestone, J.A. (2009). Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR–induced stop signal. Nat. Immunol. *10*, 1185–1192.

Figueroa, J.E., and Densen, P. (1991). Infectious diseases associated with complement deficiencies. Clin. Microbiol. Rev. *4*, 359–395.

Finne, J., Leinonen, M., and Mäkelä, P.H. (1983). ANTIGENIC SIMILARITIES BETWEEN BRAIN COMPONENTS AND BACTERIA CAUSING MENINGITIS: Implications for Vaccine Development and Pathogenesis. The Lancet *322*, 355–357.

Fischer, M., Hedberg, K., Cardosi, P., Plikaytis, B.D., Hoesly, F.C., Steingart, K.R., Bell, T.A., Fleming, D.W., Wenger, J.D., and Perkins, B.A. (1997). Tobacco smoke as a risk factor for meningococcal disease. Pediatr. Infect. Dis. J. *16*, 979–983.

Flajnik, M.F., and Kasahara, M. (2010). Origin and evolution of the adaptive immune system: genetic events and selective pressures. Nat. Rev. Genet. *11*, 47–59.

Foster, R.A., Carlring, J., McKendrick, M.W., Lees, A., Borrow, R., Read, R.C., and Heath, A.W. (2009). Evidence of a Functional B-Cell Immunodeficiency in Adults Who Experience Serogroup C Meningococcal Disease. Clin. Vaccine Immunol. CVI *16*, 692–698.

Foster, R.A., Carlring, J., Lees, A., Borrow, R., Ramsay, M., Kacsmarski, E., Miller, E., McKendrick, M.W., Heath, A.W., and Read, R.C. (2010). Functional T-Cell Deficiency in Adolescents Who Experience Serogroup C Meningococcal Disease despite Receiving the Meningococcal Serogroup C Conjugate Vaccine. Clin. Vaccine Immunol. CVI *17*, 1104–1110.

Frank, D.A., Mahajan, S., and Ritz, J. (1999). Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. Nat. Med. *5*, 444–447.

Fransen, F., Heckenberg, S.G.B., Hamstra, H.J., Feller, M., Boog, C.J.P., van Putten, J.P.M., van de Beek, D., van der Ende, A., and van der Ley, P. (2009). Naturally Occurring Lipid A Mutants in Neisseria meningitidis from Patients with Invasive Meningococcal Disease Are Associated with Reduced Coagulopathy. PLoS Pathog *5*, e1000396.

Fricke, I., Mitchell, D., Mittelstädt, J., Lehan, N., Heine, H., Goldmann, T., Böhle, A., and Brandau, S. (2006). Mycobacteria Induce IFN-γ Production in Human Dendritic Cells via Triggering of TLR2. J. Immunol. *176*, 5173–5182.

Fuks, J.M., Arrighi, R.B.G., Weidner, J.M., Kumar Mendu, S., Jin, Z., Wallin, R.P.A., Rethi, B., Birnir, B., and Barragan, A. (2012). GABAergic Signaling Is Linked to a Hypermigratory Phenotype in Dendritic Cells Infected by Toxoplasma gondii. PLoS Pathog *8*, e1003051.

Gao, Y., Nish, S.A., Jiang, R., Hou, L., Licona-Limón, P., Weinstein, J.S., Zhao, H., and Medzhitov, R. (2013). Control of T Helper 2 Responses by Transcription Factor IRF4-Dependent Dendritic Cells. Immunity *39*, 722–732.

De Gassart, A., Camosseto, V., Thibodeau, J., Ceppi, M., Catalan, N., Pierre, P., and Gatti, E. (2008). MHC class II stabilization at the surface of human

dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proc. Natl. Acad. Sci. U. S. A. *105*, 3491–3496.

Gassmann, M., Grenacher, B., Rohde, B., and Vogel, J. (2009). Quantifying Western blots: Pitfalls of densitometry. ELECTROPHORESIS *30*, 1845–1855.

Gautier, G., Humbert, M., Deauvieau, F., Scuiller, M., Hiscott, J., Bates, E.E.M., Trinchieri, G., Caux, C., and Garrone, P. (2005). A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. J. Exp. Med. *201*, 1435–1446.

Gay, N.J., and Keith, F.J. (1991). Drosophila Toll and IL-1 receptor. Nature *351*, 355–356.

Geijtenbeek, T.B.H., and Gringhuis, S.I. (2009). Signalling through C-type lectin receptors: shaping immune responses. Nat. Rev. Immunol. *9*, 465–479.

Geijtenbeek, T.B.H., Vliet, S.J. van, Koppel, E.A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C.M.J.E., Appelmelk, B., and Kooyk, Y. van (2003). Mycobacteria Target DC-SIGN to Suppress Dendritic Cell Function. J. Exp. Med. *197*, 7–17.

Germain, R.N. (2002). T-cell development and the CD4-CD8 lineage decision. Nat. Rev. Immunol. *2*, 309–322.

Gil, M.P., Bohn, E., O'Guin, A.K., Ramana, C.V., Levine, B., Stark, G.R., Virgin, H.W., and Schreiber, R.D. (2001). Biologic consequences of Stat1-independent IFN signaling. Proc. Natl. Acad. Sci. U. S. A. *98*, 6680–6685.

Ginhoux, F., and Jung, S. (2014). Monocytes and macrophages: developmental pathways and tissue homeostasis. Nat. Rev. Immunol. *14*, 392–404.

Goh, K.C., Haque, S.J., and Williams, B.R. (1999). p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. EMBO J. *18*, 5601–5608.

Gold, R., Goldschneider, I., Lepow, M.L., Draper, T.F., and Randolph, M. (1978). Carriage of Neisseria meningitidis and Neisseria lactamica in Infants and Children. J. Infect. Dis. *137*, 112–121.

Gollwitzer, E.S., Saglani, S., Trompette, A., Yadava, K., Sherburn, R., McCoy, K.D., Nicod, L.P., Lloyd, C.M., and Marsland, B.J. (2014). Lung microbiota promotes tolerance to allergens in neonates via PD-L1. Nat. Med. *20*, 642–647.

Goodbourn, S., Didcock, L., and Randall, R.E. (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J. Gen. Virol. *81*, 2341–2364.

Gorvel, L., Textoris, J., Banchereau, R., Ben Amara, A., Tantibhedhyangkul, W., von Bargen, K., Ka, M.B., Capo, C., Ghigo, E., Gorvel, J.-P., et al. (2014). Intracellular bacteria interfere with dendritic cell functions: role of the type I interferon pathway. PloS One *9*, e99420.

Guan, K.L., Broyles, S.S., and Dixon, J.E. (1991). A Tyr/Ser protein phosphatase encoded by vaccinia virus. Nature *350*, 359–362.

Guarda, G., Braun, M., Staehli, F., Tardivel, A., Mattmann, C., Förster, I., Farlik, M., Decker, T., Du Pasquier, R.A., Romero, P., et al. (2011). Type I Interferon Inhibits Interleukin-1 Production and Inflammasome Activation. Immunity *34*, 213–223.

Gunn, M.D., Kyuwa, S., Tam, C., Kakiuchi, T., Matsuzawa, A., Williams, L.T., and Nakano, H. (1999). Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J. Exp. Med. *189*, 451–460.

Hagiwara, Y., McGhee, J.R., Fujihashi, K., Kobayashi, R., Yoshino, N., Kataoka, K., Etani, Y., Kweon, M.-N., Tamura, S., Kurata, T., et al. (2003). Protective mucosal immunity in aging is associated with functional CD4+ T cells in nasopharyngeal-associated lymphoreticular tissue. J. Immunol. Baltim. Md 1950 *170*, 1754–1762.

Hallissey, C.M., Heyderman, R.S., and Williams, N.A. (2014). Human Tonsil-derived Dendritic Cells are Poor Inducers of T cell Immunity to Mucosally Encountered Pathogens. J. Infect. Dis. *209*, 1847–1856.

Hanafi, L.-A., Gauchat, D., Godin-Ethier, J., Possamaï, D., Duvignaud, J.-B., Leclerc, D., Grandvaux, N., and Lapointe, R. (2014). Fludarabine Downregulates Indoleamine 2,3-Dioxygenase in Tumors via a Proteasome-Mediated Degradation Mechanism. PLoS ONE *9*, e99211.

Handley, M.E., Thakker, M., Pollara, G., Chain, B.M., and Katz, D.R. (2005). JNK activation limits dendritic cell maturation in response to reactive oxygen species by the induction of apoptosis. Free Radic. Biol. Med. *38*, 1637–1652.

Hansson, M., Lundgren, A., Elgbratt, K., Quiding-Järbrink, M., Svennerholm, A.-M., and Johansson, E.-L. (2006). Dendritic cells express CCR7 and migrate in response to CCL19 (MIP-3beta) after exposure to Helicobacter pylori. Microbes Infect. Inst. Pasteur *8*, 841–850.

Harrison, L.H. (2010). Epidemiological Profile of Meningococcal Disease in the United States. Clin. Infect. Dis. *50*, S37–S44.

Harrison, L.H., Trotter, C.L., and Ramsay, M.E. (2009). Global epidemiology of meningococcal disease. Vaccine *27, Supplement 2*, B51–B63.

Hashimoto, S., Suzuki, T., Dong, H.-Y., Yamazaki, N., and Matsushima, K. (1999). Serial Analysis of Gene Expression in Human Monocytes and Macrophages. Blood *94*, 837–844.

Hawkins, C.A., Collignon, P., Adams, D.N., Bowden, F.J., and Cook, M.C. (2006). Profound lymphopenia and bacteraemia. Intern. Med. J. *36*, 385–388.

Hayden, M.S., and Ghosh, S. (2004). Signaling to NF-kappaB. Genes Dev. *18*, 2195–2224.

Heijtz, R.D., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H., and Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. Proc. Natl. Acad. Sci. *108*, 3047–3052.

Henderson, B., and Martin, A. (2011). Bacterial Virulence in the Moonlight: Multitasking Bacterial Moonlighting Proteins Are Virulence Determinants in Infectious Disease. Infect. Immun. *79*, 3476–3491.

Henn, V., Slupsky, J.R., Gräfe, M., Anagnostopoulos, I., Förster, R., Müller-Berghaus, G., and Kroczek, R.A. (1998). CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature *391*, 591–594.

Herman, A., Kappler, J.W., Marrack, P., and Pullen, A.M. (1991). Superantigens: Mechanism of T-Cell Stimulation and Role in Immune Responses. Annu. Rev. Immunol. *9*, 745–772.

De Heusch, M., Oldenhove, G., Urbain, J., Thielemans, K., Maliszewski, C., Leo, O., and Moser, M. (2004). Depending on their maturation state, splenic dendritic cells induce the differentiation of CD4(+) T lymphocytes into memory and/or effector cells in vivo. Eur. J. Immunol. *34*, 1861–1869.

Hilty, M., Burke, C., Pedro, H., Cardenas, P., Bush, A., Bossley, C., Davies, J., Ervine, A., Poulter, L., Pachter, L., et al. (2010). Disordered Microbial Communities in Asthmatic Airways. PLoS ONE *5*.

Hodges, A., Sharrocks, K., Edelmann, M., Baban, D., Moris, A., Schwartz, O., Drakesmith, H., Davies, K., Kessler, B., McMichael, A., et al. (2007). Activation of the lectin DC-SIGN induces an immature dendritic cell phenotype triggering Rho-GTPase activity required for HIV-1 replication. Nat. Immunol. *8*, 569–577.

Hoebe, K., Janssen, E.M., Kim, S.O., Alexopoulou, L., Flavell, R.A., Han, J., and Beutler, B. (2003). Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. Nat. Immunol. *4*, 1223–1229.

Hoffmann, J.A., and Reichhart, J.-M. (2002). Drosophila innate immunity: an evolutionary perspective. Nat. Immunol. *3*, 121–126.

Holbein, B.E. (1981). Enhancement of Neisseria meningitidis infection in mice by addition of iron bound to transferrin. Infect. Immun. *34*, 120–125.

Holten, E. (1979). Serotypes of Neisseria meningitidis isolated from patients in Norway during the first six months of 1978. J. Clin. Microbiol. *9*, 186–188.

Honda, K., Sakaguchi, S., Nakajima, C., Watanabe, A., Yanai, H., Matsumoto, M., Ohteki, T., Kaisho, T., Takaoka, A., Akira, S., et al. (2003). Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. Proc. Natl. Acad. Sci. U. S. A. *100*, 10872–10877.

Hong, B., Ren, W., Song, X.-T., Evel-Kabler, K., Chen, S.-Y., and Huang, X.F. (2009). Human suppressor of cytokine signaling 1 controls immunostimulatory activity of monocyte-derived dendritic cells. Cancer Res. *69*, 8076–8084.

Del Hoyo, G.M., Martín, P., Vargas, H.H., Ruiz, S., Arias, C.F., and Ardavín, C. (2002). Characterization of a common precursor population for dendritic cells. Nature *415*, 1043–1047.

Hu, L., Bray, M.D., Osorio, M., and Kopecko, D.J. (2006). Campylobacter jejuni induces maturation and cytokine production in human dendritic cells. Infect. Immun. *74*, 2697–2705.

Huang, S., and Terstappen, L.W. (1994). Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38- hematopoietic stem cells. Blood *83*, 1515–1526.

Huppa, J.B., and Davis, M.M. (2003). T-cell-antigen recognition and the immunological synapse. Nat. Rev. Immunol. *3*, 973–983.

Inaba, K., Steinman, R.M., Van Voorhis, W.C., and Muramatsu, S. (1983). Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and in man. Proc. Natl. Acad. Sci. U. S. A. *80*, 6041–6045.

Indramohan, M., Sieve, A.N., Break, T.J., and Berg, R.E. (2012). Inflammatory monocyte recruitment is regulated by interleukin-23 during systemic bacterial infection. Infect. Immun. *80*, 4099–4105.

Jankovic, D., Kullberg, M.C., Hieny, S., Caspar, P., Collazo, C.M., and Sher, A. (2002). In the absence of IL-12, CD4(+) T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-10(-/-) setting. Immunity *16*, 429–439.

Jerse, A.E., Cohen, M.S., Drown, P.M., Whicker, L.G., Isbey, S.F., Seifert, H.S., and Cannon, J.G. (1994). Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. J. Exp. Med. *179*, 911–920.

Jiang, X., Zhou, J., Giobbie-Hurder, A., Wargo, J., and Hodi, F.S. (2013). The activation of MAPK in melanoma cells resistant to BRAF inhibition promotes PD-L1 expression that is reversible by MEK and PI3K inhibition. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *19*, 598–609.

Jin, P., Han, T.H., Ren, J., Saunders, S., Wang, E., Marincola, F.M., and Stroncek, D.F. (2010). Molecular signatures of maturing dendritic cells: implications for testing the quality of dendritic cell therapies. J. Transl. Med. *8*, 4.

Johnson, L.M., and Scott, P. (2007). STAT1 expression in dendritic cells, but not T cells, is required for immunity to Leishmania major. J. Immunol. Baltim. Md 1950 *178*, 7259–7266.

Johswich, K.O., McCaw, S.E., Islam, E., Sintsova, A., Gu, A., Shively, J.E., and Gray-Owen, S.D. (2013). In Vivo Adaptation and Persistence of Neisseria meningitidis within the Nasopharyngeal Mucosa. PLoS Pathog *9*, e1003509.

Jones, H.E., Uronen-Hansson, H., Callard, R.E., Klein, N., and Dixon, G.L.J. (2007). The differential response of human dendritic cells to live and killed Neisseria meningitidis. Cell. Microbiol. *9*, 2856–2869.

Jones, H.E., Strid, J., Osman, M., Uronen-Hansson, H., Dixon, G., Klein, N., Wong, S.Y.C., and Callard, R.E. (2008). The role of beta2 integrins and lipopolysaccharide-binding protein in the phagocytosis of dead Neisseria meningitidis. Cell. Microbiol. *10*, 1634–1645.

Jones, H.E., Copland, A., Hamstra, H.J., Cohen, J., Brown, J., Klein, N., van der Ley, P., and Dixon, G. (2014). LOS oligosaccharide modification enhances dendritic cell responses to meningococcal native outer membrane vesicles expressing a non-toxic lipid A. Cell. Microbiol. *16*, 519–534.

De Jong, E.C., Vieira, P.L., Kalinski, P., Schuitemaker, J.H.N., Tanaka, Y., Wierenga, E.A., Yazdanbakhsh, M., and Kapsenberg, M.L. (2002). Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. J. Immunol. Baltim. Md 1950 *168*, 1704–1709.

Joshi, V.D., Kalvakolanu, D.V., Chen, W., Zhang, L., Kang, T.J., Thomas, K.E., Vogel, S.N., and Cross, A.S. (2006). A role for Stat1 in the regulation of lipopolysaccharide-induced interleukin-1beta expression. J. Interferon Cytokine Res. Off. J. Int. Soc. Interferon Cytokine Res. *26*, 739–747.

Judson, F.N., Ehret, J.M., and Eickhoff, T.C. (1978). Anogenital Infection with Neisseria meningitidis in Homosexual Men. J. Infect. Dis. *137*, 458–463.

Kadioglu, A., Coward, W., Colston, M.J., Hewitt, C.R.A., and Andrew, P.W. (2004). CD4-T-Lymphocyte Interactions with Pneumolysin and Pneumococci Suggest a Crucial Protective Role in the Host Response to Pneumococcal Infection. Infect. Immun. *72*, 2689–2697.

Kalupahana, R.S., Mastroeni, P., Maskell, D., and Blacklaws, B.A. (2005). Activation of murine dendritic cells and macrophages induced by Salmonella enterica serovar Typhimurium. Immunology *115*, 462–472.

Kamezaki, K., Shimoda, K., Numata, A., Matsuda, T., Nakayama, K.-I., and Harada, M. (2004). The role of Tyk2, Stat1 and Stat4 in LPS-induced endotoxin signals. Int. Immunol. *16*, 1173–1179.

Kanada, S., Nishiyama, C., Nakano, N., Suzuki, R., Maeda, K., Hara, M., Kitamura, N., Ogawa, H., and Okumura, K. (2011). Critical role of transcription factor PU.1 in the expression of CD80 and CD86 on dendritic cells. Blood *117*, 2211–2222.

Kaniga, K., Uralil, J., Bliska, J.B., and Galán, J.E. (1996). A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen Salmonella typhimurium. Mol. Microbiol. *21*, 633–641.

Kao, C.-Y., Chen, Y., Thai, P., Wachi, S., Huang, F., Kim, C., Harper, R.W., and Wu, R. (2004). IL-17 Markedly Up-Regulates β-Defensin-2 Expression in Human Airway Epithelium via JAK and NF-κB Signaling Pathways. J. Immunol. *173*, 3482–3491.

Kao, J.Y., Zhang, M., Miller, M.J., Mills, J.C., Wang, B., Liu, M., Eaton, K.A., Zou, W., Berndt, B.E., Cole, T.S., et al. (2010). Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. Gastroenterology *138*, 1046–1054.

Kawai, T., and Akira, S. (2006). TLR signaling. Cell Death Differ. *13*, 816–825.

Kawai, T., and Akira, S. (2007). Signaling to NF-kappaB by Toll-like receptors. Trends Mol. Med. *13*, 460–469.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999). Unresponsiveness of MyD88-Deficient Mice to Endotoxin. Immunity *11*, 115–122.

Keeney, M., Gratama, J.W., Chin-Yee, I.H., and Sutherland, D.R. (1998). Isotype controls in the analysis of lymphocytes and CD34+ stem and progenitor cells by flow cytometry--time to let go! Cytometry *34*, 280–283.

Kernbauer, E., Maier, V., Stoiber, D., Strobl, B., Schneckenleithner, C., Sexl, V., Reichart, U., Reizis, B., Kalinke, U., Jamieson, A., et al. (2012). Conditional Stat1 ablation reveals the importance of interferon signaling for immunity to Listeria monocytogenes infection. PLoS Pathog. *8*, e1002763.

Khalid, S., and Sansom, M.S.P. (2006). Molecular dynamics simulations of a bacterial autotransporter: NalP from Neisseria meningitidis. Mol. Membr. Biol. *23*, 499–508.

Khan, N., Gowthaman, U., Pahari, S., and Agrewala, J.N. (2012). Manipulation of Costimulatory Molecules by Intracellular Pathogens: Veni, Vidi, Vici!! PLoS Pathog *8*, e1002676.

Kholodenko, B.N. (2006). CELL SIGNALLING DYNAMICS IN TIME AND SPACE. Nat. Rev. Mol. Cell Biol. *7*, 165–176.

Kim, H.S., and Lee, M.-S. (2005). Essential Role of STAT1 in Caspase-Independent Cell Death of Activated Macrophages through the p38 Mitogen-Activated Protein Kinase/STAT1/Reactive Oxygen Species Pathway. Mol. Cell. Biol. *25*, 6821–6833.

Kim, M.-O., Si, Q., Zhou, J.N., Pestell, R.G., Brosnan, C.F., Locker, J., and Lee, S.C. (2002). Interferon-beta activates multiple signaling cascades in primary human microglia. J. Neurochem. *81*, 1361–1371.

Kinjyo, I., Hanada, T., Inagaki-Ohara, K., Mori, H., Aki, D., Ohishi, M., Yoshida, H., Kubo, M., and Yoshimura, A. (2002). SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity *17*, 583–591.

Klein, L., Kyewski, B., Allen, P.M., and Hogquist, K.A. (2014). Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat. Rev. Immunol. *14*, 377–391.

Klein, N.J., Ison, C.A., Peakman, M., Levin, M., Hammerschmidt, S., Frosch, M., and Heyderman, R.S. (1996). The influence of capsulation and lipooligosaccharide structure on neutrophil adhesion molecule expression and endothelial injury by Neisseria meningitidis. J. Infect. Dis. *173*, 172–179.

Koeberling, O., Seubert, A., and Granoff, D.M. (2008). Bactericidal antibody responses elicited by a meningococcal outer membrane vesicle vaccine with overexpressed factor H-binding protein and genetically attenuated endotoxin. J. Infect. Dis. *198*, 262–270.

Kolb, J.P., Casella, C.R., SenGupta, S., Chilton, P.M., and Mitchell, T.C. (2014). Type I interferon signaling contributes to the bias that Toll-like receptor 4 exhibits for signaling mediated by the adaptor protein TRIF. Sci. Signal. *7*, ra108–ra108.

Kolb-Maurer, A., Unkmeir, A., Kammerer, U., Hubner, C., Leimbach, T., Stade, A., Kampgen, E., Frosch, M., and Dietrich, G. (2001). Interaction of Neisseria meningitidis with Human Dendritic Cells. Infect. Immun. *69*, 6912–6922.

Komai-Koma, M., Gilchrist, D.S., and Xu, D. (2009). Direct recognition of LPS by human but not murine CD8+ T cells via TLR4 complex. Eur. J. Immunol. *39*, 1564–1572.

Kovarik, P., Stoiber, D., Eyers, P.A., Menghini, R., Neininger, A., Gaestel, M., Cohen, P., and Decker, T. (1999). Stress-induced phosphorylation of STAT1

at Ser727 requires p38 mitogen-activated protein kinase whereas IFN-gamma uses a different signaling pathway. Proc. Natl. Acad. Sci. U. S. A. *96*, 13956–13961.

Krawczyk, C.M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R.J., Cross, J.R., Jung, E., Thompson, C.B., Jones, R.G., et al. (2010). Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood *115*, 4742–4749.

Krishna, S.S., Tautz, L., Xu, Q., McMullan, D., Miller, M.D., Abdubek, P., Ambing, E., Astakhova, T., Axelrod, H.L., Carlton, D., et al. (2007). Crystal structure of NMA1982 from Neisseria meningitidis at 1.5 angstroms resolution provides a structural scaffold for nonclassical, eukaryotic-like phosphatases. Proteins *69*, 415–421.

Kubo, S., Yamaoka, K., Kondo, M., Yamagata, K., Zhao, J., Iwata, S., and Tanaka, Y. (2013). The JAK inhibitor, tofacitinib, reduces the T cell stimulatory capacity of human monocyte-derived dendritic cells. Ann. Rheum. Dis. annrheumdis – 2013–203756.

Kugelberg, E., Gollan, B., and Tang, C.M. (2008). Mechanisms in Neisseria meningitidis for resistance against complement-mediated killing. Vaccine *26*, I34–I39.

Kushwah, R., and Hu, J. (2010). Dendritic cell apoptosis: regulation of tolerance versus immunity. J. Immunol. Baltim. Md 1950 *185*, 795–802.

Kwon, H.-K., Lee, C.-G., So, J.-S., Chae, C.-S., Hwang, J.-S., Sahoo, A., Nam, J.H., Rhee, J.H., Hwang, K.-C., and Im, S.-H. (2010). Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc. Natl. Acad. Sci. U. S. A. *107*, 2159–2164.

Laidlaw, S.M., Robey, R., Davies, M., Giotis, E.S., Ross, C., Buttigieg, K., Goodbourn, S., and Skinner, M.A. (2013). Genetic Screen of a Mutant Poxvirus Library Identifies an Ankyrin Repeat Protein Involved in Blocking Induction of Avian Type I Interferon. J. Virol. *87*, 5041–5052.

Lambotin, M., Hoffmann, I., Laran-Chich, M.-P., Nassif, X., Couraud, P.O., and Bourdoulous, S. (2005). Invasion of endothelial cells by Neisseria meningitidis requires cortactin recruitment by a phosphoinositide-3-kinase/Rac1 signalling pathway triggered by the lipo-oligosaccharide. J. Cell Sci. *118*, 3805–3816.

Langenkamp, A., Messi, M., Lanzavecchia, A., and Sallusto, F. (2000). Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat. Immunol. *1*, 311–316.

Leahy, H.W., Stokinger, H.E., and Carpenter, C.M. (1940). The Demonstration of Phosphatase in Neisseria 1. J. Bacteriol. *40*, 435–440.

Lefebvre, S., Berrih-Aknin, S., Adrian, F., Moreau, P., Poea, S., Gourand, L., Dausset, J., Carosella, E.D., and Paul, P. (2001). A specific interferon (IFN)-stimulated response element of the distal HLA-G promoter binds IFN-regulatory factor 1 and mediates enhancement of this nonclassical class I gene by IFN-beta. J. Biol. Chem. *276*, 6133–6139.

Lehner, M., Felzmann, T., Clodi, K., and Holter, W. (2001). Type I interferons in combination with bacterial stimuli induce apoptosis of monocyte-derived dendritic cells. Blood *98*, 736–742.

Lejeune, D., Demoulin, J.B., and Renauld, J.C. (2001). Interleukin 9 induces expression of three cytokine signal inhibitors: cytokine-inducible SH2-containing protein, suppressor of cytokine signalling (SOCS)-2 and SOCS-3, but only SOCS-3 overexpression suppresses interleukin 9 signalling. Biochem. J. *353*, 109–116.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973–983.

Lewis, M., Tarlton, J.F., and Cose, S. (2007). Memory versus naive T-cell migration. Immunol. Cell Biol. *86*, 226–231.

Lewis, S.M., Khan, N., Beale, R., Treacher, D.F., and Brown, K.A. (2013). Depletion of blood neutrophils from patients with sepsis: treatment for the future? Int. Immunopharmacol. *17*, 1226–1232.

Li, N., McLaren, J.E., Michael, D.R., Clement, M., Fielding, C.A., and Ramji, D.P. (2010). ERK Is Integral to the IFN-γ–Mediated Activation of STAT1, the Expression of Key Genes Implicated in Atherosclerosis, and the Uptake of Modified Lipoproteins by Human Macrophages. J. Immunol. *185*, 3041–3048.

Li, P., Wang, N., Zhou, D., Yee, C.S.K., Chang, C.-H., Brutkiewicz, R.R., and Blum, J.S. (2005). Disruption of MHC Class II-Restricted Antigen Presentation by Vaccinia Virus. J. Immunol. *175*, 6481–6488.

Li, S., Rouphael, N., Duraisingham, S., Romero-Steiner, S., Presnell, S., Davis, C., Schmidt, D.S., Johnson, S.E., Milton, A., Rajam, G., et al. (2013). Molecular signatures of antibody responses derived from a systems biology study of five human vaccines. Nat. Immunol. *advance online publication*.

Li, Y., Zhang, Q., Winterbotham, M., Mowe, E., Gorringe, A., and Tang, C.M. (2006). Immunization with Live Neisseria lactamica Protects Mice against Meningococcal Challenge and Can Elicit Serum Bactericidal Antibodies. Infect. Immun. *74*, 6348–6355.

Liang, S., Ristich, V., Arase, H., Dausset, J., Carosella, E.D., and Horuzsko, A. (2008). Modulation of dendritic cell differentiation by HLA-G and ILT4 requires the IL-6--STAT3 signaling pathway. Proc. Natl. Acad. Sci. U. S. A. *105*, 8357–8362.

Liu, Y., and Russell, M.W. (2011). Diversion of the immune response to Neisseria gonorrhoeae from Th17 to Th1/Th2 by treatment with anti-transforming growth factor β antibody generates immunological memory and protective immunity. mBio *2*, e00095–00011.

Liu, M., John, C.M., and Jarvis, G.A. (2010). Phosphoryl moieties of lipid A from Neisseria meningitidis and N. gonorrhoeae lipooligosaccharides play an important role in activation of both MyD88- and TRIF-dependent TLR4-MD-2 signaling pathways. J. Immunol. Baltim. Md 1950 *185*, 6974–6984.

Liu, Y., Islam, E.A., Jarvis, G.A., Gray-Owen, S.D., and Russell, M.W. (2012). Neisseria gonorrhoeae selectively suppresses the development of Th1 and Th2 cells, and enhances Th17 cell responses, through TGF-β-dependent mechanisms. Mucosal Immunol. *5*, 320–331.

Liu, Y.-J., Barthélémy, C., Bouteiller, O.D., Arpin, C., Isabelle, D., and Banchereau, J. (1995). Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by Rapid Up-Regulation of B7-1 and B7-2. Immunity *2*, 239–248.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif *25*, 402–408.

Llera, A.S., Viedma, F., Sánchez-Madrid, F., and Tormo, J. (2001). Crystal structure of the C-type lectin-like domain from the human hematopoietic cell receptor CD69. J. Biol. Chem. *276*, 7312–7319.

Lo, H., Tang, C.M., and Exley, R.M. (2009). Mechanisms of avoidance of host immunity by Neisseria meningitidis and its effect on vaccine development. Lancet Infect. Dis. *9*, 418–427.

Logue, E.C., and Sha, W.C. (2004). CD28-B7 bidirectional signaling: a two-way street to activation. Nat. Immunol. *5*, 1103–1105.

Loh, E., Kugelberg, E., Tracy, A., Zhang, Q., Gollan, B., Ewles, H., Chalmers, R., Pelicic, V., and Tang, C.M. (2013). Temperature triggers immune evasion by Neisseria meningitidis. Nature *502*, 237–240.

Longhi, M.P., Trumpfheller, C., Idoyaga, J., Caskey, M., Matos, I., Kluger, C., Salazar, A.M., Colonna, M., and Steinman, R.M. (2009). Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J. Exp. Med. *206*, 1589–1602.

Longman, R.S., Braun, D., Pellegrini, S., Rice, C.M., Darnell, R.B., and Albert, M.L. (2007). Dendritic-cell maturation alters intracellular signaling networks, enabling differential effects of IFN-alpha/beta on antigen cross-presentation. Blood *109*, 1113–1122.

Lu, L.J., Sboner, A., Huang, Y.J., Lu, H.X., Gianoulis, T.A., Yip, K.Y., Kim, P.M., Montelione, G.T., and Gerstein, M.B. (2007). Comparing classical pathways and modern networks: towards the development of an edge ontology. Trends Biochem. Sci. *32*, 320–331.

Luckheeram, R.V., Zhou, R., Verma, A.D., and Xia, B. (2012). CD4+T Cells: Differentiation and Functions. Clin. Dev. Immunol. *2012*.

Lundberg, K., Albrekt, A.-S., Nelissen, I., Santegoets, S., de Gruijl, T.D., Gibbs, S., and Lindstedt, M. (2013). Transcriptional Profiling of Human Dendritic Cell Populations and Models - Unique Profiles of In Vitro Dendritic Cells and Implications on Functionality and Applicability. PLoS ONE *8*, e52875.

Luu, K., Greenhill, C.J., Majoros, A., Decker, T., Jenkins, B.J., and Mansell, A. (2014). STAT1 plays a role in TLR signal transduction and inflammatory responses. Immunol. Cell Biol. *92*, 761–769.

Mabbott, N.A., Kenneth Baillie, J., Hume, D.A., and Freeman, T.C. (2010). Meta-analysis of lineage-specific gene expression signatures in mouse leukocyte populations. Immunobiology *215*, 724–736.

Macedo, G.C., Magnani, D.M., Carvalho, N.B., Bruna-Romero, O., Gazzinelli, R.T., and Oliveira, S.C. (2008). Central Role of MyD88-Dependent Dendritic Cell Maturation and Proinflammatory Cytokine Production to Control Brucella abortus Infection. J. Immunol. *180*, 1080–1087.

Mahdavi, J., Royer, P.-J., Sjölinder, H.S., Azimi, S., Self, T., Stoof, J., Wheldon, L.M., Brännström, K., Wilson, R., Moreton, J., et al. (2013). Pro-inflammatory cytokines can act as intracellular modulators of commensal bacterial virulence. Open Biol. *3*, 130048.

Makras, P., Alexiou-Daniel, S., Antoniadis, A., and Hatzigeorgiou, D. (2001). Outbreak of Meningococcal Disease after an Influenza B Epidemic at a Hellenic Air Force Recruit Training Center. Clin. Infect. Dis. *33*, e48–e50.

Malefyt, R. de W., Abrams, J., Bennett, B., Figdor, C.G., and Vries, J.E. de (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. *174*, 1209–1220.

Mallon, D.F., Buck, A., Reece, J.C., Crowe, S.M., and Cameron, P.U. (1999). Monocyte-derived dendritic cells as a model for the study of HIV-1 infection: Productive infection and phenotypic changes during culture in human serum. Immunol. Cell Biol. *77*, 442–450.

Malu, S., Malshetty, V., Francis, D., and Cortes, P. (2012). Role of non-homologous end joining in V(D)J recombination. Immunol. Res. *54*, 233–246.

Martinez, F.O., Gordon, S., Locati, M., and Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J. Immunol. Baltim. Md 1950 *177*, 7303–7311.

Massari, P., Ho, Y., and Wetzler, L.M. (2000). Neisseria meningitidis porin PorB interacts with mitochondria and protects cells from apoptosis. Proc. Natl. Acad. Sci. U. S. A. *97*, 9070–9075.

Mattick, J.S. (2002). Type IV pili and twitching motility. Annu. Rev. Microbiol. *56*, 289–314.

McGuinness, B.T., Clarke, I.N., Lambden, P.R., Barlow, A.K., Poolman, J.T., Jones, D.M., and Heckels, J.E. (1991). Point mutation in meningococcal por A gene associated with increased endemic disease. Lancet *337*, 514–517.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394–397.

Melancon-Kaplan, J., Gronvik, K.O., and Murgita, R.A. (1986). Selective impairment of B cell function by Neisseria meningitidis. Cell. Immunol. *100*, 247–259.

Melican, K., Michea Veloso, P., Martin, T., Bruneval, P., and Duménil, G. (2013). Adhesion of Neisseria meningitidis to Dermal Vessels Leads to Local Vascular Damage and Purpura in a Humanized Mouse Model. PLoS Pathog *9*, e1003139.

Mestas, J., and Hughes, C.C.W. (2004). Of Mice and Not Men: Differences between Mouse and Human Immunology. J. Immunol. *172*, 2731–2738.

Miao, H., Hollenbaugh, J.A., Zand, M.S., Holden-Wiltse, J., Mosmann, T.R., Perelson, A.S., Wu, H., and Topham, D.J. (2010). Quantifying the Early Immune Response and Adaptive Immune Response Kinetics in Mice Infected with Influenza A Virus. J. Virol. *84*, 6687–6698.

Miller, J.C., Brown, B.D., Shay, T., Gautier, E.L., Jojic, V., Cohain, A., Pandey, G., Leboeuf, M., Elpek, K.G., Helft, J., et al. (2012). Deciphering the transcriptional network of the dendritic cell lineage. Nat. Immunol. *13*, 888–899.

Mitchell, D., Yong, M., Schroder, W., Black, M., Tirrell, M., and Olive, C. (2010). Dual Stimulation of MyD88-Dependent Toll-Like Receptors Induces Synergistically Enhanced Production of Inflammatory Cytokines in Murine Bone Marrow-Derived Dendritic Cells. J. Infect. Dis. *202*, 318–329.

Mittal, R., Bulgheresi, S., Emami, C., and Prasadarao, N.V. (2009). Enterobacter sakazakii targets DC-SIGN to induce immunosuppressive

responses in dendritic cells by modulating MAPKs. J. Immunol. Baltim. Md 1950 *183*, 6588–6599.

Mittal, R., Gonzalez-Gomez, I., and Prasadarao, N.V. (2010). Escherichia coli K1 promotes the ligation of CD47 with thrombospondin-1 to prevent the maturation of dendritic cells in the pathogenesis of neonatal meningitis. J. Immunol. Baltim. Md 1950 *185*, 2998–3006.

Miyajima, A., Kitamura, T., Harada, N., Yokota, T., and Arai, K. (1992). Cytokine Receptors and Signal Transduction. Annu. Rev. Immunol. *10*, 295–331.

Mogensen, T.H., Paludan, S.R., Kilian, M., and Østergaard, L. (2006a). Two neisseria meningitidis strains with different ability to stimulate toll-like receptor 4 through the MyD88-independent pathway. Scand. J. Immunol. *64*, 646–654.

Mogensen, T.H., Paludan, S.R., Kilian, M., and Østergaard, L. (2006b). Live Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. J. Leukoc. Biol. *80*, 267–277.

Mohamadzadeh, M., Olson, S., Kalina, W.V., Ruthel, G., Demmin, G.L., Warfield, K.L., Bavari, S., and Klaenhammer, T.R. (2005). Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. Proc. Natl. Acad. Sci. U. S. A. *102*, 2880–2885.

Mond, J.J., Vos, Q., Lees, A., and Snapper, C.M. (1995). T cell independent antigens. Curr. Opin. Immunol. *7*, 349–354.

Montoya, M., Schiavoni, G., Mattei, F., Gresser, I., Belardelli, F., Borrow, P., and Tough, D.F. (2002). Type I interferons produced by dendritic cells promote their phenotypic and functional activation. Blood *99*, 3263–3271.

Morales-Aza, B., Glennie, S.J., Garcez, T.P., Davenport, V., Johnston, S.L., Williams, N.A., and Heyderman, R.S. (2009). Impaired maintenance of naturally acquired T-cell memory to the meningococcus in patients with B-cell immunodeficiency. Blood *113*, 4206–4212.

Morelli, A.E., Larregina, A.T., Ganster, R.W., Zahorchak, A.F., Plowey, J.M., Takayama, T., Logar, A.J., Robbins, P.D., Falo, L.D., and Thomson, A.W. (2000). Recombinant Adenovirus Induces Maturation of Dendritic Cells via an NF-κB-Dependent Pathway. J. Virol. *74*, 9617–9628.

Mosleh, I.M., Huber, L.A., Steinlein, P., Pasquali, C., Günther, D., and Meyer, T.F. (1998). Neisseria gonorrhoeae Porin Modulates Phagosome Maturation. J. Biol. Chem. *273*, 35332–35338.

Moutaftsi, M., Mehl, A.M., Borysiewicz, L.K., and Tabi, Z. (2002). Human cytomegalovirus inhibits maturation and impairs function of monocyte-derived dendritic cells. Blood *99*, 2913–2921.

Murray, R.A., Siddiqui, M.R., Mendillo, M., Krahenbuhl, J., and Kaplan, G. (2007). Mycobacterium leprae inhibits dendritic cell activation and maturation. J. Immunol. Baltim. Md 1950 *178*, 338–344.

Musumeci, L., Bongiorni, C., Tautz, L., Edwards, R.A., Osterman, A., Perego, M., Mustelin, T., and Bottini, N. (2005). Low-molecular-weight protein tyrosine phosphatases of Bacillus subtilis. J. Bacteriol. *187*, 4945–4956.

Muzio, M., Bosisio, D., Polentarutti, N., D'amico, G., Stoppacciaro, A., Mancinelli, R., Veer, C. van't, Penton-Rol, G., Ruco, L.P., Allavena, P., et al. (2000). Differential Expression and Regulation of Toll-Like Receptors (TLR) in Human Leukocytes: Selective Expression of TLR3 in Dendritic Cells. J. Immunol. *164*, 5998–6004.

Nagl, M., Kacani, L., Mullauer, B., Lemberger, E.-M., Stoiber, H., Sprinzl, G.M., Schennach, H., and Dierich, M.P. (2002). Phagocytosis and Killing of Bacteria by Professional Phagocytes and Dendritic Cells. Clin. Diagn. Lab. Immunol. *9*, 1165–1168.

Najarro, P., Traktman, P., and Lewis, J.A. (2001). Vaccinia Virus Blocks Gamma Interferon Signal Transduction: Viral VH1 Phosphatase Reverses Stat1 Activation. J. Virol. *75*, 3185–3196.

Nakagawa, R., Naka, T., Tsutsui, H., Fujimoto, M., Kimura, A., Abe, T., Seki, E., Sato, S., Takeuchi, O., Takeda, K., et al. (2002). SOCS-1 Participates in Negative Regulation of LPS Responses. Immunity *17*, 677–687.

Nakahara, T., Moroi, Y., Uchi, H., and Furue, M. (2006). Differential role of MAPK signaling in human dendritic cell maturation and Th1/Th2 engagement. J. Dermatol. Sci. *42*, 1–11.

Nakao, T., Kim, S., Ohta, K., Kawano, H., Hino, M., Miura, K., Tatsumi, N., and Iwao, H. (2002). Role of mitogen-activated protein kinase family in serum-induced leukaemia inhibitory factor and interleukin-6 secretion by bone marrow stromal cells. Br. J. Pharmacol. *136*, 975–984.

Napolitani, G., Bortoletto, N., Racioppi, L., Lanzavecchia, A., and D'Oro, U. (2003). Activation of src-family tyrosine kinases by LPS regulates cytokine production in dendritic cells by controlling AP-1 formation. Eur. J. Immunol. *33*, 2832–2841.

Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A., and So, M. (1993). Antigenic variation of pilin regulates adhesion of Neisseria meningitidis to human epithelial cells. Mol. Microbiol. *8*, 719–725.

Newland, S.A., Phillips, J.M., Mastroeni, P., Azuma, M., Zaccone, P., and Cooke, A. (2011). PD-L1 blockade overrides Salmonella typhimurium-mediated diabetes prevention in NOD mice: No role for Tregs. Eur. J. Immunol. *41*, 2966–2976.

Niu, H., Cattoretti, G., and Dalla-Favera, R. (2003). BCL6 Controls the Expression of the B7-1/CD80 Costimulatory Receptor in Germinal Center B Cells. J. Exp. Med. *198*, 211–221.

Noske, N., Kämmerer, U., Rohde, M., and Hammerschmidt, S. (2009). Pneumococcal Interaction with Human Dendritic Cells: Phagocytosis, Survival, and Induced Adaptive Immune Response Are Manipulated by PavA. J. Immunol. *183*, 1952–1963.

Oftung, F., Løvik, M., Andersen, S.R., Frøholm, L.O., and Bjune, G. (1999). A mouse model utilising human transferrin to study protection against Neisseria meningitidis serogroup B induced by outer membrane vesicle vaccination. FEMS Immunol. Med. Microbiol. *26*, 75–82.

Olesky, M., Zhao, S., Rosenberg, R.L., and Nicholas, R.A. (2006). Porin-mediated antibiotic resistance in Neisseria gonorrhoeae: ion, solute, and antibiotic permeation through PIB proteins with penB mutations. J. Bacteriol. *188*, 2300–2308.

Oliver, K.J., Reddin, K.M., Bracegirdle, P., Hudson, M.J., Borrow, R., Feavers, I.M., Robinson, A., Cartwright, K., and Gorringe, A.R. (2002). Neisseria lactamica Protects against Experimental Meningococcal Infection. Infect. Immun. *70*, 3621–3626.

O'Neill, L.A.J., Golenbock, D., and Bowie, A.G. (2013). The history of Toll-like receptors — redefining innate immunity. Nat. Rev. Immunol. *13*, 453–460.

Opitz, H.G., Opitz, U., Lemke, H., Hewlett, G., Schreml, W., and Flad, H.D. (1977). The role of fetal calf serum in the primary immune response in vitro. J. Exp. Med. *145*, 1029–1038.

Osorio, F., and Reis e Sousa, C. (2011). Myeloid C-type Lectin Receptors in Pathogen Recognition and Host Defense. Immunity *34*, 651–664.

O'Sullivan, B.J., and Thomas, R. (2002). CD40 Ligation Conditions Dendritic Cell Antigen-Presenting Function Through Sustained Activation of NF-κB. J. Immunol. *168*, 5491–5498.

Øvstebø, R., Brandtzaeg, P., Brusletto, B., Haug, K.B.F., Lande, K., Høiby, E.A., and Kierulf, P. (2004). Use of Robotized DNA Isolation and Real-Time PCR To Quantify and Identify Close Correlation between Levels of Neisseria meningitidis DNA and Lipopolysaccharides in Plasma and Cerebrospinal Fluid from Patients with Systemic Meningococcal Disease. J. Clin. Microbiol. *42*, 2980–2987.

Pan, J., Zhang, M., Wang, J., Wang, Q., Xia, D., Sun, W., Zhang, L., Yu, H., Liu, Y., and Cao, X. (2004). Interferon-gamma is an autocrine mediator for dendritic cell maturation. Immunol. Lett. *94*, 141–151.

Pantel, A., Teixeira, A., Haddad, E., Wood, E.G., Steinman, R.M., and Longhi, M.P. (2014). Direct Type I IFN but Not MDA5/TLR3 Activation of Dendritic Cells Is Required for Maturation and Metabolic Shift to Glycolysis after Poly IC Stimulation. PLoS Biol *12*, e1001759.

Pappu, R., Rutz, S., and Ouyang, W. (2012). Regulation of epithelial immunity by IL-17 family cytokines. Trends Immunol. *33*, 343–349.

Park, H., Shin, J.W., Park, S.-G., and Kim, W. (2014). Microbial Communities in the Upper Respiratory Tract of Patients with Asthma and Chronic Obstructive Pulmonary Disease. PLoS ONE *9*, e109710.

Pathan, N., Faust, S., and Levin, M. (2003). Pathophysiology of meningococcal meningitis and septicaemia. Arch. Dis. Child. *88*, 601–607.

Pattison, M.J., MacKenzie, K.F., Elcombe, S.E., and Arthur, J.S.C. (2013). IFNβ autocrine feedback is required to sustain TLR induced production of MCP-1 in macrophages. Febs Lett. *587*, 1496–1503.

Pauli, E.-K., Schmolke, M., Wolff, T., Viemann, D., Roth, J., Bode, J.G., and Ludwig, S. (2008). Influenza A Virus Inhibits Type I IFN Signaling via NF-κB-Dependent Induction of SOCS-3 Expression. PLoS Pathog *4*, e1000196.

Perreau, M., Vigano, S., Bellanger, F., Pellaton, C., Buss, G., Comte, D., Roger, T., Lacabaratz, C., Bart, P.-A., Levy, Y., et al. (2014). Exhaustion of bacteria-specific CD4 T cells and microbial translocation in common variable immunodeficiency disorders. J. Exp. Med. *211*, 2033–2045.

Peters, M.J., Ross-Russell, R.I., White, D., Kerr, S.J., Eaton, F.E.M., Keengwe, I.N., Tasker, R.C., Wade, A.M., and Klein, N.J. (2001). Early severe neutropenia and thrombocytopenia identifies the highest risk cases of severe meningococcal disease. Pediatr. Crit. Care Med. J. Soc. Crit. Care Med. World Fed. Pediatr. Intensive Crit. Care Soc. *2*, 225–231.

Peterson, P.K., Verhoef, J., Schmeling, D., and Quie, P.G. (1977). Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leukocytes and monocytes. J. Infect. Dis. *136*, 502–509.

Pfistershammer, K., Klauser, C., Pickl, W.F., Stöckl, J., Leitner, J., Zlabinger, G., Majdic, O., and Steinberger, P. (2006). No evidence for dualism in function and receptors: PD-L2/B7-DC is an inhibitory regulator of human T cell activation. Eur. J. Immunol. *36*, 1104–1113.

Platanias, L.C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat. Rev. Immunol. *5*, 375–386.

Polak, M.E., Borthwick, N.J., Gabriel, F.G., Jager, M.J., and Cree, I.A. (2008). Activation of tonsil dendritic cells with immuno-adjuvants. BMC Immunol. *9*, 10.

Pollara, G., Handley, M.E., Kwan, A., Chain, B.M., and Katz, D.R. (2006). Autocrine Type I Interferon Amplifies Dendritic Cell Responses to Lipopolysaccharide via the Nuclear Factor-κB/p38 Pathways. Scand. J. Immunol. *63*, 151–154.

Pollard, A.J., Galassini, R., Rouppe van der Voort, E.M., Booy, R., Langford, P., Nadel, S., Ison, C., Kroll, J.S., Poolman, J., and Levin, M. (1999a). Humoral Immune Responses to Neisseria meningitidis in Children. Infect. Immun. *67*, 2441–2451.

Pollard, A.J., Galassini, R., Voort, E.M.R. van der, Hibberd, M., Booy, R., Langford, P., Nadel, S., Ison, C., Kroll, J.S., Poolman, J., et al. (1999b). Cellular Immune Responses to Neisseria meningitidis in Children. Infect. Immun. *67*, 2452–2463.

Precious, B.L., Carlos, T.S., Goodbourn, S., and Randall, R.E. (2007). Catalytic turnover of STAT1 allows PIV5 to dismantle the interferon-induced anti-viral state of cells. Virology *368*, 114–121.

Pridmore, A.C., Wyllie, D.H., Abdillahi, F., Steeghs, L., Ley, P. van der, Dower, S.K., and Read, R.C. (2001). A Lipopolysaccharide-Deficient Mutant of Neisseria meningitidis Elicits Attenuated Cytokine Release by Human Macrophages and Signals via Toll-like Receptor (TLR) 2 but Not via TLR4/MD2. J. Infect. Dis. *183*, 89–96.

Putonti, C., Nowicki, B., Shaffer, M., Fofanov, Y., and Nowicki, S. (2013). Where does Neisseria acquire foreign DNA from: an examination of the source of genomic and pathogenic islands and the evolution of the Neisseria genus. BMC Evol. Biol. *13*, 184.

Qin, H., Wilson, C.A., Lee, S.J., Zhao, X., and Benveniste, E.N. (2005). LPS induces CD40 gene expression through the activation of NF-κB and STAT-1α in macrophages and microglia. Blood *106*, 3114–3122.

Raetz, C.R.H. (1990). Biochemistry of Endotoxins. Annu. Rev. Biochem. *59*, 129–170.

Rahman, M.M., and McFadden, G. (2011). Modulation of NF-κB signalling by microbial pathogens. Nat. Rev. Microbiol. *9*, 291–306.

Rajagopal, D., Paturel, C., Morel, Y., Uematsu, S., Akira, S., and Diebold, S.S. (2010). Plasmacytoid dendritic cell–derived type I interferon is crucial for the adjuvant activity of Toll-like receptor 7 agonists. Blood *115*, 1949–1957.

Rawlings, J.S., Rosler, K.M., and Harrison, D.A. (2004). The JAK/STAT signaling pathway. J. Cell Sci. *117*, 1281–1283.

Re, F., and Strominger, J.L. (2004). IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are

specifically induced by TLR4 or TLR3 in human dendritic cells. J. Immunol. Baltim. Md 1950 *173*, 7548–7555.

Read, R.C. (2014). Neisseria meningitidis; clones, carriage, and disease. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. *20*, 391–395.

Reis e Sousa, C. (2006). Dendritic cells in a mature age. Nat. Rev. Immunol. *6*, 476–483.

Reller, L.B., MacGregor, R.R., and Beaty, H.N. (1973). Bactericidal Antibody after Colonization with Neisseria meningitidis. J. Infect. Dis. *127*, 56–62.

Rescigno, M., Martino, M., Sutherland, C.L., Gold, M.R., and Ricciardi-Castagnoli, P. (1998). Dendritic Cell Survival and Maturation Are Regulated by Different Signaling Pathways. J. Exp. Med. *188*, 2175–2180.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., and Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat. Immunol. *2*, 361–367.

Rissoan, M.-C., Soumelis, V., Kadowaki, N., Grouard, G., Briere, F., Malefyt, R. de W., and Liu, Y.-J. (1999). Reciprocal Control of T Helper Cell and Dendritic Cell Differentiation. Science *283*, 1183–1186.

Robbins, J.B., Schneerson, R., Xie, G., Hanson, L.Å., and Miller, M.A. (2011). Capsular polysaccharide vaccine for Group B Neisseria meningitidis, Escherichia coli K1, and Pasteurella haemolytica A2. Proc. Natl. Acad. Sci. *108*, 17871–17875.

Robinson, K., Neal, K.R., Howard, C., Stockton, J., Atkinson, K., Scarth, E., Moran, J., Robins, A., Todd, I., Kaczmarski, E., et al. (2002). Characterization of Humoral and Cellular Immune Responses Elicited by Meningococcal Carriage. Infect. Immun. *70*, 1301–1309.

Rodionova, E., Conzelmann, M., Maraskovsky, E., Hess, M., Kirsch, M., Giese, T., Ho, A.D., Zöller, M., Dreger, P., and Luft, T. (2007). GSK-3 mediates differentiation and activation of proinflammatory dendritic cells. Blood *109*, 1584–1592.

Rodríguez-García, M., Porichis, F., de Jong, O.G., Levi, K., Diefenbach, T.J., Lifson, J.D., Freeman, G.J., Walker, B.D., Kaufmann, D.E., and Kavanagh, D.G. (2011). Expression of PD-L1 and PD-L2 on human macrophages is up-regulated by HIV-1 and differentially modulated by IL-10. J. Leukoc. Biol. *89*, 507–515.

Rosenstein, N.E., Perkins, B.A., Stephens, D.S., Popovic, T., and Hughes, J.M. (2001). Meningococcal Disease. N. Engl. J. Med. *344*, 1378–1388.

Ross, S.C., Rosenthal, P.J., Berberich, H.M., and Densen, P. (1987). Killing of Neisseria meningitidis by human neutrophils: implications for normal and complement-deficient individuals. J. Infect. Dis. *155*, 1266–1275.

Rydström, A., and Wick, M.J. (2009). Monocyte and neutrophil recruitment during oral Salmonella infection is driven by MyD88-derived chemokines. Eur. J. Immunol. *39*, 3019–3030.

Rytkönen, A., Albiger, B., Hansson-Palo, P., Källström, H., Olcén, P., Fredlund, H., and Jonsson, A.-B. (2004). Neisseria meningitidis Undergoes PilC Phase Variation and PilE Sequence Variation during Invasive Disease. J. Infect. Dis. *189*, 402–409.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J. Exp. Med. *179*, 1109–1118.

Sallusto, F., Lenig, D., Förster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708–712.

Schneider, M.C., Exley, R.M., Ram, S., Sim, R.B., and Tang, C.M. (2007). Interactions between Neisseria meningitidis and the complement system. Trends Microbiol. *15*, 233–240.

Schoen, C., Blom, J., Claus, H., Schramm-Glück, A., Brandt, P., Müller, T., Goesmann, A., Joseph, B., Konietzny, S., Kurzai, O., et al. (2008). Whole-genome comparison of disease and carriage strains provides insights into virulence evolution in Neisseria meningitidis. Proc. Natl. Acad. Sci. U. S. A. *105*, 3473–3478.

Schreiber, H.A., Hulseberg, P.D., Lee, J., Prechl, J., Barta, P., Szlavik, N., Harding, J.S., Fabry, Z., and Sandor, M. (2010). Dendritic Cells in Chronic Mycobacterial Granulomas Restrict Local Anti-Bacterial T Cell Response in a Murine Model. PLoS ONE *5*, e11453.

Schroder, K., Spille, M., Pilz, A., Lattin, J., Bode, K.A., Irvine, K.M., Burrows, A.D., Ravasi, T., Weighardt, H., Stacey, K.J., et al. (2007). Differential effects of CpG DNA on IFN-beta induction and STAT1 activation in murine macrophages versus dendritic cells: alternatively activated STAT1 negatively regulates TLR signaling in macrophages. J. Immunol. Baltim. Md 1950 *179*, 3495–3503.

Schubert-Unkmeir, A., Konrad, C., Slanina, H., Czapek, F., Hebling, S., and Frosch, M. (2010). Neisseria meningitidis induces brain microvascular endothelial cell detachment from the matrix and cleavage of occludin: a role for MMP-8. PLoS Pathog. *6*, e1000874.

Sedwick, C. (2006). The Education of Mr. T. PLoS Biol *4*, e117.

Seguín-Estévez, Q., Dunand-Sauthier, I., Lemeille, S., Iseli, C., Ibberson, M., Ioannidis, V., Schmid, C.D., Rousseau, P., Barras, E., Geinoz, A., et al. (2014). Extensive remodeling of DC function by rapid maturation-induced transcriptional silencing. Nucleic Acids Res. *42*, 9641–9655.

Severa, M., Remoli, M.E., Giacomini, E., Ragimbeau, J., Lande, R., Uzé, G., Pellegrini, S., and Coccia, E.M. (2006). Differential responsiveness to IFN-α and IFN-β of human mature DC through modulation of IFNAR expression. J. Leukoc. Biol. *79*, 1286–1294.

Shannon, J.G., Howe, D., and Heinzen, R.A. (2005). Virulent Coxiella burnetii does not activate human dendritic cells: Role of lipopolysaccharide as a shielding molecule. Proc. Natl. Acad. Sci. U. S. A. *102*, 8722–8727.

Shea, M.W. (2013). The Long Road to an Effective Vaccine for Meningococcus Group B (MenB). Ann. Med. Surg. *2*, 53–56.

Shen, H., Tesar, B.M., Walker, W.E., and Goldstein, D.R. (2008). Dual signaling of MyD88 and TRIF is critical for maximal TLR4-induced dendritic cell maturation. J. Immunol. Baltim. Md 1950 *181*, 1849–1858.

Shen, L., Evel-Kabler, K., Strube, R., and Chen, S.-Y. (2004). Silencing of SOCS1 enhances antigen presentation by dendritic cells and antigen-specific anti-tumor immunity. Nat. Biotechnol. *22*, 1546–1553.

Sheppard, K.-A., Fitz, L.J., Lee, J.M., Benander, C., George, J.A., Wooters, J., Qiu, Y., Jussif, J.M., Carter, L.L., Wood, C.R., et al. (2004). PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. FEBS Lett. *574*, 37–41.

Shi, C., and Pamer, E.G. (2011). Monocyte recruitment during infection and inflammation. Nat. Rev. Immunol. *11*, 762–774.

Shi, H., Kokoeva, M.V., Inouye, K., Tzameli, I., Yin, H., and Flier, J.S. (2006). TLR4 links innate immunity and fatty acid–induced insulin resistance. J. Clin. Invest. *116*, 3015–3025.

Shortman, K., Lahoud, M.H., and Caminschi, I. (2009). Improving vaccines by targeting antigens to dendritic cells. Exp. Mol. Med. *41*, 61–66.

Shuai, K., and Liu, B. (2003). Regulation of JAK-STAT signalling in the immune system. Nat. Rev. Immunol. *3*, 900–911.

Shuai, K., Stark, G.R., Kerr, I.M., and Darnell, J.E. (1993). A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. Science *261*, 1744–1746.

Sica, A., and Mantovani, A. (2012). Macrophage plasticity and polarization: in vivo veritas. J. Clin. Invest. *122*, 787–795.

Sikorski, K., Chmielewski, S., Olejnik, A., Wesoly, J.Z., Heemann, U., Baumann, M., and Bluyssen, H. (2012). STAT1 as a central mediator of IFN? and TLR4 signal integration in vascular dysfunction. JAK-STAT *1*, 241–249.

Singleton, T.E., Massari, P., and Wetzler, L.M. (2005). Neisserial porin-induced dendritic cell activation is MyD88 and TLR2 dependent. J. Immunol. Baltim. Md 1950 *174*, 3545–3550.

Smith, H., Rogers, S.L., Smith, H.V., Gillis, D., Siskind, V., and Smith, J.A. (2013). Virus-associated apoptosis of blood neutrophils as a risk factor for invasive meningococcal disease. J. Clin. Pathol. *66*, 976–981.

Smyth, C., Logan, G., Weinberger, R.P., Rowe, P.B., Alexander, I.E., and Smythe, J.A. (1998). Identification of a dynamic intracellular reservoir of CD86 protein in peripheral blood monocytes that is not associated with the Golgi complex. J. Immunol. Baltim. Md 1950 *160*, 5390–5396.

Soares, H., Waechter, H., Glaichenhaus, N., Mougneau, E., Yagita, H., Mizenina, O., Dudziak, D., Nussenzweig, M.C., and Steinman, R.M. (2007). A subset of dendritic cells induces CD4+ T cells to produce IFN-γ by an IL-12–independent but CD70-dependent mechanism in vivo. J. Exp. Med. *204*, 1095–1106.

Sokolova, O., Heppel, N., Jägerhuber, R., Kim, K.S., Frosch, M., Eigenthaler, M., and Schubert-Unkmeir, A. (2004). Interaction of Neisseria meningitidis with human brain microvascular endothelial cells: role of MAP- and tyrosine kinases in invasion and inflammatory cytokine release. Cell. Microbiol. *6*, 1153–1166.

Sompayrac, L. (2012). How the immune system works (John Wiley & Sons).

Spinosa, M.R., Progida, C., Talà, A., Cogli, L., Alifano, P., and Bucci, C. (2007). The Neisseria meningitidis Capsule Is Important for Intracellular Survival in Human Cells. Infect. Immun. *75*, 3594–3603.

Srinivasan, A., Salazar-Gonzalez, R.-M., Jarcho, M., Sandau, M.M., Lefrancois, L., and McSorley, S.J. (2007). Innate immune activation of CD4 T cells in salmonella-infected mice is dependent on IL-18. J. Immunol. Baltim. Md 1950 *178*, 6342–6349.

Staab, J., Herrmann-Lingen, C., and Meyer, T. (2013). Clinically Relevant Dimer Interface Mutants of STAT1 Transcription Factor Exhibit Differential Gene Expression. PLoS ONE *8*, e69903.

Steeghs, L., den Hartog, R., den Boer, A., Zomer, B., Roholl, P., and van der Ley, P. (1998). Meningitis bacterium is viable without endotoxin. Nature *392*, 449–449.

Steeghs, L., van Vliet, S.J., Uronen-Hansson, H., van Mourik, A., Engering, A., Sanchez-Hernandez, M., Klein, N., Callard, R., van Putten, J.P.M., van der

Ley, P., et al. (2006). Neisseria meningitidis expressing lgtB lipopolysaccharide targets DC-SIGN and modulates dendritic cell function. Cell. Microbiol. *8*, 316–325.

Steeghs, L., Keestra, A.M., Mourik, A. van, Uronen-Hansson, H., Ley, P. van der, Callard, R., Klein, N., and Putten, J.P.M. van (2008). Differential Activation of Human and Mouse Toll-Like Receptor 4 by the Adjuvant Candidate LpxL1 of Neisseria meningitidis. Infect. Immun. *76*, 3801–3807.

Stefka, A.T., Feehley, T., Tripathi, P., Qiu, J., McCoy, K., Mazmanian, S.K., Tjota, M.Y., Seo, G.-Y., Cao, S., Theriault, B.R., et al. (2014). Commensal bacteria protect against food allergen sensitization. Proc. Natl. Acad. Sci. *111*, 13145–13150.

Steigbigel, R.T., Lambert, L.H., and Remington, J.S. (1974). Phagocytic and bacterial properties of normal human monocytes. J. Clin. Invest. *53*, 131–142.

Steinman, R.M., and Cohn, Z.A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. J. Exp. Med. *139*, 380–397.

Stent, G., Reece, J.C., Baylis, D.C., Ivinson, K., Paukovics, G., Thomson, M., and Cameron, P.U. (2002). Heterogeneity of freshly isolated human tonsil dendritic cells demonstrated by intracellular markers, phagocytosis, and membrane dye transfer. Cytometry *48*, 167–176.

Stephanou, A., Scarabelli, T.M., Brar, B.K., Nakanishi, Y., Matsumura, M., Knight, R.A., and Latchman, D.S. (2001). Induction of Apoptosis and Fas Receptor/Fas Ligand Expression by Ischemia/Reperfusion in Cardiac Myocytes Requires Serine 727 of the STAT-1 Transcription Factor but Not Tyrosine 701. J. Biol. Chem. *276*, 28340–28347.

Stephens, D.S., Hajjeh, R.A., Baughman, W.S., Harvey, R.C., Wenger, J.D., and Farley, M.M. (1995). Sporadic meningococcal disease in adults: results of a 5-year population-based study. Ann. Intern. Med. *123*, 937–940.

Stephenson, H.N., Mills, D.C., Jones, H., Milioris, E., Copland, A., Dorrell, N., Wren, B.W., Crocker, P.R., Escors, D., and Bajaj-Elliott, M. (2014). Pseudaminic Acid on Campylobacter jejuni Flagella Modulates Dendritic Cell IL-10 Expression via Siglec-10 Receptor: A Novel Flagellin-Host Interaction. J. Infect. Dis. jiu287.

Stevanin, Tâ.M., Moir, J.W.B., and Read, R.C. (2005). Nitric Oxide Detoxification Systems Enhance Survival of Neisseria meningitidis in Human Macrophages and in Nasopharyngeal Mucosa. Infect. Immun. *73*, 3322–3329.

Stockwin, L.H., McGonagle, D., Martin, I.G., and Blair, G.E. (2000). Dendritic cells: Immunological sentinels with a central role in health and disease. Immunol. Cell Biol. *78*, 91–102.

Stoiber, D., Stockinger, S., Steinlein, P., Kovarik, J., and Decker, T. (2001). Listeria monocytogenes Modulates Macrophage Cytokine Responses Through STAT Serine Phosphorylation and the Induction of Suppressor of Cytokine Signaling 3. J. Immunol. *166*, 466–472.

Stollenwerk, N., and Jansen, V.A.A. (2003). Meningitis, pathogenicity near criticality: the epidemiology of meningococcal disease as a model for accidental pathogens. J. Theor. Biol. *222*, 347–359.

Su, L.F., Kidd, B.A., Han, A., Kotzin, J.J., and Davis, M.M. (2013). Virus-Specific CD4+ Memory-Phenotype T Cells Are Abundant in Unexposed Adults. Immunity *38*, 373–383.

Summers, K.L., Hock, B.D., McKenzie, J.L., and Hart, D.N.J. (2001). Phenotypic Characterization of Five Dendritic Cell Subsets in Human Tonsils. Am. J. Pathol. *159*, 285–295.

SWAIN, C.L., and MARTIN, D.R. (2007). Survival of meningococci outside of the host: implications for acquisition. Epidemiol. Infect. *135*, 315–320.

Tammen, H., Schulte, I., Hess, R., Menzel, C., Kellmann, M., Mohring, T., and Schulz-Knappe, P. (2005). Peptidomic analysis of human blood specimens: comparison between plasma specimens and serum by differential peptide display. Proteomics *5*, 3414–3422.

Tanimoto, K., Saito, Y., Hamanaka, I., Kuwahara, K., Harada, M., Takahashi, N., Kawakami, R., Nakagawa, Y., Nakanishi, M., Adachi, Y., et al. (2005). SOCS1/JAB likely mediates the protective effect of cardiotrophin-1 against lipopolysaccharide-induced left ventricular dysfunction in vivo. Circ. J. Off. J. Jpn. Circ. Soc. *69*, 1412–1417.

Tettelin, H., Saunders, N.J., Heidelberg, J., Jeffries, A.C., Nelson, K.E., Eisen, J.A., Ketchum, K.A., Hood, D.W., Peden, J.F., Dodson, R.J., et al. (2000). Complete genome sequence of Neisseria meningitidis serogroup B strain MC58. Science *287*, 1809–1815.

Tezera, L.B., Hampton, J., Jackson, S.K., and Davenport, V. (2011). Neisseria lactamica attenuates TLR-1/2-induced cytokine responses in nasopharyngeal epithelial cells using PPAR-γ. Cell. Microbiol. *13*, 554–568.

Thorburn, K., Baines, P., Thomson, A., and Hart, C.A. (2001). Mortality in severe meningococcal disease. Arch. Dis. Child. *85*, 382–385.

Trez, C.D., Pajak, B., Brait, M., Glaichenhaus, N., Urbain, J., Moser, M., Lauvau, G., and Muraille, E. (2005). TLR4 and Toll-IL-1 Receptor Domain-Containing Adapter-Inducing IFN-β, but Not MyD88, Regulate Escherichia coli-Induced Dendritic Cell Maturation and Apoptosis In Vivo. J. Immunol. *175*, 839–846.

Troncoso, G., Sánchez, S., Moreda, M., Criado, M.T., and Ferreirós, C.M. (2000). Antigenic cross-reactivity between outer membrane proteins of Neisseria meningitidis and commensal Neisseria species. FEMS Immunol. Med. Microbiol. *27*, 103–109.

Tunbridge, A.J., Stevanin, T.M., Lee, M., Marriott, H.M., Moir, J.W.B., Read, R.C., and Dockrell, D.H. (2006). Inhibition of Macrophage Apoptosis by Neisseria meningitidis Requires Nitric Oxide Detoxification Mechanisms. Infect. Immun. *74*, 729–733.

Tunis, M.C., and Marshall, J.S. (2014). Toll-Like Receptor 2 as a Regulator of Oral Tolerance in the Gastrointestinal Tract. Mediators Inflamm. *2014*, e606383.

Türeci, O., Bian, H., Nestle, F.O., Raddrizzani, L., Rosinski, J.A., Tassis, A., Hilton, H., Walstead, M., Sahin, U., and Hammer, J. (2003a). Cascades of transcriptional induction during dendritic cell maturation revealed by genome-wide expression analysis. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *17*, 836–847.

Türeci, Ö., Bian, H., Nestle, F.O., Raddrizzani, L., Rosinski, J.A., Tassis, A., Hilton, H., Walstead, M., Sahin, U., and Hammer, J. (2003b). Cascades of transcriptional induction during dendritic cell maturation revealed by genome-wide expression analysis. FASEB J. *17*, 836–847.

Turnquist, H.R., Cardinal, J., Macedo, C., Rosborough, B.R., Sumpter, T.L., Geller, D.A., Metes, D., and Thomson, A.W. (2010). mTOR and GSK-3 shape the CD4+ T-cell stimulatory and differentiation capacity of myeloid DCs after exposure to LPS. Blood *115*, 4758–4769.

Tze, L.E., Horikawa, K., Domaschenz, H., Howard, D.R., Roots, C.M., Rigby, R.J., Way, D.A., Ohmura-Hoshino, M., Ishido, S., Andoniou, C.E., et al. (2011). CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. J. Exp. Med. *208*, 149–165.

Ueno, H., Palucka, A.K., and Banchereau, J. (2010). The expanding family of dendritic cell subsets. Nat. Biotechnol. *28*, 813–815.

Ungureanu, D., Saharinen, P., Junttila, I., Hilton, D.J., and Silvennoinen, O. (2002). Regulation of Jak2 through the Ubiquitin-Proteasome Pathway Involves Phosphorylation of Jak2 on Y1007 and Interaction with SOCS-1. Mol. Cell. Biol. *22*, 3316–3326.

Uronen-Hansson, H., Steeghs, L., Allen, J., Dixon, G.L.J., Osman, M., van der Ley, P., Wong, S.Y.C., Callard, R., and Klein, N. (2004a). Human dendritic cell activation by Neisseria meningitidis: phagocytosis depends on expression of lipooligosaccharide (LOS) by the bacteria and is required for optimal cytokine production. Cell. Microbiol. *6*, 625–637.

Uronen-Hansson, H., Allen, J., Osman, M., Squires, G., Klein, N., and Callard, R.E. (2004b). Toll-like receptor 2 (TLR2) and TLR4 are present inside human dendritic cells, associated with microtubules and the Golgi apparatus but are not detectable on the cell surface: integrity of microtubules is required for interleukin-12 production in response to internalized bacteria. Immunology *111*, 173–178.

Vallis, A.J., Yahr, T.L., Barbieri, J.T., and Frank, D.W. (1999). Regulation of ExoS Production and Secretion byPseudomonas aeruginosa in Response to Tissue Culture Conditions. Infect. Immun. *67*, 914–920.

Vercammen, E., Staal, J., and Beyaert, R. (2008). Sensing of Viral Infection and Activation of Innate Immunity by Toll-Like Receptor 3. Clin. Microbiol. Rev. *21*, 13–25.

Verdijk, P., van Veelen, P.A., de Ru, A.H., Hensbergen, P.J., Mizuno, K., Koerten, H.K., Koning, F., Tensen, C.P., and Mommaas, A.M. (2004). Morphological changes during dendritic cell maturation correlate with cofilin activation and translocation to the cell membrane. Eur. J. Immunol. *34*, 156–164.

Verhaar, A.P., Hoekstra, E., Tjon, A.S.W., Utomo, W.K., Deuring, J.J., Bakker, E.R.M., Muncan, V., and Peppelenbosch, M.P. (2014). Dichotomal effect of space flight-associated microgravity on stress-activated protein kinases in innate immunity. Sci. Rep. *4*.

Verma, B., Neethling, F.A., Caseltine, S., Fabrizio, G., Largo, S., Duty, J.A., Tabaczewski, P., and Weidanz, J.A. (2010). TCR mimic monoclonal antibody targets a specific peptide/HLA class I complex and significantly impedes tumor growth in vivo using breast cancer models. J. Immunol. Baltim. Md 1950 *184*, 2156–2165.

Vieira, S.M., Lemos, H.P., Grespan, R., Napimoga, M.H., Dal-Secco, D., Freitas, A., Cunha, T.M., Verri, W.A., Souza-Junior, D.A., Jamur, M.C., et al. (2009). A crucial role for TNF-alpha in mediating neutrophil influx induced by endogenously generated or exogenous chemokines, KC/CXCL1 and LIX/CXCL5. Br. J. Pharmacol. *158*, 779–789.

Virji, M. (2009). Pathogenic neisseriae: surface modulation, pathogenesis and infection control. Nat. Rev. Microbiol. *7*, 274–286.

Virji, M., Saunders, J.R., Sims, G., Makepeace, K., Maskell, D., and Ferguson, D.J. (1993). Pilus-facilitated adherence of Neisseria meningitidis to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. Mol. Microbiol. *10*, 1013–1028.

Vogel, U., Schoen, C., and Elias, J. (2010). Population Genetics of Neisseria meningitidis. In Bacterial Population Genetics in Infectious Disease, D.A.

Robinson, D. Falush, and E.J. Feil, eds. (John Wiley & Sons, Inc.), pp. 247–267.

Wang, J., Gray-Owen, S.D., Knorre, A., Meyer, T.F., and Dehio, C. (1998). Opa binding to cellular CD66 receptors mediates the transcellular traversal of Neisseria gonorrhoeae across polarized T84 epithelial cell monolayers. Mol. Microbiol. *30*, 657–671.

Weatherill, A.R., Lee, J.Y., Zhao, L., Lemay, D.G., Youn, H.S., and Hwang, D.H. (2005). Saturated and polyunsaturated fatty acids reciprocally modulate dendritic cell functions mediated through TLR4. J. Immunol. Baltim. Md 1950 *174*, 5390–5397.

Welsch, J.A., and Granoff, D. (2007). Immunity to Neisseria meningitidis Group B in Adults despite Lack of Serum Bactericidal Antibody. Clin. Vaccine Immunol. CVI *14*, 1596–1602.

Wetzler, L.M., Ho, Y., Reiser, H., and Wetzler, L.W. (1996). Neisserial porins induce B lymphocytes to express costimulatory B7-2 molecules and to proliferate. J. Exp. Med. *183*, 1151–1159.

Whitmore, S.E., and Lamont, R.J. (2012). Tyrosine phosphorylation and bacterial virulence. Int. J. Oral Sci. *4*, 1–6.

Wiesemann, E., Deb, M., Trebst, C., Hemmer, B., Stangel, M., and Windhagen, A. (2008). Effects of interferon-beta on co-signaling molecules: upregulation of CD40, CD86 and PD-L2 on monocytes in relation to clinical response to interferon-beta treatment in patients with multiple sclerosis. Mult. Scler. Houndmills Basingstoke Engl. *14*, 166–176.

Wilson, J.E., Katkere, B., and Drake, J.R. (2009). Francisella tularensis induces ubiquitin-dependent major histocompatibility complex class II degradation in activated macrophages. Infect. Immun. *77*, 4953–4965.

Wölfle, S.J., Strebovsky, J., Bartz, H., Sähr, A., Arnold, C., Kaiser, C., Dalpke, A.H., and Heeg, K. (2011). PD-L1 expression on tolerogenic APCs is controlled by STAT-3. Eur. J. Immunol. *41*, 413–424.

Wong, H.E.E., Li, M.-S., Kroll, J.S., Hibberd, M.L., and Langford, P.R. (2011). Genome Wide Expression Profiling Reveals Suppression of Host Defence Responses during Colonisation by Neisseria meningitides but not N. lactamica. PLoS ONE *6*, e26130.

Woods, C.W., Armstrong, G., Sackey, S.O., Tetteh, C., Bugri, S., Perkins, B.A., and Rosenstein, N.E. (2000). Emergency vaccination against epidemic meningitis in Ghana: implications for the control of meningococcal disease in West Africa. The Lancet *355*, 30–33.

Worbs, T., and Förster, R. (2009). T cell migration dynamics within lymph nodes during steady state: an overview of extracellular and intracellular

factors influencing the basal intranodal T cell motility. Curr. Top. Microbiol. Immunol. *334*, 71–105.

Yoneyama, M., and Fujita, T. (2009). RNA recognition and signal transduction by RIG-I-like receptors. Immunol. Rev. *227*, 54–65.

You, M., Flick, L.M., Yu, D., and Feng, G.S. (2001). Modulation of the nuclear factor kappa B pathway by Shp-2 tyrosine phosphatase in mediating the induction of interleukin (IL)-6 by IL-1 or tumor necrosis factor. J. Exp. Med. *193*, 101–110.

Youlyouz-Marfak, I., Gachard, N., Le Clorennec, C., Najjar, I., Baran-Marszak, F., Reminieras, L., May, E., Bornkamm, G.W., Fagard, R., and Feuillard, J. (2007). Identification of a novel p53-dependent activation pathway of STAT1 by antitumour genotoxic agents. Cell Death Differ. *15*, 376–385.

Yu, Q., Chow, E.M.C., McCaw, S.E., Hu, N., Byrd, D., Amet, T., Hu, S., Ostrowski, M.A., and Gray-Owen, S.D. (2013). Association of Neisseria gonorrhoeae Opa(CEA) with dendritic cells suppresses their ability to elicit an HIV-1-specific T cell memory response. PloS One *8*, e56705.

Zavascki, A.P., Fritscher, L., Superti, S., Dias, C., Kroth, L., Traesel, M.A., Antonello, I.C.F., and Saitovitch, D. (2006). First Case Report of Neisseria lactamica Causing Cavitary Lung Disease in an Adult Organ Transplant Recipient. J. Clin. Microbiol. *44*, 2666–2668.

Zhang, P., Snyder, S., Feng, P., Azadi, P., Zhang, S., Bulgheresi, S., Sanderson, K.E., He, J., Klena, J., and Chen, T. (2006). Role of N-acetylglucosamine within core lipopolysaccharide of several species of gram-negative bacteria in targeting the DC-SIGN (CD209). J. Immunol. Baltim. Md 1950 *177*, 4002–4011.

Zhang, Y., Lu, Y., Ma, L., Cao, X., Xiao, J., Chen, J., Jiao, S., Gao, Y., Liu, C., Duan, Z., et al. (2014). Activation of Vascular Endothelial Growth Factor Receptor-3 in Macrophages Restrains TLR4-NF-κB Signaling and Protects against Endotoxin Shock. Immunity *40*, 501–514.

Zheng, J., Liang, H., Xu, C., Xu, Q., Zhang, T., Shen, T., and Lu, F. (2014). An unbalanced PD-L1/CD86 ratio in CD14++CD16+ monocytes is correlated with HCV viremia during chronic HCV infection. Cell. Mol. Immunol. *11*, 294–304.

Zhu, J., and Paul, W.E. (2009). Heterogeneity and plasticity of T helper cells. Cell Res. *20*, 4–12.

Zhu, W., Ventevogel, M.S., Knilans, K.J., Anderson, J.E., Oldach, L.M., McKinnon, K.P., Hobbs, M.M., Sempowski, G.D., and Duncan, J.A. (2012). Neisseria gonorrhoeae suppresses dendritic cell-induced, antigen-dependent CD4 T cell proliferation. PloS One *7*, e41260.

Zubler, R.H. (2001). Naive and memory B cells in T-cell-dependent and T-independent responses. Springer Semin. Immunopathol. *23*, 405–419.

Zughaier, S.M., Kandler, J.L., and Shafer, W.M. (2014). Neisseria gonorrhoeae Modulates Iron-Limiting Innate Immune Defenses in Macrophages. PLoS ONE *9*, e87688.

Zuniga, E.I., McGavern, D.B., Pruneda-Paz, J.L., Teng, C., and Oldstone, M.B.A. (2004). Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection. Nat. Immunol. *5*, 1227–1234.