Dendritic Cell Interactions with *Neisseria meningitidis*

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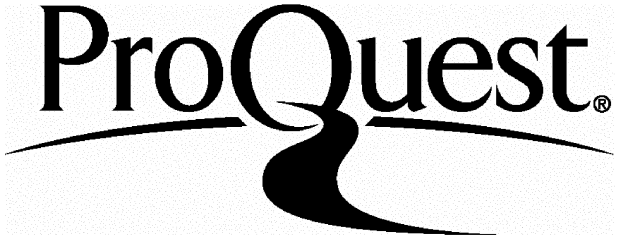

Abstract

Group B *Neisseria meningitidis* is a human pathogen, for which a vaccine is still not available. Immune responses to bacteria are initiated by dendritic cells (DCs), which phagocytose and process bacterial antigens for presentation to T cells. In this study, interactions between human monocyte derived DCs and *N. meningitidis* were investigated.

Maximal cytokine production by DCs in response to *N. meningitidis* was shown to depend on physical contact and internalisation of the bacteria by DCs. The majority of DCs making cytokines had internalised *N. meningitidis* and inhibition of phagocytosis impaired cytokine production, especially IL-12. Phagocytosis of *N. meningitidis* was shown to depend on LPS expressed by the bacteria. Phagocytosis of the LPS deficient mutant bacteria was poor and occurred at a very low rate. By regulating the expression of LPS, it was shown that restoration of bacterial LPS biosynthesis restored both phagocytosis and cytokine production by DCs. In addition, optimal phagocytosis and cytokine production required the presence of LPS binding protein. DC responses to novel bacterial mutants expressing LPS with modified oligosaccharide core or lipid A were examined. Optimal phagocytosis and cytokine production were shown to depend on the precise oligosaccharide and lipid A structures.

The expression and localisation of Toll-like receptors (TLR) 2 and 4 in human DCs was investigated. TLR2 and TLR4 were not present on the surface but were detected inside the DCs in association with tubulovesicular structures close to the Golgi complex. Co-localisation of TLR2 and TLR4 with DC microtubules was observed. Depolymerisation of the microtubule network disrupted intracellular TLR2 and TLR4 and inhibited IL12 production in response to *N. meningitidis* but did not prevent phagocytosis. These results suggested that TLR activation by *N. meningitidis* required for IL12 production occurred inside DCs and not on the cell surface. These findings have important implications for designing vaccines that will induce protective immune responses to group B *N. meningitidis.*

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Heli,
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Abbreviations

APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CR3	Complement Receptor 3
CTL	Cytotoxic T Lymphocyte
DAG	Diacyl Glycerol
DC	Dendritic Cell
DC-SIGN	Dendritic Cell Specific ICAM3 Grabbing Non-integrin
DD	Death Domain
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethenediamine Tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorter
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
GPI	Glycosylphosphatidyl Inositol
GTP	Guanine Triphosphate
HBSS	Hanks Balanced Salt Solution
HEPES	Hydroxyethylpiperazine-N'-2-ethansulfonic acid
ICAM	Intracellular Adhesion Molecule

Ig	Immunoglobulin
IL	Interleukin
IκB	Inhibitory κB
IRAK	Interleukin-1 Receptor-Associated Kinase
kDa	Kilo Dalton
LBP	Lipopolysaccharide Binding Protein
LPS	Lipopolysaccharide
M	Molar
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
MTOC	Microtubule Organising Centre
MYD88	Myeloid Differentiation Factor 88
NFκB	Nuclear Factor κB
OD	Optical Density
OM	Outer Membrane
OMC	Outer Membrane Complex
OMP	Outer Membrane Protein
OMV	Outer Membrane Vesicle
P	Probability
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PFA	Paraformaldehyde
PI	Phosphatidyl Inositol
PI3K	Phosphoinositide 3-kinase

PI(3)P	Phosphatidyl Inositol 3-phosphate
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PKC	Protein Kinase C
PLC	Phospholipase C
PRR	Pattern Recognition Receptor
RPMI	Roswell Park Memorial Institute
TCR	T cell receptor
Th Cell	T helper Cell
Th1 Cell	T helper type 1 Cell
Th2 Cell	T helper type 2 Cell
TIR	Toll IL-1 Receptor
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor alpha
TRAF	TNF-Receptor Associated Protein
WASp	Wiscott-Aldrich Syndrome proteins
WT	Wild Type

Declaration

The experimental work, as well as the analysis and interpretation of the data presented in this thesis were performed by the candidate with the following exceptions:

Chapter 3

The experiments with live meningococci were performed in collaboration with Ms Theresa Singleton from Boston University School of Medicine, Boston, USA.

Chapter 4

The experiments with the *lpxA* regulatory strain HA3003 were performed in collaboration with Dr Liana Steeghs from University Medical Centre Utrecht, Utrecht, The Netherlands.

Chapter 6

All experimental work was carried out in collaboration with Dr Liana Steeghs.

Chapter 1

Introduction

1.1 The immune system

Protective immunity results from the interplay of two systems, antigen specific adaptive immunity and innate immunity. The cells of the adaptive immune system, B and T lymphocytes, consist of large number of clones expressing distinct receptors that recognise antigens in a highly specific manner. Following recognition of antigen, clonal expansion of the responding lymphocyte population occurs and both effector and memory cells can be formed.

Innate defence mechanisms evolved early in the phylogeny of the immune system and feature in plants as well as in invertebrate and vertebrate immunity. Innate immunity lies behind most inflammatory responses and is important to survival as defects in the innate mechanisms are relatively rare. Innate reactions are mediated by the action of phagocytic leucocytes, such as granulocytes and macrophages, dendritic cells (DC), mast cells and natural killer (NK) cells. These cells have the ability to distinguish foreign molecules from self molecules by bearing innate or germ-line encoded recognition receptors, but do not have memory, a feature characteristic of B and T cell responses. The cells of the innate immune system are able to convey information about the nature of the pathogenic challenge to the cells of the adaptive immune system. T cells can only recognise antigenic peptides bound to major histocompatibility complex (MHC) class I or class II molecules. The MHC-peptide complexes are recognised when present on the surface of antigen presenting cells (APC). Non-professional APCs are only able to stimulate previously activated T cells, whereas professional APCs are able to both present MHC-peptide complexes and express cell surface proteins that generate the necessary secondary signals needed to activate naive T cells. DCs are

considered to be the professional APCs most capable of initiating immune responses because of their ability to supply co-stimulatory signals and also because of their efficiency at antigen processing, presentation and their tissue distribution in *vivo* (Palucka and Banchereau, 1999) .

1.2 Dendritic cells

DCs are highly potent APCs required for most primary immune responses. They have the ability to capture antigen and then mature into cells capable of efficient T cell activation. Because of their unique characteristics, DCs are often referred to as being the essential link between the adaptive and innate immune systems (Banchereau *et al.*, 2003;Palucka and Banchereau, 1999).

1.2.1 Origin and cell lineages of dendritic cells

In vivo, DCs are typically found in the peripheral tissues and organs, the skin, mucosae of the airways and in the gastrointestinal tract, which are continuously exposed to a variety of antigens and micro-organisms. DCs can be classified based on their location in the body (Kuby J, 1998):

-*Langerhans cells* (found in epidermis and mucous membranes)

-*Interstitial DCs* (populate most tissues such as skin dermis)

-*Interdigitating DCs* (present in T cell areas of secondary lymphoid tissue and thymus)

-*Circulating DCs* (in blood and lymph)

DCs in different locations display several morphologic and functional differences. Nevertheless, all DCs are considered as highly potent APCs that are able to present high levels of co-stimulatory and MHC Class II molecules.

DCs *in vivo* are derived from CD34+ hematopoietic stem cells within the bone marrow (Ardavin *et al.*, 2001;Liu *et al.*, 2001;Liu, 2001) (Figure 1.1). The common myeloid CD34+ progenitor cells differentiate into cells that give rise to Langerhans cell in the skin epidermis (in the presence of TGF-β) and interstitial DCs in skin dermis and other tissues. DCs can also develop from CD14+ monocytes, which *in vitro* can be cultured by addition of exogenous cytokines GM-CSF and IL4. Under these condition, monocytes develop into a homogenous population of DCs without cell division (Sallusto and Lanzavecchia, 1994). The *in vitro* culture of monocyte-derived DCs is now a widely used method and has enabled extensive studies of many aspects of DC biology. Monocytes can also differentiate into DCs following transendothelial migration and phagocytosis of bacteria in the absence of additional cytokines (Randolph *et al.*, 1998;Randolph *et al.*, 1999).

DCs were originally thought to be derived from myeloid precursors but it is clear now that they can develop from both myeloid and lymphoid precursor cells (Figure 1.1) (Cella *et al.*, 1997;Liu *et al.*, 2001). Lymphoid-derived DCs express lymphoid markers and develop in the absence of GM-CSF. These cells may have different functional properties compared with those of myeloid DCs (Cella *et al.*, 1997;Pulendran *et al.*, 2001). Recently, plasmacytoid DCs were described in humans (Siegal *et al.*, 1999). These cells are CD11c- and produce significant

amounts of type I interferons. Plasmacytoid DCs are present in lymphoid tissue and blood and are considered to be important in viral infections. An equivalent DC subtype has been described in mice (Asselin-Paturel *et al.*, 2001). In addition, at least three additional phenotypically defined populations have been recognised in the murine spleen (CD11c+CD11b+CD4+, CD11c+CD11b+CD4- and CD11c+CD11b-CD8α+) (Henri *et al.*, 2001). Lymph nodes, Peyers patches and mesenteric lymph nodes contain two additional subsets (CD11c+CD11b+CD8α-CD4- and CD11c+langerin+) (Iwasaki and Kelsall, 2001). Whether these subsets have human equivalents is currently not known.

Due to current controversial and conflicting data, no definitive conclusions can be drawn about the origin of the different DC subpopulations (Shortman and Liu, 2002). It is also not known why so many subsets are required. Specific locations in the body may require different function of the DCs. It has also been suggested that different subsets of DCs may have evolved to respond to different pathogens (Pulendran *et al.*, 2001).

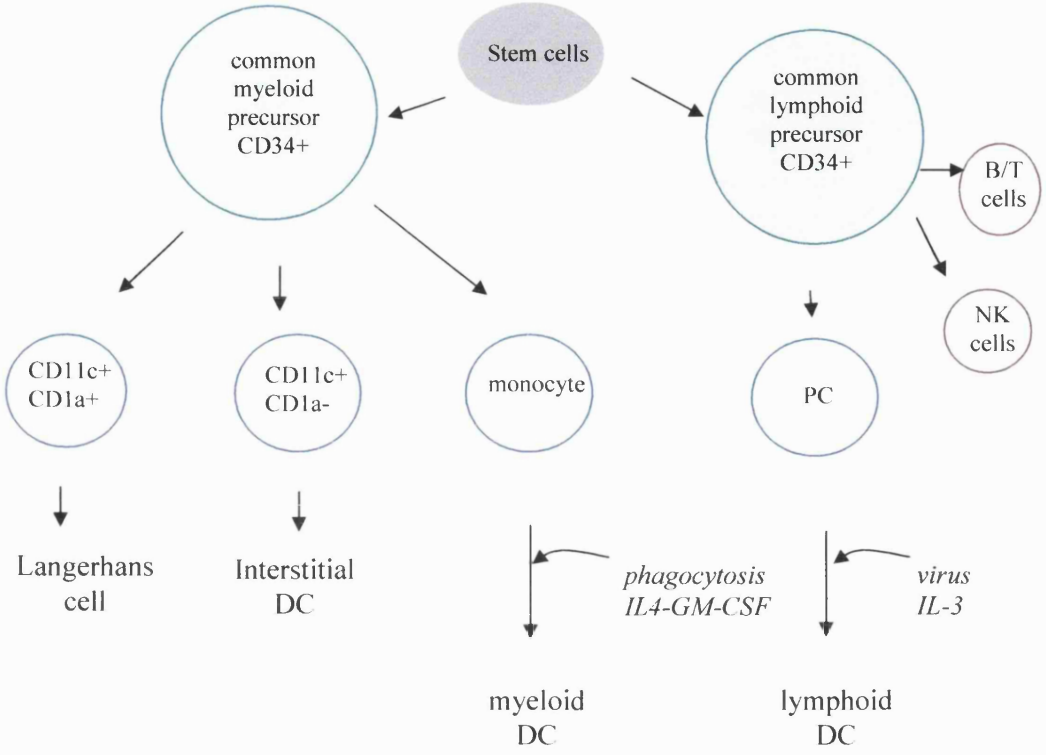

Figure 1.1 Origin and differentiation of dendritic cells. Common CD34+ myeloid progenitors give rise to cells, which differentiate into precursors of Langerhans cells (LC) and interstitial DCs. Peripheral monocytes develop into either immature DCs or macrophages, depending upon the influence of different cytokines or bacterial stimuli. Plasmacytoid cells (PC) develop from the common lymphoid progenitors, which also give rise to NK, B and T cells. (Modified from (Cella *et al.*, 1997;Liu, 2001)).

1.2.2 Antigen capture by dendritic cells

DCs act as sentinels of the immune system and constantly sample their microenvironment for foreign antigens. DCs have developed specialised mechanisms relevant to antigen uptake, of which macropinocytosis, receptor-mediated endocytosis and phagocytosis are the most important (Mellman and Steinman, 2001).

Macropinocytosis by immature DCs is a constitutive, non-selective form of uptake by which large volumes of solute can be internalised. It has been estimated that in one hour, a DC can take up as much fluid as its own cell volume (Sallusto *et al.*, 1995). It was recently shown that aquaporins, which are membrane water channels that play critical roles in controlling the water contents of cells, play an essential role in the process of macropinocytosis and fluid homeostasis of DCs (de Baey and Lanzavecchia, 2000). Macropinocytosis is mediated by the actin cytoskeleton and is independent of clathrin and membrane receptors (Garrett *et al.*, 2001;Lanzavecchia, 1996).

In receptor-mediated endocytosis, ligands, such as viral and bacterial antigens are internalised via a clathrin coat dependent mechanism (Garrett *et al.*, 2001). Membrane receptors (such as Fc receptors as well as several types of lectin and complement receptors (CR), cluster in clathrin-coated pits together with the ligand, pinch off as coated vesicles inside the cell and then traffic through the endosomal-lysosomal pathway. These receptors are described in more detail in section 1.3.1. Once the receptors have been internalised, they dissociate from their ligands and can be either recycled back to the cell surface or degraded in the

lysosomes. Antigens that are taken up in the fluid phase accumulate in the endocytic compartments where they are loaded onto newly synthesized MHC Class II molecules (Inaba *et al.*, 1998;Mellman and Steinman, 2001;Turley *et al.*, 2000).

Phagocytosis is a receptor-mediated, actin- and ATP-driven process that allows internalisation of large particles approximately 0.5-6μm of size (Chavrier, 2001). Phagocytosis is complex and involves a variety of molecular and morphological processes. Receptor clustering upon particle binding generates a phagocytic signal that in turn leads to local polymerisation of actin and cytoskeletal movement, the key events during particle internalisation. The important role of actin can be demonstrated by using cytochalasin D, a fungal toxin from *Zygosporium mansonii* that inhibits actin polymerisation by binding to the plus end of the actin filament, thus preventing the addition of globular (G) actin. A myriad of accessory proteins are known to be involved in the process of internalisation including actin binding proteins, regulators of membrane traffic, ion channels, kinases and lipases (Underhill and Ozinsky, 2002). In addition, hundreds of proteins are localised within the phagosome and only a fraction of them have a known function (Garin *et al.*, 2001).

1.2.2.1 Molecular mechanisms of phagocytosis

The signalling pathways activated during phagocytosis are not completely understood. Signalling in response to CR3 and Fcγ mediated phagocytosis have been studied the most although the basic processes of cytoskeletal rearrangement and membrane trafficking are thought to operate in other forms of phagocytosis,

such as non-opsonic uptake of bacteria (Greenberg, 2001). Nevertheless, four molecules stand out as major participants in signalling during phagocytosis of bacteria:

1) Phosphoinositide 3-kinase (PI3K)

2) Phospholipase C (PLC)

3) Protein kinase C (PKC)

4) Rho guanine triphosphate (GTP)ases

PI3K are a family of enzymes that catalyze the phosphorylation of phosphatidylinositol (PI) lipids to generate PI-3-phosphate PI(3)P, PI 3,4-biphosphate $PI(3,4)P_2$ and PI 3,4,5-triphospate $PI(3,4,5)P_3$. These phospholipids are involved in the recruitment of kinases and other accessory proteins to specific regions of membranes and play an important role during phagocytosis (Kwiatkowska and Sobota, 1999;Leevers *et al.*, 1999). Phagocytosis is inhibited by PI3K inhibitors, such as Wortmannin (Botelho *et al.*, 2000). It is now thought that PI3K are essential for pseudopod extension and phagosome closure during phagocytosis, as inhibition of the PI3K blocks membrane extension and fusion around the particle (Botelho *et al.*, 2000;Leevers *et al.*, 1999).

Phosphoinositide specific PLC mediates cleavage of PIP_2, which results in the generation of two second messengers: IP_3 and DAG. These molecules mobilise intracellular Ca^{2+} stores and activate PKC, respectively (Cox and Greenberg, 2001). PKC is involved in intracellular signalling pathways in response to a variety of stimuli, including phagocytosis (Kwiatkowska and Sobota, 1999).

Inhibition of PKC isoforms results in blockade of particle internalisation as well as the subsequent cytokine production (Shapira *et al.*, 1994;Zheleznyak and Brown, 1992).

Members of the Rho subfamily of GTPases (Ras superfamily) have been implicated in a number of processes involving actin cytoskeleton rearrangements and are considered to be the key regulators of actin polymerisation during phagocytosis, adhesion, membrane ruffling and stress fibre formation (Greenberg and Grinstein, 2002;Hall, 1998). These proteins act as small molecular switches that control actin nucleation and polymerisation at the site of phagocytosis (Castellano *et al.*, 2001). *Clostridium difficile* toxin B is a powerful inhibitor of Rho GTPases and is used to block phagocytosis (Just *et al.*, 1995;Leverrier and Ridley, 2001). In DCs, dendrite formation, but not their maintenance, depends on the activity of Rho GTPases (Swetman *et al.*, 2002). In addition, Rho GTPases control the characteristic shape and immunogenic capacity of DCs (Kobayashi *et al.*, 2001).

The Rho subfamily of GTPases includes Rho, Rac and Cdc42, all of which control signal transduction pathways that link membrane receptors to actin cytoskeleton. Although the regulation of the actin cytoskeleton has been most extensively studied in fibroblasts, it is now clear that most cells use similar mechanisms for its control (Hall, 1998). Micro-injected Rho and Cdc42 have been shown to induce the formation of filopodia and lamellipodial protrusions by macrophages (Swetman *et al.*, 2002). Interestingly, activation of Rho GTPases is dependent on the type of phagocytic receptor engaged. For example, Fc receptor-

mediated phagocytosis does not involve Rho, whereas complement-opsonized particle internalisation does not involve active Cdc42 or Rac (Caron and Hall, 1998).

The biochemical pathways regulated by Rho GTPases are still not clear and the downstream targets of these proteins are under investigation. The effects of GTPases are mediated by multimolecular complexes and the precise downstream effects are complex and remain to be discovered. For example, the relationship between GTPases and PI3K is complicated by the fact that the latter can act both upstream and downstream of Ras and Rho (Bar-Sagi and Hall, 2000). The Wiscott-Aldrich Syndrome proteins (WASp) are thought to be key regulators of actin polymerisation downstream of Cdc42 and Rac. Active WASp brings together an actin monomer and Arp2/3 complex, which then initiates the growth of a new actin filament (Pollard *et al.*, 2001). Actin associated proteins, such as ezrin, radixin, moesin, gelsolin and vinculin, may also interact with Rho GTPases either directly or indirectly, adding to the complexity of actin regulation.

1.2.3 Antigen processing by dendritic cells

After antigen capture, DCs migrate to secondary lymphoid organs, which may take several days. In order to delay antigen processing over such extended periods, DCs have the ability to retain protein antigens in their endocytic system (Lutz *et al.*, 1997). This is achieved by acidification of the endosomes in the so called 'retention vesicles', in which the antigen can be stored intact during DC migration. Antigen processing after antigen uptake is tightly controlled and may

be arrested in immature DCs until after receipt of a strong activation stimulus, such as LPS (Turley *et al.*, 2000).

After antigen processing has started in the endocytic system, the internalised antigen vesicles or phagosomes are fused with early endosomes. The early endosomes have acidic pH, which allows for dissociation of the ligands from their receptors. After the early endosomes have accumulated ligands, they fuse with late endosomes, which contain even lower pH than early endosomes. The late endosomes contain active hydrolases, which initiate the degradation of the protein antigens. This degradation process is continued in lysosomes, where the pH 5 is optimal for hydrolytic enzyme digestion. Lysosomes also contain cysteine proteases of the cathepsin family, which act optimally at acidic pH and have broad substrate specificity. In DCs, the late endocytic compartments (often referred to as MHC Class II compartments), contain newly synthesised MHC class II molecules, which form a part of the MHC-peptide complexes. In stimulated DCs, these complexes are transported to the cell surface by tubulovesicular structures that fuse with the plasma membrane (Chow *et al.*, 2002).

In addition to presentation of exogenous antigens to CD4+ T helper cells, DCs employ mechanisms that allow cross-priming, *i.e* presentation of endogenous antigen on MHC class I molecules (Thery and Amigorena, 2001). Endogenous antigens are normally produced inside the cytosol after infection and then presented on the cell surface to CD8+ cytotoxic T cells (CTL). Most viral and tumour antigens presented by DCs are not, however, derived from DCs

themselves. The mechanisms of cross-priming are not fully understood, but there are several possibilities how endogenous antigens gain access to MHC class I on DCs. Loading of the peptide could take place in the endosomes, where low levels of MHC I are present, or in the endoplasmic reticulum (ER), where synthesis of new MHC class I molecules occurs. Internalised antigens may access the cytosol for processing by the proteasome and loading in the ER by an unconventional cytosolic processing pathway. Unique transport mechanisms may therefore be involved in the transport of antigen from endosomes to the cytosol (Lanzavecchia, 1996;Thery and Amigorena, 2001)

1.2.4 Dendritic cell maturation

After internalisation of antigens by endocytosis or phagocytosis, DCs undergo a maturation process from immature DCs into potent T-cell stimulating mature DCs. Maturation involves a decrease in antigen uptake by downregulation of antigen uptake receptors and a marked increase in the surface expression of MHC Class II and co-stimulatory molecules, such as CD86 (Table 1.1) (Mellman and Steinman, 2001). Activated DCs also start to secrete a variety of cytokines, such as IL12 (Aliberti *et al.*, 2000;Dixon *et al.*, 2001;Huang *et al.*, 2001a;Kolb-Maurer *et al.*, 2001b).

The increased expression of co-stimulatory molecules CD80 and CD86 is important for generating optimal T cell responses. Both CD80 and CD86 bind to CD28 and cytotoxic T lymphocyte antigen-4 (CTLA-4) on the surface of T cells. While engagement of CD28 enhances proliferation and cytokine production by T cells, the ligation of CTLA-4 has an inhibitory effect and serves to regulate the T

cell response (Chambers, 2001). In addition, activated DCs express CD40, which binds to CD40 ligand transiently expressed by activated T cells.

After receipt of a maturation signal, such as bacteria or their products, DCs start to secrete a variety of cytokines. The pro-inflammatory cytokines TNF-α, IL1-α, IL1-β and IL6 are released early following stimulation and aid the local inflammatory response at the site of infection. For example, TNF-α activates endothelial cells allowing increased leukocyte infiltration to the site of infection. The systemic release of IL6 and IL1 induce fever and are important growth factors for B and T cells. DCs can also produce IL10, a major regulatory cytokine that has been shown to inhibit DC maturation and differentiation (Allavena *et al.*, 1998;De Smedt *et al.*, 1997). The cytokines IL12 and IL18 are produced at high levels by activated DCs and play an important role in the activation of naïve T helper cells, as discussed below.

Table 1.1 Properties of immature and mature dendritic cells

Immature DC	**Mature DC**
Macropinocytosis +++	Macropinocytosis +
Phagocytosis +++	Phagocytosis +
Surface MHC Class II +	Surface MHC Class II +++
CD86 +	CD86 +++
CD40+	CD40+++
CD83-	CD83 ++
CCR5++	CCR7++
Cytokines -	Cytokines +++

1.2.5 Dendritic cell migration

Maturing DCs migrate from peripheral tissues to secondary lymphoid organs, where interaction with T cells can occur. Migration involves pronounced changes in cell morphology and surface expression of several adhesion molecules and chemokine receptors (Palucka and Banchereau, 1999).

Chemokines constitute a superfamily of small cytokines that are involved in a variety of immune and inflammatory responses including cell migration (Luster, 2002). Chemokines are classified into two families, CXC and CC, according to the position of their first two cysteine residues. The receptors are correspondingly classified CXCR or CCR. It is now clear that the selective expression of these chemokines and their receptors on DCs is a critical determinant of their migratory capacity, and with it the ability to generate an effective immune response (Sozzani *et al.*, 1999).

Monocytes and immature DCs express CCR1 and CCR5, which bind to inflammatory chemokines MIP-1α (CCL3) and RANTES (CCL5) that help them to extravasate and reach the source of inflammatory stimulus. Chemokines MIP-1 and RANTES are produced by the cells at the site of infection, including macrophages and DCs themselves (Sallusto and Lanzavecchia, 2000).

Following antigenic stimulation, DCs migrate from inflamed tissues to the draining lymph nodes, where they localise in T cell areas. This migration involves a switch in chemokine receptor expression by the maturing DCs. After exposure to maturation stimuli, DCs downregulate their expression of CCR1 and CCR5 and

become unresponsive to their ligands MIP-1 and RANTES (Sallusto *et al.*, 1998;Sallusto and Lanzavecchia, 2000). Maturing DCs increase their expression of CCR7 and become responsive to chemokines MIP-3β and 6Ckine, which are expressed in T cell zones of lymph nodes by endothelial cells (Kellermann *et al.*, 1999). In addition, the maturing DCs produce chemokines, such as MIP-1, RANTES and IL8 that are released at the site of infection boosting further recruitment of immature DCs and other immune cells such as neutrophils (Sallusto and Lanzavecchia, 2000).

Migrating DCs must cross both the extracellular matrix of local tissues as well as pass through endothelial barriers. In addition to chemokines and their receptors, adhesion molecules play an important role during DC migration. DC binding and migration across endothelium involves CD11a, CD11b, CD18 and VLA-4 (Brown *et al.*, 1997;D'amico *et al.*, 1998). Langerhans cells in the skin express the intracellular adhesion molecule (ICAM)-1, which has been shown to be important for their migration from the skin to lymph nodes (Xu *et al.*, 2001).

1.2.6 Dendritic cell interactions with T cells

After antigen degradation and peptide loading to MHC molecules, DCs serve as the principal APCs for T cell priming. Following migration to the T cell areas of secondary lymphoid organs, DCs act as potent inducers of CD4+ and CD8+ primary responses (Guermonprez *et al.*, 2002). In addition to peptide-loaded MHC molecules, mature DCs express high levels of cell surface costimulatory molecules CD40, CD80, CD86 as well as adhesion and other activation markers, which interact with their counter receptors on the surface of T cells and form the

'immunological synapse' (Davis, 2002). Mature DCs also secrete several cytokines necessary for efficient T cell priming. The role of DC-derived cytokines is highlighted in the process of CD4+ T cell polarisation, after which T helper cells acquire either Th1 or Th2 phenotype. For example, DCs can be induced to secrete high levels of IL12 in response to bacteria, intracellular parasites or their products or by ligation of CD40 with CD40L expressed on activated T cells (DeKruyff et al., 1996; Kennedy et al., 1996). As one of its main activities, bioactive IL-12 p70 promotes the differentiation of naïve CD4+ Th cells into IFN-γ producing Th1 effector cells while inhibiting the Th2 phenotype. IFN-γ, produced in response to IL-12, favours differentiation into Th1 phenotype partly by enhancing IL-12 secretion by DCs and partly by maintaining the expression of IL-12 receptors on Th cells (Abbas et al., 1996). The important role of IL-12 in cell mediated immune responses has been shown in studies with IL-12 knockout mice, which are defective in IFN-γ production and cell mediated immunity (Magram *et al.*, 1996;Watford *et al.*, 2003).

1.3 Dendritic cell interactions with pathogens

Several types of micro-organism, such as *Mycobacterium tuberculosis* (Giacomini *et al.*, 2001;Henderson *et al.*, 1997), *Listeria monocytogenes* (Kolb-Maurer *et al.*, 2001a), *Salmonellae* (Yrlid and Wick, 2002) *Neisseria meningitidis* (Dixon *et al.*, 2001;Kolb-Maurer *et al.*, 2001b) protozoan parasite *Leishmania donovani* (Gorak *et al.*, 1998) as well as the fungus *Candidia albicans* (Newman and Holly, 2001) have been shown to induce DC maturation *in vitro*. Activation of DCs can also occur in response to purified microbial products, such as LPS (Cella *et al.*, 1996;Dixon *et al.*, 2001;Riva *et al.*, 1996b;Sallusto *et al.*, 1995), lipoteichoic acid (Riva *et al.*, 1996a), bacterial CpG DNA (Jakob *et al.*, 1999) and viruses, such as HIV and measles (Klagge and Schneider-Schaulies, 1999). DC activation in response to microbial stimuli may also be indirectly mediated by cytokines, such as IL-1 and TNF-α secreted either by other cells or by DCs themselves (Reis e Sousa *et al.*, 1999;Tsuji *et al.*, 2000). DC activation is generally measured by the expression levels of surface markers and cytokines. It is important to note that DCs are extremely plastic and able to respond appropriately in different ways to a wide variety of pathogens. Several studies have shown that DCs exhibit stimulus-specific maturation and activation. For example, the unicellular yeast *C. albicans* induces DC maturation and IL12 production supporting a Th1 response (d'Ostiani *et al.*, 2000). In contrast, *C. albicans* filamentous hyphae do not induce IL12 by DCs and drive a Th2 response. When exposed to diverse set of organisms, such as bacteria, fungus and yeast, a unique profile of genes are regulated by each pathogen as measured by gene microarray techniques (Huang *et al.*, 2001b). DC maturation cannot

therefore be defined simply by the modulation of a standard set of markers. In addition, although many purified microbial components are potent inducers of DC maturation, there is evidence that DC responses to whole organisms may be different (Dixon *et al.*, 2001;Hofer *et al.*, 2001;Rescigno *et al.*, 2002). Additional pathways activated during events such as phagocytosis are likely to affect the resulting DC response by changes in cell signalling.

The co-ordinated events during DC maturation leading to surface marker expression and cytokine production to different pathogens are a product of complex interactions between different pattern recognition receptors, which together generate physiologically relevant responses by DCs. To date, numerous studies have revealed the antigenic complexity of bacteria, their protective structures and strategies to evade the immune system. Thus, understanding the interactions of bacteria with DCs may shed some light on these complex mechanisms involving initiation of an immune response as well as on aspects of invasiveness and pathogenicity of many bacteria.

1.3.1 Dendritic cells and pattern recognition receptors

The innate immune system does not recognise every possible antigen. Instead, it is designed to recognise a few highly conserved structures present in many different micro-organisms. Pattern recognition receptors (PRR) expressed by the cells of the innate immune system recognise these conserved molecular motifs that are not expressed by mammalian cells. DCs express a broad spectrum of receptors, such as Fc receptors, complement receptors, lectins, integrins and Toll-like receptors (TLR) that have been shown to interact with micro-organisms.

Some receptors bind to particles directly, whereas others require opsonins on the microbial surface. Many different receptors may recognise the same pathogen simultaneously. Microbial binding to multiple PRRs expressed by DCs often leads to internalisation of the pathogen. Some receptors, however, participate in binding only, whereas others are also involved in internalisation either directly or indirectly through additional receptors (Underhill and Ozinsky, 2002).

Microbial contact and internalisation is often accompanied by the production of inflammatory mediators, such as cytokines, as well as other anti-microbial responses. Chemokine and cytokine production can be triggered directly through engagement of phagocytic receptors or, as is often the case, by co-operation with additional receptors (Underhill, 2003). It is important to note that activation of one response, such as phagocytosis does not necessarily activate other responses, such as cytokine production. For example, apoptotic cells, although phagocytosed, induce modified responses by DCs that may be involved in the generation of tolerance (Inaba *et al.*, 1998;Morelli *et al.*, 2003).

A schematic representation of the interaction between receptors, signalling molecules and the cellular response after phagocytosis is shown in figure 1.2. The main PPRs expressed by DCs are described below.

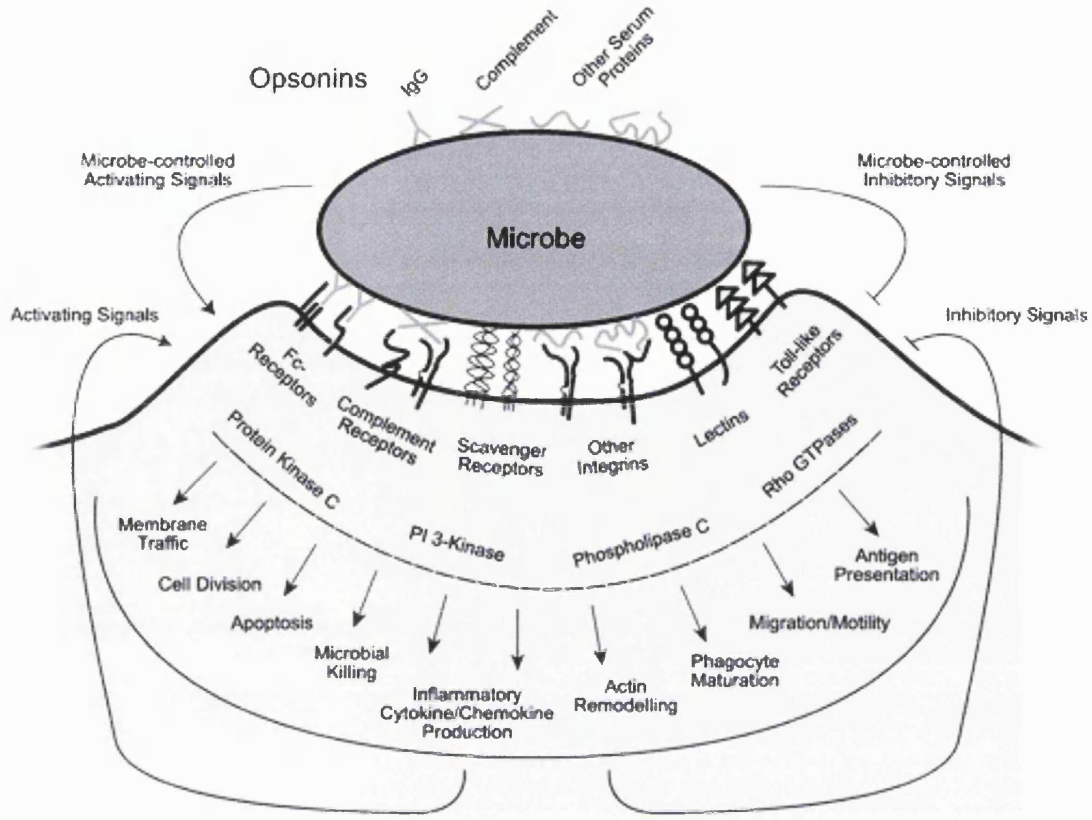

Figure 1.2 Complexity of interactions during phagocytosis. Binding of microbes to cells is mediated by multiple receptors, which can bind the same pathogen simultaneously. After receptor binding, intracellular signalling molecules transduce the information from the receptors by a complex network of signalling pathways. Finally, these co-ordinated events generate cellular responses that ultimately ensure the eradication of the pathogen. From (Underhill and Ozinsky, 2002).

1.3.1.1 Fc receptors

Fc-mediated phagocytosis has been the most extensively studied model for particle internalisation. Particles opsonized with immunoglobulin (Ig) are recognised by receptors that bind to the Fc fragment of the Ig molecule. Two different types of Fcγ receptors have been described on DCs: FcγRI (CD64) and

FcγRII (CD32), which bind to particles coated with IgG. The expression of these receptors may differ between DC subsets and depends on their maturation state (Fanger *et al.*, 1996;Fanger *et al.*, 1997). Triggering via immunreceptor tyrosine based activation motifs (ITAM) on Fcγ receptors activates cell signalling cascades that lead to internalisation directly through actin filamentation and particle engulfment by the cell (Underhill and Ozinsky, 2002).

1.3.1.2 Complement receptors

Serum complement proteins are activated through a highly regulated enzymatic cascade (Kuby J, 1998). As particles become coated with the reaction products of the activated complement system, they become more readily phagocytosed. Opsonised microbes are internalised via specific complement receptors (CR) such as CR3 expressed by phagocytic cells, including DCs (Underhill and Ozinsky, 2002). The CR3 belongs to the β_2 integrin subfamily and is composed of CD18, which is then paired with CD11b. CR3 plays an important role in opsonophagocytosis as it binds particles coated with iC3b (Agramonte-Hevia *et al.*, 2002). In addition, CR3 plays a major role in non-opsonic phagocytosis of bacteria. Interestingly, CR3 has been described as a lipopolysaccharide (LPS) binding receptor (Wright and Jong, 1986). It has been suggested that CR3 participates in binding preferentially when LPS is presented in particulate form, such as whole bacteria or LPS-coated beads (Flo *et al.*, 2000;Moore *et al.*, 2000).

1.3.1.3 Lectins

Carbohydrate-binding C-type lectins, such as mannose receptor (MR), DC specific ICAM-3-grabbing non-integrin (DC-SIGN) and Dectin-1 are expressed

by DCs and play a role both in cell adhesion and phagocytosis (Cambi and Figdor, 2003). These receptors mediate binding of glycosylated particulate or soluble molecules with terminal mannose, fucose and N-acetyl glucosamine residues, which are then internalised through the endocytic and phagocytic pathways. DCs express substantial amounts of mannose receptor (MR), a lectin that recognises specific carbohydrate structures on the surface of micro-organisms (Engering *et al.*, 1997). Diverse pathogens, such as *C. albicans*, *Leishmania donovani* and *Pneumocystis carinii* have all been shown to interact with the MR (Newman and Holly, 2001;Stahl and Ezekowitz, 1998). DC-SIGN is a novel C-type lectin expressed by DCs, which supports early, antigen-nonspecific contact between T cells and DCs (Engering *et al.*, 2002). In addition, DC-SIGN has been shown to bind mycobacteria (Tailleux *et al.*, 2003). Dectin-1, a lectin receptor for β-glucan, is expressed by DCs and mediates the attachment and uptake of fungi and yeast (Brown *et al.*, 2002;Cambi *et al.*, 2003;Gordon, 2002;Taylor *et al.*, 2002).

1.3.1.4 Scavenger receptors

Scavenger receptors (SRs) were originally described on macrophages as surface glycoproteins with an ability to bind modified lipoproteins (Peiser *et al.*, 2002). In addition to macrophages, endothelial cells and smooth muscle cells express SRs. There are several members of the SR-A family, including SR-AI, SR-AII and MARCO. In addition, another family of receptors called SR-C has been described. Only a few studies have shown a role for SRs in human DC biology. Recently, MARCO was shown to be expressed by DCs and involved in actin cytoskeleton rearrangements and the downregulation of antigen uptake during DC

maturation (Granucci *et al.*, 2003;Grolleau *et al.*, 2003). Indirect evidence of their role in DCs from a recent study showed that SR-A are involved in DC 'cell nibbling' (Harshyne *et al.*, 2003). In these experiments, polyanionic ligands (inhibitors of SR) were shown to inhibit DC antigen capture from other live cell membranes, a process referred to as nibbling. MARCO and SR-A have been directly linked to phagocytosis by macrophages and bind both Gram-positive and Gram-negative bacteria although SRs appear to be involved in binding only and do not convey signals for internalisation (Peiser *et al.*, 2002).

1.3.1.5 Toll-like receptor family

Toll was first identified in *Drosophila* in the mid 1980's as a gene involved in dorsal-ventral patterning during fly embryogenesis. Later on it became clear that the *Drososophila* Toll also regulates the expression of anti-microbial peptides and hence plays an additional role in the adult fly immune system (Hoffmann and Reichhart, 2002). To date, 10 different mammalian homologues of toll, TLRs, have been described in humans and mice. The TLR family of innate immunity receptors are involved in the recognition of pathogen associated molecular patterns (PAMPs), which represent highly conserved molecular structures on a given microbe. After binding to their ligands, TLRs trigger a variety of signalling pathways, which help to orchestrate the appropriate immune response to a pathogen (Janeway, Jr. and Medzhitov, 2002).

DCs express the full repertoire of 10 different TLRs that have been shown to recognise a broad spectrum of ligands. In most cases, direct binding of microbial ligands to TLRs still has to be demonstrated. For example, TLR4 has been shown

to recognise bacterial LPS whereas TLR2 responds to peptidoglycan and lipteichoic acid (Hirschfeld *et al.*, 1999). Double stranded RNA, a product of most viruses, is recognised by TLR3 (Alexopoulou *et al.*, 2001), whereas TLR5 responds to the structural component of bacterial flagella, flagellin (Hayashi *et al.*, 2001). Bacterial DNA contains unmethylated CpG motifs not present in mammalian DNA and is recognised by TLR9 (Hemmi *et al.*, 2000). It is thought that the ligand specificity of each TLR ensures that a micro-organism will be detected early on in the course of infection and that an infection cannot pass unnoticed by the immune system. In addition, activation of the innate defence mechanisms via different TLRs orchestrates the development of the subsequent adaptive responses.

DCs are not the only cells to express TLRs. Neutrophils, B cells, CD25+ T cells, macrophages, astrocytes, hepatic and epithelial have all been shown to express different TLRs, although the amounts of particular TLRs expressed may vary according to the cell type (Bourke *et al.*, 2003;Bowman *et al.*, 2003;Hayashi *et al.*, 2003;Hornef *et al.*, 2002;Marsik *et al.*, 2003;Paik *et al.*, 2003). In phagocytic cells, TLRs are not generally thought be involved in the phagocytic process itself. Rather, they serve as pattern recognition molecules that help to generate and define the nature of the effector response by inducing distinct signalling pathways (Underhill, 2003). The fact that receptors for phagocytosis as well as TLRs operate close to each other suggest that they do interact in important ways. This was recently shown by two groups who demonstrated that Dectin-1, a β-glucan binding receptor, co-operates with TLR2 for the induction of inflammatory response (Brown *et al.*, 2003;Gantner *et al.*, 2003). It has also been shown that the

signalling pathways in operation during phagocytosis may influence the TLR signalling pathways. For example, PI3K and PLC, which are required in the mechanical aspect of particle uptake can influence the subsequent inflammatory response, such as cytokine production (Aderem, 2003;Fukao and Koyasu, 2003;Ojaniemi *et al.*, 2003).

Toll-like receptor signalling

All human TLRs are type I transmembrane proteins similar in structure to IL-1 receptor (IL-1R). The intracellular domain of both IL-1R and TLRs contain a Toll-IL-1R (TIR) domain, whereas the extracellular domains differ substantially. TLRs contain a leucine rich extracellular domain, whereas IL-1R contains immunoglobulin-like domains (Akira *et al.*, 2001). All TLRs activate a common signalling pathway that culminates in the activation of nuclear factor kappa B (NF-kB) transcription factors as well as protein kinases such as MAPK, ERK and JNK (Barton and Medzhitov, 2003). NF-kB plays a central role in regulation of genes involved in inflammatory responses, such as cytokines and co-stimulatory molecules expressed by DCs. Signal transduction in response to TLR triggering resembles that of IL-1R signalling (O'Neill and Dinarello, 2000). Both pathways include ligand induced receptor dimerisation and it has been shown that all ten TLRs can form homodimers (Zhang *et al.*, 2002). In addition, TLR2 can form heterodimers with TLR1 and TLR6 (Ozinsky *et al.*, 2000). All TLRs, as well as IL-1R, use a common adaptor protein called MyD88, which associates with the TLRs through homophilic interactions of their TIR domains (Figure 1.3). Important differences exist, however, in IL-1R and TLR signalling. In fact, individual TLRs can induce TLR specific signal transduction and new members

mediating these pathways are being discovered. An additional adaptor molecule, TIRAP, has homology to MyD88 and is required for TLR2 and TLR4 signalling, but not IL-1R (Henneke and Golenbock, 2001). Most TLR responses depend on MyD88 but TLR3 and TLR4 are capable of inducing responses, such as IFN-β production and CD86 expression by DCs independently of MyD88. Adaptor molecules called TRIF and TRAM may operate in the absence of MyD88 and TIRAP and may mediate such MyD88 independent responses (Yamamoto *et al.*, 2003a;Yamamoto *et al.*, 2003b).

MyD88 adaptor protein contains a death domain (DD), which recruits IL-1R kinase (IRAK) family through homophilic interaction via death domains. IRAK1 and IRAK4 are serine-threonine kinases involved in the phosphorylation and activation of tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6) (Figure 1.3). Activated TRAF6 serves as ubiquitin ligase, but the direct targets of the ubiquitination complex remain unknown. It is known, however, that TRAF6 activates a MAP kinase (MAPK) kinase (MAPKK) called TAK-1, which in turn activates MKK3 and MKK6, kinases upstream of p38 MAPK and JNK (Barton and Medzhitov, 2003). In addition, TAK-1 can activate inhibitory (I)kB kinase complex (IKK), although the precise mechanism remain unknown. The transcription factor NF-kB is activated by the degradation and release of IkB allowing NF-kB to translocate to the nucleus. IKK plays a central role in the phosphorylation of IkB (Figure 1.4) (Barton and Medzhitov, 2003).

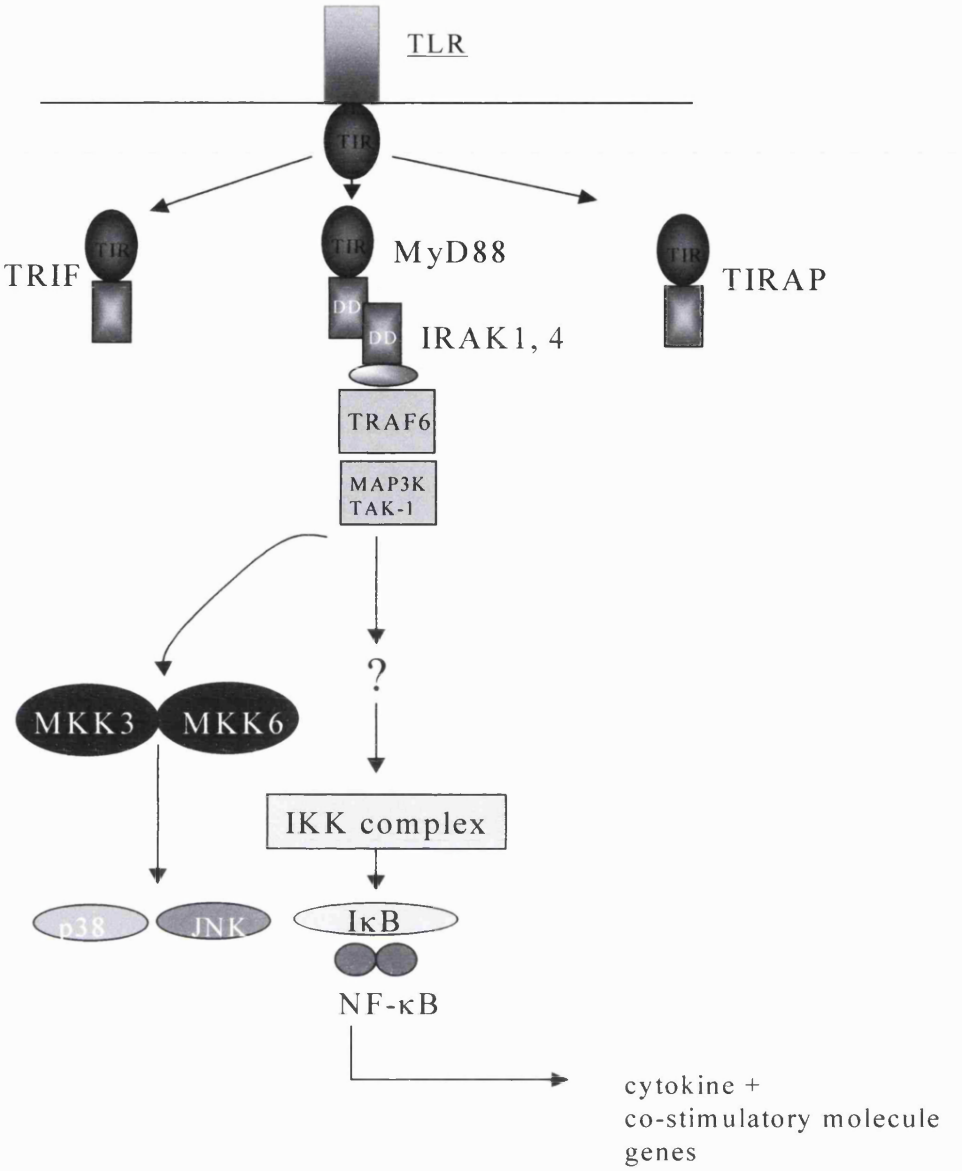

Figure 1.3 Toll-like receptor signalling pathways. All TLRs use the MyD88 dependent signalling pathway shown here, which is initiated by homophilic binding of TIR domains. TLR3 and TLR4 can also use TRIF for MyD88 independent production of IFN-β, an important cytokine mediating antiviral response. TIRAP functions after specific triggering of TLR2 and TLR4. TLR signalling culminates in the release of transcription factors that control genes involved in inflammatory responses, such as cytokines and co-stimulatory molecules. Modified from (Barton and Medzhitov, 2003).

Lipopolysaccharide signalling complex

LPS is a component of all Gram-negative bacterial outer membranes and is considered to be the major cause of bacterial sepsis and multi organ failure. In recent years, the mechanisms by which cells respond to LPS have become clearer. In particular, the discovery of TLRs helped to understand how the innate immune system recognises such a powerful stimulus. More than ten years ago, a Glycosylphosphatidyl Inositol (GPI) anchored membrane protein CD14 was shown to confer LPS responsiveness, but the signalling pathways remained unclear. CD14 has no cytoplasmic domain and therefore cannot signal intracellularly (Wright *et al.*, 1990). Subsequently, a point mutation in an LPS unresponsive mouse strain, C3H/Hej, was found in the ^{d}*lps* locus, which mapped to a single mutation in the TLR4 gene locus (Poltorak *et al.*, 1998). Confirmation that TLR4 is required for LPS signalling came from TLR4 knock-out mice that were hyporesponsive to LPS (Hoshino *et al.*, 1999). Hence, TLR4 was identified as the missing component able to transduce intracellular signals in response to LPS.

It is now known that the 'LPS receptor signalling complex' consists of several accessory proteins necessary for both binding and signalling (Figure 1.4). Multiple studies have shown that CD14 binds to LPS, but does not participate in signalling. It has been suggested that CD14 may facilitate the contact between LPS and TLR4, but there is controversy as to whether or not LPS actually interacts directly with TLR4 (Jiang *et al.*, 2000). LPS recognition by CD14 is highly amplified in the presence of LPS binding protein (LBP), which promotes rapid binding of LPS by either membrane bound or soluble CD14 (Hailman *et al.*,

1994). Interestingly, LBP has been shown to transfer LPS directly from Gram-negative bacterial membranes (Vesy *et al.*, 2000). In addition, LBP was shown to increase phagoctosis of *E. coli* whole bacteria by macrophages (Klein *et al.*, 2000). A recently discovered molecule called MD-2, which physically associates with TLR4, is an additional candidate for binding and signalling function associated with TLR4 (Nagai *et al.*, 2002).

In addition to TLR4/MD-2/LBP/CD14 complex, several other membrane proteins have been implicated in LPS binding and signal transduction. The CR3 (or Mac-1, CD11b/CD18) has been shown to bind LPS and contributes to LPS induced inflammatory signalling (Wright *et al.*, 1989). Fcγ receptors, as well as heat shock proteins and chemokine receptor CXCR4 have also been shown to interact with the LPS receptor complex (Triantafilou *et al.*, 2001). The data to date therefore strongly suggest that LPS-induced recognition and signalling is a product of a multicomponent receptor complex, which may vary according to cell type and function.

DCs have been shown to respond to LPS by increasing cell surface marker expression and by production of cytokines (Cella *et al.*, 1996;Sallusto *et al.*, 1995). The response is mediated at least in part via TLR4, as LPS-induced maturation is abolished in DCs from TLR4 deficient mice (Kaisho and Akira, 2001). Immature DCs express only very small amounts, if any, of CD14 and is not therefore considered to play a major role in LPS signalling complex in DCs. In contrast, monocytes express high levels of CD14, which is important for LPS

signalling as well as phagocytosis of Gram-negative bacteria (Grunwald *et al.*, 1996).

Recently, a new family of leucine repeat containing molecules was described and named as the NOD family of proteins. These proteins were found to be strikingly similar to TLRs and IL-1R and suggested a role in pathogen detection (Inohara and Nunez, 2003). NOD1 and NOD2 were shown to induce NF-kB signalling in response to bacterial peptidoglycan and LPS (Inohara *et al.*, 2001). Interestingly, the NOD proteins are expressed inside the cell and are therefore implicated on intracellular detection of PAMPs. Although the TLRs are assumed to reside on the cell membrane, some have also been found intracellularly. For example, TLR9 has been found in macrophage lysosomes, where it recognises CpG DNA (Ahmad-Nejad *et al.*, 2002). In addition, TLR2, TLR3 and TLR4 have been found intracellularly in macrophages, DCs and epithelial cells, respectively, suggesting that TLRs may also play a role in intracellular recognition of pathogens (Hornef *et al.*, 2002;Matsumoto *et al.*, 2003;Underhill *et al.*, 1999).

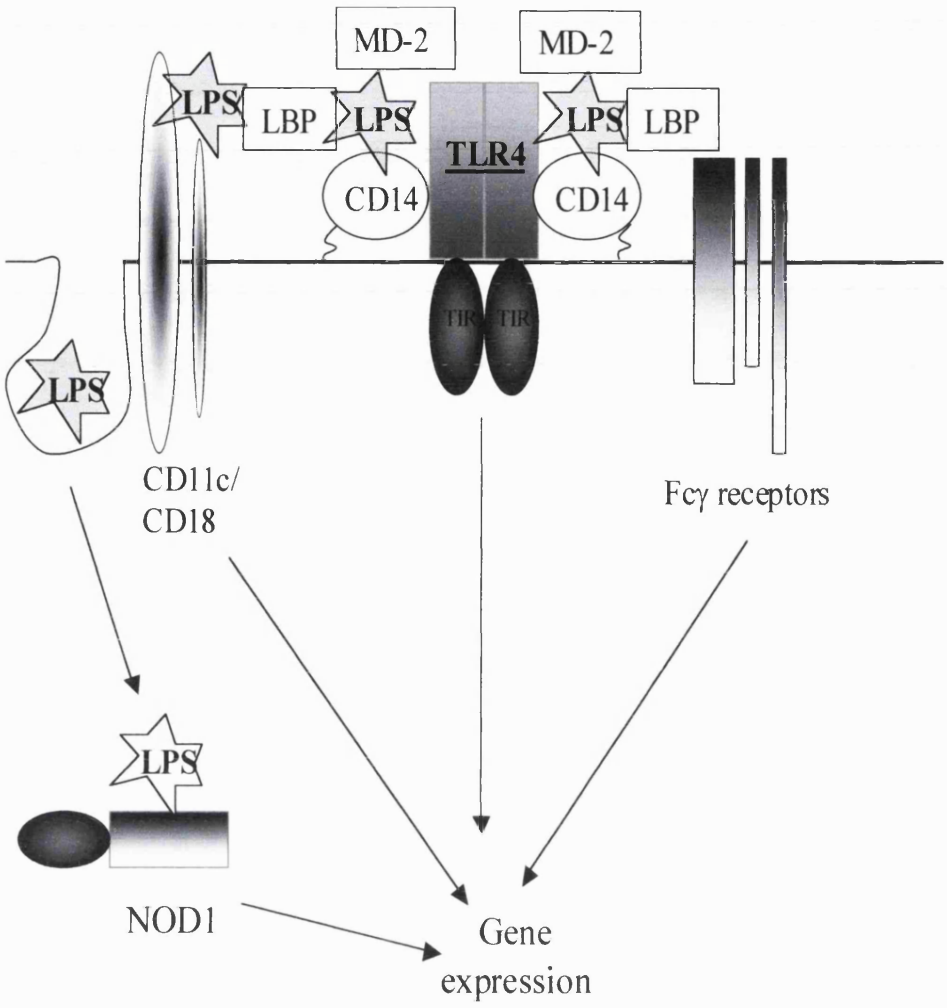

Figure 1.4 Lipopolysaccharide receptor signalling complex. This schematic picture shows some of the known proteins recruited into the LPS signalling complex. LPS is bound by LBP and CD14 on the cell surface, which facilitates interaction with the TLR4/MD-2 complex. LPS may also be internalised and interact with intracellular NODs. Ultimately, signalling leads to activation of transcription factors that regulate genes involved in the inflammatory cascades.

1.4 *Neisseria meningitidis*

1.4.1 Meningococcal infection

Neisseria meningitidis was identified as the causative agent of bacterial meningitis at the end of the 19th century. Infections caused by this bacterium still remain an important cause of mortality and morbidity world-wide (van Deuren *et al.*, 2000).

N. meningitidis is a Gram-negative diplococcus that is an exclusive human pathogen (Rosenstein *et al.*, 2001). It is grouped according to its polysaccharide capsule of which the main disease causing serogroups are A, B, C, Y and W135. Group B *N. meningitidis* is the most common in the UK, whereas in the USA and Canada, group C organisms cause most of the invasive disease (van Deuren *et al.*, 2000). Meningococci are transferred from one individual to another by close direct contact, such as nasal secretions. In fact, approximately 10% of people harbour the bacteria in the nasooropharyngeal mucosa, although the carriage may increase up to 100% during epidemic outbreaks. From early age, children will be colonised in the nasopharynx with *N. meningitidis* but only a small number will develop invasive disease. Young children under the age of four, the elderly and teenagers are amongst the high incidence group.

The clinical manifestations of the invasive meningococcal disease range from primary meningitis, to sepsis through to fulminant septicaemia. Meningococcal meningitis has a relatively good prognosis, whereas severe cases of sepsis and meningococcal disease have a rapidly progressive course and can be fatal.

Mortality from meningococcal disease has been reduced in recent years, but for patients presenting with severe shock, mortality still remains as high as 50%. In addition, patients who survive may have extensive tissue injury sometimes requiring amputation and/or skin grafting.

The rapid onset of meningococcal disease and high fatality rate make vaccine development against this pathogen of utmost importance. The surface antigens of *N. meningitidis* have been therefore studied in great detail.

1.4.2 The structure of *Neisseria meningitidis*

N. meningitidis group B organisms express an α-2,8 linked polysialic acid capsule that is anchored in the outer membrane (OM) of the bacteria. The polysaccharide capsule renders the bacteria resistant to host attack mechanisms, such as complement-mediated phagocytosis. Recently, Kolb-Maurer *et al.* (2001b) studied the interactions of group B *N. meningitidis* with human DCs. In this study, adhesion and phagocytosis were compared between encapsulated bacteria and a mutant unencapsulated *N. meningitidis*. Expression of the capsule significantly impaired neisserial adherence and phagocytic killing. In another study, the influence of the polysialic acid capsule on the interaction of *N. meningitidis* with human monocyte-derived macrophages was investigated (Read *et al.*, 1996). In this study the results indicated that the polysialic acid capsule modifies the interaction of meningococci with human macrophages at multiple steps, including adherence to the macrophage surface and phagosome-lysosome fusion.

Underneath the capsule lies the bacterial cell surface, which is composed of the OM and inner membrane (IM) separated by the periplasmic space (PS) (Figure 1.5). Type IV pili, composed of subunits called pilins, protrude through the membranes and the surrounding capsule. Pili are implicated in DNA uptake and twitching motility of meningococci. The tip of the pili consists of PilC protein, which has been shown to be involved in the initial adhesion of *Neisseria* to epithelial and endothelial cells. Recently, the complement regulatory protein CD46 was identified as a receptor for pilus binding (Nassif, 1999).

The OM is an asymmetric lipid bi-layer consisting mainly of LPS and proteins found on the outer leaflet, whereas the inner leaflet consists mainly of phospholipids. Bacterial outer membrane proteins (OMP) function in nutrient metabolism and protein secretion, as well as in invasion of host tissues. The major OMPs consist of porins and opacity proteins. PorA and PorB form trimeric cation- and anion-selective pores on the bacterial OM, respectively (Massari *et al.*, 2003). Variability in the porins forms the basis for *Neisseria* serosubtyping (Rosenstein *et al.*, 2001). The opacity proteins Opa and Opc are involved in adhesion and invasion and have been shown to mediate attachment to host cells via CD66 receptor from the carcinoembryonic antigen related cell adhesion (CEACAM) family (de Vries *et al.*, 1998). The reduction-modifiable protein M (RmpM) is constitutively expressed and antigenically invariant among different strains. Other membrane proteins, such as haem, lactoferrin and transferrin receptors may be expressed by meningococci in iron-limiting conditions.

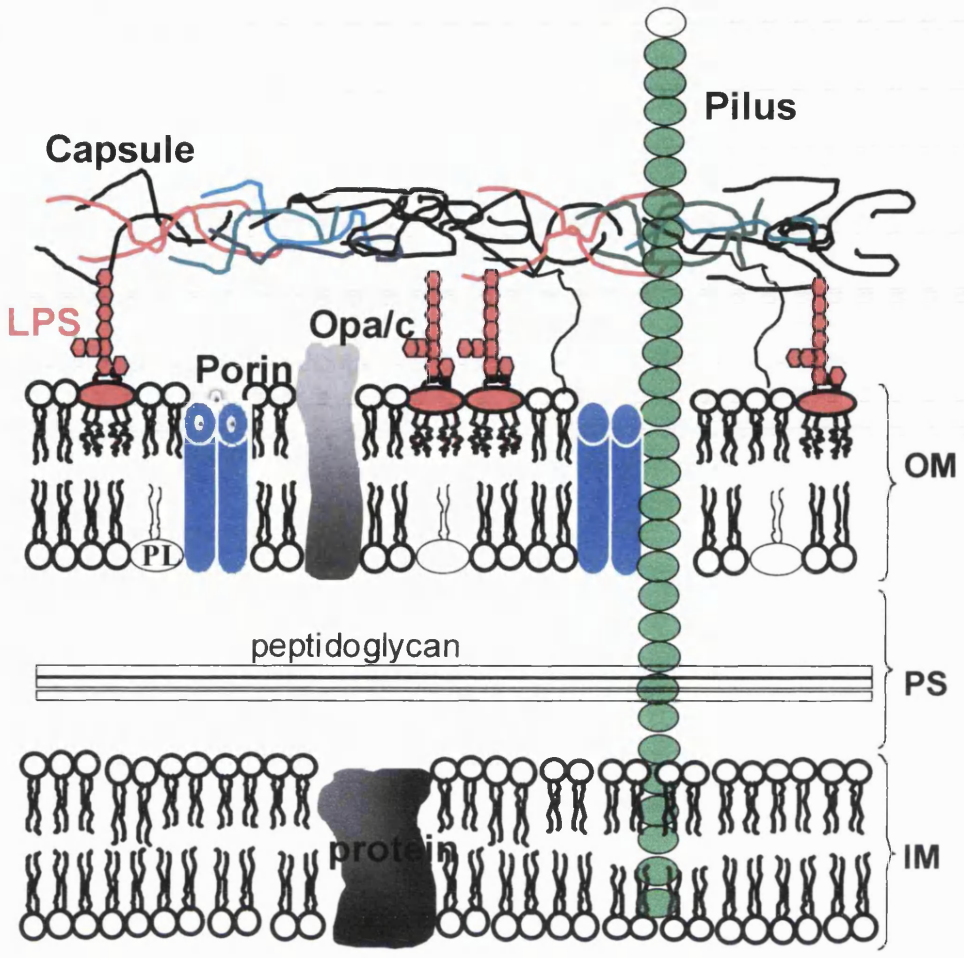

Figure 1.5 Surface structures of *Neisseria meningitidis.* The bacterium is surrounded by a capsule consisting of polysialic acid. The cell envelope consists of an inner membrane (IM), outer membrane (OM) and the periplasmic space (PS). The capsule is anchored to the OM, which harbours many integral proteins such as porins and Opa. LPS is present on the outer leaflet of the OM, whereas the inner leaflet consists of phospholipids (PL). The IM contains proteins and a symmetrical lipid bi-layer consisting of PL. Modified from (Steeghs, 2001)

1.4.2.1 Structure and biosynthesis of *Neisseria meningitidis* LPS

LPS is the major component of the outer leaflet of Gram-negative bacterial OM. Although the basic structure of LPS is the same in all Gram-negative bacteria, the structure and biological activity vary between different bacterial species (Erridge *et al.*, 2002). Meningococcal LPS consists of a hydrophobic lipid A part and a hydrophilic oligosaccharide core (Figure 1.6). The lipid A anchors the molecule in the OM and is generally relatively conserved among Gram-negative bacteria. The oligosaccharide, or sugar, core is exposed to the surface of the bacteria and is highly variable. *Neisseriae* LPS lacks the highly variable O-antigen on LPS found in other Gram-negative bacteria, such as *Salmonellae*, and is often referred to as lipo-oligosaccharide (LOS) (Erridge *et al.*, 2002).

Lipid A biosynthesis is the first step in the formation of LPS by *N. meningitidis* (Kahler and Stephens, 1998). The *lpxA* gene codes for the lpxA acyltransferase, which catalyses the first transfer of an acyl chain to UDP-N-acetyl glucosamine (UDP-GlcNac)(Figure 1.7). Inactivation of the *lpxA* gene by insertion of an inactivation cassette resulted in an LPS-deficient mutant of *N. meningitidis* (Steeghs *et al.*, 1998). This mutant was found to be viable, although reduced growth rate was observed. In addition, all major OMPs were unaffected, whereas changes in the phospholipid composition were reported. Interestingly, expression of the polysialic acid capsule was required for viability of the *lpxA*- knockout mutant (Steeghs *et al.*, 2001).

Several additional acyltransferases and other enzymes, such as LpxD and LpxB, are involved in the subsequent steps in the lipid A biosynthesis pathway (see

detailed review in (Kahler and Stephens, 1998). The lpxL2 and lpxL1 (in that order) acyloxyacyl transferases function in the symmetrical distribution of secondary fatty acyl chains on the lipid A molecule (Van Der Ley P. *et al.*, 2001a) (Figure 1.7 and 1.8).

The enzyme activities of *rfaC, icsA, icsB, lgtA, lgtB, lst* gene products are shown in figure 1.6. These enzymes are involved in the biosynthesis of the oligosaccharide core region by sequentially and specifically adding sugar residues to the growing oligosaccharide chain (Kahler and Stephens, 1998). Based on the structure of the oligosaccharide chain, *N. meningitidis* is grouped into 12 different immunotypes. The LPS of *N. meningitidis* strain H44/76 in figure 1.6 expresses the L3 immunotype. Several additional enzymes are involved in the LPS biosynthesis of the immunotypes L1-6 and L8. Figure 1.8 shows the LPS structures of immunotypes L1, L3 and L8.

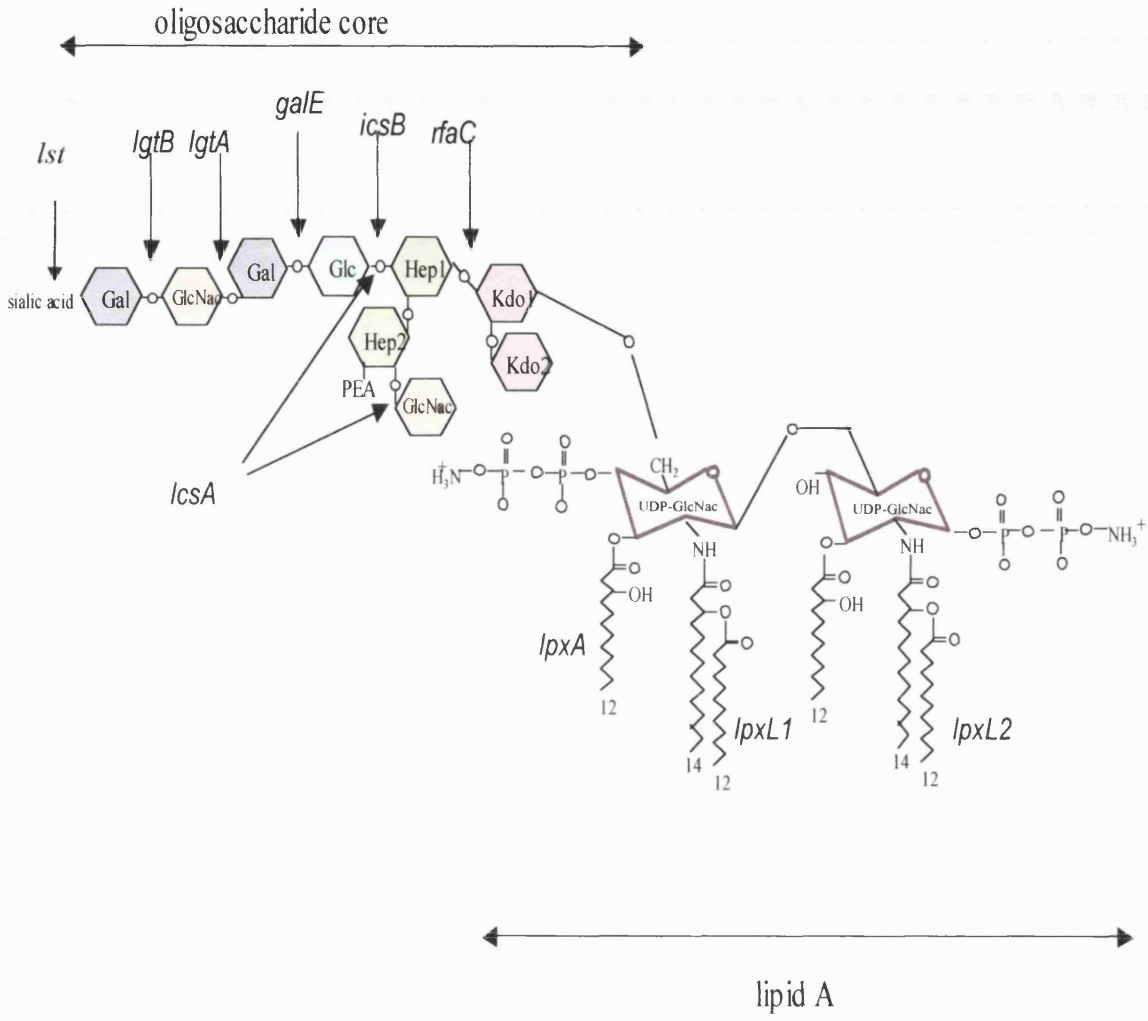

Figure 1.6 Structure of *Neisseria meningitidis* LPS immunotype L3. LPS consists of the lipid A moiety and the oligosachharide core. The enzyme activities of the *rfaC, icsA, icsB, galE, lgtA, lgtB, lst, lpxA, lpxL1 and lpxL2* gene products are shown. Gal=galactose, GlcNac= N-acetylglucosamine, Glc=glucose, Hep=heptose, KDO=2-keto-3-deoxyoctulosonic acid PEA=phosphoethanolamine sialic acid=N-acetyneuraminic acid. Modified from (Steeghs, 2001).

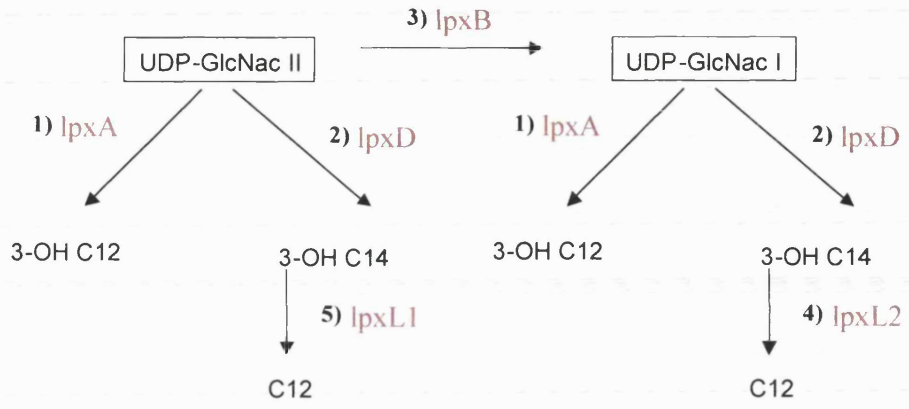

Figure 1.7 Meningococcal lipid A biosynthesis pathway. After acylation of UDP-GlcNac by lpxA and lpxD, dimerisation of UDO-GlcNac takes place by activation of lpxB disaccharide synthetase. Secondary fatty acid chains C12 are added by lpxL2 and lpxL1 (in this order) to the N-linked 3-OH C14. From (van der Ley P. *et al.*, 2001b).

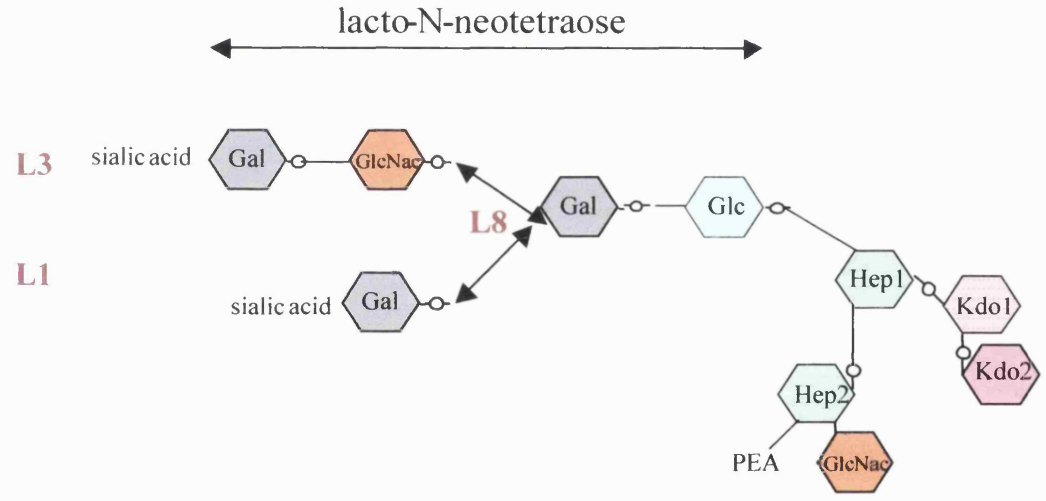

Figure 1.8 Structure of the oligosaccharide core of immunotypes L1, L3 and L8 of *N. meningitidis*. The L3 and L1 immunotypes are an extension of the L8 basal structure. Lacto-N-neotetraose (Gal/GlcNac/Gal/Glc) is expressed by the majority of the meningococcal strains including H44/76 used in this thesis. Gal=galactose, GlcNac= N-acetylglucosamine, Glc=glucose, Hep=heptose, KDO=2-keto-3-deoxyoctulosonic acid PEA=phosphoethanolamine sialic acid=N-acetylneuraminic acid

1.4.3 The host response

After crossing the nasopharyngeal mucosa, meningococci enter the blood stream and may cause invasive disease depending on the immune status of the host and the bacterial strain (Pathan *et al.*, 2003). Whilst the pathogenesis of meningococcal sepsis is complex, authorities consider that LPS is the principal inducer of the host inflammatory response seen in this condition (Rosenstein *et al.*, 2001). Severity of meningococcal disease correlates with the concentration of LPS in the plasma (Brandtzaeg *et al.*, 2001). A recent study has indicated a far higher bacterial load in patients than previously estimated in meningococcal sepsis (Hackett *et al.*, 2002). In patients with severe disease this reached as high as 10^8 organisms/ml of blood. High levels of circulating bacteria, as well as released blebs and LPS stimulate the production of pro- and anti-inflammatory cytokines, such as TNF-α, IL6, IL1, IL10 and IL12 (Brandtzaeg *et al.*, 2001). Elevated levels of these pro-inflammatory cytokines have been observed in patients with severe meningococcal disease (Prins *et al.*, 1998).

Despite evidence for the role of LPS in the catastrophic activation of host inflammatory response in meningococcal disease, other *N. meningitidis* surface structures than LPS, such as porins and opacity proteins Opc, have also been shown to activate host cells (Makepeace *et al.*, 2001;Massari *et al.*, 2002). Furthermore, non-LPS components of *N. meningitidis* have been shown to induce monocyte activation as well as DC maturation and cytokine production (Dixon *et al.*, 2001;Kolb-Maurer *et al.*, 2001b;Sprong *et al.*, 2001;Uronen *et al.*, 2000). In these studies, LPS-deficient meningococci were used to stimulate cells in order to study the contribution of non-LPS components in cell activation in response to *N.*

meningitidis. Although capable of inducing pro-inflammatory cytokine production, the potency of the LPS-deficient meningococci was approximately 10-100 lower than the parent strain containing LPS.

1.4.4 Vaccine development against group B *Neisseria meningitidis*

Purified polysaccharide vaccines against group A, C, Y and W-135 *N. meningitidis* have been developed in recent years. Protective immune responses have been obtained with these vaccines in adults but little protection is seen in children under the age of two who are known to be most prone to meningococcal infections. Attempts to generate a polysaccharide vaccine to group B organisms have proven unsuccessful. The group B polysaccharide vaccine is poorly immunogenic probably due to structural similarities with polysialic acid antigen structures present in human neural cell adhesion molecules (NCAM) (Finne *et al.*, 1987;Rosenstein *et al.*, 2001). To improve the immunogenicity, polysaccaharide conjugate vaccines against serogroup A and C have been developed, of which the group C vaccine has been introduced successfully in the UK (Miller *et al.*, 2001). The group B polysaccharide remained poorly immunogenic both in its native and conjugated form.

As the group B capsular polysaccharide antigens turned out to be poor vaccine candidates, OMPs were recognised as potentially interesting vaccine components. PorA is a highly immunogenic component of *N. meningitidis* and has been used in group B vaccine preparations as it elicits strong bactericidal responses (Cartwright *et al.*, 1999). Due to its highly strain specific variation, PorA containing vaccines showed strain-limited responses and have therefore lost some

of the vaccine potential. In turn, PorB has adjuvant properties and has therefore been included in poorly immunogenic vaccine preparations (Fusco *et al.*, 1997). Many other OMPs, such as opacity proteins, show high antigenic variation and are therefore not considered to be prominent vaccine candidates. Nevertheless, targeting conserved domains in these highly variable OMPs may prove useful in vaccine design against *N. meningitidis.*

Recently, a more promising approach in meningococcal vaccine development has been taken in the form of OM vesicle (OMV) vaccines. *N. meningitidis* bacteria are naturally able to release membrane 'blebs' or vesicles in culture (Bjerre *et al.*, 2000) (Figure 1.9).

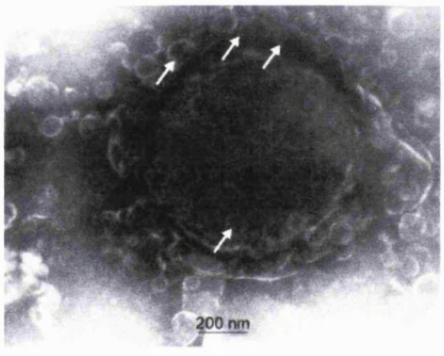

Figure 1.9 'Blebbing' meningococcus. Release of "blebs" (magnified ×65000, arrows) from rapidly growing meningococci is thought to contribute to the very high levels of LPS in plasma, which characterise fatal meningococcal septicaemia in patients. The blebs are currently used as vaccine candidates. From: (Namork and Brandtzaeg, 2002).

Isolation of OMVs in native form and their 'detoxification' by depletion of LPS in vesicles resulted in a vaccine with increased immunogenicity and low toxicity. A new hexavalent OMV vaccine was developed in order to prevent strain limited

immune responses. This OMV vaccine has now been shown to protect from the dominant serosubtypes of group B organisms isolated from both the UK and the Netherlands, although the requirement of four doses is its major drawback (Cartwright *et al.*, 1999;Van Der Ley P. *et al.*, 1995).

The relatively low immunogenicity of the OMV vaccines could be improved by inclusion of potent adjuvants. LPS is recognised as a powerful adjuvant and deserves serious consideration as a component in meningococcal vaccines (Verheul *et al.*, 1993). It is naturally present in the bacterial OM and therefore OMVs, but its harmful effects have limited its use so far. Immunisations with the OMVs from LPS deficient strain *lpxA*-, created by Steeghs and colleagues (2000), have proved inefficient in murine studies (Steeghs *et al.*, 1999). Inclusion of exogenous LPS in the LPS deficient OMVs was shown to restore their immunogenicity. Alterations in the composition of the LPS molecule and therefore in its biological (endotoxic) activity could provide useful in vaccine design against group B *N. meningitidis.*

1.4.5 Dendritic cell interactions with *Neisseria meningitidis*

To date, only a few studies have investigated human DC interactions in response to group B *N. meningitidis.* Dixon *et al.* (2001) were the first to study how the meningococcal structure, especially LPS, would affect DC activation and maturation. Three major findings came out of these studies. Firstly, purified LPS was found to be a poor inducer of DC cytokine production compared to whole organisms. Secondly, DCs could be activated by LPS-deficient organisms, showing that components of *N. meningitidis* other than LPS can induce DC

maturation. Thirdly, the presence of LPS within the bacteria was required for optimal DC cytokine production, in particular IL12. Other studies have later confirmed the critical requirement of LPS in cytokine production by DCs in response to *N meningitidis* (Kolb-Maurer *et al.*, 2001b;Unkmeir *et al.*, 2002).

DCs have been shown to phagocytose and kill group B *N. meningitidis* (Kolb-Maurer *et al.*, 2001;Unkmeir *et al.*, 2002). In these studies, adhesion and phagocytosis of live encapsulated and a mutant unencapsulated *N. meningitidis* were compared. Expression of the capsule significantly impaired adherence of bacteria and phagocytosis by DC and resulted in reduced cytokine production. Sialylation of LPS was also shown to decrease phagocytosis of *N. meningitidis* by human DCs.

1.5 Aims

The different aspects of DC activation (surface marker expression, cytokines, migration and T cell responses) can be used as a measure of immunogenicity. In this thesis, the role of LPS as a component of the bacterial outer membrane in the immunogenicity of *N. meningitidis* was studied. The main aims of this thesis were:

1) To explore human DC activation following stimulation with *N. meningitidis*
2) To investigate phagocytosis of *N. meningitidis* and its role in DC activation
3) To study the role of LPS and its structure in bacterial internalisation, DC maturation and cytokine production

The results of this thesis hope to add to the understanding of DC biology during interactions with *N. meningitidis* and provide novel information for future vaccine development against this organism.

Chapter 2

Materials and General Methods

2.1 Introduction

General methods are outlined here, whereas methods specific to a particular chapter are covered in each of the results chapters. All reagents, including antibodies and solutions as well as equipment used are listed in this section.

2.2 Reagents and Equipment

2.2.1 General Reagents

Reagent	Supplier
Agar for gonococci and meningococci	DIFCO, BD bioscience, Oxford, UK
Bovine Serum Albumin (BSA)	Sigma, Poole, UK
Bovine Serum Albumin cell culture tested	Sigma, Poole, UK
Brefeldin A	Sigma, Poole, UK
Cellfix	BD bioscience, Oxford, UK
Citi-fluor	Citi-fluor LtD, UK
Colcemid	Invitrogen, Paisley, UK
Cytochalasin D	Sigma, Poole, UK
Cytidine 5'-monophosphoN-acetylneuraminic acid (CMP-Nana)	Sigma, Poole, UK
Dimethylsulphoxide (DMSO)	ICN Biomedical, Basingstoke, UK
Ethanol	Sigma, Poole, UK
Fluorescein isothiocyanate (FITC)	Sigma, Poole, UK
Foetal Calf Serum (FCS)	Hyclone, Cramlington, UK
Glycerol	Sigma, Poole, UK
Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF)	Schering-Plough, Henilworth, NJ, USA
Hank's Balanced Salt Solution, without Phenol Red (HBSS)	Invitrogen, Paisley, UK

Reagent	Supplier
Interleukin 4 (IL4)	Schering-Plough, Henilworth, NJ, USA
isopropyl-beta-D-thiogalactopyranoside	Roche, UK
N-2-Hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES)	Invitrogen, Paisley, UK
Kanamycin	Sigma, Poole, UK
L-Glutamine	Invitrogen, Paisley, UK
Lipopolysaccharide binding protein, human recombinant	Biometec, Greifswald, Germany
Lipopolysaccharide (E.coli) Alexa 488 conjugated	Molecular probes, Eugene, OR, USA
Lymphoprep	Nycomed (Amersham), Little Chalfont, UK
Methanol	Sigma, Poole, UK
Muller-Hinton broth	DIFCO, BD bioscience, Oxford, UK
Monoparin sodium heparin	CP Pharmaceuticals, Wrexham, UK
Paraformaldehyde $(CH_2O)_n$ (PFA)	Sigma, Poole, UK
Percoll	Amersham, Little Chalfont, UK
Penicillin/Streptomycin	Invitrogen, Paisley, UK
Permeabilisation Solution	Caltag, Burlingame, CA, USA
Phosphate Buffered Saline tablets (PBS)	Oxoid, Basingstoke, UK
Potassium Chloride (KCl)	BDH Merck, Poole, UK
RPMI 1640 medium with 10mM L-glutamine	Invitrogen, Paisley, UK
RPMI 1640 medium without phenol red	Invitrogen, Paisley, UK
Saponin	Sigma, Poole, UK
Sodium Azide (NaN_3)	BDH Merck, Poole, UK
Sodium Chloride (NaCl)	BDH Merck, Poole, UK
Sodium Hydroxide (NaOH)	BDH Merck, Poole, UK
TMP solution	Europa Bioproducts, Cambridge, UK
To-pro-3 iodide (642/661)	Molecular probes, Eugene, OR, USA
Toxin B	Ann Ridley, Ludwig Institute, London
Trypan Blue	Sigma, Poole, UK
Tween 20 (Polyoxethylene sorbitan monolaurate)	Sigma, Poole, UK
VITOX	Oxoid, Basingstoke, UK

2.2.2 Antibodies for flow cytometry and confocal microscopy

2.2.2.1 Primary antibodies

All primary antibodies listed here were mouse monoclonal anti-human antibodies, except the TLR antibodies which were rabbit polyclonal anti-human antibodies.

Antibody	Conjugation	Isotype	Clone	Supplier
CD3	FITC	Mouse IgG2a	S4.1	Caltag, Burlingame, CA, USA
CD14	FITC	Mouse IgG2a	Tük 4	Caltag, Burlingame, CA, USA
CD19	FITC	Mouse	HIB19	BD bioscience, Oxford, UK
CD40	FITC	Mouse IgM	14G7	Caltag, Burlingame, CA, USA
CD83	FITC	Mouse IgG2b	HB15	Caltag, Burlingame, CA, USA
CD83	PE	Mouse IgG2b	HB15	Caltag, Burlingame, CA, USA
CD86	FITC	Mouse IgG1	BU63	Caltag, Burlingame, CA, USA
CD107a (LAMP-1)	purified	Mouse	H4A3	Pharmingen, BD Bioscience, Oxford, UK
Golgin-97	purified	Mouse IgG1	CDF4	Molecular Probes, Eugene, OR,USA
HLA-DR, DP, DQ	Purified	Mouse IgG1	CR3/43	Dako, Cambridge, UK
MHC Class II	FITC	Mouse IgG2b	TÜ 36	Caltag, Burlingame, CA, USA
MHC Class II	PE	Mouse IgG2b	TÜ 36	Caltag, Burlingame, CA, USA
IL1β	PE	Mouse IgG1	AS5	BD bioscience, Oxford, UK
IL6	PE	Mouse IgG1	AS12	BD bioscience, Oxford, UK
IL12 (p40/p70)	PE	Mouse IgG1	C11.5	Pharmingen, BD Bioscience, Oxford, UK
TNF-α	PE	Mouse IgG1	6401.1111	BD bioscience, Oxford, UK

Antibody	Conjugation	Isotype	Clone	Supplier
Porin P1.7 *N. meningitidis* group B	purified	mouse IgG2a	MN14C11.6	National Institute for Biological Standards and Control (NIBSC), Hertfordshire, UK
TLR2	purified	rabbit polyclonal IgG		GlaxoSmithKline
TLR4	purified	rabbit polyclonal IgG		GlaxoSmithKline
α-tubulin	purified	Mouse	TAT-1	Dr Sue Hall, Vascular Biology, ICH, London UK

2.2.2.2 Secondary antibodies

All secondary antibodies listed here were raised in goat.

Antibody	Conjugation	Supplier
mouse IgG	Alexa 488	Molecular Probes, Eugene, OR,USA
mouse IgG	Alexa 568	Molecular Probes, Eugene, OR,USA
mouse IgG	FITC	Molecular Probes, Eugene, OR,USA
mouse IgG	Texas-red X	Molecular Probes, Eugene, OR,USA
rabbit IgG	biotinylated	Dako, Cambridge, UK
rabbit IgG	FITC	Sigma, Poole, UK
rabbit IgG	TRITC	Sigma, Poole, UK
rabbit IgG F(ab)2	FITC	Caltag, Burlingame, CA, USA
streptavidin	PE-Cy5	Pharmingen, BD Bioscience, Oxford, UK

2.2.2.3 Isotype control antibodies

Isotype	Conjugation	Clone	Supplier
Mouse IgG1	purified	-	Dako, Cambridge, UK
Mouse IgG1	FITC	MOPC-21	Caltag, Burlingame, CA, USA
Mouse IgG1	PE	MOPC-21	Caltag, Burlingame, CA, USA
Mouse IgG2a	FITC	5.205	Caltag, Burlingame, CA, USA
Mouse IgG2a	PE	5.205	Caltag, Burlingame, CA, USA
Mouse IgG2b	FITC	MOPC-195	Caltag, Burlingame, CA, USA
Mouse IgG2b	PE	MOPC-195	Caltag, Burlingame, CA, USA
Mouse IgM	FITC	MOPC-104E	Caltag, Burlingame, CA, USA
Mouse IgG1	PE	X40	BD bioscience, Oxford, UK
Mouse IgG1	PE	MOPC-21	Pharmingen, BD Bioscience, Oxford, UK
Mouse IgG2a	PE	G155-178	Pharmingen, BD Bioscience, Oxford, UK
Rabbit IgG, normal	purified		Caltag, Burlingame, CA, USA
Goat serum			Caltag, Burlingame, CA, USA

2.2.3 Antibodies and standards for ELISA

Antibody pairs and standards (recombinant cytokines) for human IL1β, IL6, IL10, IL12 and TNF-α were purchased as CytoSets™ ELISA kits from BioSource for determination of soluble cytokines.

2.2.4 Consumables

Below is a list of general plastic ware and other small pieces of equipment used.

Equipment	Supplier
Bijou 7 ml	SLS, Wilford, Nottingham, UK
Coverslips (for microscopy)	BDH Merck, Poole, UK
Dako pen for immunohistochemistry	Dako, Cambridge, UK
Filter Eppendorf tubes 1.5ml (Ultrafree-MC)	Millipore, Watford, UK
FACS tubes, small LT2	Life Sciences International
Falcon tubes 5 ml (FACS)	BD Falcon, BD Bioscience, Oxford, UK
Falcon tubes 15 ml	TPP, Trasadingden, Switzerland
Falcon tubes 50 ml	TPP, Trasadingden, Switzerland
Nail varnish (for micrscopy)	Boots, UK
Maxisorb ELISA plates - 96 well	Nalgene Nunc, Rochester, NY, USA
Slides, poly-L-lysine coated	BDH Merck, Poole, UK
Slides for adhesion, 12-wells (for microscopy)	Paul Marienfeld GMBH & Co., Germany
Syringe filters 0.22μm	Millipore, Watford, UK
Tissue culture flask T75 (for blood)	Helena Biosciences, UK
Tissue culture plates - 6 well	Corning (Costar), Corning, NY, USA
Tissue culture plates - 24 well	Corning (Costar), Corning, NY, USA
Transwells 6.5 mm Polycarbonate membrane (0.1 μm pore)	Corning (Costar), Corning, NY, USA
Universal tubes 20 ml	Helena Bioscience, UK
96 well round bottom plates for FACS staining	Greiner Bio-one, Fricekenhausen, Germany

2.2.5 Equipment

Equipment	Supplier
FACScalibur Flow Cytometer	Becton Dickinson, (BD Bioscience), Oxford, UK
Confocal Microscope	Leica Microsystems UK, Milton Keynes, UK
MRX ELISA microplate reader	Dynatech Laboratories, Chantilly, VA, USA
ELISA plate washer	Aw1, UK

2.2.6 General buffers, solutions and culture media

The following solutions were prepared with Milli Q purified water as indicated.

'A' mix solution for FITC conjugation
5.8ml of 5.3% (w/v) Na_2CO_3 (anhydrous)
into
10ml of 4.2% (w/v) $NaHCO_3$

Conjugation buffer (FITC), pH 9.5
1 part 'A' mix solution
9 parts 0.1M NaCl

DC Complete medium for culture
RPMI 1640 w L-glutamine
10% FCS (HI)
2.4 mM L-glutamine
100 U/ml Penicillin/Streptomycin
(with 100 ng/ml rGM-CSF and
50 ng/ml rIL-4 as indicated)

ELISA Buffer A, pH 7.4
8.0 g NaCl
1.42 g $Na_2HPO_4.H_2O$
0.2 g KH_2PO_4
0.2 g KCl
1000 ml H_2O

ELISA Block buffer
Buffer A
0.5% BSA

ELISA Diluent
Buffer A
0.5% BSA
0.1% Tween 20

ELISA Wash buffer
9.0g NaCL
1 ml Tween 20
1000 ml H_2O

FACS wash buffer
1x PBS
0.02% Sodium Azide
0.5 % BSA

General cell wash medium
RPMI 1640 w L-glutamine
5% FCS (heat inactivated [HI])

Phosphate Buffered Saline (PBS)
1 tablet of PBS to 100 ml H_2O

10x PBS, pH 4.6
67.5 g NaCl
0.625 g Na_2HPO_4
10.5 g KH_2PO_4
1000 ml H_2O

Saponin permeabilisation buffer/wash
HBSS
0.1% saponin
2mM Hepes buffer
0.05% Sodium Azide

2.2.7 FITC Conjugation of P1.7 antibody

The antibody P1.7 specifically binds to Porin A subtype called P1.7 present on meningococci of strain H44/76. Lyophilisate from 100µl of mouse ascites fluid containing the antibody was reconstituted in 1ml of PBS. Large eppendorf tubes containing detachable filters were used to spin down 0.5 ml of antibody in PBS at low speed setting (3500rpm/1200g in a microfuge). After carefully recovering the antibody from the filter membrane by gentle pipetting, the volume was adjusted back to 0.5ml with conjugation buffer. FITC powder at 1mg/ml was dissolved in the conjugation buffer and filtered through a 0.22µm Millipore syringe filter in order to remove large undissolved aggregates of FITC. Next, 150µl of FITC conjugation buffer was added to 0.5 ml of antibody solution in conjugation buffer and the mixture incubated for 3h at RT in the dark. Unbound FITC was removed by thorough washing with PBS in filtered eppendorfs at low speed until running buffer was clear. A final concentration of 0.18mg/ml of protein was calculated from the equation OD 280 - (OD 495 x 0.35)/1.35mg/ml. (OD 280nm= 0.25 and OD 495nm= 0.269). The conjugate was stored at 4°C in the dark with addition of 0.1% sodium azide. Unconjugated antibody was aliquoted and stored frozen at -70°C.

2.3 Cell preparation and culture

2.3.1 Separation of peripheral blood mononuclear cells

Venous blood (60-150ml) obtained from healthy volunteers was collected into heparinised (10 U/ml of blood) tissue culture flasks. The blood was then diluted 1:1 in RPMI-1640 medium, layered onto Lymphoprep (at a density of 1.077 g/ml) and centrifuged for 25 min. at 400g at room temperature. Mononuclear cells were recovered from the interface and washed in cell wash medium once for 10 min. at 400g and then two times for 10 min. at 200g. Cell viability was determined on a haemocytometer with trypan blue and was typically >95%.

2.3.2 Isolation of monocytes

Monocytes have a lower nuclear to cytoplasm ratio than peripheral blood lymphocytes and can therefore be separated by density centrifugation on a discontinuous percoll gradient (Hilkens *et al.*, 1997).

Standard Isotonic Percoll (SIP) was prepared by adding 1 part 10x PBS to 9 parts percoll (density of 1.793 g/ml). The SIP was then diluted with cell wash medium to give three different densities: 34% SIP (1.045 g/ml), 47.5% SIP (1.059 g/ml) and 60% SIP (1.076 g/ml). The PBMC pellet (obtained as in 2.3.1) was resuspended in 8-12 ml of 60% SIP, and 2 ml of this suspension was placed into each 15ml polypropylene tube. Next, 4.5 ml of 47.5% SIP was layered on top of the 60% SIP-cell suspension, followed by 2 ml of the 34% SIP. The gradients were centrifuged at 1750 g for 45 minutes at room temperature (Fig 2.1).

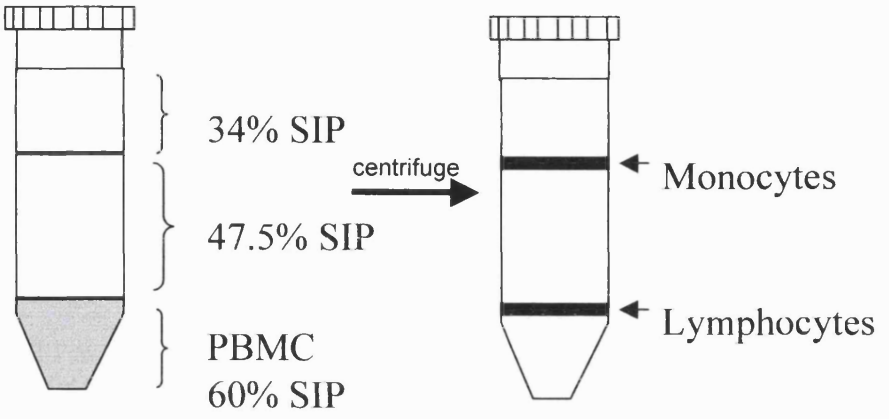

Figure 2.1 Separation of monocytes by percoll gradient

Monocytes were separated from PBMCs by centrifugation on a discontinuous percoll gradient. After centrifugation, monocytes formed a layer at the interface between the 34% and 47.5% SIP. The lymphocytes formed a layer at the interface between 47.5% and 60% SIP. Platelets formed a suspension in the 34% SIP and any contaminating red blood cells and cell debris pelleted at the bottom of the tube.

Following centrifugation, monocytes were harvested from the upper interface with a sterile Pasteur pipette. Monocytes were washed once for 10 min. at 400g and then two times for 10 min. at 200g in ice cold cell wash medium, counted and then resuspended to a final concentration of 0.5 $x10^6$ cells/ml in complete culture medium. Monocytes were used as indicated in chapter 7. The purity of the monocyte preparation was analysed by flow cytometry for contaminating lymphocytes using CD3, CD14 and CD19 antibodies (Figure 2.2). Whole blood contained approximately 5% and PBMC 10-20% of $CD14^+$ monocytes. After purification on the discontinuous percoll gradient, the upper fraction contained >90% $CD14^+$ cells and <5% T and B cells.

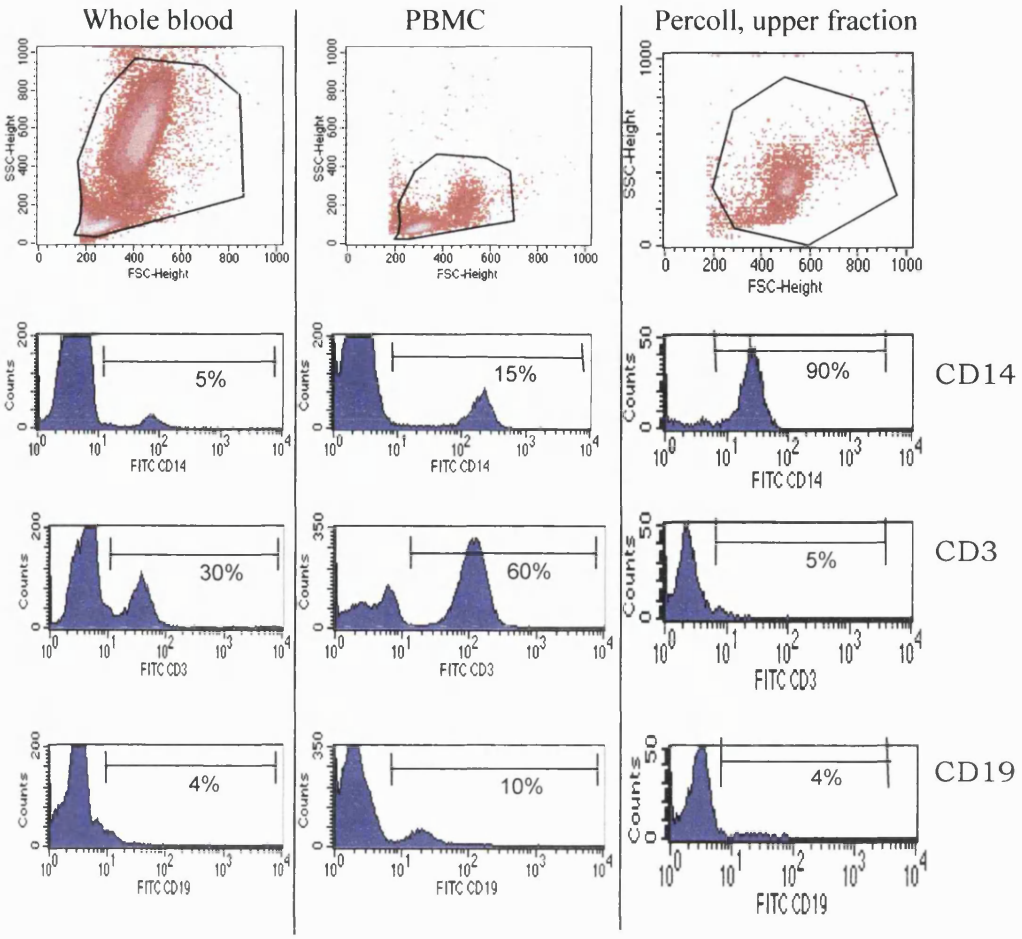

Figure 2.2 Monocyte purification by discontinous percoll density gradient.
The relative proportion of CD14+, CD3+ and CD19+ cell populations in whole blood, PBMCs or after separation on a percoll gradient. The cells were stained with the appropriate monoclonal antibodies, gated and analysed by FACS.

2.3.3 Dendritic cell preparation

Dendritic cells (DCs) were generated by culture of blood monocytes with GM-CSF and IL4 as described previously (Sallusto and Lanzavecchia, 1994). Monocytes were resuspended to a concentration of 10^5 cells/ml in complete culture medium supplemented with 100ng/ml of human recombinant GM-CSF and 50ng/ml of human recombinant IL-4. The cells were aliquoted into 6 well

tissue culture plates at $2x10^6$ cells/well (4 ml/well) and cultured for 5-7 days at 37 °C in an atmosphere of 5% CO_2 in air. The DCs were then gently washed by centrifugation for 5 min at 200g and resuspended in fresh DC complete medium (without GM-CSF or IL4) to a concentration of 10^5-10^6 cells/ml. Careful selection of LPS-free products, particularly the FCS, proved important for DC culture.

2.3.3.1 Phenotypic characteristics of dendritic cells

On day 5-7, the DCs were examined by light microscopy for typical DC morphology of large, irregular shaped veiled cells. On flow cytometry, DCs were easily identified by their distinct forward and right angle scatter characteristics due to their large size and granularity. They formed a distinct population with high forward and right angle scatter on a density plot (Fig 2.3). The identity of this DC population was confirmed by moderate/high levels of MHC Class II (DR, DP, DQ) and CD86 and low levels of CD14 and CD83.

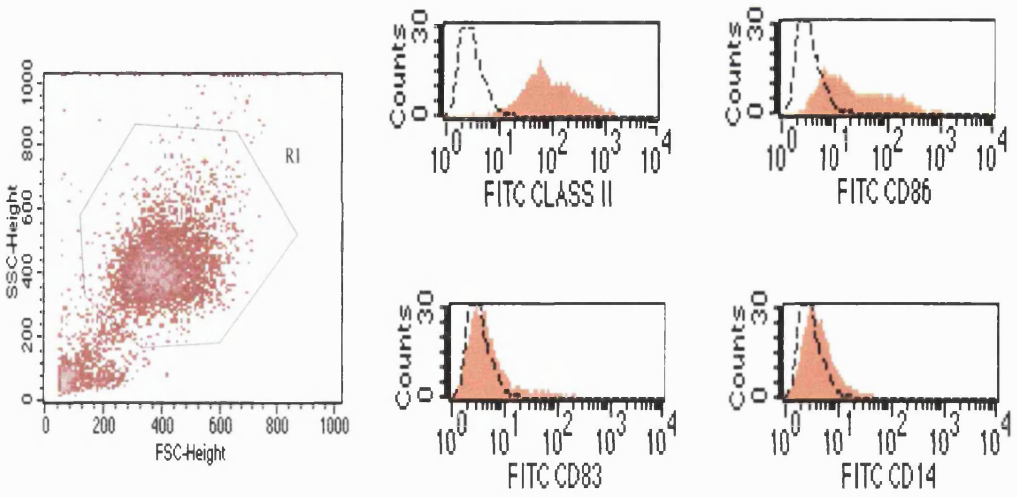

Figure 2.3 Phenotypic characteristics of DCs. DCs were gated according to their forward and side scatter properties (R1) and stained for MHC Class II, CD86, CD83 and CD14 (Filled histograms). Unfilled histograms show the isotype control staining.

2.4 Cell staining

2.4.1 Staining for cell surface antigens

For cell surface staining, 100µl of cells were aliquoted into a 96 well plate (~10^4-$5x10^4$cells/well) and incubated with purified or directly conjugated monoclonal antibodies (5µg/ml unless otherwise stated) for 30min on ice in the dark. The cells were then washed with cold FACS wash buffer and fixed in 200µl Cellfix. If a second antibody layer was required, cells were incubated on ice in the dark for 30 minutes with appropriate secondary antibodies, washed by centrifugation at 200 g for 5 minutes and then fixed in Cellfix. Appropriate isotype controls were used to detect any nonspecific binding.

2.4.2 Intracellular staining

When intracellular cytokines were to be measured, the protein transport inhibitor Brefeldin A was added to the cultures at 10μg/ml for 18h before harvesting. One hundred microliters (~10-50000 cells) of cells were aliquoted into the wells of a 96 well plate, fixed by addition of 100μl 4% paraformaldehyde (PFA) in PBS for 15 minutes and washed in FACS washing buffer by centrifugation at 200 g for 5 minutes. The cells were then permeabilised in 25μl of permeabilisation solution and the specific antibodies and the relevant isotype controls added for 30 minutes in the dark at room temperature. Finally, the cells were washed in FACS wash, fixed in 200μl of Cellfix and analysed by flow cytometry.

2.4.3 Flow Cytometry

Fixed cells were analysed on a FACScalibur flow cytometer using Cellquest software. Voltage and compensation were set using antibody staining controls. In each acquisition, at least 3000 gated events were collected.

2.4.4 Preparation of human serum

Human serum was used to prevent nonspecific binding of antibodies. Venous blood from a healthy volunteer was allowed to clot at room temperature for 1h in a sterile plastic container and the serum collected and then centrifuged for 10 min. at 250g to remove any contaminating cells. Finally, the serum was filtered twice through a 0.45μm Millipore membrane, aliquoted and stored at –20°C.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

For analysis of soluble cytokine secretion by DCs, human IL12, IL1β, IL6, IL10 and TNF-α CytoSets™ ELISA kits were used. High binding 96-well plates were coated with 100μl/well of coating antibody at 1μg/ml for 18h at +4°C, washed twice in washing buffer and then blocked for 2h on a rotation platform at RT by adding 300μl blocking buffer. After 4 washes, standards and samples were added at 100μl/well in duplicates. As standards, serial 1:2 dilutions of human recombinant IL1β (concentration range from 5000pg/ml to 2.5pg/ml), IL12 (from 10000pg/ml to 5pg/ml), IL6 (from 5000pg/ml to 2.5pg/ml), IL10 (from 5000pg/ml to 2.5pg/ml), and TNF-α (from 10000pg/ml to 5pg/ml) were used (Fig. 2.4). In order to read the OD values from the linear part of the standard curve, samples were diluted accordingly in diluent. As negative controls, diluent or RPMI-1640 were added to two wells. The plates were incubated for 18h at +4°C and then washed 4 times followed by addition of 50μl of biotinylated detection antibodies specific to cytokines. After 2h rotation, the plates were washed 4 times and 100μl of streptavidin-horseradish peroxidase conjugate added to each well. The plates were then incubated for 45min protected from light. After 4 washes, 100μl of the Chromogen TMB substrate buffer was added to the plates and incubated in the dark until sufficient colour development was reached (between 20-40min). The reaction was stopped by addition of 100μl/well of stop solution (1.8N H_2SO_4). Optical density was measured at 450nm (reference filter 650nm) on a Dynatech MRX microplate reader and analysed using the Revelation software.

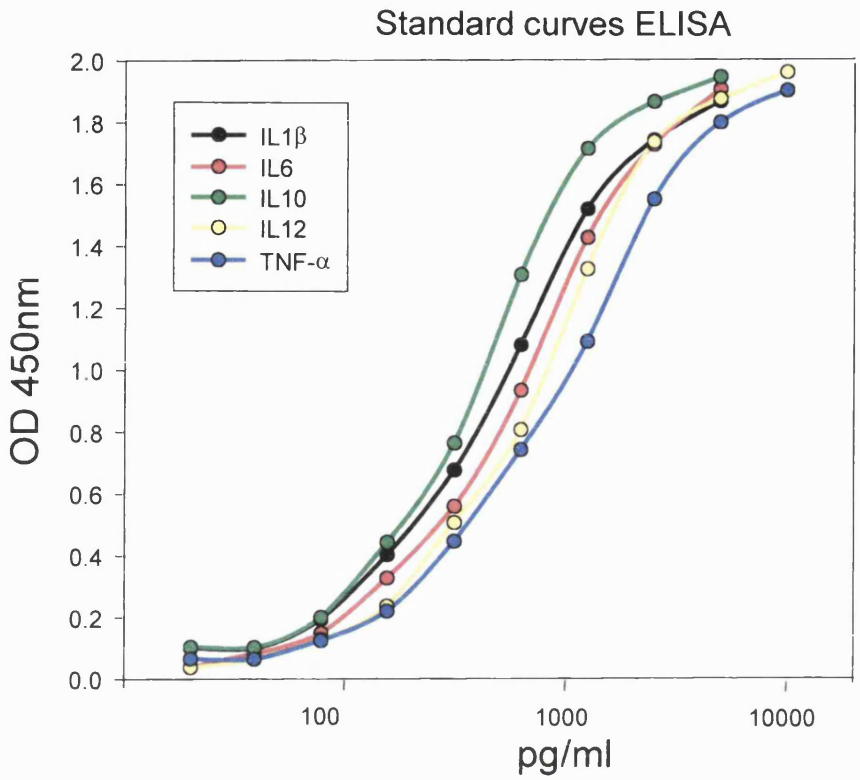

Figure 2.4 ELISA standard curves. Representative standard curves for cytokine ELISAs read on the Dynatech MRX microplate reader at 450nm (ref filter 650nm). The standard curves started at 10,000 pg/ml for TNFα and IL12 and 5,000 pg/ml for IL1β, IL6 and IL10.

2.6 Preparation of *Neisseria menigitidis*

2.6.1 Bacterial Strains

All bacterial strains used in this thesis, except *N. meningitidis* group B M992 strain, are derived from a group B *N. menigitidis*, strain H44/76 (sero (sub)-type (B:15:P1.7,16), ET-5 complex), which is a Norwegian isolate from a case of fatal septicaemia (Andersen *et al.*, 1995). All strains were kindly provided by Dr Peter van der Ley, RIVM, Bilthoven, and Dr Liana Steeghs, University Medical Centre Utrecht, the Netherlands. Several isogenic mutant strains have been described using the H44/76 WT background. The viable, but completely LPS deficient isogenic mutant, *lpxA-*, was constructed by insertional inactivation of the *lpxA* gene with a kanamycin cassette as described (Steeghs *et al.*, 1998). The protein product of *lpxA* is an enzyme required for the first committed step in lipid A biosynthesis. The absence of LPS was previously confirmed by Limulus amebocyte lysate (LAL) assay, whole cell ELISA using LPS specific monoclonal antibodies and gas chromatography/mass spectrometry (Steeghs *et al.*, 1998). The *lpxA-* isogenic mutant and the parent both showed similar binding patterns to monoclonal antibodies for outer membrane proteins (Steeghs *et al.*, 2001). Purity of the *lpxA-* mutant was maintained by growth on agar plates containing kanamycin (100 μg/ml). In addition to the WT and LPS fdeficient *lpxA-* strains of *N. meningitidis*, LPS regulatory strain HA3003 were used. *N. meningitidis* strain HA3003 has been described (Steeghs *et al.*, 2001). The HA3003 strain carries the *lpxA* gene under control of an Isopropyl-BD-Thiogalactopyranoside (IPTG)-inducible promoter. When this strain is grown in the absence of IPTG, LPS is barely detectable on the outer membrane of the bacteria but in the presence of

IPTG the biosynthesis of LPS is restored. Lipid A and oligosaccharide core mutant strains prepared by Liana Steeghs and Peter van der Ley were also used and are described in detail in Chapter 6.

2.6.2 Culture of *Neisseria meningitidis*

Culture and handling of live *N. meningitidis* was carried out in a Class I safety cabinet housed in a category 3 containment facility under negative pressure at the Great Ormond Street Hospital. All strains of *N. meningitidis* were stored as frozen stocks in Mueller-Hinton broth with 15% glycerol at -80°C. When needed, aliquots of frozen bacteria were plated on freshly prepared gonococcal agar plates supplemented with the growth medium Vitox. Organisms were cultured at 35°C in an atmosphere of 6% CO_2 and used in stationary phase after 16-18h of growth. Fixed or live organisms were used as indicated. When live organisms were used, the bacteria were grown overnight on gonococcal agar plates, from which a few colonies were selected for cultures the next day. The bacteria were grown to log-phase in liquid cultures in Muller-Hinton broth for 5h in 50ml falcon tubes in a rotation incubator, washed in RPMI 1640 without phenol red.

To inactivate bacteria, several individual colonies were collected from plates with sterile cotton wool swabs into RPMI 1640 (without phenol red) containing 0.5% PFA. After fixing and careful washing in RPMI 1640 (without phenol red), the optical density (OD) of the bacterial suspension was adjusted to an OD of 1.0 by using a spectrophotometer reading at 540nm. It was shown by serial dilutions, plating and viability counts, that a suspension of organisms in stationary phase at OD 1.0 contains around 10^9 CFU/ml (colony forming units) (G. Dixon personal

communication). Aliquots of fixed bacteria at a concentration of 10^9 CFU/ml were stored at -80°C or used fresh as indicated.

HA3003 strain was grown overnight on gonococcal agar plates in the presence or absence of 2μM, 10μM or 50μM IPTG. The level of LPS expression was verified by SDS-PAGE and silver staining (Fig. 2.5). As expected, when HA3003 was grown in the absence of IPTG, LPS was barely detectable. LPS deficient *lpxA*- bacteria were used as a control. In contrast, an IPTG dose-dependent increase in LPS expression was found when IPTG concentrations up to 50μM were used. At 10μM IPTG or more, LPS biosynthesis in strain HA3003 was restored to the WT level.

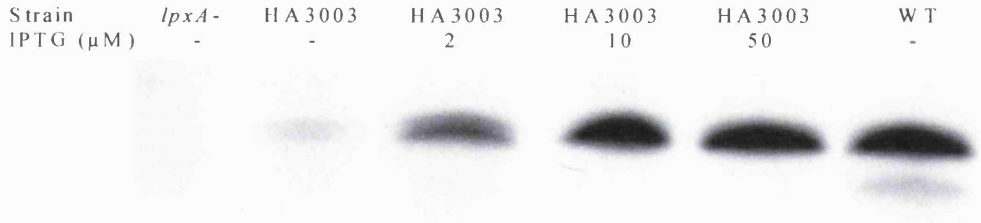

Figure 2.5 Analysis of LPS content of the regulatory strain HA3003. Strain HA3003 bacteria were grown in the absence or presence of 2μM, 10μM or 50μM IPTG. The *lpxA*- and WT bacteria are shown for comparison. The LPS content was analyzed in whole bacterial cell lysates by Tricine SDS-PAGE and silver staining (kindly performed by Dr Liana Steeghs, Utrecht Medical Centre, the Netherlands).

2.6.3 Lipopolysaccharide from *Neisseria meningitidis*

Meningococcal LPS from *N. meningitidis* serogroup B strain H44/76 was prepared by Dr. Svein Andersen (Edward Jenner Institue for Vaccine Research, Newbury, UK) by hot aqueous phenol extraction, ultra-centrifugation, gel filtration and cold ethanol NaCl precipitation. The final product contained <0.3% protein and was without detectable nucleic acids (Andersen *et al.*, 1995).

2.6.4 Preparation of FITC-labelled *Neisseria meningitidis*

Bacteria were grown and fixed as in 2.6.2. The bacterial suspension was washed once with RPMI without Phenol Red and the OD was adjusted roughly to 1. The bacteria were then incubated with 0.5mg/ml of FITC for 20 min at 37°C on a rotation platform. After 4 washes with clear RPMI, the OD at 540nm was adjusted to 1 and aliquots of the FITC labelled bacteria were stored in -80°C or used fresh as indicated. Flow cytometry was used to verify the level of FITC labelling of the bacteria. Bacteria were identified by forward and right angle scattering by flow cytometry (Fig. 2.6). All strains of bacteria were shown to bind FITC equally.

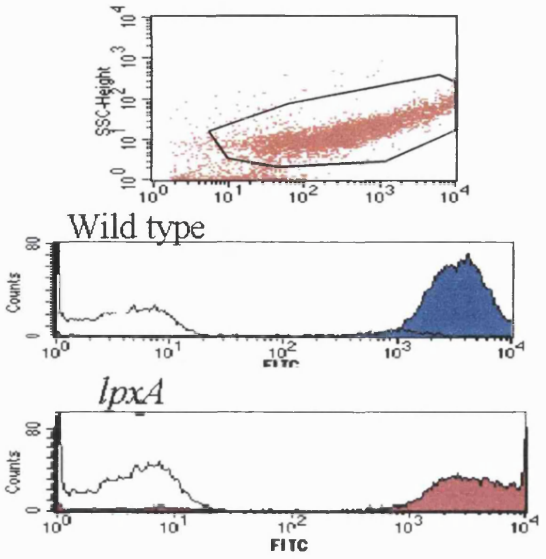

Figure 2.6 FITC labelling of *Neisseria meningitidis* wild type and *lpxA*-organisms. Bacteria were gated by flow cytometry according to their forward and side scatter properties. WT bacteria are shown in blue fill and *lpxA*- in red. Unfilled histograms represent unlabelled bacteria.

2.7 Phagocytosis assay

Binding and phagocytosis of bacteria was determined by a combination of flow cytometry and confocal microscopy. For detection by flow cytometry, DCs were incubated with FITC labelled bacteria for periods of time between 1 and 48 hours, fixed in CellFix, washed in FACS wash and analysed on a flow cytometer. DCs associated with bacteria were easily identified by fluorescence within the DC gated population. In preliminary studies, control phagocytosis experiments were carried out on ice in order to judge the extent of surface binding of the bacteria to DCs. Very little binding was found in these conditions. For confocal microscopy, DCs stimulated with FITC labelled *N. meningitidis* were allowed to adhere to adhesion slides as described in 2.8.

2.8 Immunostaining of slides for confocal microscopy

DCs were cultured in separate culture dishes with the appropriate stimuli. A 20μl aliquot of the cells was collected and allowed to adhere to an adhesion slide for 10 min. The cells were then fixed in 4% PFA for 10min, air dried and kept frozen at -80C until used. On the day of microscopical analysis, the slides were defrosted and rehydrated for 5min with PBS. The slides were first incubated with 5% goat serum to block any nonspecific binding. After one wash with PBS, DCs were incubated with 10μl of unconjugated MHC Class II antibodies at a concentration of 5μg/ml for 1h. After careful washing in PBS, 10μl of Texas red-X conjugated goat anti-mouse secondary antibodies at 5μg/ml were added for 1h. The slides were washed three times in PBS, mounted in Citifluor and the coverslip attached by using nail varnish.

2.9 Confocal microscopy

Confocal images were obtained using a Leica SP2 confocal laser scanning microscope system fitted with appropriate filter sets. Typically, 15-25 optical sections (0.2-0.5μm) spanning the entire DC were projected and superimposed with Leica confocal imaging software. All images with multiple fluorochromes were acquired in sequential scan mode and extra care was taken to minimize and control for emission spectral overlap (bleedthrough).

2.10 Statistical methods

The results were analysed by paired t-test using Sigmaplot 2000 analysis package. P values <0.05 were considered statistically significant.

Chapter 3

Activation of dendritic cells by *Neisseria meningitidis*

3.1 Introduction

Contact between pathogens and immature DCs triggers a series of events, which may lead to activation and maturation of DCs. These activated DCs modulate the subsequent immune response by expressing high levels of cell surface molecules such as Class II, CD40 and CD86, which are involved in the antigen presentation and T cell activation. Activated DCs also secrete a variety of cytokines and chemokines, which play an important role in T cell differentiation and migration (Moll, 2003). Whole Gram-positive and Gram-negative bacteria, protozoa, fungi and different bacterial components such as LPS and lipoproteins have been all shown to activate DCs *in vitro* and *in vivo*.

In this chapter, DCs were stimulated with meningococcal LPS and a clinical isolate of *N. meningitidis* bacteria and the subsequent activation of the cells, as determined by surface marker expression and cytokine production, was investigated. A unique mutant *N. meningitidis, lpxA-*, completely deficient in LPS was also used to study the relative contribution of meningococcal LPS on DC activation and maturation.

3.2 Materials and methods

3.2.1 Dendritic cell culture and activation with *Neisseria meningitidis*

Immature DCs at day 7 were cultured at a concentration of 10^6/ml in the presence of 10^8 organisms/ml (1:100 DC/bacteria ratio) of fixed WT and *lpxA- N. meningitidis.* When cytokines were measured, Brefeldin A at 10μg/ml was added to the cultures.

Live bacteria were added to the DCs at the multiplicity of infection (MOI) of 2,5 (1:2,5 DC/bacteria ratio at the start of infection) and co-cultured for 14-16h.

3.3 Results

3.3.1 Surface marker expression by dendritic cells

3.3.1.1 Maturation of dendritic cells in response to *N. meningitidis*

DC maturation in response to *N. meningitidis* was assessed by culture of immature day 7 DCs with WT, *lpxA-* (1:100 DC/bacteria ratio; corresponding to 10^8/ml) or purified meningococcal LPS (100ng/ml) for 24 hours. The DCs were then stained for surface markers MHC Class II, CD86, CD40 and CD83. In initial experiments, a dose dependent increase was found in DC maturation with *N. meningitidis* (Jennifer Allen, PhD thesis 2004 and Dixon *et al*, 2001). In these experiments, WT bacteria at concentrations of 10^5-10^7 organisms/ml induced greater DC maturation than the *lpxA-*. In addition, *lpxA-* only induced an increase in DC surface marker expression at 10^6 organisms/ml and above. In the present study, DC maturation was studied by using a high concentration of bacteria (1:100 DC/bacteria ratio) and LPS (100ng/ml).

Representative FACS histograms are shown in figure 3.1. Unstimulated cells expressed high levels of MHC Class II and CD86 and moderate levels of CD40. CD83 is a specific marker for DC activation and was expressed at very low levels by immature DC. Expression of MHC Class II, CD86, CD40 and CD83 was enhanced by stimulation both with the whole bacteria and purified LPS. Upon stimulation, CD83 was expressed at moderate levels in response to all stimuli.

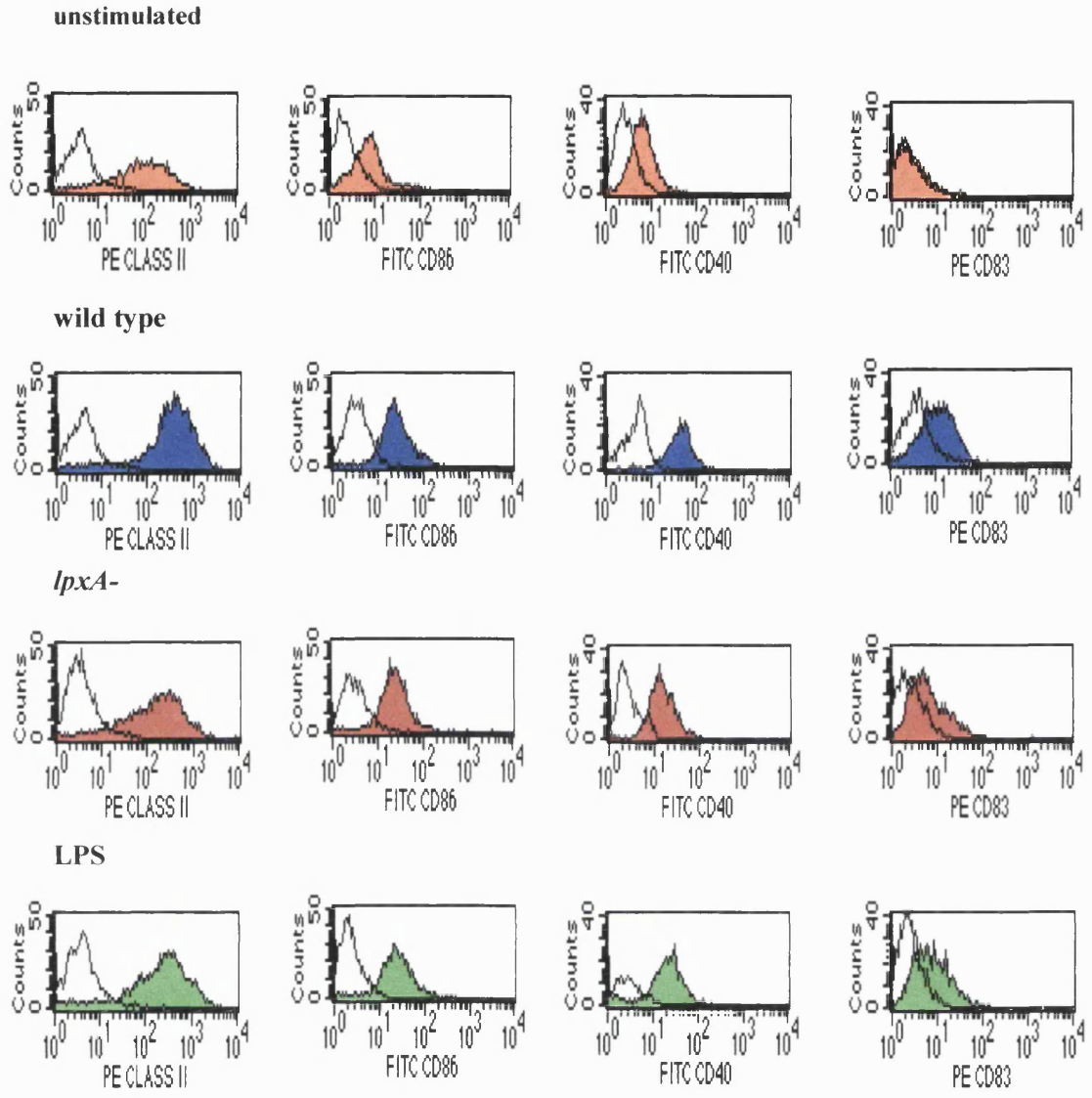

Figure 3.1 Expression of cell surface markers by dendritic cells. DCs were cultured with WT and *lpxA*- bacteria at 1:100 ratio of DC/bacteria or meningococcal LPS at 100ng/ml for 24h. The cells were then stained for MHC Class II, CD86, CD40 and CD83. DC were gated as a single population on forward and size scatter and analysed by flow cytometry. A representative of 5 experiments is shown.

For quantification of the apparent differences in DC maturation induced by the different stimuli, combined data from five separate experiments are shown in figure 3.2. All the stimuli used induced surface antigen expression above the levels seen in unstimulated DCs (p=<0.05). The WT bacteria were the most potent inducers of CD40 and CD83 expression by DCs compared to both the *lpxA-* and purified LPS (p=<0.05). LPS induced similar levels of MHC Class II and CD86 compared to WT. Expression of MHC Class II, CD40 and CD83 were lower in response to the LPS deficient organisms compared to the WT (p=<0.05), whereas expression of CD86 was similar after all stimuli used, including *lpxA-*.

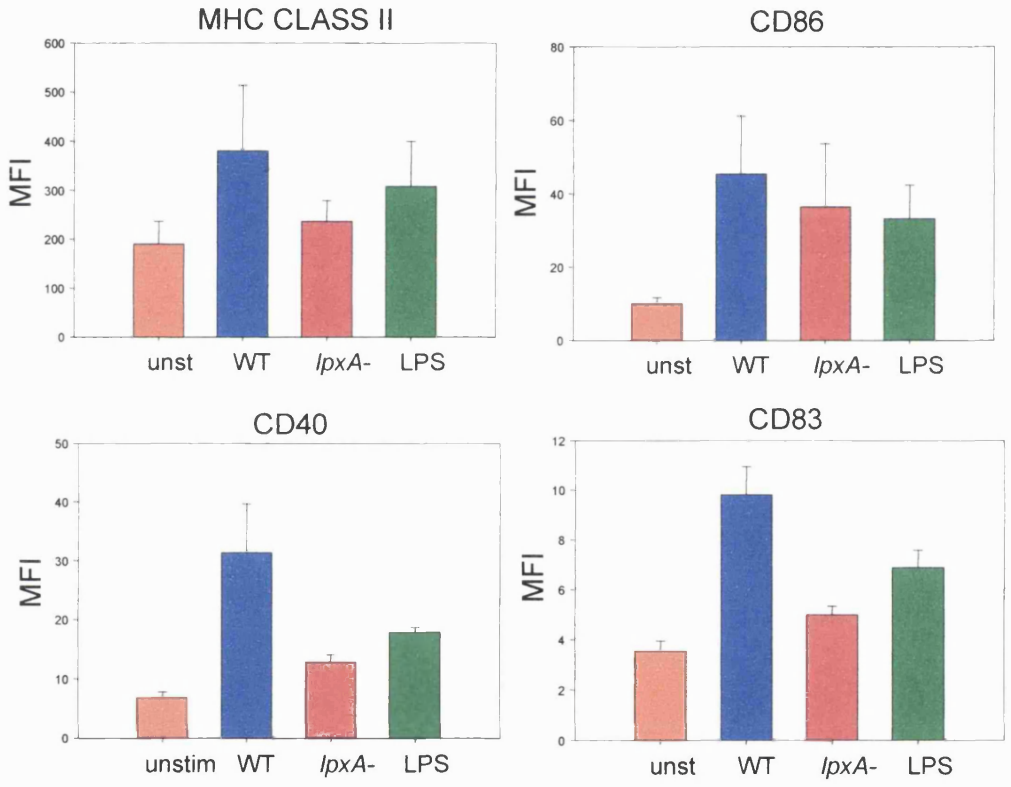

Figure 3.2 Expression of cell surface markers by dendritic cells. DCs were stimulated with WT and *lpxA-* bacteria (1:100 DC/bacteria) or purified meningococcal LPS at 100ng/ml for 24h. The median fluorescence intensity (MFI) of MHC Class II, CD86, CD40 and CD83 expression was analysed by flow cytometry. The mean and SEM of 5 individual experiments is shown.

3.3.1.2 Kinetics of dendritic cell maturation in response to *N. meningitidis*

To establish the kinetics for the expression of cell surface markers, DCs were stimulated with WT and *lpxA- N. meningitidis* (1:100 DC/bacteria) for a time period between 3h and 48h and then stained for CD86, CD40 and CD83 (Figure 3.3). Although the levels of surface markers expressed in response to the *lpxA-* organisms were lower compared to the WT, the kinetics of the response were found to be similar. All surface activation markers were found to be expressed at high levels after 24h of stimulation with the bacteria.

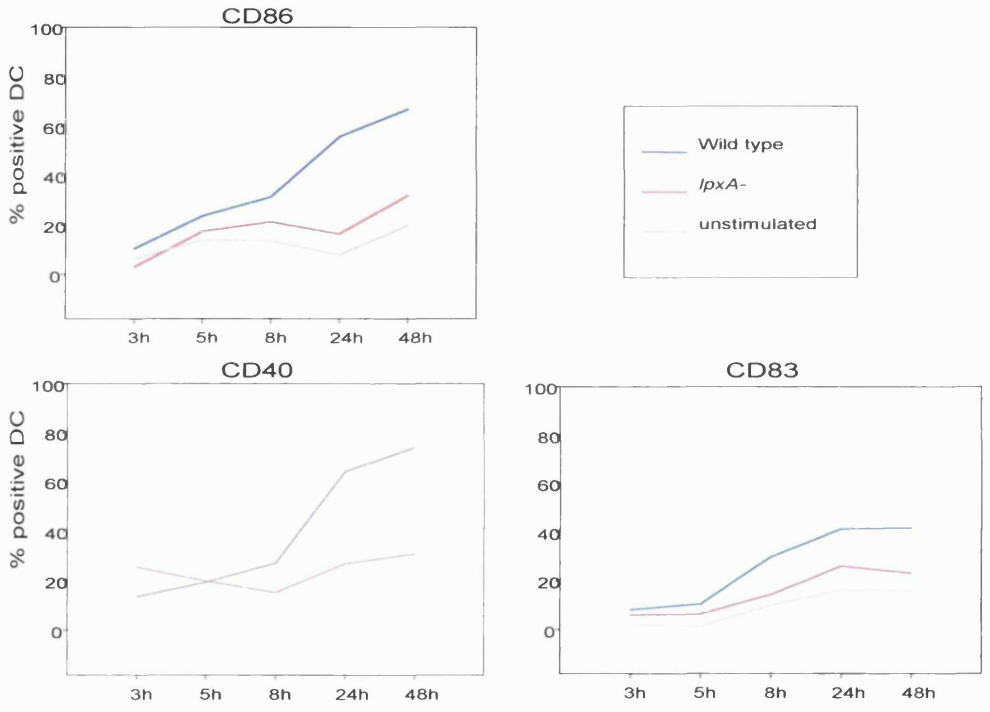

Figure 3.3 Kinetics of cell surface marker expression by dendritic cells. The cells were cultured with WT or *lpxA- N. menigitidis* at 1:100 ratio for 3h-48h and stained for CD86, CD40 and CD83. DC were gated according to forward and size scatter and analysed by flow cytometry. A representative of 2 experiments is shown.

3.3.2 Dendritic cell cytokine production in response to *Neisseria meningitidis*

3.3.2.1 Intracellular cytokine production by dendritic cells

To investigate intracellular cytokine production in response to *N. meningitidis* whole bacteria and purified LPS, DCs were cultured for 18 hours with WT or *lpxA*- at 1:100 DC/bacteria or with meningococcal LPS at 100 ng/ml (Figure 3.4). The WT bacteria were a potent inducer of IL12, TNF-α and IL6 production by the DCs. In contrast, both the *lpxA*- bacteria and purified LPS were poor inducers of cytokines compared to the WT with both inducing similar cytokine levels.

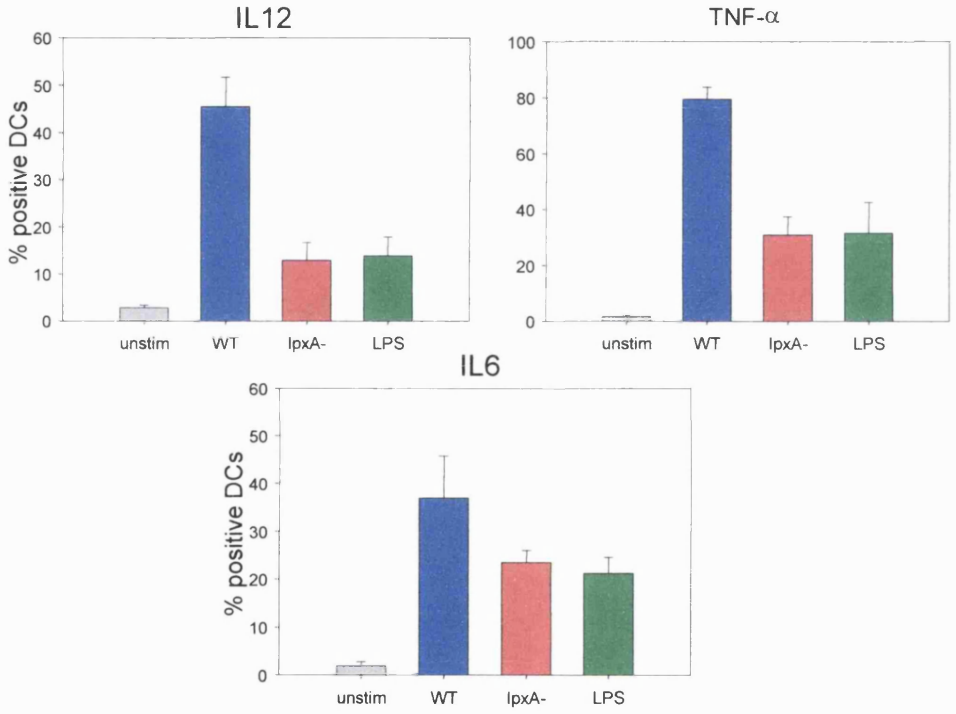

Figure 3.4 Intracellular cytokine production by dendritic cells. DCs were cultured with 10^8/ml WT and *lpxA*- bacteria or 100ng/ml of purified LPS in the presence of Brefeldin A for 18h. Intracellular cytokines were stained after fixation and permeabilisation of the DCs. Mean and SEM from 5 experiments is shown.

3.3.2.2 Effect of bacterial concentration on dendritic cell cytokine production

To determine the effect of bacterial concentration on DC cytokine production, DCs were stimulated with WT and *lpxA*- bacteria at low (1:10) and high (1:100) DC/bacteria ratio for 18h.

DCs responded to WT and *lpxA*- bacteria in a dose dependent manner with a significant ($p<0.05$) increase in both IL12 and TNF-α production corresponding to an increase in bacterial concentration (Figure 3.5). At both concentrations of bacteria, the WT bacteria induced higher levels of cytokines than the *lpxA*-, which failed to induce significant IL12.

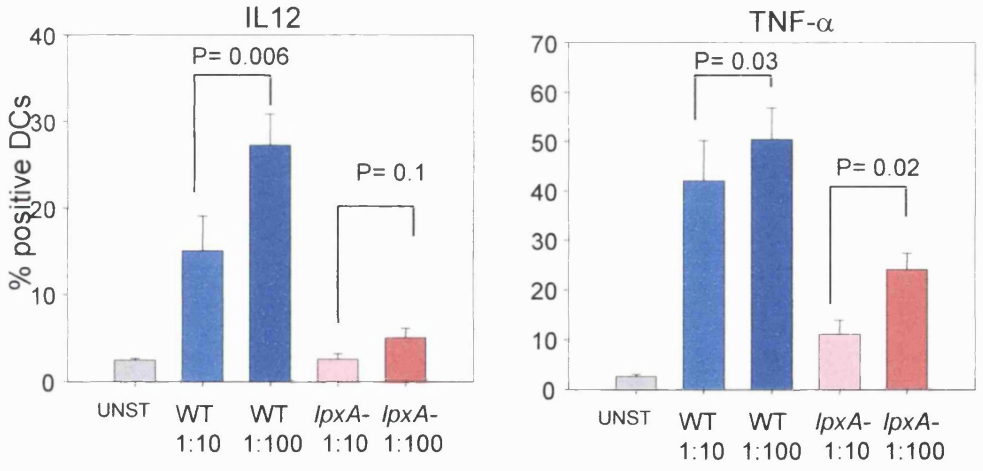

Figure 3.5 Intracellular cytokine production by dendritic cells in response to different concentrations of WT and *lpxA*- *N. meningitidis*. DCs were stimulated for 18 hours in the presence of WT and *lpxA*- *N. meningitidis* at 1:10 or 1:100 ratio of DC/bacteria. Intracellular IL12 and TNF-α were stained after fixation and permeabilisation of the DCs. Mean and SEM from 5 experiments is shown.

3.3.2.3 Kinetics of intracellular cytokine production by dendritic cells

To investigate the kinetics of cytokine production in response to *N. meningitidis*, DCs were stimulated with WT or *lpxA*- bacteria and with purified LPS in the presence of Brefeldin A and IL12, TNF-α and IL6 production determined by intracellular staining at various time points between 3-18h. A typical FACS histogram of IL12 production in response to the whole WT and *lpxA*- bacteria is shown in figure 3.6. Cytokine production increased in a time dependent fashion in response to the WT bacteria (Figure 3.6A). Significantly less IL12 was produced in response to *lpxA*- bacteria throughout the time course (Figure 3.6B). After 8h of stimulation, approximately 30% of the DCs produced IL12 in response to the WT bacteria, whereas only 10% or less had responded to *lpxA*-. Maximal (>50%) IL12 production was detected only after 18h of stimulation. The ability of DCs to produce IL12 was highly donor dependent. Some individuals responded very well, whereas others produced little IL12 even after 18h of stimulation with the WT bacteria. In all cases, *lpxA*- bacteria induced significantly less IL12 compared to WT bacteria. The addition of exogenous meningococcal LPS did not restore cytokine production in response to LPS deficient *lpxA*- bacteria to that observed with the WT bacteria (not shown).

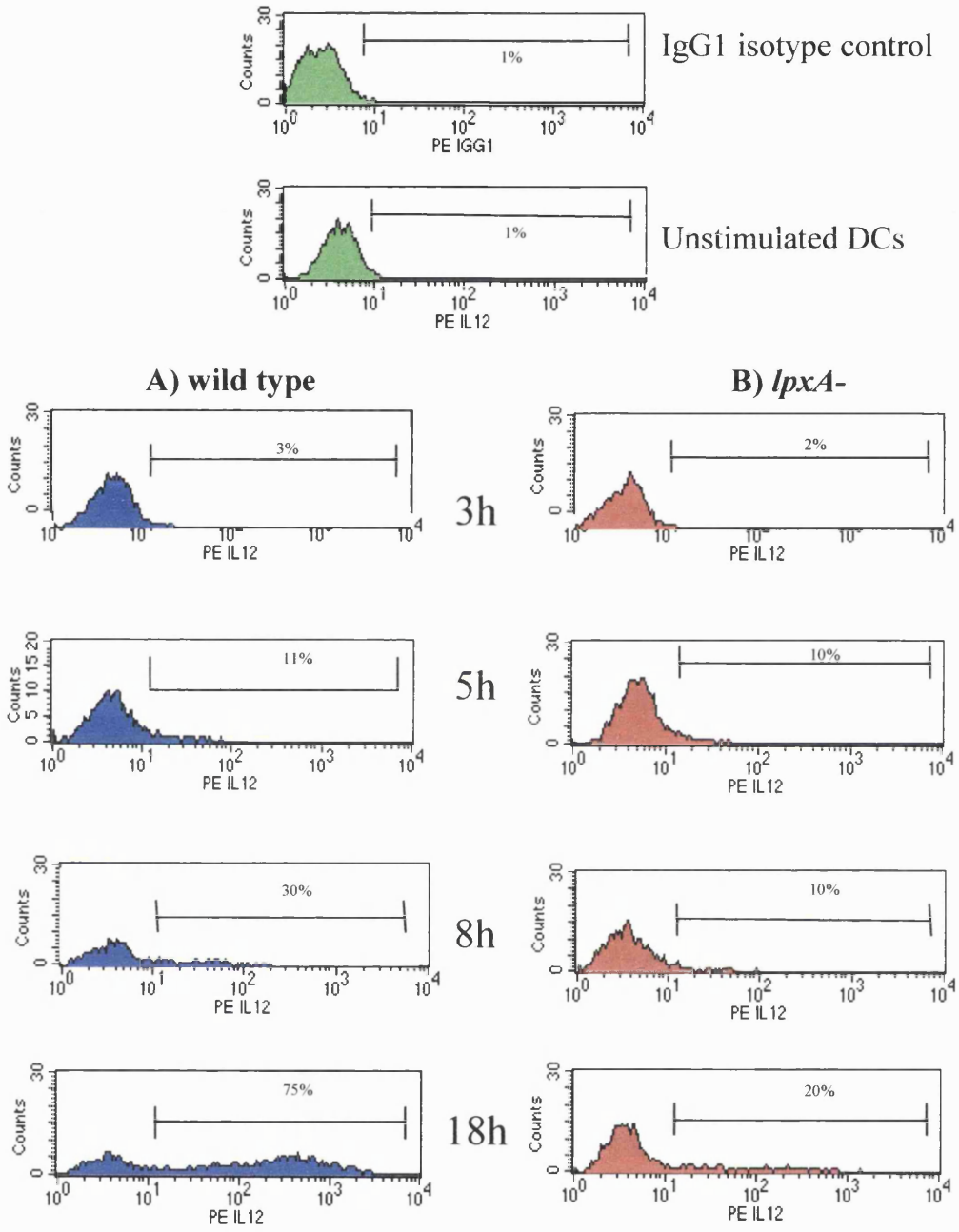

Figure 3.6 Kinetics of intracellular IL12 production by dendritic cells. DCs were stimulated for 3, 5, 8 and 18h in the presence of the WT (blue) and *lpxA*- (red) *N. meningitidis* and Brefeldin A. The cells were then fixed and permeabilised and IL12 stained intracellularly. The isotype control (green) and unstimulated (green) cells showed no staining. Representative histograms from three experiments are shown.

The combined data for intracellular IL12, TNF-α and IL6 of three separate experiments from three different donors are shown in figure 3.7. Both TNF-α and IL6 production in response to the WT bacteria displayed fast kinetics and were detected already after 3h of stimulation, whereas IL12 production showed slower kinetics and was typically detected at maximal level only after 18h. The levels of IL12, TNF-α and IL6 produced in response to the *lpxA*- bacteria were lower than the WT bacteria at 18h.

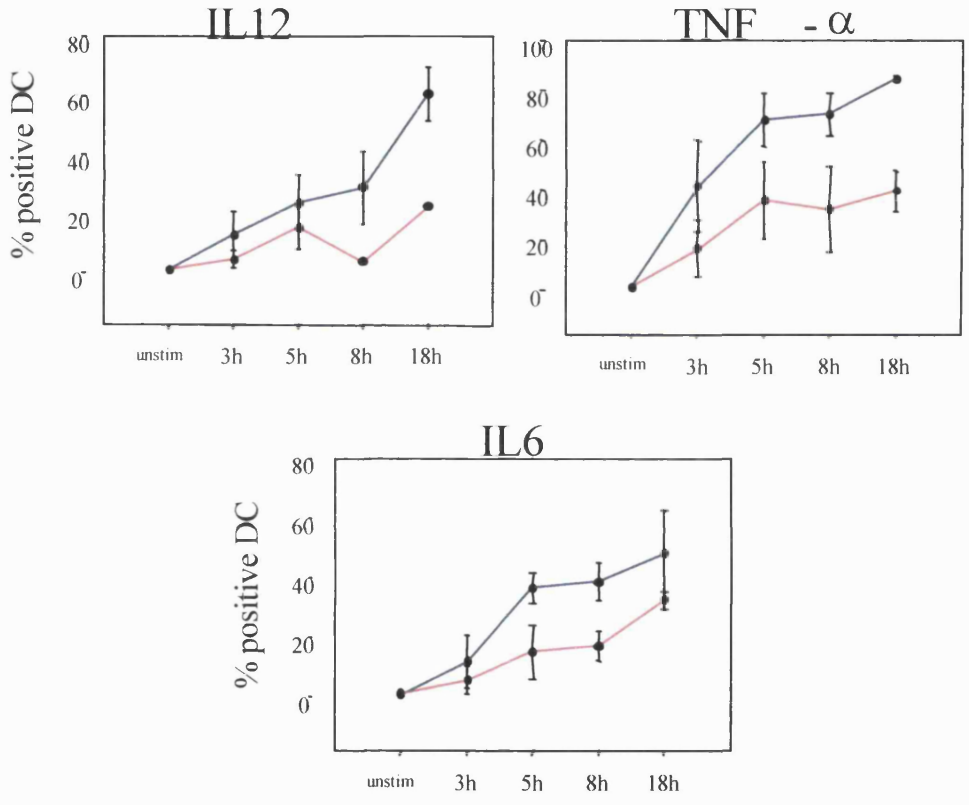

Figure 3.7 Kinetics of intracellular IL12, TNF-α and IL6 production by dendritic cells. DCs were incubated with WT (blue) and *lpxA*- (red) *N. meningitidis* at 1:100 ratio for time periods shown in the presence of Brefeldin A and the cytokines stained intracellularly. Mean and SEM from three experiments with three different donors are shown.

3.3.2.3 Kinetics of cytokine secretion by dendritic cells

Intracellular cytokine production allowed for a detailed study of cytokines produced by gated, specific cell populations. However, this method required the use of brefeldin A, which is toxic to cells if used for extended periods of time. In addition, detection of IL10 by the intracellular staining method was difficult. In order to measure soluble cytokine production between 3h-48h, DCs were stimulated with WT and *lpxA- N. meningitidis* and the culture supernatants collected and analysed for IL12, TNF-α, IL6 and IL1-β by ELISA (Figure 3.8). The data obtained by ELISA were generally consistent with intracellular cytokine assays. Production of IL12 was induced after 8h of stimulation and reached maximal level at 24h. The levels of IL12 after stimulation with *lpxA-* were significantly lower than the WT bacteria. More TNF-α was produced in response to the WT bacteria than with the *lpxA-* mutant. After 8h of incubation with the WT bacteria, a drop in TNF-α production was seen. This could have been due to protein degradation in the supernatant. The production of IL6 started after 5h of stimulation by both organisms and reached similar levels by 24-48h. The production of the pro-inflammatory cytokine IL1-β by DCs was also studied in the supernatants. IL1-β was detected in the cultures after 5h of stimulation. The levels of IL1-β were significantly lower in response to the *lpxA-* mutant compared to the WT. After 24h of stimulation with the bacteria, the levels of all cytokines, except IL6, had reached a plateau. Cytokine production in response to *lpxA-* bacteria did not reach the level of WT even after 48 hours of incubation. Finally, IL10 production was analysed in the culture supernatants after 24h of stimulation with the WT and *lpxA-* bacteria (Figure 3.7). As shown, significantly reduced levels of IL10 were detected in response to *lpxA-* bacteria compared to the WT.

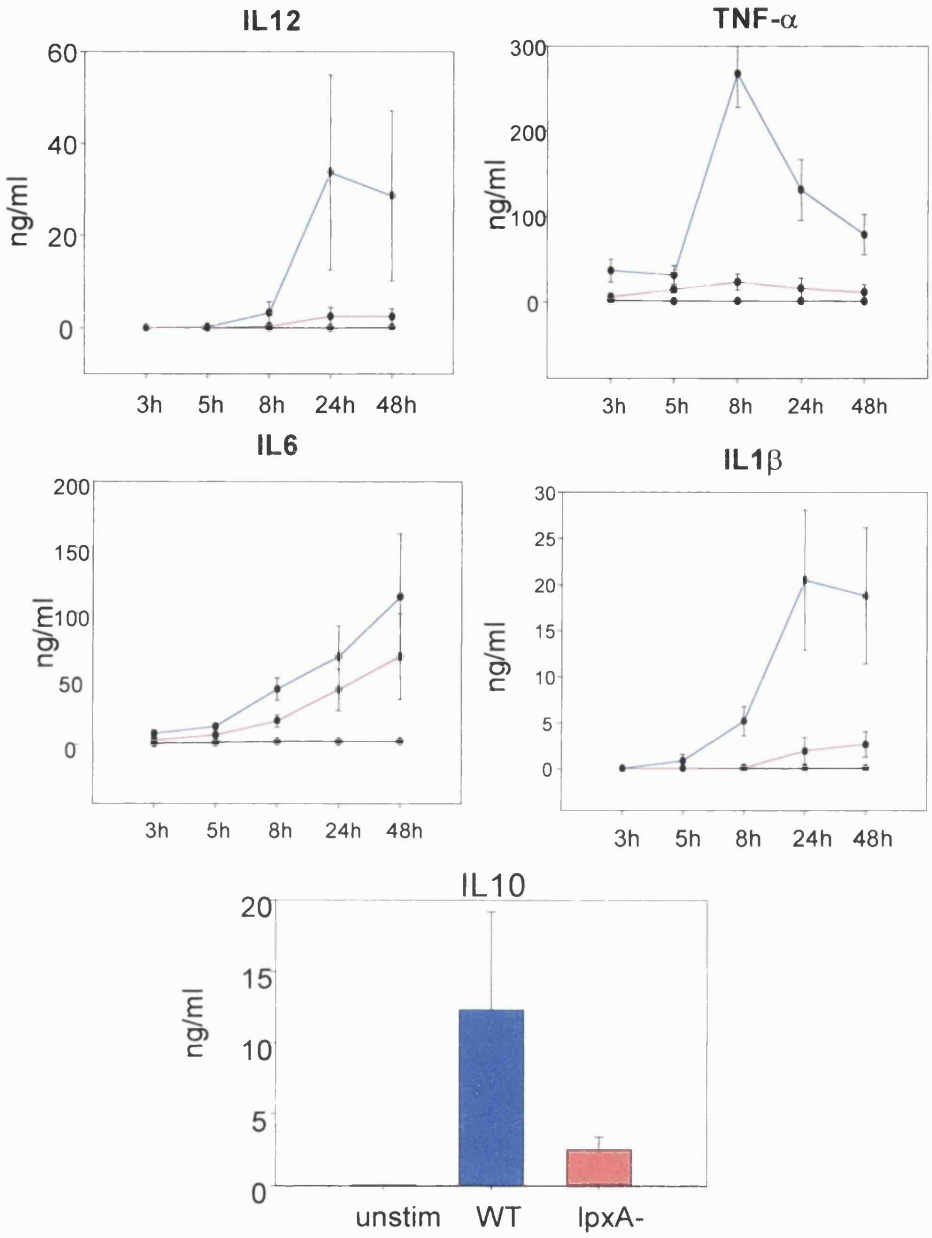

Figure 3.8 Kinetics of secreted cytokines by dendritic cells measured by ELISA. DCs were cultured for 3-48h in the presence of WT (blue) or *lpxA*- (red) *N. meningitidis* at 1:100 DC/bacteria ratio or medium only (black). The culture supernatants were collected and analysed for IL12, TNF-α IL6 and IL1-β. The data shows the mean and SEM of three experiments amongst three different donors. IL10 was analysed after 24h of stimulation and the results shown as mean and SEM from 4 separate experiments. The levels of cytokines were calculated with revelation software.

3.3.3 Activation of dendritic cells by live *Neisseria meningitidis*

The experiments above used paraformaldehyde fixed, killed *N. meningitidis*. A set of experiments was therefore designed to study the surface marker expression and cytokine production with live WT and *lpxA*- organisms. DCs were grown for 14h in the presence of live bacteria at multiplicity of infection (MOI) 2.5. When intracellular cytokines were to be measured, brefeldin A was added in the cultures. Figure 3.8 shows typical histograms obtained from these experiments.

Both the WT and *lpxA*- live bacteria were able to induce enhanced expression of cell surface markers MHC Class II, CD86 (B7.2), CD40 and CD83 (Fig. 3.9A), although the levels of expression in the response to *lpxA*- bacteria were typically lower than with the WT *N. meningitidis*. Intracellular production of IL12, TNF-α, IL6, IL1-β and IL8 in response to WT bacteria is shown in Fig. 3.9B, left panel. High levels of all cytokines were detected after 14h of stimulation. In contrast, the *lpxA*- mutant bacteria were able to induce minimal amounts of these cytokines (Fig. 3.9B).

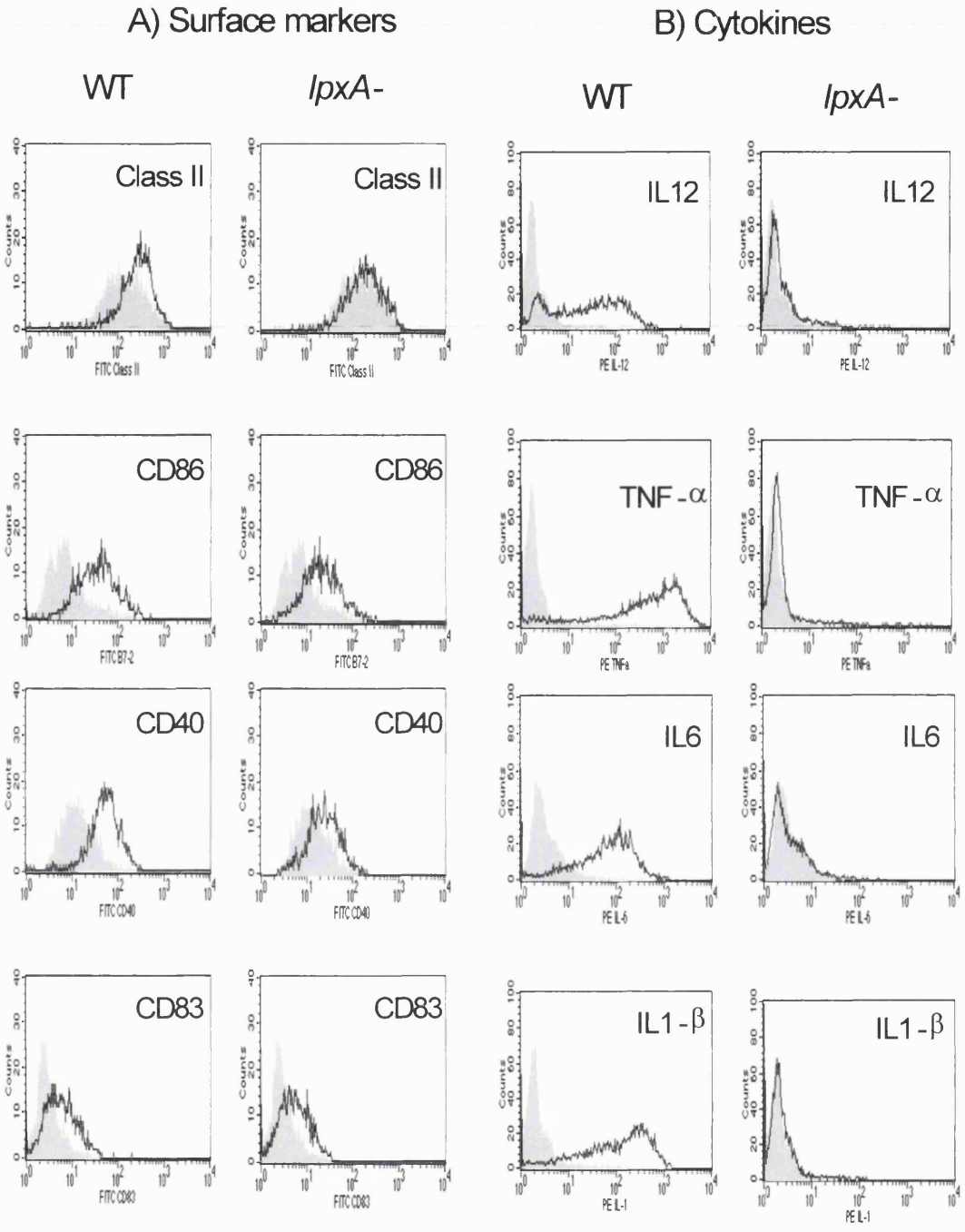

Figure 3.9 Activation of dendritic cells by live *N. meningitidis*. DCs were stimulated with live WT or *lpxA*- bacteria at the multiplicity of infection of 2.5 for 14h. Cell surface markers are shown in **(A).** Intracellular cytokines were stained after culture in brefeldin A followed by cell fixation and permeabilisation **(B)**. Grey fill = unstimulated cells. Representative of three separate experiments with three different donors is shown.

3.4 Discussion

Following stimulation, DCs mature, a process associated with several phenotypic and functional changes. Mature DCs increase the cell surface expression of activation and co-stimulatory molecules that are needed in the subsequent interactions with T cells. In addition, activated DCs produce cytokines that regulate the inflammatory response at the site of infection as well as have a major influence in the T cell response. In this chapter, the activation of DCs in response to whole *N. meningitidis* bacteria and purified meningococcal LPS was studied. The expression of cell surface markers and production of cytokines was measured and considered as indicative of cellular activation. The use of the LPS deficient *lpxA*- deficient mutant bacteria allowed for a study of the relative contribution of non-LPS components in the activation of DCs in response to *N. meningitidis*. Both the WT and mutant LPS deficient bacteria, as well as LPS, induced enhanced expression of MHC Class II, CD86, CD40 and CD83 above the levels of unstimulated cells indicating maturation of the DCs. The WT bacteria were the most potent stimulators of surface marker expression, whereas the *lpxA*- bacteria where the least effective. The response to LPS was generally found to be between the WT and *lpxA*-. These results confirm the findings of Dixon *et al.* (2001) and our previous studies on monocytes (Uronen *et al.*, 2000) that components of *N. meningitidis* other than LPS can induce activation of DCs and monocytes.

The outer membrane proteins (OMP) of the *lpxA*- mutant are unaffected by the absence of LPS (Steeghs *et al.*, 2001). Thus bacterial components such as PorA and PorB may be involved in the LPS independent activation of DCs. In fact, PorB has been shown to signal via the TLR2 MyD88 dependent pathway and induces CD86

expression in B cells (Massari *et al.*, 2002). In addition, bacterial DNA, has been shown to stimulate DCs and may account for the DC activation in response to *lpxA-* (Sparwasser *et al.*, 1998).

The level of DC derived cytokines induced by WT and *lpxA-* were strikingly different, although the kinetics of cytokine production induced by the two strains were found to be similar. WT bacteria were clearly the most potent inducers of cytokine production as measured by both intracellular staining and ELISA analysis of soluble cytokines. Cytokines derived from DCs play a major role during immune responses to bacteria. Proinflammatory cytokines such as TNF-α, IL6 and IL1 act locally at the site of infection by activating the local endothelium and recruiting other cell types, such as neutrophils and monocytes. The production of IL12 by DCs *in vivo* is a hallmark for the generation of Th1 cells. The *lpxA-* mutant of *N. meningitidis* induced consistently lower levels of IL12 production by the DCs. TNF-α production was also reduced in response to the LPS deficient mutant. The levels of IL6 were not statistically different between the WT and *lpxA-* although reduced levels in response to *lpxA-* were seen in some experiments. Other groups have also compared DC cytokine production in response to WT and *lpxA- N. meningitidis*. In all studies it was concluded that the *lpxA-* mutant bacteria were poor inducers of cytokines by DCs compared to the WT *N. meningitidis* suggesting that LPS is the main component of bacterial OM to induce cytokines (Dixon *et al.*, 2001;Unkmeir *et al.*, 2002). Steeghs *et al.* (1999) showed that outer membrane complexes or whole *lpxA-* bacteria failed to induce bactericidal antibodies in mice indicating that the immunogenicity of the LPS free organisms is poor also *in vivo*.

It was of interest that the *lpxA*- mutant induced similar cytokine response to purified LPS and that the cytokine response was not restored by addition of exogenous LPS (Jennifer Allen, thesis 2004, Dixon *et al.* 2001). It could be argued that the amount of purified LPS added to DCs was in general less than in whole WT bacteria. There are approximately 1.5 x 10^5 molecules of LPS per *Neisseria* bacterium based on LPS specific sugar 2-keto-3-deoxyoctonic acid (KDO) measurement. LPS at 100ng/ml is therefore equivalent to about 10^8 bacteria (Liana Steeghs and Peter van der Ley, personal communication). It was therefore unlikely that the differences in cytokine production induced in response to either purified LPS or WT bacteria were due to quantitative differences. It is interesting that meningococcal LPS seems to be such a poor stimulus for cytokine induction. This is in contrast to other studies, which have shown good DC activation and cytokine production in response to LPS from other species (Rescigno *et al.*, 1998;Verhasselt *et al.*, 1997). In this thesis, a highly purified LPS preparation was used containing less than 0.3% protein (Andersen *et al.*, 1995). Commercially obtained LPS has been shown to contain impurities and may induce cell signalling by several pathways (Yang *et al.*, 1999). In particular, this has been the case for *E.coli* LPS, which is widely used model for DC activation. In addition, the structure of meningococcal LPS differs substantially from *E. coli* LPS, which contains the O-antigen (Erridge *et al.*, 2002).

In the study by Dixon *et al.* (2001) and Jennifer Allen (PhD thesis, 2004), bacterial concentrations between 10^5- 10^7/organisms/ml were used and were shown to induce a dose dependent increase in cytokine production and DC maturation. The higher bacterial concentration of 10^8/organisms/ml (or 1:100 DC/bacteria ratio) used in this chapter induced similar patterns of DC maturation and cytokine production in

response to WT and *lpxA*- bacteria. Recently, it was shown that the bacterial load in patients with severe meningococcal disease reached as high as 10^8 organisms/ml of blood (Hackett *et al.*, 2002). The highest dose of bacteria (10^8/ml, 1:100 ratio) used in this chapter would therefore represent the load of bacteria (and LPS) present in the severest of cases.

The intracellular cytokine staining method has been extensively used in our laboratory (Uronen *et al.*, 2000;Uronen and Callard, 2000;Uronen-Hansson *et al.*, 2003). Brefeldin A inhibits the activity of the Golgi Apparatus and was used as an inhibitor of protein secretion to enhance intracellular cytokine staining. This reagent is toxic to cells if used for prolonged periods of time. For the IL12 assay, 18h stimulation in the presence of brefeldin A gave best results and was shown not to affect cell viability. Brefeldin A may, however, have affected the cytokine response by DCs subsequent to activation. To test this, culture supernatants were analysed for cytokines by ELISA, which does not involve addition of brefeldin A. In addition, longer time points could be analysed by this methods. Similar patterns of cytokine production using either intracellular or ELISA cytokine detection were obtained. In addition, the results did not suggest a difference in kinetics even after 48h of stimulation with the WT and *lpxA*- bacteria.

Finally, surface marker expression and cytokine production by DCs in response to live meningococci were studied. It was important to establish, whether DCs could also be activated by live *N. meningitidis*. DCs were shown to be activated at least as efficiently with live WT bacteria as with killed organisms as judged by surface marker expression and cytokine production. The response to *lpxA*- was reduced

compared to the WT confirming that LPS present on the OM of live bacteria is necessary for full activation. In particular, very poor cytokine response was obtained by live *lpxA*- bacteria. The experiments were performed over 14-16h, after which the cultures were overtaken by the dividing bacteria. Longer incubations resulted in deterioration of cell viability. Of note, the experiments with live bacteria were carried out at the MOI of 2,5 at the start of the infection. As bacteria divided over the culture period, the final concentration of the organisms was not measured. It has been shown that the *lpxA*- organisms have slower growth compared to the WT (Steeghs *et al.*, 1998). A low final concentration of the *lpxA*- bacteria could explain the minimal cytokine response obtained with live *lpxA*- organisms.

The results presented in this chapter established conditions for DC activation by *N. meningitidis*. DCs were shown to be very sensitive to stimulation with WT *N. meningitidis*. The maturation process and cytokine secretion was initiated after only a few hours in the presence of the bacterial stimuli. Cytokine production, particularly IL12, required the presence of LPS within the intact bacteria. The results here suggested that DCs may interact with whole bacteria in a different way to soluble stimuli, such as LPS. In addition, the presence or absence of LPS in whole bacteria may critically alter the way DCs deal with *N. meningitidis*. The experiments in the next chapter in this thesis were designed to investigate binding and internalisation of *N. meningitidis* by DCs. In particular, the role of LPS was studied in detail.

Chapter 4

Phagocytosis of *Neisseria meningitidis* by dendritic cells

4.1 Introduction

Immature DCs are highly endocytic and phagocytic cells. They express a large selection of surface pattern recognition receptors that bind to microbes and microbial products, which are then internalized and processed for presentation to T cells (Guermonprez *et al.*, 2002;Palucka and Banchereau, 1999). LPS is an important trigger of the pro-inflammatory response in meningococcal disease and it is generally believed to be the main mediator of cell activation and inflammation by Gram negative bacteria (Brandtzaeg *et al.*, 2001;Brandtzaeg and van Deuren, 2002). As was shown in Chapter 3, DCs activated with the LPS deficient *lpxA-* isogenic mutant of *N. meningitidis* failed to make significant amounts of IL12 and production of the other cytokines was significantly reduced. These striking differences between the two strains of bacteria suggested that the DC interaction with the bacteria may differ depending on the presence of LPS within the intact bacterium. To date, very little is known about the role of LPS in phagocytosis of *N. meningitidis* by DCs. In this chapter, internalisation of *N. meningitidis* by DCs was studied and the role of LPS in phagocytosis investigated.

4.2 Materials and methods

4.2.1 Phagocytosis assay

DC binding and phagocytosis of bacteria was determined by a combination of flow cytometry and confocal microscopy. For detection by flow cytometry, DCs were incubated with FITC labelled bacteria at a ratio of 1:1, 1;10, 1:50, 1:100 and

1:200 for periods of time between 30 min. to 48 hours, fixed in CellFix, washed and analysed on a FACSCalibur. DCs associated with bacteria were easily identified by fluorescence within the DC gated population. For confocal microscopy, DCs were stimulated with FITC labelled *N. meningitidis* at 1:100 or 1:200 ratio (DC/bacteria) and the cells allowed to adhere to 12-well adhesion slides. To stop phagocytosis, the cells were fixed with 4% PFA. In some experiments, intracellular bacteria and the nuclei of DCs were stained on slides with the cell permeable DNA stain To-Pro3 (1:1000) for 10 min. Extracellular bacteria were identified by staining with 1μg/ml of FITC conjugated P1.7 antibodies specific for *N. meningitidis* for 30 min on slides. Visualisation of DCs was enhanced by staining with 5μg/ml of MHC Class II antibodies and Texas red conjugated secondary antibodies as in 2.5. The slides were washed, mounted and confocal images obtained using a confocal microscope.

With a confocal microscope, it is possible to obtain thin (0.2-0.5μm) optical sections through a specimen. Subsequently, it allows the visualisation of the internalisation of particles, such as bacteria, by the cell. Confocal images were obtained by taking 15-25 optical sections (0.2-0.5μm) spanning the entire DC. The images were then projected and superimposed with Leica confocal imaging software.

4.2.2 Staining of bacteria with FITC conjugated P1.7 antibody

An aliquot of 10^7 WT and *lpxA- N. meningitidis* or *Streptococcus pneumoniae* (Supplied by Mrs Ulrike Meltzer, Institute of Child Health) bacteria were stained in 1.5ml eppendorf tubes with FITC conjugated P1.7 monoclonal antibody in

1:1000 dilution for 30 min. The bacteria were washed in HBSS by centrifugation at 6500 rpm (4000g) for 3 min, resuspended in Cellfix and analysed by FACS.

The FITC conjugated P1.7 monoclonal antibody specific to *N. meningitidis* porin was used to identify bacteria that had not been internalised by the DCs. Figure 4.1 shows that the binding of P1.7 antibody was specific to *N. meningitidis* and did not stain other bacteria, such as *Streptococcus pneumonia*. Unstained WT bacteria were used as a control without antibody. Importantly, both the WT and *lpxA*- were stained equally well by the P1.7 antibody.

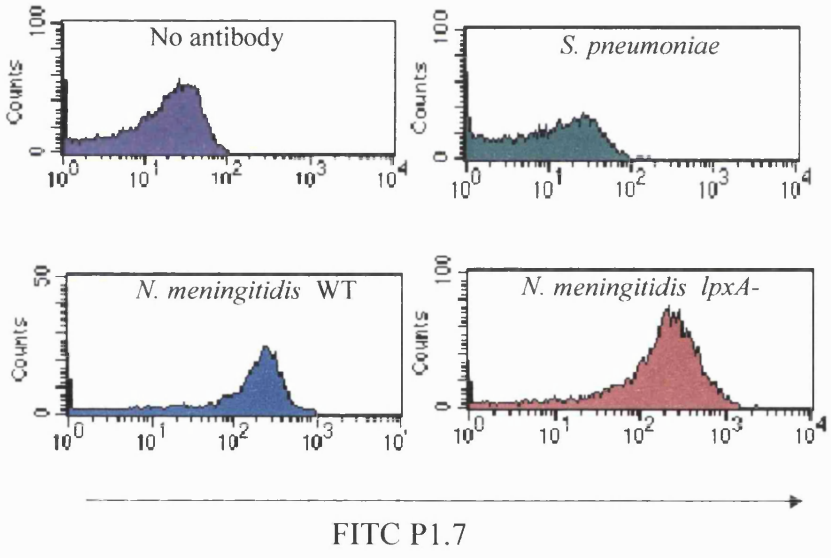

Figure 4.1 Specific binding of P1.7 antibody to *Neisseria meningitidis*. Bacteria (10^7) were stained with FITC conjugated P1.7 monoclonal antibodies for 30 min. After washing, the bacteria were gated and analysed by FACS. Unstained WT bacteria were used as a control (purple). P1.7 Mab binding to *Streptococcus pneumoniae* (green), WT (blue) and *lpxA*- (red) *N. meningitidis*

4.3 Results

4.3.1 Phagocytosis of *Neisseria meningitidis* by dendritic cells

4.3.1.1 Association of dendritic cells with FITC conjugated *Neisseria meningitidis*

DCs are highly phagocytic cells and have been shown to internalise both Gram-negative and Gram-positive bacteria. In order to study the phagocytosis of *N. meningitidis,* DCs were stimulated with FITC labelled WT and *lpxA*- bacteria at DC/bacteria ratio of 1:1, 1:10 and 1:100 (low, medium and high dose, respectively) for 1h. The DC population was gated and then analysed by flow cytometry for the presence of associated bacteria (FITC). In these experiments, 'association' indicated both adherence and internalisation of the bacteria by DCs.

No FITC staining of the DCs could be seen in unstimulated cells (Figure 4.2 grey fill) whereas a dose dependent increase in association of the WT bacteria with the DCs was found (Figure 4.2 blue fill). After culture with FITC labelled WT bacteria at 1:1 DC/bacteria ratio, approximately 9% of the DCs were associated with the WT bacteria after 1h. With DC/bacteria ratio of 1:10, approximately 45% of the cells were associated with the FITC bacteria. At the highest concentration used (1:100), 80% of the DCs were associated with the bacteria after 1h of stimulation. A dose dependent increase in the median fluorescence intensity (MFI) was also observed indicating an increase in the number of bacteria associated with each DC.

The response to *lpxA*- bacteria is also shown in figure 4.2 (red fill). In contrast to the WT bacteria, only a few *lpxA*-bacteria were associated with the DCs at any of the concentrations studied. After stimulation with 1:1 and 1:10 organisms/DC, only approximately 2-3% of the DCs were FITC positive, i.e. associated with the bacteria. At the highest concentration used (1:100 ratio), 15% of the DCs were associated with the bacteria but the histograms showed only a slight whole peak shift, a pattern different to that observed with WT bacteria.

As the *lpxA*- mutant completely lacks LPS on its outer membrane, addition of purified soluble meningococcal LPS together with the *lpxA*- bacteria may have provided the necessary signals for efficient phagocytosis. DCs were therefore co-cultured with the *lpxA*- mutant bacteria at the ratio of 1:1, 1:10 or 1:100 in the presence or absence of 1, 10 or 100ng/ml of exogenous meningococcal LPS, respectively. Addition of purified LPS did not enhance the association between DC and *lpxA*- bacteria as measured by flow cytometry (Figure 4.2 green dashed line right panel). These data altogether suggested that the presence of LPS on the membrane of intact whole *N. meningitidis* is necessary for association with the DCs.

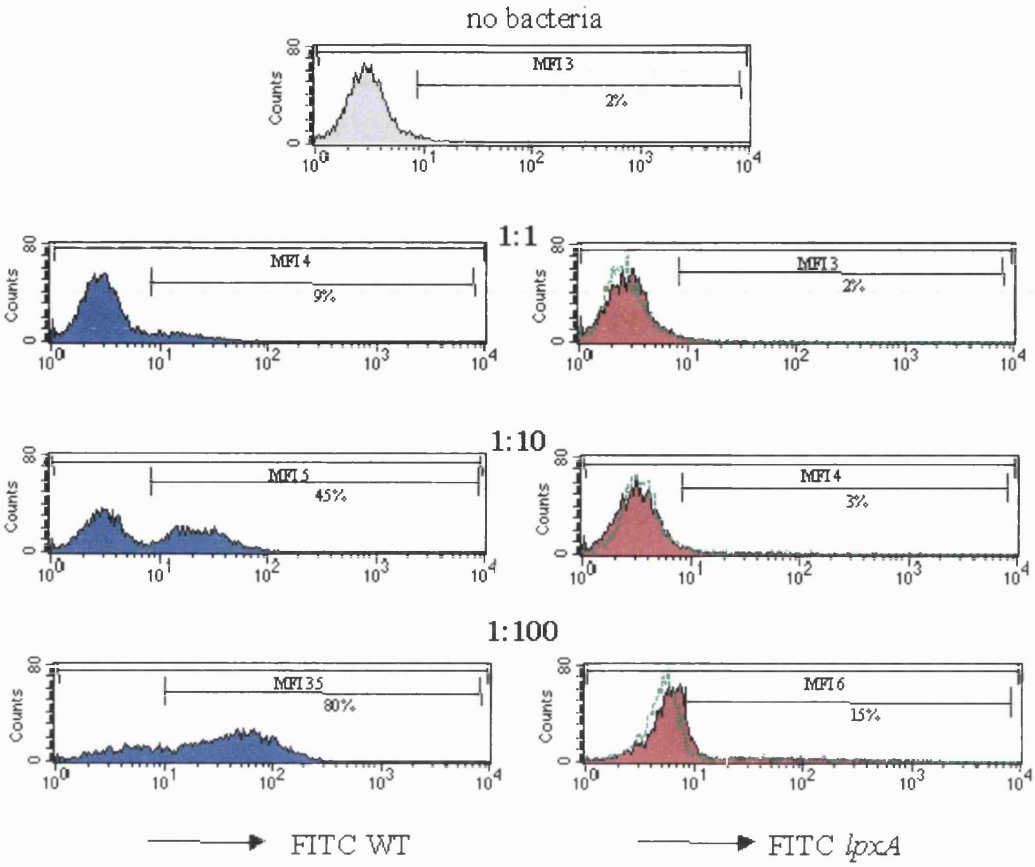

Figure 4.2 Association of dendritic cells with *Neisseria meningitidis*. DCs were incubated with FITC labelled WT and *lpxA- N. meningitidis* at DC/bacteria ratio of 1:1, 1:10 and 1:100 for 1h. DCs were gated on forward and side scatter on flow cytometry and analysed for FITC fluorescence. Unstimulated cells (grey fill),WT (blue) and *lpxA-* (red). Addition of purified LPS did not enhance the association between DCs and *lpxA-* bacteria (green dashed line). Representative histograms from three experiments are shown.

4.3.1.2 Kinetics of dendritic cell association with FITC conjugated *Neisseria meningitidis*

To establish the kinetics of DC association with WT and *lpxA*- bacteria, immature DCs were stimulated with FITC labelled bacteria (1:100 ratio) for times between 30min and 48h. Representative FACS histograms are shown in figure 4.3. A time dependent increase of DCs associated with FITC labelled WT bacteria was found (histograms with blue fill). In this experiment, >60% of the DCs were associated with FITC labelled WT bacteria within 1h. The response to the *lpxA*- mutant was very different as very few bacteria were associated with the DCs in the first 8h. Only after 24h was there significant association of *lpxA*- bacteria with the DCs. Although this proportion rose slowly over time, it always remained much less than the proportion of DCs with associated WT bacteria up to 24h. In addition, the MFI of the DCs in response to *lpxA*-was very low for 24h and reached high levels only after 48h of incubation. A summary of 10 similar experiments is shown in Figure 4.4. It is clear from these results that both the MFI (amount of bacteria/cell) and percentage positivity (% cells containing bacteria) of the DCs in response to *lpxA*- mutant bacteria was much lower than WT and reached high levels only after over night co-culture.

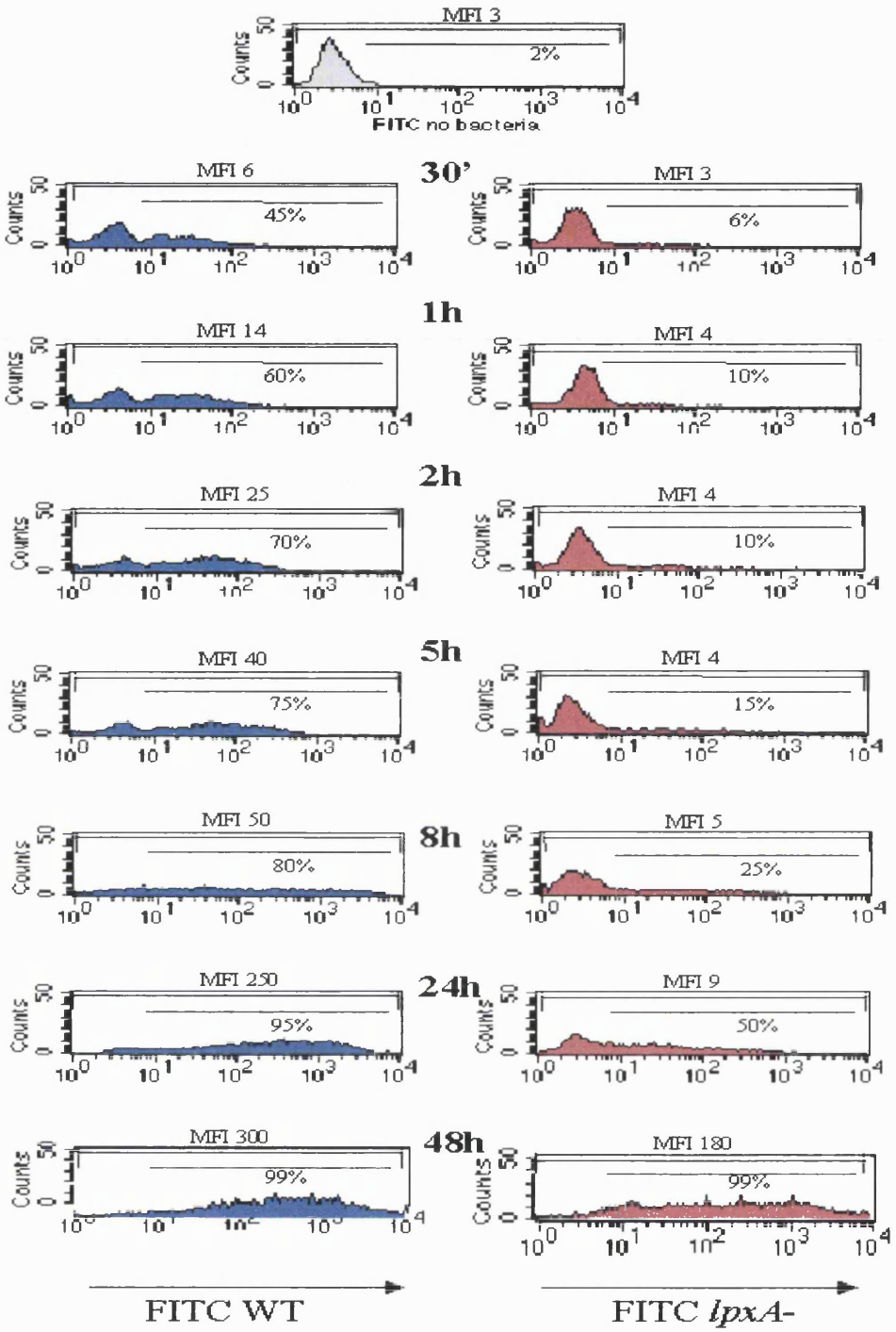

Figure 4.3 Kinetics of *Neisseria meningitidis* association with dendritic cells. DCs were co-cultured with WT and *lpxA- N. meningitidis* at the concentration of 1:100 DC/bacteria for time periods between 30 min up until 48h. The cells were fixed and analysed by flow cytometry. Unstimulated (grey fill), WT (blue) and *lpxA-* (red). Representative of at least 10 separate experiments is shown.

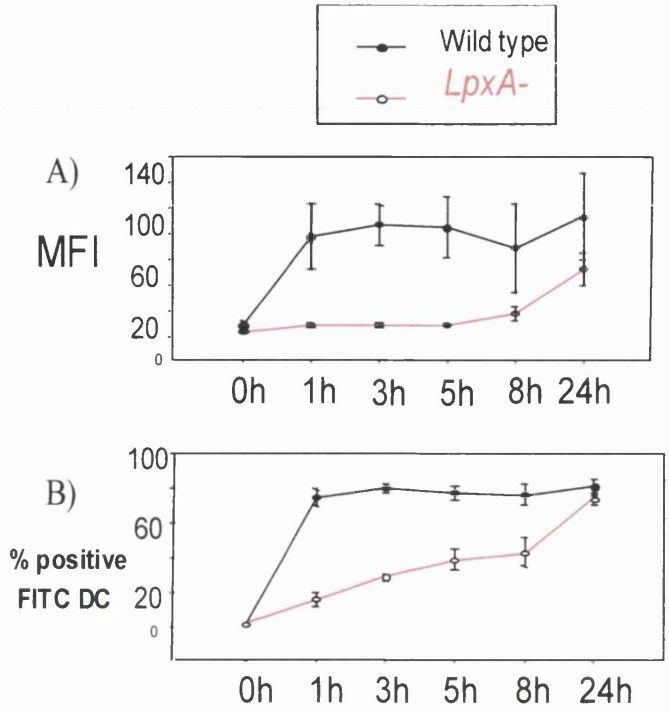

Figure 4.4 Combined time course data of bacterial association with dendritic cells. DCs were incubated with FITC labelled WT and *lpxA- N. meningitidis* for time periods between 1-24h. Association of the bacteria with DCs is expressed as A) the MFI and B) percentage of positive DCs as read from FACS histograms. Data are expressed as the mean and SEM of ten separate experiments.

4.3.1.3 Effect of inactivation of *Neisseria meningitidis* on phagocytosis by DCs

In the experiments described so far, *N. meningitidis* bacteria were inactivated with 0.5% PFA for 30 minutes before labelling the bacteria with FITC and performing phagocytosis assays. PFA fixation could have altered the OM composition of the bacteria and therefore affect the phagocytosis by DCs. Therefore, 70% ethanol

and heat inactivation were compared with PFA fixation. DCs were cultured with PFA, ethanol and heat (56°C) inactivated FITC labelled WT and *lpxA*- bacteria at the ratio of 1:100. As shown in figure 4.5, similar results were obtained regardless of the method used to kill the bacteria. It was therefore concluded that inactivation by PFA did not change the bacteria in a particular way and was used throughout this thesis in phagocytosis assays.

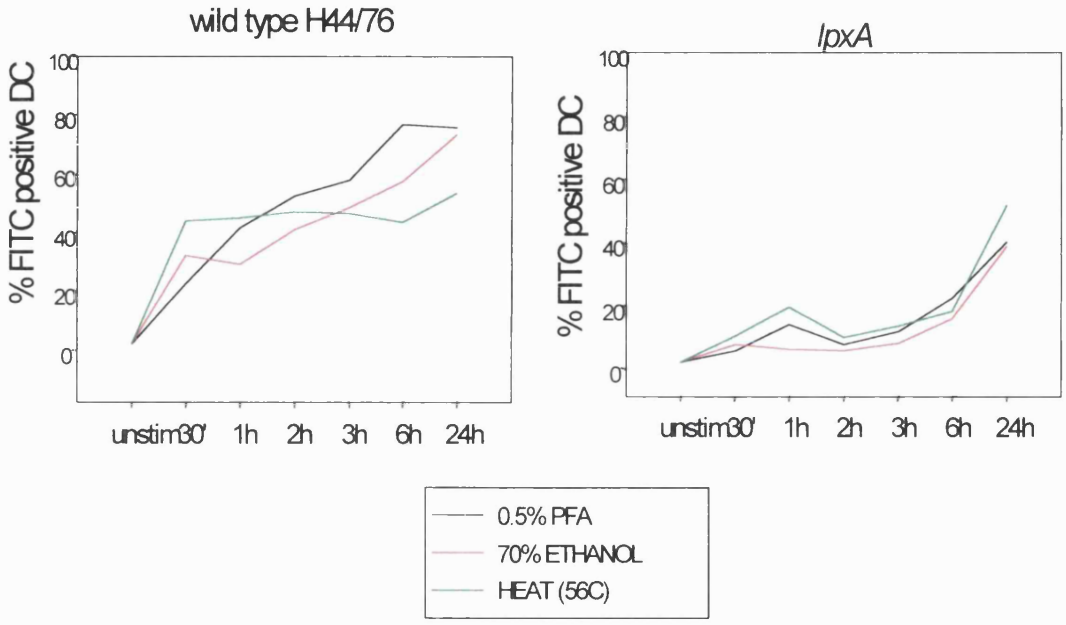

Figure 4.5 Effect of inactivation method of bacteria on phagocytosis of *Neisseria meningitidis*. DCs were stimulated at 1:100 ratio with PFA, ethanol and heat inactivated FITC labelled WT or *lpxA*- bacteria. At the time points shown, an aliquot of DCs was fixed in Cellfix, washed and analysed by FACS. A representative experiment of 2 performed is shown.

4.3.1.4 Phagocytosis of *Neisseria meningitidis* by dendritic cells analysed by confocal microscopy

The results in section 4.3.1 showed a rapid association of WT bacteria with DCs whereas the response to *lpxA*- mutant bacteria was severely delayed. These results were obtained by using a flow cytometric technique. FACS analysis did not, however, allow surface bound and internalised bacteria to be distinguished. To confirm that the bacteria were internalised and not just adhered to the cell surface, confocal microscopy analysis was performed. This allowed for a simultaneous analysis of DCs and the position of the bacteria within the cells.

To study the kinetics of the phagocytosis of WT and *lpxA*- bacteria, DCs were incubated with DC/bacteria ratio of 1:200 over a time period between 30 min and 24h and the results analysed by confocal microscopy. Figure 4.6 shows confocal microscopy images of FITC labelled WT *N. meningitidis*. WT bacteria were rapidly bound and internalised by the DCs. After 30 min. and 1h of stimulation, FITC labelled WT bacteria appeared mostly adherent to the cell surface and only a few were internalised by the DCs. Phagocytosis increased in a time dependent manner and by 24h, the cytoplasms of the DCs were filled with FITC labelled bacteria. By this time, a typical localisation of the internalised bacteria next to the nuclei was observed. After 4-6h of co-culture, clustering (several DCs adhered together) of the activated DCs was typical.

In contrast to the WT bacteria, the *lpxA*- appeared poorly attached or internalised by the DCs (Fig. 4.7), although a time dependent increase in binding of the *lpxA*- bacteria was observed. After 24h of stimulation, a low proportion of DCs had internalised *lpxA*- organisms.

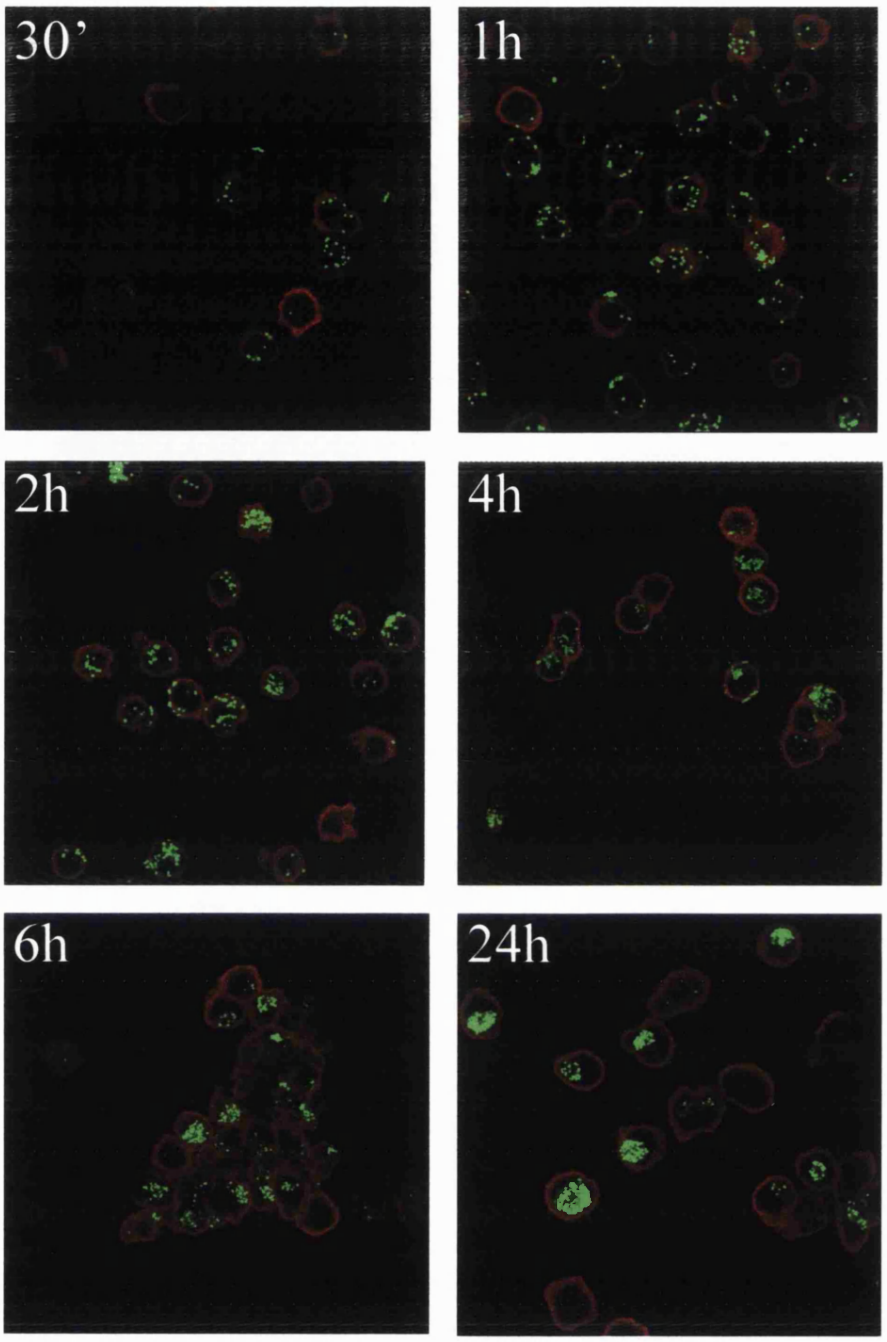

Figure 4.6 Kinetics of dendritic cell phagocytosis of FITC conjugated WT *Neisseria meningitidis* analysed by confocal microscopy. Confocal image of DCs after co-culture with FITC labelled WT *N. meningitidis* at 1:200 DC/bacteria ratio. After each time point, the cells were allowed to adhere to glass slides and then stained for MHC Class II (Texas Red). A representative experiment of more than 10 separate experiments is shown.

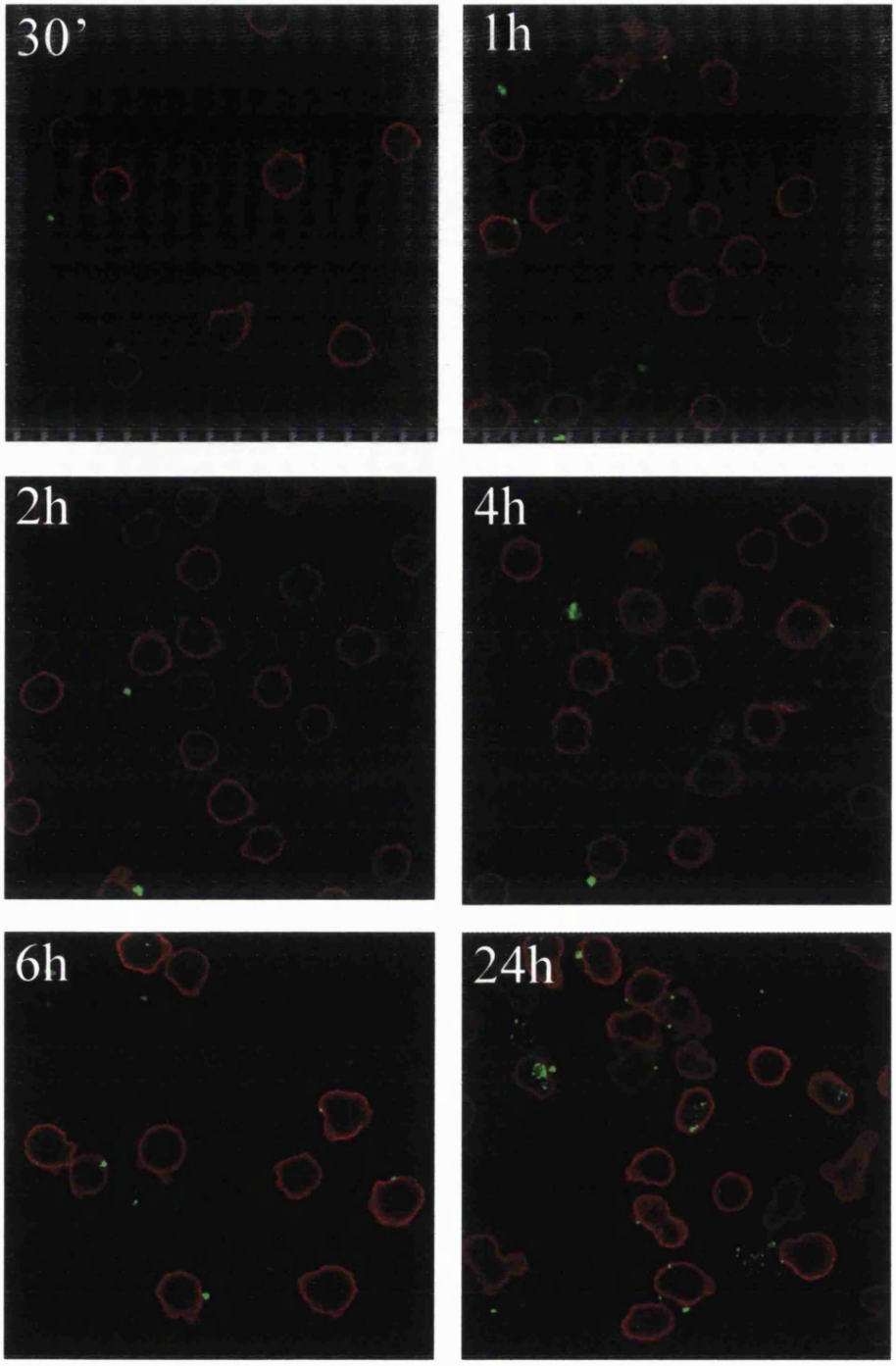

Figure 4.7 Kinetics of dendritic cell phagocytosis of FITC conjugated *lpxA-Neisseria meningitidis* analysed by confocal microscopy. Confocal image of DCs after co-culture with FITC labelled *lpxA- N. meningitidis*. The cells were allowed to adhere to glass slides and then stained for MHC Class II (Texas Red). A representative experiment of more than 10 separate experiments is shown.

4.3.1.5 Binding and internalisation of *Neisseria meningitidis* by dendritic cells

Confirmation that the bacteria had been internalised by the DCs and not just surface bound, was obtained by a labelling technique in combination with confocal microscopy. DCs were incubated with (not FITC conjugated) *N. meningitidis* for 24 hours and then stained with cell-permeable To-Pro3 to label all DNA, including DC nuclei and the bacteria. Bacteria on the DC surface exposed bacteria were then detected with FITC conjugated P1.7 antibody. As shown in figure 4.8, the majority of the WT bacteria were found to be inside the DCs after 24h and only a few adhered to the cell surface as detected. In contrast, the majority of the *lpxA*- bacteria were attached to the cell surface and stained positive with the FITC P1.7 antibody. Only a few *lpxA*- bacteria were detected inside the DCs. These experiments showed that the flow cytometric technique used was detecting mostly internalised WT bacteria, whereas the majority of *lpxA*- bacteria we bound to the cell surface.

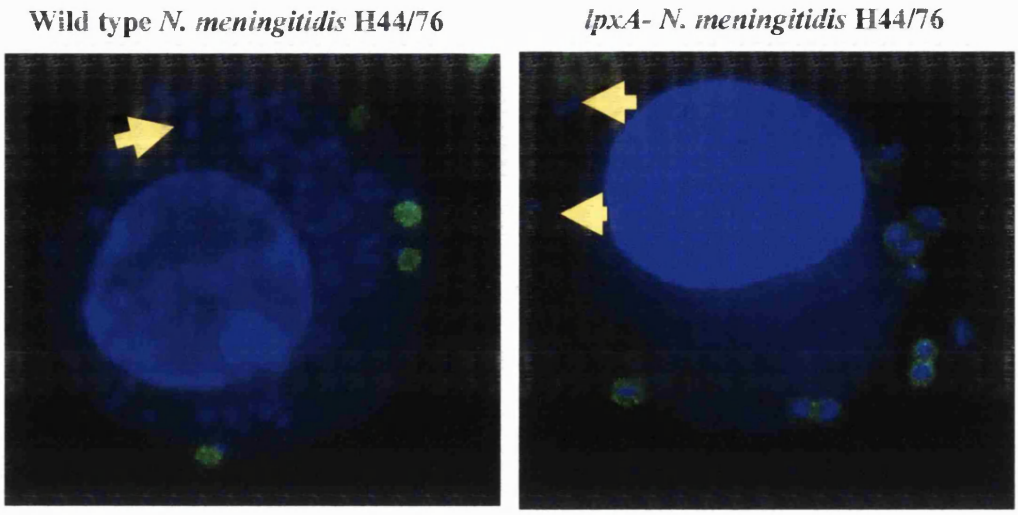

Figure 4.8 Binding and internalisation of *Neisseria meningitidis* by dendritic cells. DCs were incubated with WT or *lpxA*- bacteria at a ratio of 1:100 for 24h and analysed by confocal microscopy. Confocal image of DCs and bacteria is shown in a reconstructed image from 15 optical slices spanning the entire DC. After co-culture with the bacteria, DCs were allowed to adhere to slides. After fixation, the large nuclei of DCs and small bacteria were stained using differential staining with To-Pro3 (blue) and FITC conjugated P1.7 antibodies (green) specific for *N. meningitidis* to distinguish between internalised and externally bound bacteria. The yellow arrows point to internalised bacteria.

4.3.2 Role of LPS binding protein in phagocytosis of *Neisseria meningitidis* by dendritic cells

The results with the *lpxA*- mutant bacteria pointed towards an essential requirement for LPS expressed by the bacteria for optimal phagocytosis by DCs. Confirmation of a requirement for LPS was obtained by examining phagocytosis

in the absence of LPS binding protein (LBP). LBP is a soluble protein that binds to LPS. Binding of the LPS/LBP complex to receptors, such as CD14 or CD18, expressed on the cell surface is important for internalisation of LPS and cell activation (Tobias *et al.*, 1999;Ulevitch and Tobias, 1999).

Phagocytosis of *N. meningitidis* by DCs cultured in serum free medium, serum free medium with added recombinant LBP and medium with FCS was compared. Figure 4.9A shows typical FACS histograms obtained in these experiments. As expected, most DCs cultured in FCS, which contains free LBP, rapidly internalised the bacteria. In serum free conditions, however, very few bacteria were associated with the DCs. When the cells were cultured in serum free conditions but with added recombinant LBP, a pronounced increase in bacterial association with DCs was observed by flow cytometry. The analysis of the same cells by confocal microscopy revealed similar results and showed increased binding as well as internalisation of the bacteria (Fig. 4.9B). In serum free conditions, fewer bacteria were associated by the DCs and were typically bound to the cell surface with very few internalised. Addition of LBP increased both surface binding and internalisation of *N. meningitidis* by DCs. Figure 4.10 shows a representative time course of DC association with the bacteria analysed by FACS. Results for both percentage positivity and MFI are shown for comparison. The removal of serum resulted in reduced phagocytosis compared to 10% FCS over the entire time course. Addition of LBP restored the high level of phagocytosis. In most cases, the presence of recombinant LBP resulted in slightly increased phagocytosis compared to the levels when FCS was used. In these experiments, DCs were cultured for a maximum of 7 hours because culture for

longer than this time in serum free conditions resulted in lower viability of the cells. Nevertheless, the results clearly demonstrate a crucial role for LBP in the phagocytosis of *N. meningitidis* by DCs.

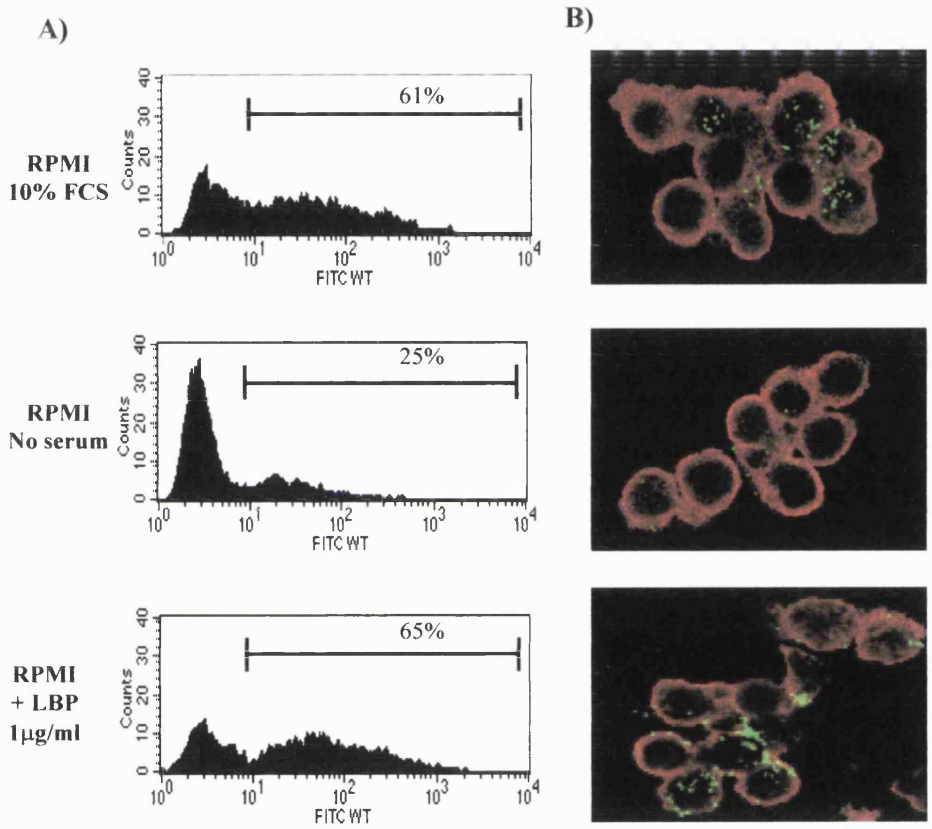

Figure 4.9 Role of LBP in phagocytosis of *Neisseria meningitidis* by dendritic cells. DCs were cultured with *N. meningitidis* at a ratio of 1:100 in RPMI 1640/10% FCS, RPMI/2.5% BSA or RPMI/2.5% BSA with 1µg/ml of recombinant human LBP. Phagocytosis was measured by FACS **(A)** and confocal microscopy **(B)** after 7h. Results shown are representative of of 3 individual experiments.

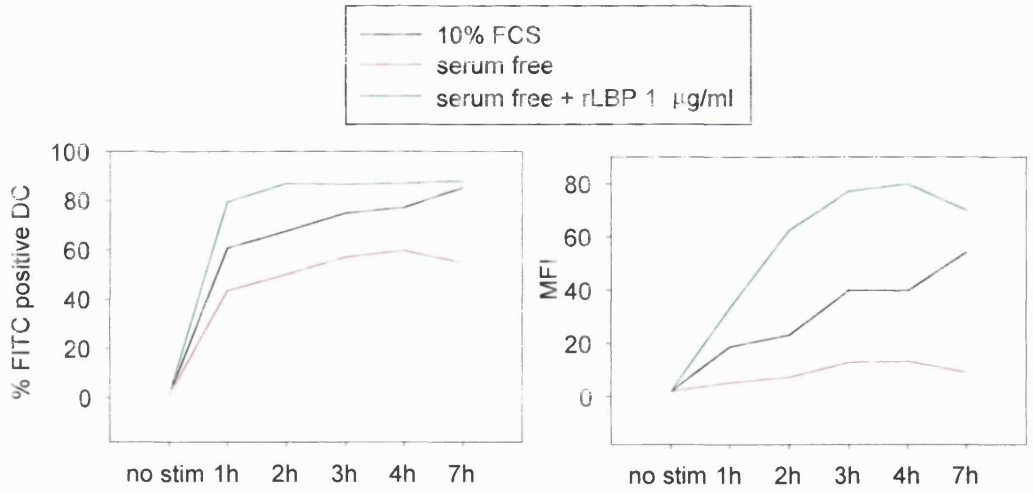

Figure 4.10 Role of LBP in phagocytosis of *Neisseria meningitidis* by dendritic cells. DCs were cultured with *N. meningitidis* at a ratio of 1:100 in RPMI 1640/10% FCS, RPMI/2.5% BSA or RPMI/2.5% BSA with 1µg/ml of recombinant human LBP. A time course of phagocytosis between 1-7h is shown. Results shown are representative of 3 individual experiments.

4.3.3 Dendritic cell phagocytosis of *Neisseria meningitidis* LPS regulatory strain HA3003

The data in the previous sections of this chapter have shown an important role of LPS in the phagocytosis of *N. meningitidis* by DCs. To further provide proof of this concept, strain HA3003 derived from the *N. menigitidis* strain H44/76 was used. The HA3003 strain carries the *lpxA* gene under control of an IPTG-inducible promoter. When this strain is grown in the absence of IPTG, LPS is barely detectable on the outer membrane of the bacteria. In contrast, in the presence of IPTG the biosynthesis of LPS is restored (see Figure 2.5 in General Materials and Methods). To regulate the expression of LPS on the surface of the bacteria, HA3003 strain was grown in the absence and presence of increasing

concentrations of IPTG and phagocytosis analysed by FACS and confocal microscopy.

Figure 4.11 shows the phagocytosis time course of HA3003 strain grown in the absence or presence of 2μM, 10μM, 50μM or 250μM IPTG. The WT and *lpxA*- strains are also shown for comparison. The results show that phagocytosis of the HA3003 is increased in a time dependent manner with increasing concentrations of IPTG. Confirmation that the bacteria had been internalised by the DCs was done by confocal microscopy (data not shown). These results suggest that the more LPS the bacteria expressed on the outer membrane, the more they were phagocytosed. The *lpxA*- mutant was poorly phagocytosed compared to the WT bacteria confirming earlier results in this chapter.

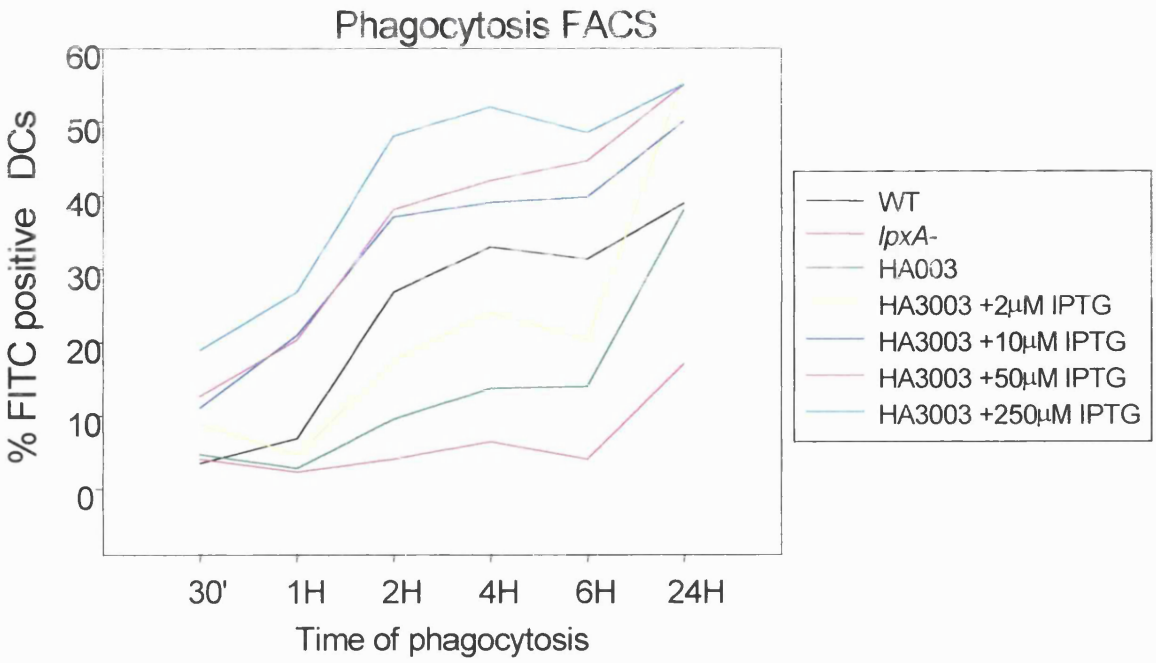

Figure 4.11 Phagocytosis of *Neisseria meningitidis lpxA* regulatory strain HA3003 by dendritic cells. Strain HA3003 bacteria were grown in the absence or presence of 2μM, 10μM, 50μM or 250μM IPTG, fixed and FITC labelled. Phagocytosis by DCs was then analysed by FACS after 30' up until 24h co-culture with the bacteria at 1:100 ratio DC:bacteria. WT and *lpxA*- bacteria were used for comparison. A representative experiment of three performed is shown.

4.4. Discussion

Immature DCs constantly sample their environment for antigens by micropinocytosis and endocytosis (Mellman and Steinman, 2001). Internalisation of antigens, processing and subsequent presentation to T cells is a hallmark in the generation of adaptive immune responses. Whereas macrophages are mostly known to initiate microbial death by ingesting microbes, DCs utilise phagocytosis to direct antigens to both MHC I and II compartments. Phagocytosis therefore serves a dual role: it can function as innate immune effector mechanism as well as a bridge between innate and acquired immunity (Greenberg and Grinstein, 2002).

In this chapter, DCs were shown to be extremely efficient at internalisation of intact WT *N. meningitidis* bacteria. A FACS based method was developed to assess both binding and internalisation of the bacteria by DCs. This method was combined with confocal microscopy, which allowed the binding and internalisation of the bacteria to be distinguished. Finally, an adaptation of the confocal method was used, where nuclear staining was combined with antibody staining of the bacteria to confirm bacterial internalisation by the DCs. The critical requirement of membrane bound LPS for phagocytosis was first observed with the LPS deficient *lpxA-* bacteria. The results from many experiments showed that phagocytosis of the *lpxA-* mutant was much slower than phagocytosis of WT bacteria. Even after 24 hours, only a few DCs had phagocytosed *lpxA-* compared to WT bacteria. In addition, exogenous LPS did not enhance the phagocytosis of the *lpxA-* mutant bacteria. It was therefore concluded that the presence of LPS in the outer membrane of *N. meningitidis* was critical for phagocytosis by DCs.

The use of the *lpxA* regulatory strain HA3003 provided direct evidence that phagocytosis of *N. meningitidis* by DCs depends on the expression of LPS by the bacteria. When these bacteria were grown in the presence of IPTG, the *lpxA* gene under the $lacI^q$-tac promoter was activated and the biosynthesis of LPS reinitiated. In the absence of IPTG, HA3003 bacteria did not express LPS and were phagocytosed to similar extent as the *lpxA*- mutant bacteria.

A crucial role for LPS expressed by the bacteria for phagocytosis and cytokine production was confirmed by the dependence of DC phagocytosis and cytokine production on LBP. Internalisation of LPS has been shown previously to depend on LPS complexed with LBP binding to CD14 (Tobias *et al.*, 1999). The reduction in phagocytosis by DCs in media without LBP and its restoration by addition of recombinant LBP is consistent with a requirement for LPS expressed by the bacteria. This is interesting in view of the fact that DCs express only low levels of CD14, which is lost on activation by the bacteria. It is possible that the low amount of CD14 expressed by the immature DCs, or soluble CD14, is sufficient for the response. Alternatively, it may be operating through a CD14 independent pathway. LBP can potentiate LPS responses in the absence of CD14, especially in response to whole bacteria (Klein *et al.*, 2000;Moore *et al.*, 2000). CR3 (CD11b/CD18) has been shown to bind bacterial LPS and may provide such a pathway (Wright *et al.*, 1989).

This is the first time that the importance of LPS in phagocytosis of *N. meningitidis* by human DCs has been shown. In a previous study by Peiser *et al.* (2002), the scavenger receptor A (SR-A) was shown to be a major receptor for *N.*

meningitidis in mouse macrophages. Phagocytosis of *N. meningitidis* by macrophages from SR-A knockout mice was completely abolished but this process was shown to be LPS-independent since the *lpxA*- bacteria were taken up as well as the WT bacteria. In these experiments, flow cytometry was used to study association of FITC labelled organisms with macrophages and it remains unclear whether the *lpxA*- bacteria were internalised or just surface bound to the macrophages. In general, these results highlight the differences between species and the different functions of macrophages and DCs. Recently, reduced adherence of *lpxA*- bacteria to epithelial cells was demonstrated indicating a role of LPS in the binding of *N. meningitidis* to epithelium (Albiger *et al.*, 2003). This study used a new group C *lpxA*- mutant showing a critical role for LPS in the binding of meningococci with a different capsular structure.

Other groups have studied the interactions of group B *N. meningitidis* with human DCs (Kolb-Maurer *et al.*, 2001;Unkmeir *et al.*, 2002). In these experiments, DCs were shown to internalise live WT bacteria, albeit at reduced level compared to findings in the present chapter. Phagocytosis of live meningococci was determined by gentamicin killing assay where bacterial colonies were counted after lysis of DCs. In addition, adhesion and phagocytosis of encapsulated bacteria and a mutant unencapsulated *N. meningitidis* were compared. Removal of the capsule significantly increased adherence of bacteria and phagocytosis by DCs. It is possible that LPS is more accessible to the DCs in the unencapsulated mutant and could account for the increased phagocytosis.

Overall, DCs were found to be very effective at internalising *N. meningitidis.* The experiments shown in this chapter provided convincing evidence that LPS expressed by the bacteria is required for rapid phagocytosis of *N. meningitidis* by DCs. In particular, the reduced capacity of *lpxA*- to induce cytokine production and the inability of DCs to rapidly phagocytose the mutant bacteria raised the possibility that ingestion of the bacteria may be a critical event for DC cytokine production. In the next chapter, the role of phagocytosis of *N. meningitidis* in the activation and cytokine production of DCs was therefore investigated.

Chapter 5

Role of phagocytosis in the activation of dendritic cells in response to *Neisseria meningitidis*

5.1 Introduction

DCs express a variety of phagocytic receptors that recognise molecular patterns on the surface of micro-organisms. Some of these receptors are capable of transmitting signals that trigger internalisation, while others appear primarily to facilitate or participate in binding (Underhill and Ozinsky, 2002). In general, multiple receptors simultaneously recognise microbes and generate complex intracellular signals. Some of these signalling molecules are not only required for the mechanical aspects of particle internalisation, but also regulate pro-inflammatory signalling so that phagocytosis is often coupled to a inflammatory response. Certain phagocytic receptors, such as Fcγ receptors, trigger inflammatory responses directly, whereas others such as complement receptors are regulated by additional receptors that are not phagocytic themselves. These additional receptors include TLRs, which have been shown to co-operatively evoke inflammatory signals with phagocytic receptors. Recruitment of TLRs to phagosomes therefore provides a mechanism by which phagocytosis and inflammatory responses can be linked (Underhill and Ozinsky, 2002).

The results in Chapter 3 showed that co-culture of WT *N. meningitidis* with human DCs results in IL1, IL6, IL8, TNF-α and IL12 production as well as enhanced expression of surface activation markers. In Chapter 4, DCs were found to be very effective at internalising *N. meningitidis.* In addition, LPS expressed by the bacteria was found to be critical for internalisation. The mutant *lpxA-* bacteria induced significantly reduced IL12 and TNF-α production as well as being poorly phagocytosed. In addition, activation of LPS biosynthesis of the HA3003 *N.*

meningitidis strain restored phagocytosis to WT level. These findings together raised the possibility that ingestion of the bacteria may be a critical event for DC cytokine production. To investigate this possibility, the role of phagocytosis of *N. meningitidis* in the activation of the DCs was studied.

5.2 Materials and methods

5.2.1 Dendritic cell culture and stimulation with *Neisseria meningitidis*

DCs were cultured for a concentration of 1:100 DC/bacteria ratio with *N. meningitidis* H44/76 and *lpxA*- mutant bacteria or with 100ng/ml purified LPS for 1-18h. In inhibition experiments, 500ng/ml of cytochalasin D or 10ng/ml toxin B were added to DCs 30 min prior to the addition of the bacteria. In serum free experiments, DCs were cultured in RPMI with 2.5% BSA (LPS free). Recombinant human lipopolysaccharide binding protein (LBP) was added at 1μg/ml as indicated.

5.2.2 Stimulation of dendritic cells with polystyrene beads

Alexa 488 conjugated FluoSpheres® fluorescent microbeads were used to study phagocytosis and cytokine production in the absence of inflammatory stimulus such as bacteria. The size of the beads (i.e. 1μm) was chosen to be similar to the size of the bacteria used in this thesis. Beads were washed once by centrifugation at 400g for 2 minutes and resuspended in RPMI medium. The beads were then cultured with the DCs at 1:100 ratio (5μl bead solution was equivalent to 10^8 beads) for indicated time periods. For confocal microscopy, the cells were stained

for surface MHC Class II as described in 2.5. Intracellular cytokine were stained as in 2.3.6.

5.2.3 Staining of *Neisseria meningitidis* P1.7 on slides after inhibition of phagocytosis by cytochalasin D

DCs were cultured with FITC labelled *N. meningitidis* in the presence or absence of cytochalasin D for 18h. DCs were adhered on slides, followed by a secondary staining of bacteria by using purified mouse anti P1.7 antibody in 1:1000 dilution for 1h. After three washes in PBS, the slides were incubated with 10μg/ml goat anti mouse Texas Red-X conjugated, washed carefully and mounted in Citifluor. The slides were analysed by confocal microscopy.

5.3 Results

5.3.1 Cytokine production by dendritic cells in response to *lpxA* regulatory strain HA3003

The results of the previous chapter with the LPS deficient mutant and the *lpxA* regulatory strain HA3003 clearly showed that LPS expressed by the bacteria was necessary for DC phagocytosis of *N. meningitidis*. The LPS deficient mutant also failed to induce high levels of cytokines, IL12 in particular. These results suggested that internalisation may be a critical event for DC cytokine production, To study the role of internalisation in cytokine production, LPS regulatory strain HA3003 was grown in the presence (LPS biosynthesis -on) and absence (LPS biosynthesis -off) of IPTG and the cytokine production by DCs analysed. Figure

5.1A shows DC association with FITC bacteria after 2h of co-culture with HA3003 strain grown in the absence or presence of 2μM to 250μM of IPTG. Similarly to *lpxA*-, HA3003 grown in the absence of IPTG (expressing very low levels of LPS) was poorly associated with the DCs. However, restoration of LPS biosynthesis by IPTG restored DC association of HA3003 *N. meningitidis* to levels comparable with the WT bacteria. Moreover, the LPS dependent increase in association of HA3003 with DC also restored internalisation of the bacteria as studied by confocal microscopy (data not shown)

Restoration of LPS biosynthesis resulted in a concomitant increase in cytokine production (Figure 5.1B). Similar levels of IL12, IL10, TNF-α and IL6 were produced by DCs in response to either the HA3003 strain grown in the presence of IPTG as the WT bacteria after 18h of co-culture as measured by ELISA. In contrast, *lpxA*- and HA3003 grown in the absence of IPTG induced less IL12, IL10, TNF-α and IL6 compared to the WT or the LPS-expressing HA3003 strain. Similar results were obtained by intracellular staining of cytokines (not shown). These data confirm the requirement of LPS for both the internalisation of *N. meningitidis* and subsequent cytokine response. In addition, these data suggested a link between phagocytosis and cytokine production by DCs in response to *N. meningitidis*.

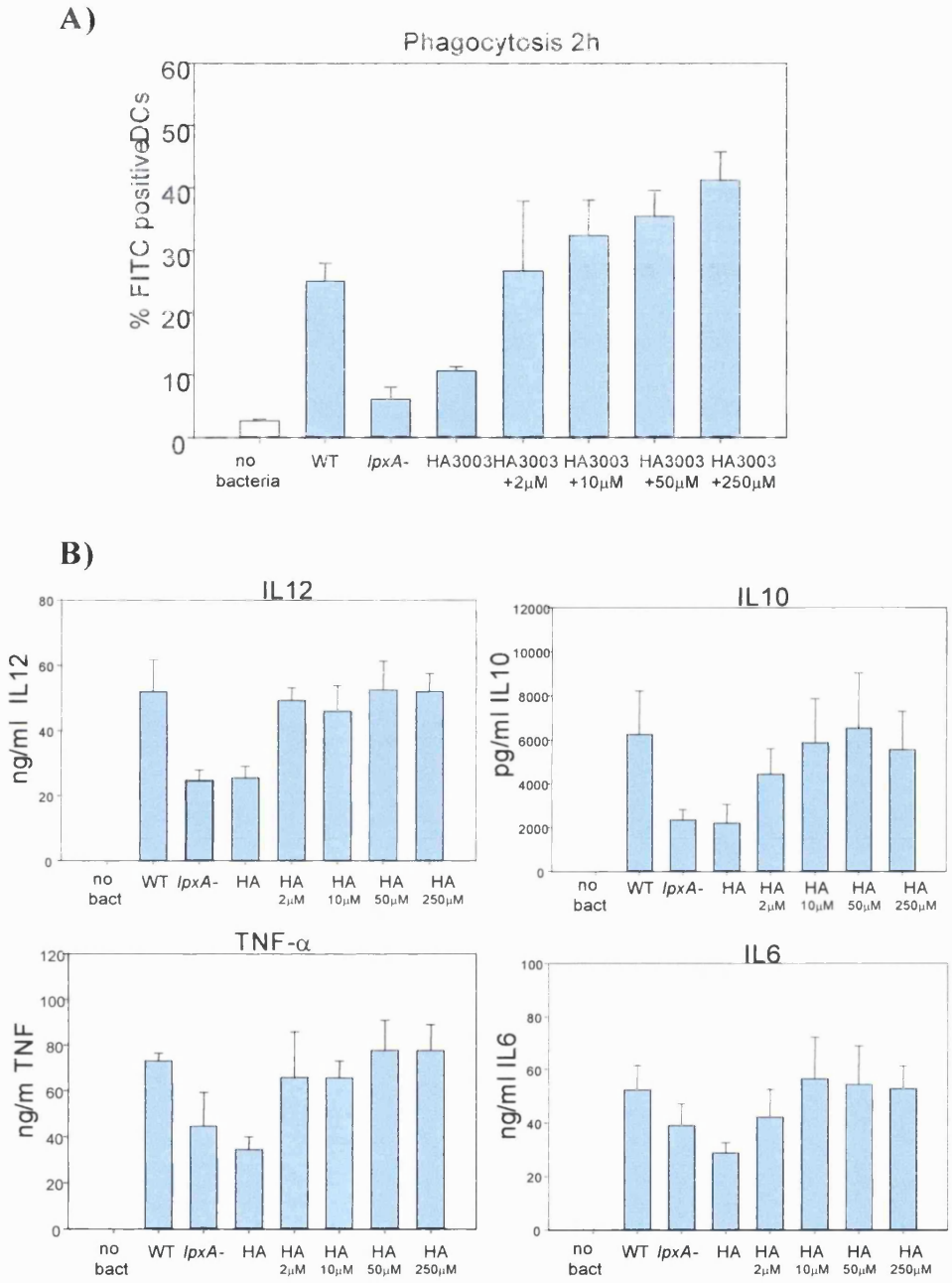

Figure 5.1 Phagocytosis and cytokine production by dendritic cells in response to *lpxA* regulatory strain HA3003. Strain HA3003 bacteria were grown in the absence or presence of 2μM, 10μM, 50μM or 250μM IPTG and fixed in 0.5% PFA. DCs and bacteria were cultured at 1:100 ratio (DC:bacteria). Phagocytosis by DCs was analysed by FACS after 2h **(A)**. Cytokines were measured by ELISA after 18h of co-culture. Mean and SEM of three separate experiments is shown.

5.3.2 Dendritic cell stimulation with *Neisseria meningitidis* in Transwells

To determine whether DC activation, as measured by surface markers and cytokines, required contact between DCs and bacteria, a transwell system was used, in which DCs were physically separated from the bacteria by 0.1μm porous membranes (Fig. 5.2). Under these conditions, soluble components such as DNA, outer membrane particles, LPS etc released by the bacteria would pass through the membrane and made contact with the DCs, whereas the intact bacteria could not.

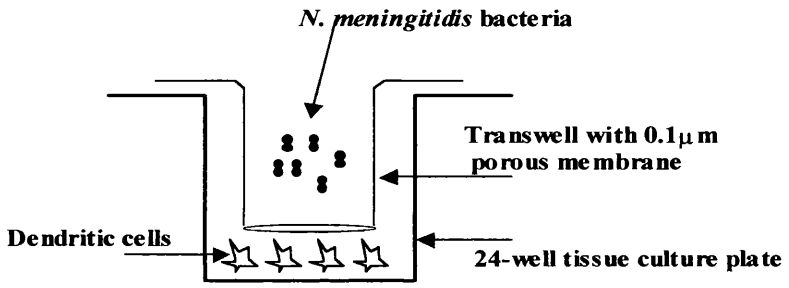

Figure 5.2 Transwell™ system. Transwell inserts were sterilely placed into wells of a 24-well tissue culture plate and used to separate DCs and the bacteria. DCs were added to the lower chamber and bacteria added to the top chamber. After culture, the inserts were removed and DCs recovered from the lower chamber for analysis.

DCs were cultured with WT and *lpxA*- bacteria for 18h with or without Transwell inserts. The production of IL12, TNF-α and IL6 was measured by intracellular staining and the surface marker expression investigated. Cytokine production in response to 100ng/ml of meningococcal LPS was used as a control as LPS should pass the transwell pores. Combined results from three experiments are shown in

Figure 5.3. Separation of DCs from WT bacteria in transwells resulted in a significant (P=<0.05) reduction in IL12 and TNF-α production, whereas IL6 production was marginally reduced. (Fig. 5.3 blue fill). A significant reduction (P=<0.05) in the production of all cytokines was found in transwells separating the DCs and *lpxA*- bacteria (Fig. 5.3. red fill). As expected, cytokine production to LPS was unaffected by the presence of transwells (Fig. 5.3 green fill). These results suggested that physical contact between bacteria and DCs was necessary for maximal cytokine production in response to *N. meningitidis*. The results also suggest that LPS is the main soluble component inducing cytokine production in response to the WT bacteria as the levels of cytokines were severely reduced in transwells combined with the *lpxA*- bacteria.

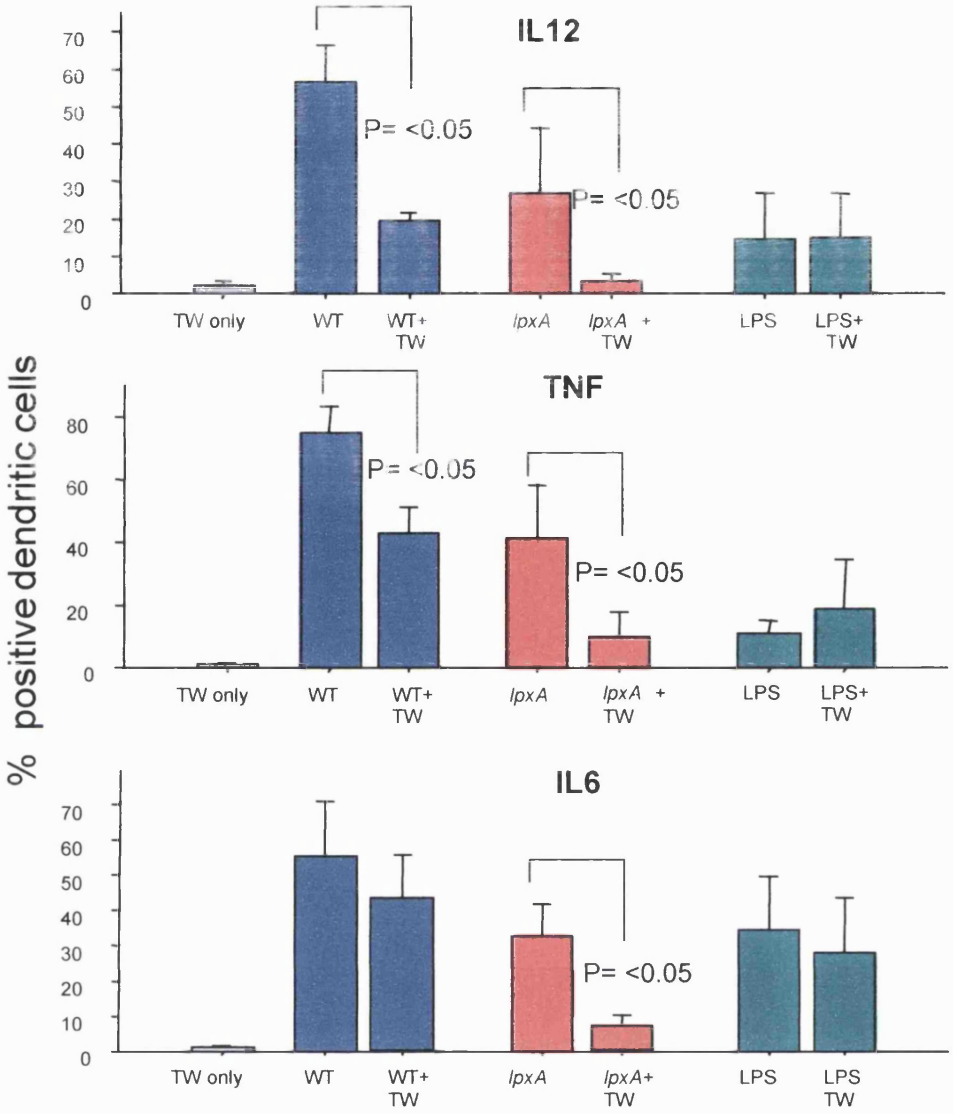

Figure 5.3 Intracellular cytokine production by dendritic cells stimulated with *Neisseria meningitidis* H44/76 in transwell™ cultures. DCs cultured in the lower chamber of a transwell were separated from the bacteria added to the upper chamber by a membrane with 0.1μm pores. Intracellular IL12, TNF-α and IL6 were measured after 18h of incubation in the presence of brefeldin A. The cells were permeabilised and analysed by flow cytometry for intracellular cytokine. Purified LPS was used as a control. The results are expressed as the mean +/- SEM. of % positive DCs from combined data of three separate experiments. TW = transwell

The transwell system was used to address whether freely diffusible LPS or other bacterial components in the culture could induce DC maturation as judged by surface marker expression or whether a contact between the bacteria and the DCs was necessary. DCs were cultured for 18h in the transwell system in the presence of WT and *lpxA*- bacteria, or purified LPS. Enhanced expression of MHC Class II, CD86 and CD83 was observed in the presence of all stimuli compared to the unstimulated cells (Figure 5.4). The expression of both MHC Class II and CD86 by DCs was unchanged when DCs were separated from the WT bacteria in transwells. Similarly, the expression of CD86 in response to *lpxA*- was unaffected by transwells. MHC Class II expression remained very low after *lpxA*- stimulation and was not significantly different from unstimulated cells. Curiously, the expression of CD83 in response to both WT and *lpxA*- bacteria was significantly reduced in the presence of transwells. As expected, transwells made no difference to the expression of surface markers induced by purified LPS. These results suggested that a contact between the bacteria and DCs may not have been necessary for the induction of surface MHC Class II and CD86 expression, but was needed for upregulation of CD83. It also seemed that soluble components other than LPS were able to induce CD86 expression by the DCs, as the presence of transwells with the *lpxA*- bacteria made no difference to its expression.

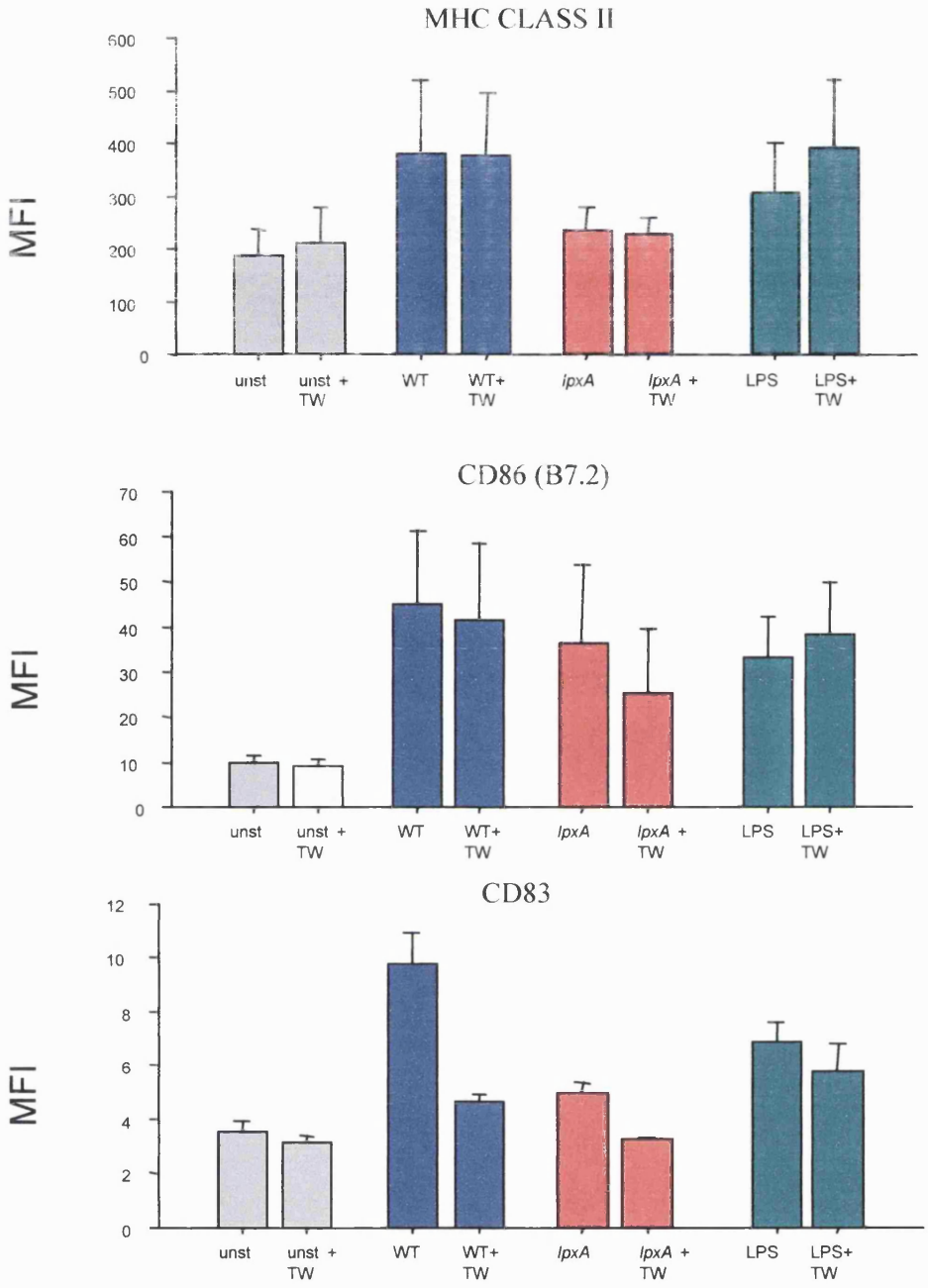

Figure 5.4 Expression of surface markers by DCs stimulated with *Neisseria. meningitidis* H44/76 in transwell™ cultures. DCs cultured in the lower chamber of a transwell were separated from the bacteria added to the upper chamber by a membrane with 0.1μm pores. MHC Class II, CD86 and CD83 were measured after 18h by flow cytometry. Purified LPS was used as a control. The results are expressed as the mean +/- SEM. MFI of DCs from combined data of three separate experiments. TW= transwell.

5.3.3 Phagocytosis and cytokine production by dendritic cells in response to polystyrene beads

DCs employ several methods to internalise antigen from the environment. Phagocytosis is a form of endocytosis by which DCs capture large insoluble particles up to 6μm in size. Phagocytosis triggers a myriad of cellular processes ranging from cytoskeletal rearrangements to the production of inflammatory cytokines. In order to study phagocytosis-induced cell activation, DCs were allowed to phagocytose polystyrene beads in the absence of an inflammatory stimulus. DCs were cultured in the presence of Alexa 488 conjugated 1μm beads at a DC/bead ratio of 1:100. Phagocytosis was analysed after 30 min, 3h and 18h by confocal microscopy (Fig. 5.5A). Similarly to FITC labelled bacteria, a time dependent increase in internalisation of the beads by DCs was observed. After 18h, the cytoplasms of the DCs were filled with fluorescent beads. Intracellular cytokine production was then analysed by FACS after 18h of stimulation with the beads (Fig. 5.5B). No IL12, TNF-α or IL1β production was observed in response to the beads showing that the process of phagocytosis on its own was not sufficient to induce cytokine production by DCs. Similar results were obtained by ELISA analysis of soluble cytokines (not shown).

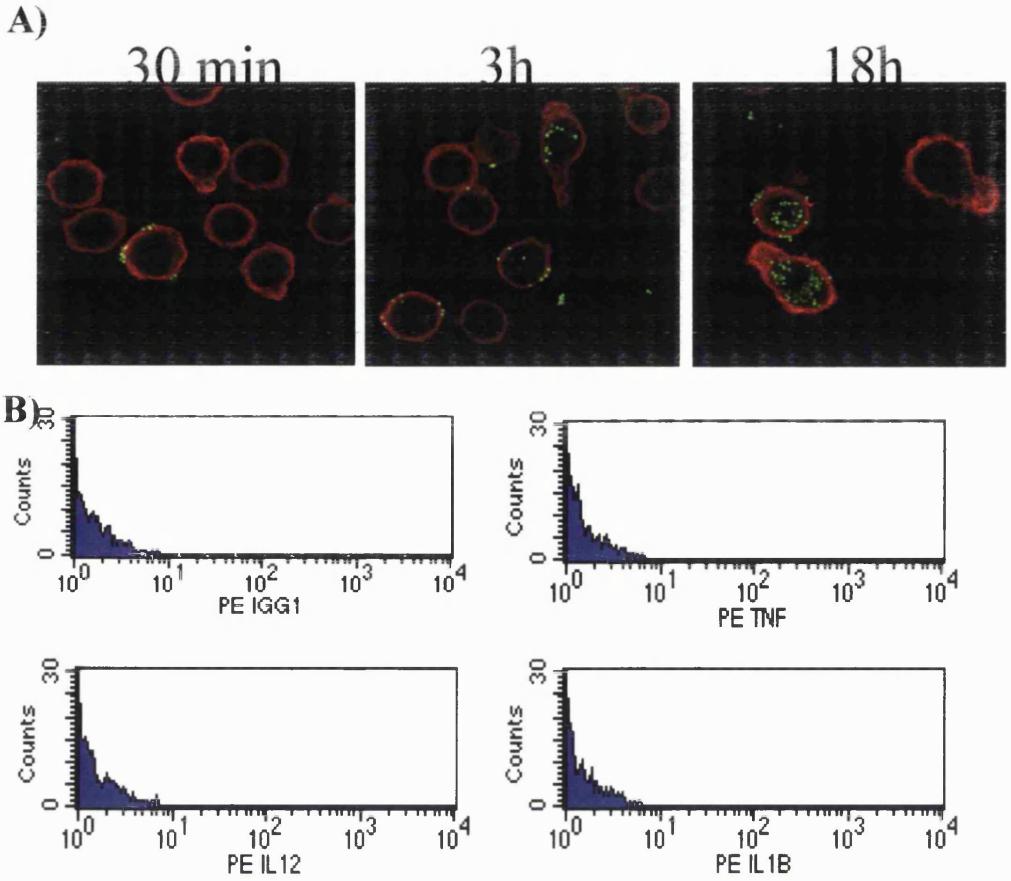

Figure 5.5 Phagocytosis and intracellular cytokine production in response to fluorescent polystyrene beads. DCs were cultured with Alexa 488 conjugated polystyrene beads and analysed for phagocytosis by confocal microscopy (A) and intracellular cytokines by FACS (B). Representative results from 2 experiments are shown.

5.3.4 Simultaneous analysis of phagocytosis and intracellular cytokine production by flow cytometry

To investigate the relationship between phagocytosis of bacteria and cytokine production, the flow cytometric technique for measuring internalisation of bacteria by DCs was adapted to analyse simultaneously phagocytosis and intracellular cytokine production. DCs were co-cultured with FITC labelled bacteria for 24 hours and then stained for intracellular cytokine production. The results of one such experiment are given in Figure 5.6. The quadrants were placed to separate cells that had phagocytosed FITC bacteria from cells that had not. In this experiment, 71% (57%/80%) of DCs that had phagocytosed WT bacteria were also making TNFα and 42% (33%/78%) were making IL12. Only 25% of DCs that had not phagocytosed bacteria made TNF-α and 18% made IL12. In comparison, 38% of DCs that had phagocytosed FITC labelled *lpxA-* bacteria produced TNF-α and 8% produced IL12. A higher percentage of cells that had not phagocytosed *lpxA-* produced TNF-α (27%) and IL12 (5%) compared to the WT bacteria. These results were typical of several experiments showing that DCs, which had phagocytosed WT bacteria were 2-3 times more likely to make TNFα and IL12 than those that had not phagocytosed bacteria. This was only partially true for *lpxA-* as a higher proportion of cells that had not phagocytosed *lpxA-* bacteria also produced cytokines.

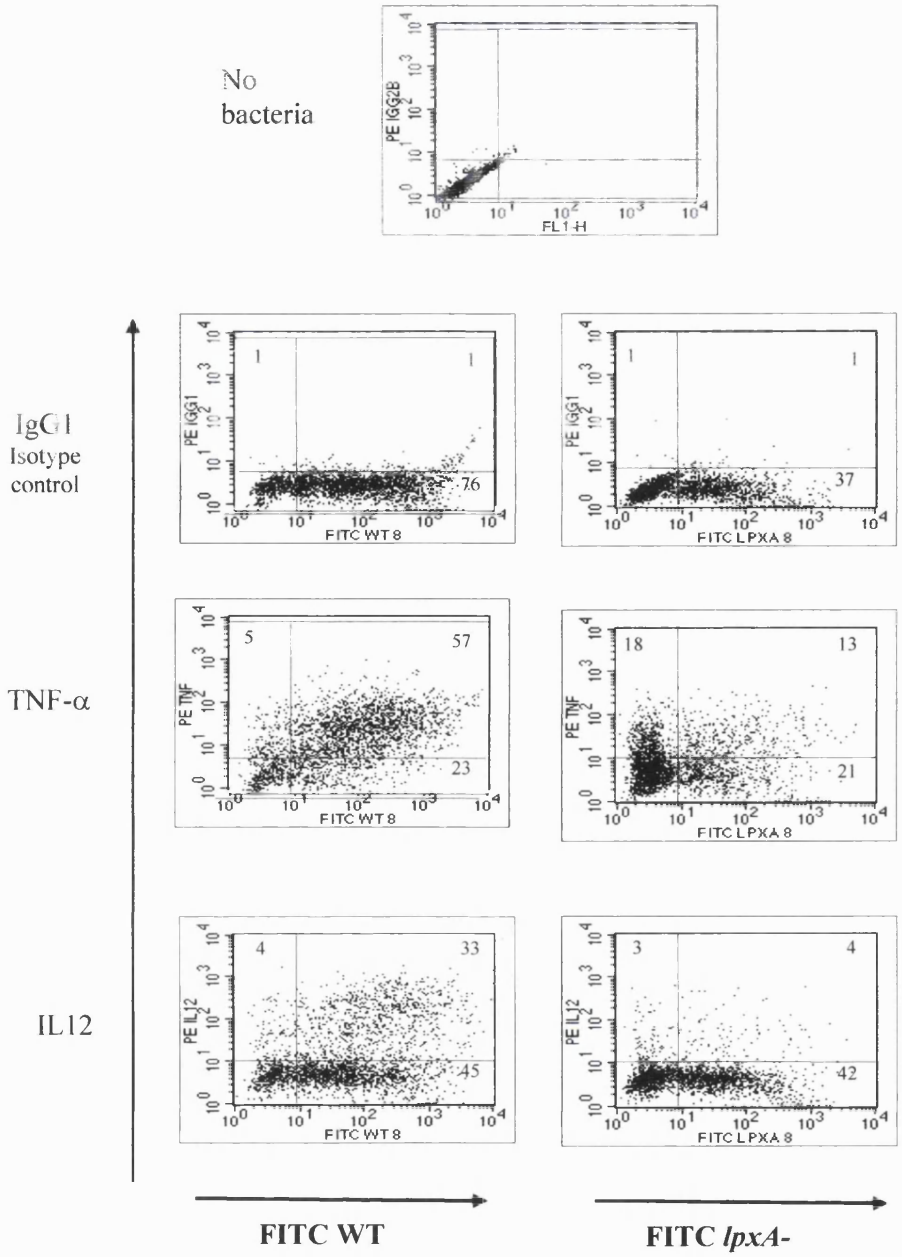

Figure 5.6 Intracellular cytokine production and phagocytosis of FITC labelled bacteria by dendritic cells. DCs were stimulated with FITC labelled bacteria for 18h in the presence of brefeldin A and then stained for intracellular TNF-α and IL12. The cells were then analysed by FACS. The dot plots show association of FITC bacteria and intracellular cytokines. A representative of 3 experiments is shown.

Figure 5.7 shows a time course of phagocytosis and TNF-α production analysed simultaneously. DCs were incubated with FITC labelled WT or *lpxA*- bacteria for 1, 3, 5 and 8h and the intracellular production of TNF-α detected by flow cytometry. Already after 1h stimulation with WT bacteria, a small proportion of cells had started to produce TNF-α. These cells were also the most FITC positive (mostly associated with FITC bacteria). The proportion of cells that produced TNF-α rose over time and by 8h, DCs that had associated with the most bacteria (the most FITC positive), were clearly the same ones that produced the cytokine.

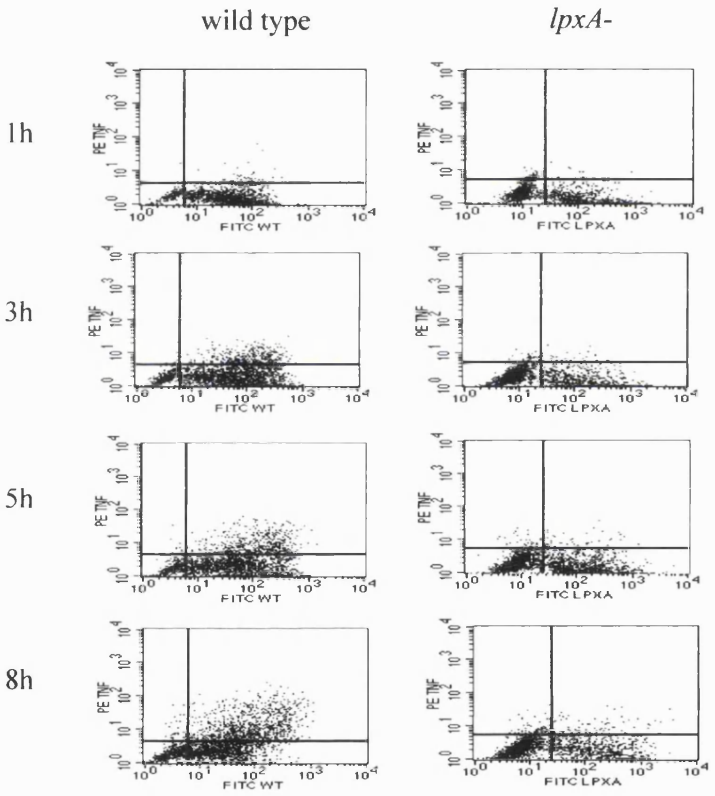

Figure 5.7 Time course of intracellular TNF-α production and phagocytosis of FITC labelled bacteria. DCs were stimulated with FITC labelled WT and *lpxA*- bacteria for 1,3,5 and 8h in the presence of brefeldin A and then stained for intracellular TNF-α. The cells were analysed by FACS. A representative of 3 experiments is shown.

5.3.5 Inhibition of phagocytosis

5.3.5.1 Inhibition of *Neisseria meningitidis* phagocytosis by cytochalasin D and toxin B

The role of phagocytosis in DC cytokine production was examined further by inhibiting internalisation of the bacteria with cytochalasin D and toxin B. Cytochalasin D is a fungal toxin from *Zygosporium mansonii* that inhibits actin polymerisation, an event required for phagocytosis. *Clostridium difficile* toxin B is an inhibitor for endogenous Rho GTPases, which have been widely implicated in the regulation of the actin cytoskeleton (Hall, 1998). DCs were cultured for 18h with WT *N. meningitidis* in the presence or absence of cytochalasin D or toxin B. As shown by confocal microscopy, both these agents completely inhibited internalisation but not binding of the bacteria by DCs (Figure 5.8). Optical sectioning by confocal microscopy verified that the great majority of the bacteria had not been internalised by the DCs after cytochalasin D treatment. These results showed the requirement for actin filaments in the phagocytosis of *N. meningitidis*.

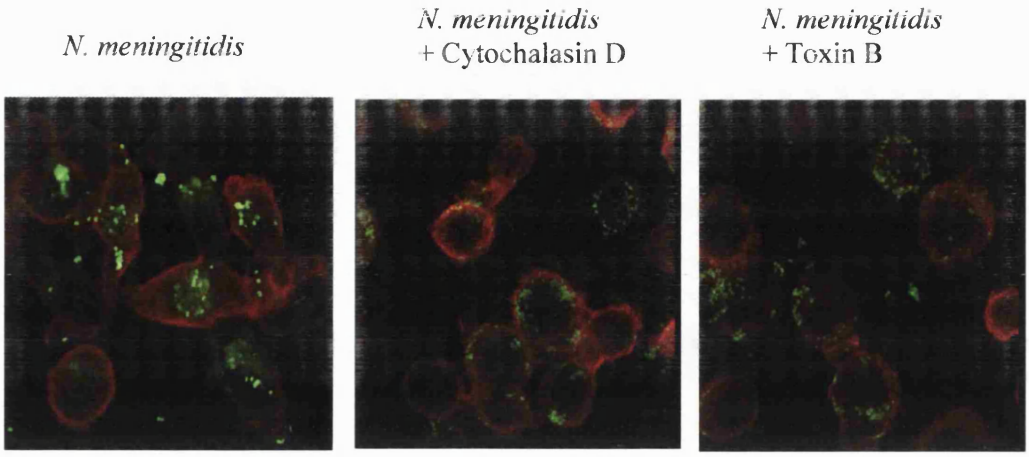

Figure 5.8 Inhibition of bacterial internalisation by cytochalasin D. DCs were incubated with cytochalasin D (500ng/ml) or Toxin B (10ng/ml) for 30 min and then cultured with FITC labelled bacteria at a ratio 1:100 for 18h. The cells were then adhered to slides, stained for MHC Class II (Texas Red) and examined by confocal microscopy. Bacteria appear as green (FITC) particles. Note the typical circle-like formations of the bacteria on the surface of the individual DCs.

The confocal images showed that WT bacteria were adhered to the surface of DCs, but not internalised, in the presence of cytochalasin D. To further verify whether inhibition of phagocytosis by cytochalasin D completely prevented the internalisation of the bacteria, a staining protocol was used in which surface bound bacteria were distinguished from internalised *N. meningitidis*. FITC labelled bacteria were first incubated with the cells as previously. After co-culture, a second staining of *N. meningitidis* by P1.7 antibody was performed in order to detect cell surface bound FITC bacteria. Figure 5.9A shows a *N. meningitidis* diplococcus stained with both FITC-labelling and texas-red conjugated secondary antibody detecting the P1.7 antibody. The merged images

are shown far right. In figures 5.8B and C, a DC was focused in the middle of the image with the help of transmitted light and the bacteria revealed by double staining. After stimulation with *N. meningitidis*, most of the bacteria fluoresced only FITC and were not stained with P1.7 antibody, which indicated that the majority of them had been phagocytosed by the DC (Fig. 5.9B). In this example, one bacterium was detected clearly double stained and was therefore bound to the cell surface (Fig. 5.9A). In contrast, after incubation with cytochalasin D, all bacteria were labelled double positive (Fig. 5.9C). Cytochalasin D therefore completely prevented internalisation of the bacteria by the DC but allowed binding to DC surface.

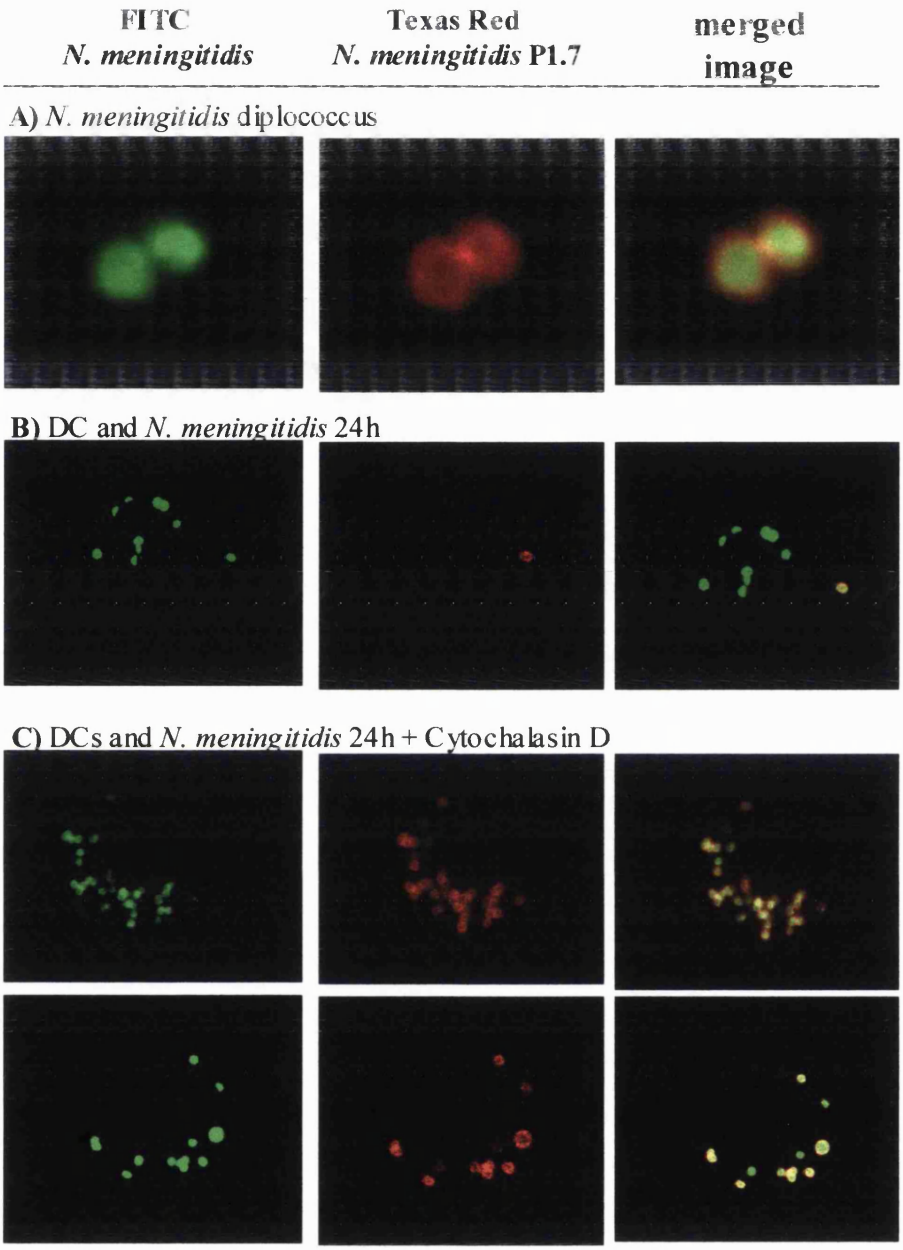

Figure 5.9 Inhibition of phagocytosis by cytochalasin D. DCs were incubated with FITC labelled *N. meningitidis* for 18h in the presence or absence of cytochalasin D. The cells were then allowed to adhere to slides and the bacteria stained with mouse P1.7 antibody to meningococci and Texas red- conjugated secondary antibodies. **A)** *N. meningitidis* diplococcus **B)** DC and *N. meningitidis* 18h **C)** DC and bacteria + cytochalasin D. Each image was focused on one DC.

5.3.5.2 Cytokine production by dendritic cells after inhibition of *Neisseria meningitidis* phagocytosis

After establishing that the inhibition protocol was working as required, DCs were cultured with WT *N. meningitidis* in the presence or absence of cytochalasin D and the intracellular cytokines measured by flow cytometry and ELISA. A timecourse of intracellular IL12 and TNF-α production after inhibition of phagocytosis with cytochalasin D is shown in figure 5.10. Inhibition of cytokine production by cytochalasin D was detected already after 2h of stimulation with the WT bacteria. The inhibitory effect of cytochalasin D lasted throughout the entire timecourse.

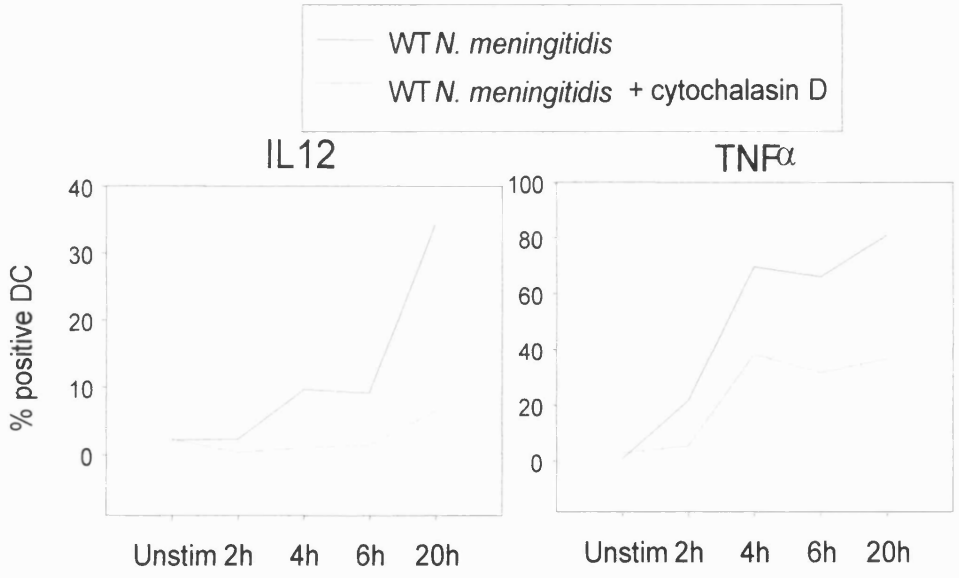

Figure 5.10 Effect of cytochalasin D on kinetics of intracellular cytokine production by dendritic cells. DCs were incubated with 500ng/ml cytochalasin D for 30 min and then cultured with WT *N. meningitidis* at a ratio of 1:100 for 2, 4, 6 and 20h. IL12 and TNF-α were then measured by intracellular staining. Representative results from 2 experiments performed are shown.

Combined data from 5 experiments for cytokine production in the presence of cytochalasin D is shown in figure 5.11. Cytochalasin D significantly ($p=<0.05$) inhibited production of both IL12 and TNF-α, but had only a slight effect on IL6, by DCs in response to WT *N. meningitidis* as analysed by intracellular staining (Fig. 5.11). Only IL12 production was significantly ($p=<0.05$) reduced in response to *lpxA-* bacteria. Similar results were obtained when cytokines were assayed by ELISA (Fig 5.11 right panel). IL12 and TNF-α production in response to both WT and *lpxA-* were significantly reduced ($p=<0.05$) in the presence of cytochalasin D. Inhibition by cytochalasin D had no effect in IL6 production induced either by WT or *lpxA-* bacteria. In addition, cytochalasin D did not induce cytokine production on its own.

The results for intracellular cytokine production in the presence of another inhibitor of phagocytosis, toxin B, are also shown in figure 5.12. Four individual experiments with different donors are shown. Toxin B significantly reduced both IL12 and TNF-α ($p=<0.05$) in response to the WT *N. meningitidis.*

Taken together, the inhibition of phagocytosis by using two different inhibitors, cytochalasin D and toxin B, proved to be an efficient way to inhibit cytokine production. This clearly suggested a critical role for phagocytosis in the activation of DCs.

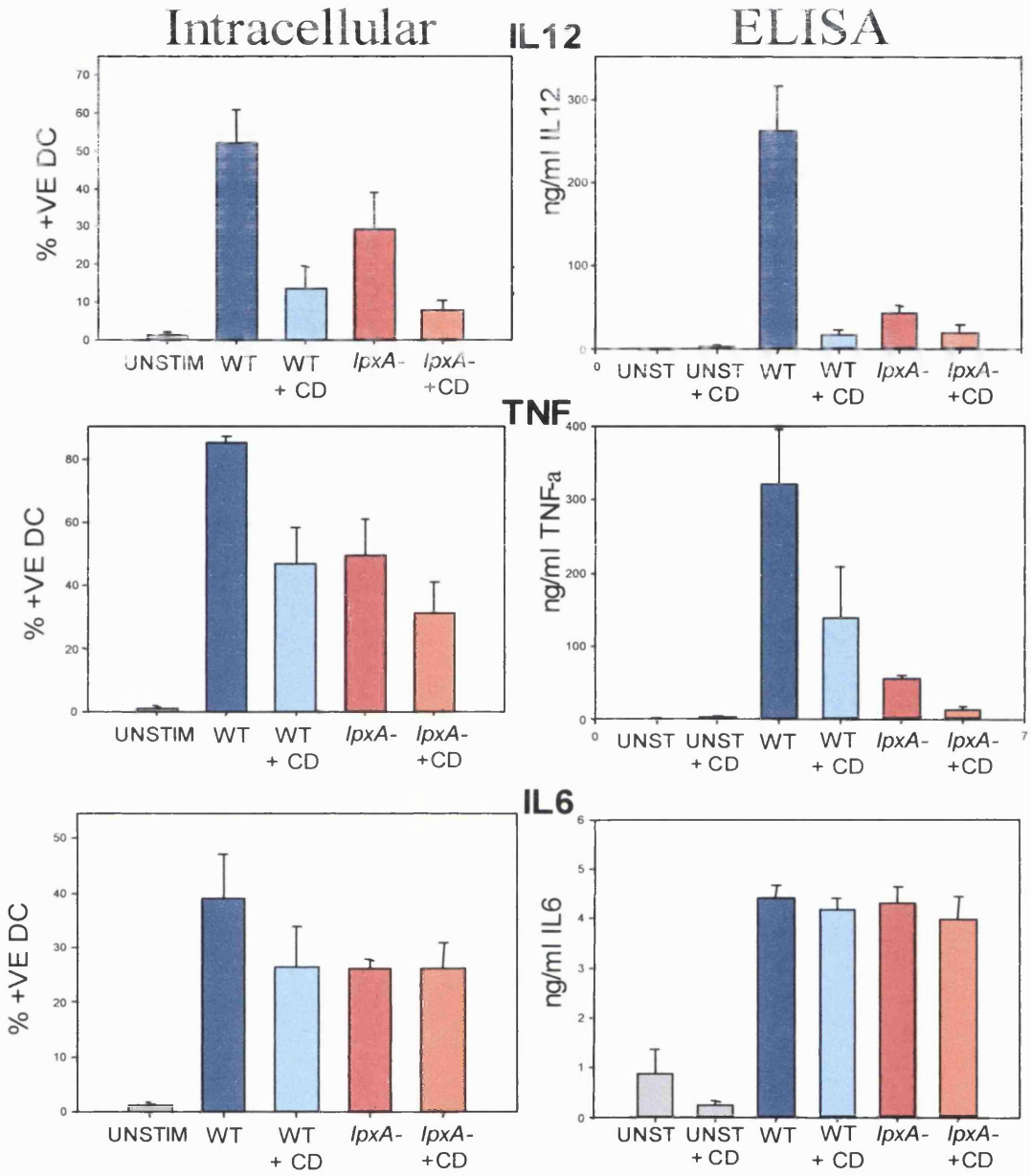

Figure 5.11 Cytokine production by dendritic cells after inhibition of phagocytosis with cytochalasin D. DCs were incubated with 500ng/ml cytochalasin D for 30 min and then cultured with WT or *lpxA- N. meningitidis* at a ratio of 1:100 for 18h and IL12, TNF-α and IL6 measured by intracellular staining and ELISA. Results are expressed as the mean and SEM of 5 (FACS) or 4 (ELISA) individual experiments. CD= cytochalasin D

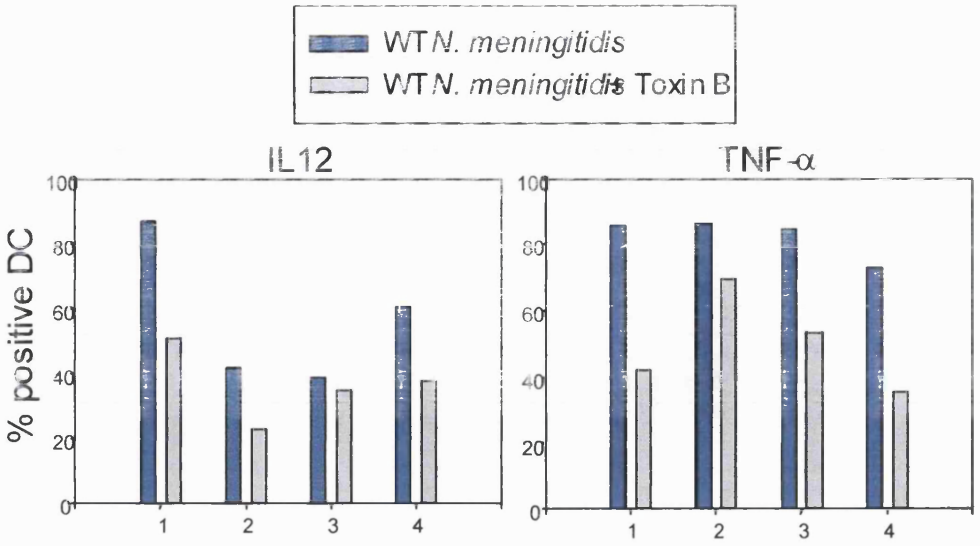

Figure 5.12 Intracellular cytokine production after inhibition of phagocytosis with toxin B. DCs were incubated with 10ng/ml toxin B for 30 min and then cultured with WT *N. meningitidis* at a ratio of 1:100 for 20h and intracellular IL12 and TNF-alpha measured by FACS after cell permeabilisation. Results show 4 individual experiments (experiments 1-4).

5.3.5.2 Surface marker expression by dendritic cells after inhibition of *Neisseria meningitidis* phagocytosis by cytochalasin D

The results obtained with the transwell system in section 5.3.2.2 suggested that the expression of surface markers was not as dependent on physical contact between the bacteria and the DCs as cytokine production. It was therefore of interest to study the effect of cytochalasin D treatment on the surface marker expression. DCs were stimulated with WT or *lpxA- N. meningitidis* and cultured in the presence or absence of cytochalasin D. The expression of cell surface CD83, Class II and CD86 was determined by flow cytometry. The increased expression of DC surface activation markers induced by *N. meningitidis* was not

affected by cytochalasin D (Figure 5.13). In fact, when unstimulated cells were cultured with cytochalasin D, a slight increase in surface marker expression was sometimes found. Interestingly, CD83 expression, which was reduced in transwells, was not affected by cytochalasin D treatment suggesting that binding of the bacteria to the cell surface, but not internalisation, is necessary for its up-regulation.

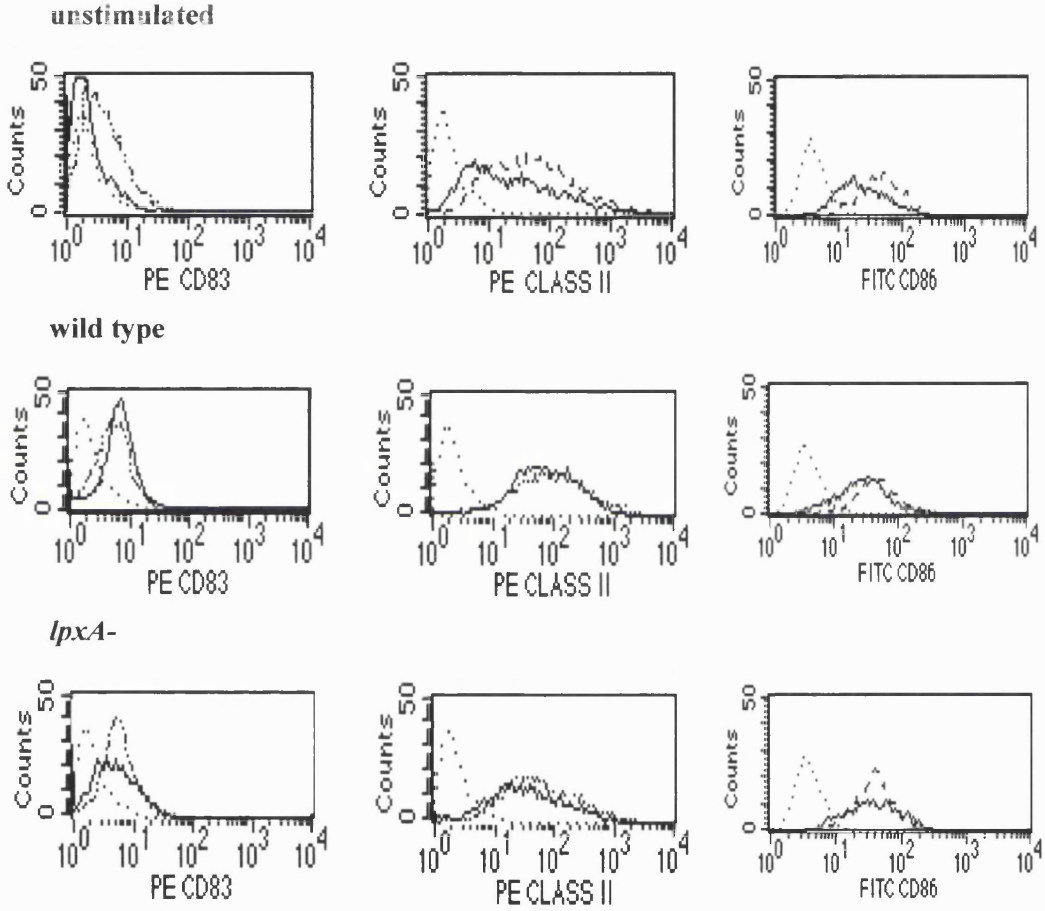

Figure 5.13 Effect of cytochalasin D treatment on surface marker expression. DCs were first incubated with cytochalasin D and then stimulated with WT and *lpxA- N. meningitidis* for 18h and the expression of CD83, MHC Class II and CD86 analysed by FACS. The histograms shown are representative of 5 experiments. Dotted line= isotype control, straight line= without cytochalasin D ,dashed line= cytochalasin D treated.

5.3.6 Role of LBP in phagocytosis and cytokine production by dendritic cells

The results in chapter 4 showed a crucial role for LBP in the phagocytosis of *N. meningitidis*. The role of LBP was next studied by simultaneous analysis of phagocytosis and cytokine production by DCs. In these experiments, phagocytosis together with intracellular cytokine production were analysed in serum free medium, serum free medium with added recombinant LBP and medium with FCS (Fig. 5.14). In serum free conditions very few bacteria were phagocytosed by the DCs. When the cells were cultured in serum free conditions but with added recombinant LBP, an increase in binding and phagocytosis was observed both by flow cytometry and confocal microscopy (see chapter 4). Figure 5.14 shows typical dot plots obtained from these experiments. In serum free conditions, the proportion of DCs making TNFα and IL12 was much lower than DCs cultured in medium with FCS. Addition of recombinant LBP to the serum free cultures partially restored both TNF-α and IL12 production. As already seen in earlier results shown in this chapter, a higher percentage of DCs that had internalised *N. meningitidis* was producing cytokines compared to the DCs that had not phagocytosed FITC bacteria indicating an important role for phagocytosis in cytokine production. In these experiments, DCs were cultured for a maximum of 7 hours because culture for longer than this time in serum free conditions resulted in lower viability of the DCs. The production of TNFα at this time was already high and easily detectable whereas the levels of IL12 production were significantly less than typically obtained after overnight culture.

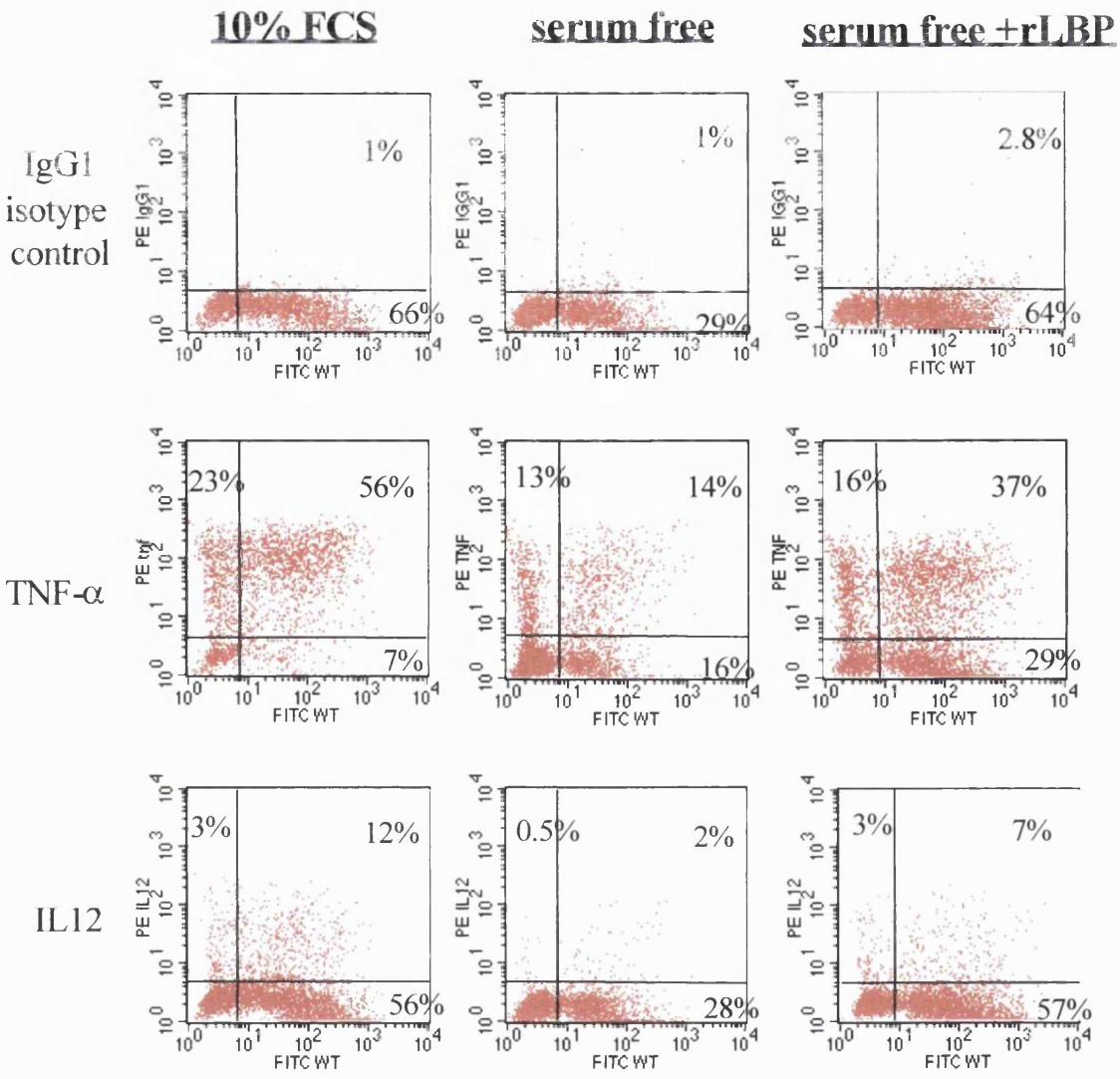

Figure 5.14 Role of LBP in phagocytosis and cytokine production by dendritic cells. DCs were cultured with *N. meningitidis* at a ratio of 1:100 in RPMI 1640/10% FCS, RPMI/2.5% BSA or RPMI/2.5% BSA with 1μg/ml of recombinant human LBP. The dot plots show phagocytosis and intracellular TNF-α and IL12 production analysed simultaneously by FACS after 7h. The quadrants were placed to separate cells that had associated with FITC bacteria from cells that had not phagocytosed FITC bacteria. Representative of 3 separate experiments is shown.

5.4 Discussion

The results of this thesis confirm that Group B *N. meningitidis* bacteria are potent activators of human DCs inducing expression of surface activation markers CD40, CD80, CD83 and CD86 and production of pro-inflammatory cytokines TNFα, IL1, IL6, IL8 and IL12 (Dixon *et al.*, 2001;Kolb-Maurer *et al.*, 2001;Unkmeir *et al.*, 2002). Various bacterial components have been implicated in DC activation but LPS is generally thought to be the most potent (Kadowaki *et al.*, 2001;Riva *et al.*, 1996;Verhasselt *et al.*, 1997). Purified LPS from *N. meningitidis* was however unable to stimulate optimal cytokine production, particularly IL12, suggesting that other bacterial components either alone or together with LPS may be required. In addition, direct contact between DCs and the bacteria may be required. To investigate this question, a transwell membrane culture system was used to separate bacteria from DCs. Under these conditions, diffusible components such as DNA, outer membrane particles, LPS etc released during storage of the bacteria will pass through the membrane and make contact with the DCs, whereas the intact bacteria cannot. In these experiments, IL12 and TNFα production was reduced to less than half compared to co-culture of bacteria and DCs allowing contact and internalisation. Curiously, IL6 production seemed to be more easily induced by soluble bacterial products such as LPS. The cytokine response to *lpxA-* was almost completely abolished in transwell cultures suggesting that LPS was the major soluble component inducing cytokine production in this system. In contrast, separation of intact bacteria and DCs in the transwell cultures did not have any effect on the increase in expression of surface activation markers CD86 and MHC Class II. These observations suggested that direct association of the bacteria with DCs was necessary for maximal cytokine

production but not costimulatory molecule expression. Interestingly, expression of CD83 was the only exception to this as greatly reduced expression of this marker was found in transwell experiments. Another explanation is that bacterial contact was indeed needed for full maturation of the DCs, as judged by the expression of CD83. Experiments with cytochalasin D showed that allowing bacterial contact, but no internalisation, was enough to induce maximal CD83 expression. It is of note that expression of CD83 was observed to be the most 'difficult' to induce and may therefore reflect special functions of this molecule. In fact, very little is known about its role in DC biology but it is one of the very few surface molecules considered to be expressed selectively by mature DCs. Both immunostimulatory as well as regulatory effects of the DC expressed CD83 molecule have been reported (Lechmann *et al.*, 2002a;Lechmann *et al.*, 2002b). Recent reports have however shown that CD83 is not strictly DC specific as activated T and B cells can also express it (Mahanonda *et al.*, 2002;McKinsey *et al.*, 2000).

In chapter 4, DCs were found to be very effective at internalising *N. meningitidis* raising the possibility that ingestion of the bacteria may be an important event for DC cytokine production. In addition, the restoration of phagocytosis *and* cytokine production in the LPS expressing HA3003 bacteria suggested a link between these two events (Fig. 5.1). This was addressed in several ways. First, intracellular cytokine staining of DCs co-cultured with FITC labelled *N. meningitidis* showed that cytokine production was confined mostly to DCs that has phagocytosed bacteria. Although the flow cytometric technique used in these experiments did not distinguish between bacteria on the cell surface and bacteria

inside the cell, confocal microscopy in chapter 4 showed that the majority of WT bacteria after co-culture with DCs had been phagocytosed. Secondly, cytochalasin D or toxin B blocking of phagocytosis inhibited IL12 and TNF-α production. Both of these agents dramatically reduced phagocytosis, but not binding, of the bacteria and inhibited cytokine production without an effect on DC viability. They also did not have an effect on DC expression of surface activation molecules in response to WT bacteria consistent with the notion that surface markers can be induced by purified LPS or by bacterial binding to the DC, whereas maximal IL12 and TNF require phagocytosis (Fig 5.4). Third, a crucial role for LPS expressed by the bacteria for phagocytosis and cytokine production was confirmed by the dependence of DC phagocytosis and cytokine production on LBP (Fig 5.14). A restoration of both phagocytosis and cytokine production by recombinant LBP suggests that these two events were linked.

Several groups have studied the role of phagocytosis and cytokine production by different cell types. Inhibition of phagocytosis by cytochalasins reduced cytokine production by epithelial and endothelial cells in response to both Gram-positive and Gram-negative bacteria (Kang and Kuramitsu, 2002;Schilling *et al.*, 2001;Yao *et al.*, 1995). Fulton *et al.* (1996) studied the role of phagocytosis in IL12 production by monocytes in response to *Mycobacterium tuberculosis* and showed that induction of IL12 was reduced by treatment with cytochalasin D. Interestingly, phagocytosis of large latex beads also induced significant IL12 in monocytes (Fulton *et al.*, 1996). In contrast, no cytokine production by DCs was found in response to polystyrene beads despite very efficient internalisation of the beads (Fig 5.5). These differences suggest different down-stream signalling

events in monocytes and DCs followed by phagocytosis and also highlight the importance of phagocytosis in IL12 production in general. Studies in murine macrophages have yielded very different data. No reduction in cytokine production was reported after inhibition of phagocytosis of *Listeria monocytogenes*, *Streptococcus suis* or *N. meningitidis* by macrophages (Demuth *et al.*, 1996;Peiser *et al.*, 2002;Segura *et al.*, 1999). It is likely that macrophages and DCs differ in their response to microbes. In one study, macrophages and DCs showed differential cytokine expression in response to Mycobacteria that modulated the subsequent T cell response (Giacomini *et al.*, 2001). Interestingly, only DCs, and not macrophages, were shown to produce IL12. In another study by Moore *et al.*(2000), CD14 negative macrophages were shown to respond to whole bacteria, but not soluble LPS. More importantly, inhibition of phagocytosis by cytochalasin abrogated the induction of cytokines in CD14 null cells, whereas no effect of cytochalasin was found in 'normal' macrophages expressing CD14 (Moore *et al.*, 2000). One of the characteristics of monocyte differentiation into DCs is the loss of CD14 and therefore the CD14 deficient macrophages in this study may have become more 'DC like'.

Very few reports have studied the role of phagocytosis in DC activation. Yrlid and Wick (2002) studied the importance of phagocytosis of *Salmonella typhimurium* in cytokine production by murine splenic and mesenteric lymph node DC subsets. The authors concluded that bacterial contact, but not phagocytosis, was necessary for cytokine production. Almost complete inhibition of cytokines was seen in transwell system similar to the one used in this thesis whereas a partial reduction (about 30-50%) was found after inhibition with

cytochalasin D (Yrlid and Wick, 2002). The similar data in this thesis led to the interpretation that phagocytosis of *N. meningitidis* was necessary for maximal cytokine production, although some cytokine production was also observed in transwell experiments as well as after cytochalasin D treatment. These results suggested that internalisation is not absolutely required, but leads to optimal cytokine production in response to *Salmonella*. A recent report by Colino and Snapper (2002) showed that inhibition of phagocytosis of *S. pneumoniae* by cytochalasin D resulted in more than 80% reduction in IL12 production by DCs, whereas IL6 was unaffected. In addition, surface marker expression was unaffected by cytochalasin treatment, a finding similar to the data shown in this chapter. These results show that production of IL12 by human DCs in response to a Gram-positive extracellular bacterium *S. pneumoniae* also requires internalisation of the bacteria.

The requirement for internalisation of bacteria is consistent with the report that human toll-like receptors (TLRs) may be expressed inside cells rather than on the cell membrane. TLR2 is not required for phagocytosis but is recruited specifically to macrophage phagosomes where it is thought to signal the cell for cytokine production by sampling the content of the phagosome (Underhill *et al.*, 1999). It has been shown recently that TLR9 is expressed in macrophage endosomes/lysosomes where it signals in response to CpG DNA (Ahmad-Nejad *et al.*, 2002;Heil *et al.*, 2003). Similarly, LPS has been shown to co-localise with TLR4 within the Golgi complex of epithelial cells (Hornef *et al.*, 2002). Intact Gram-negative bacteria are more potent inducers of NF-κB translocation than purified LPS indicating that phagocytosis of whole bacteria leads to increased cell

signalling compared to purified bacterial components (Hofer *et al.*, 2001). This suggests that the signalling events might not occur on the cell surface, but rather in the phagosome or lysosome after bacterial internalisation. The role of TLRs in DC responses to meningococcal bacteria was studied in this thesis and the results presented in chapter 7.

The results presented in this chapter may be of particular importance in the design of future *N. meningitidis* group B vaccines. Current strategies are predominantly directed at identifying immunogenic bacterial sub-units or outer membrane vesicle (OMV) vaccines. However, the processing of such vaccines by DCs will be critical for generating an effective and long lasting immune response. Consideration of how and what to deliver to DCs to achieve this aim will be crucial to the development of a successful *N. meningitidis* group B vaccine. Meningococcal LPS has received attention as a potential vaccine candidate and has in particular been recognized as a very strong adjuvant. However, the endotoxic properties of LPS are the major drawbacks for using it in human vaccines. In the next chapter, truncated oligosaccharide chain and lipid A mutants were used to study the relative role of sugar residues and lipid A in phagocytosis and cytokine production by DCs.

Chapter 6

Role of *Neisseria meningitidis* LPS oligosaccharide core and lipid A in phagocytosis and cytokine production by dendritic cells

6.1 Introduction

Capsular polysaccharide vaccines against *N. meningitidis* group B organisms have failed due to poor immunogenicity and vaccines based on outer membrane vesicles (OMV) are currently under investigation. Recently, a new hexavalent OMV vaccine was developed in the Netherlands and has now been shown to protect from the dominant serosubtypes of group B organisms isolated from both the UK and the Netherlands (Cartwright *et al.*, 1999;Van Der Ley P. *et al.*, 1995). Although this OMV vaccine seems promising in combating meningococcal disease, the requirement of four doses remains its major drawback. The relatively low immunogenicity could, however, be expected to be improved by inclusion of a potent adjuvant. Meningococcal LPS has received attention as a potential vaccine candidate due to its capacity to induce LPS-specific immune responses as well as being a potent adjuvant. The endotoxic effects of LPS are however its major drawback for use in human vaccines. In addition to its role as a virulence factor, LPS is held responsible for the severe pathophysiological reactions during host response to this organism.

Meningococcal LPS is composed of a hydrophobic lipid A portion that anchors it to the bacterial outer membrane and a hydrophilic oligosaccharide core, which is exposed to the bacterial surface (Figure 6.1 and Erridge *et al.*, 2002). Inclusion of the native oligosaccharide chain in vaccine preparations is unadvisable due to its structural similarity with host glycolipid antigens (Mandrell and Apicella, 1993). Genetic engineering of the LPS oligosaccharide biosynthesis pathways has enabled the production of *N. meningitidis* mutants expressing truncated oligosaccharide chains (Fig. 6.1). The majority of the endotoxic and adjuvant

activity of LPS appear to be determined by its lipid A part (Galanos *et al.*, 1985). A unique set of *N. meningitidis* lipid A mutants with highly improved pharmacological properties have been generated by Steeghs and collegues. Replacement of the meningococcal *lpxA* gene with its *Pseudomonas aeruginosa* or *Escherichia coli* homologue resulted in lipid A with shorter or longer O-linked fatty acids, respectively, that showed reduced toxicity with retained adjuvant activity (Figure 6.2 and Steeghs *et al.*, 2002). Furthermore, inactivation of the *lpxL1* and *lpxL2* genes, involved in lipid A acyloxyacylation, resulted in mutant bacterial species with penta- or tetra-acylated lipid A, respectively, showing altered biological activity (Fig. 6.2 and van der Ley *et al.*, 2001). In addition, a *N. meningitidis* mutant completely lacking LPS due to inactivation of the meningococcal *lpxA* gene was isolated. Thus, modification of the lipid A portion as well as the oligosaccharide portion of meningococcal LPS has opened new possibilities for improvement of meningococcal vaccine efficacy.

Dendritic cells have become of major interest because of their role in initiation of immune responses both during natural infection and in response to vaccination. Immune responses to bacteria are initiated by DCs, which phagocytose and process bacterial antigens for presentation to T cells. The results in this thesis have shown that *N. meningitidis* LPS plays a major role during interactions with human DCs. By using the LPS-deficient *N. meningitidis* mutant, it was shown in chapter 4 that the whole bacteria must express LPS in order to be internalised. Moreover, this internalisation was demonstrated to be required for optimal IL12 and TNF-α production (chapter 5). To determine which part of LPS is required for the internalisation and cytokine production by DCs, a set of *N. meningitidis*

oligosaccharide and lipid A mutants was tested. The results of the chapter hope to provide novel information for the development of future meningococcal vaccines.

6.2 Materials and methods

6.2.1 Bacterial strains and culture with dendritic cells

The oligosaccharide core (Jennings *et al.*, 1993;Jennings *et al.*, 1995;Pavliak *et al.*, 1993;Stojiljkovic *et al.*, 1997;van der Ley *et al.*, 1997) and lipid A (Steeghs *et al.*, 1998;Steeghs *et al.*, 2002;van der Ley *et al.*, 2001) mutant bacterial strains have been described previously. Structures of the oligosaccharide core mutants used in this chapter are shown in figure 6.1 and the lipid A mutants in figure 6.2. In addition, *N. meningitidis* strain M992 expressing the L6 LPS immunotype was used as indicated. All strains were grown overnight, fixed with 0.5% PFA and labelled with FITC.

DCs were incubated with the strains at 1:50, 1:100 or 1:200 DC:bacteria ratio as indicated. Phagocytosis and cytokine production were analysed as described in general materials and methods chapter.

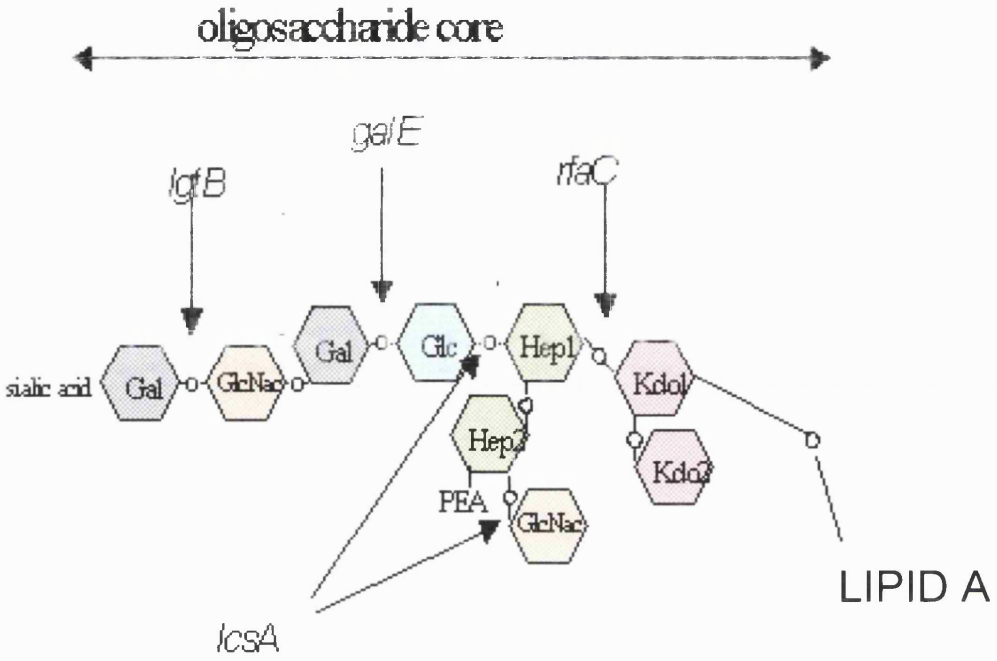

Figure 6.1 Oligosaccharide core mutant *Neisseria meningitidis* H44/76 strains used in this study. The WT expresses the full length sugar chain. Sialylation of the WT LPS is only obtained by growing the bacteria in CMP-Nana supplemented agar plates. The *lgtB*, *galE*, *icsA* and *rfaC* strains were obtained by genetic engineering to inactivate the genes required for oligosaccharide chain biosynthesis. In addition, M992 strain was used, which is naturally occurring strain with L6 LPS immunotype and expresses GlcNac as its terminal sugar.

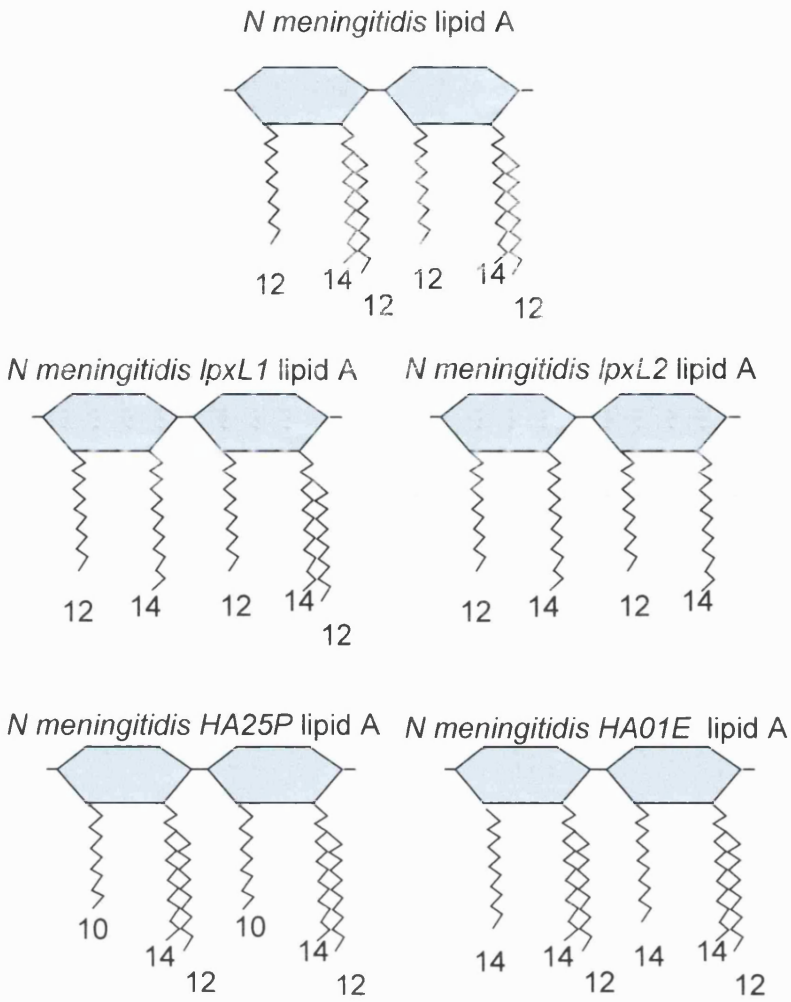

Figure 6.2 Lipid A mutant strains derived from *Neisseria meningitidis* H44/76 used in this study. The schematic pictures show the length and distribution of fatty acyl chains in the *N. meningitidis* strains used in this thesis. An additional mutant, *lpxL1galE* was also used. This strain has the same lipid A structure as the *lpxL1* mutant but expresses a truncated *galE* oligosaccharide chain, as did the *lpxL2* (see Fig 6.1). The WT *N. meningitidis* expresses a symmetrical, hexa acylated lipid A and is shown on top. Numbers indicate the quantity of carbon atoms in each fatty acid chain.

6.3 Results

6.3.1 Phagocytosis of oligosaccharide core mutant strains of *Neisseria meningitidis* by dendritic cells

The effect of the individual meningococcal LPS oligosaccharides on phagocytosis by DCs was studied by using a set of mutants expressing truncated oligosaccharide chains. The flow cytometric technique together with confocal microscopy was used to investigate binding and internalisation of oligosaccharide mutants by human DCs. Immature DCs were co-cultured with FITC labelled mutant strains derived from the WT *N. meningitidis* H44/76 at a DC/bacteria ratio of 1:50 and 1:200 and analysed by FACS for the presence of DC associated FITC bacteria (Figure 6.3).

Time and dose dependent increase in DC association with WT and oligosaccharide mutants was observed. Interestingly, the *lgtB* mutant showed consistently higher DC association than the WT or all the other sugar mutants at both concentrations used. Compared to the WT, no difference in DC association was observed with the *icsA* mutant. In contrast, the *galE* and *rfaC* mutant were slightly reduced in DC association at the lower concentration, however at high concentration no difference was seen.

To discriminate between bacterial binding and internalisation, confocal microscopy was used (Figure 6.4). As compared to the WT bacteria, the majority of the DC had internalised *lgtB* mutant and each DC had more bacteria inside

already after 1h of incubation confirming the results by FACS. In addition to high level of internalisation, surface adherence remained typically high for the *lgtB* mutant bacteria throughout the time course. In the case of the *galE* and *icsA* mutant, DC internalisation was less efficient as observed for the WT. Whereas the WT was mostly internalised at 4h, a large proportion of the *galE* and *icsA* mutant remained attached to the cell surface. Still, this difference in internalisation efficacy at the early time points did not result in reduced internalisation after 24h. In contrast, binding and internalisation of the *rfaC* mutant by DCs was lower as with the WT throughout the entire time course.

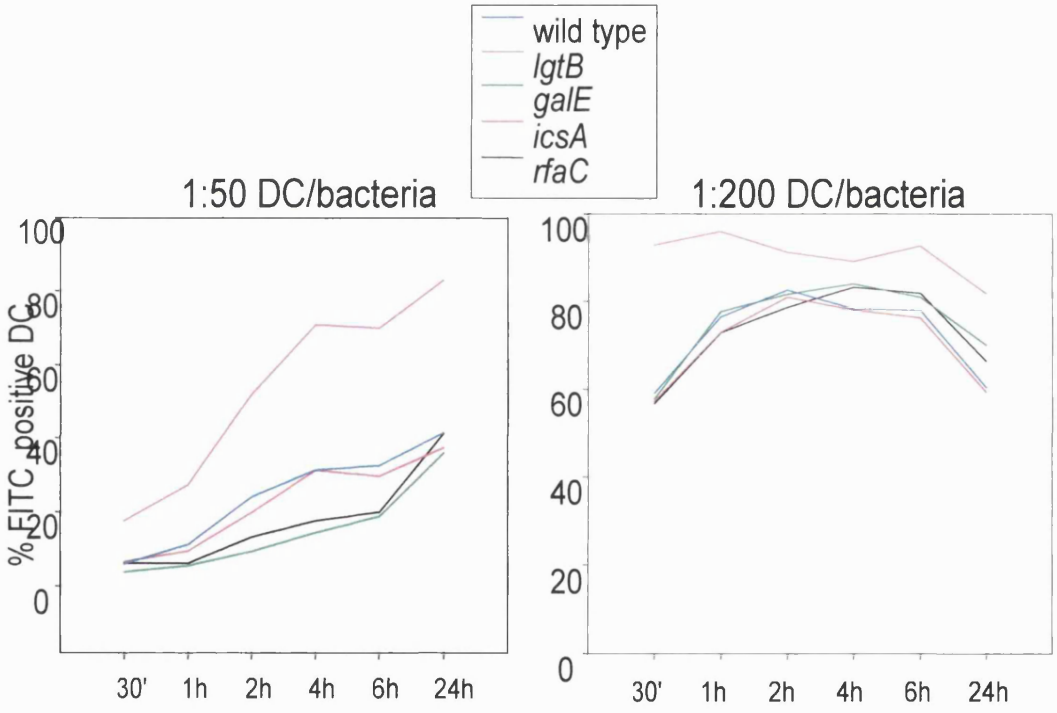

Figure 6.3 Binding and internalisation of *Neisseria meningitidis* oligosaccharide mutants by dendritic cells. DC were co-cultured with FITC labelled bacteria at 1:50 and 1:200 ratio. The cells were then fixed, washed and analysed by flow cytometry. Representative results from a minimum of three separate experiments are shown.

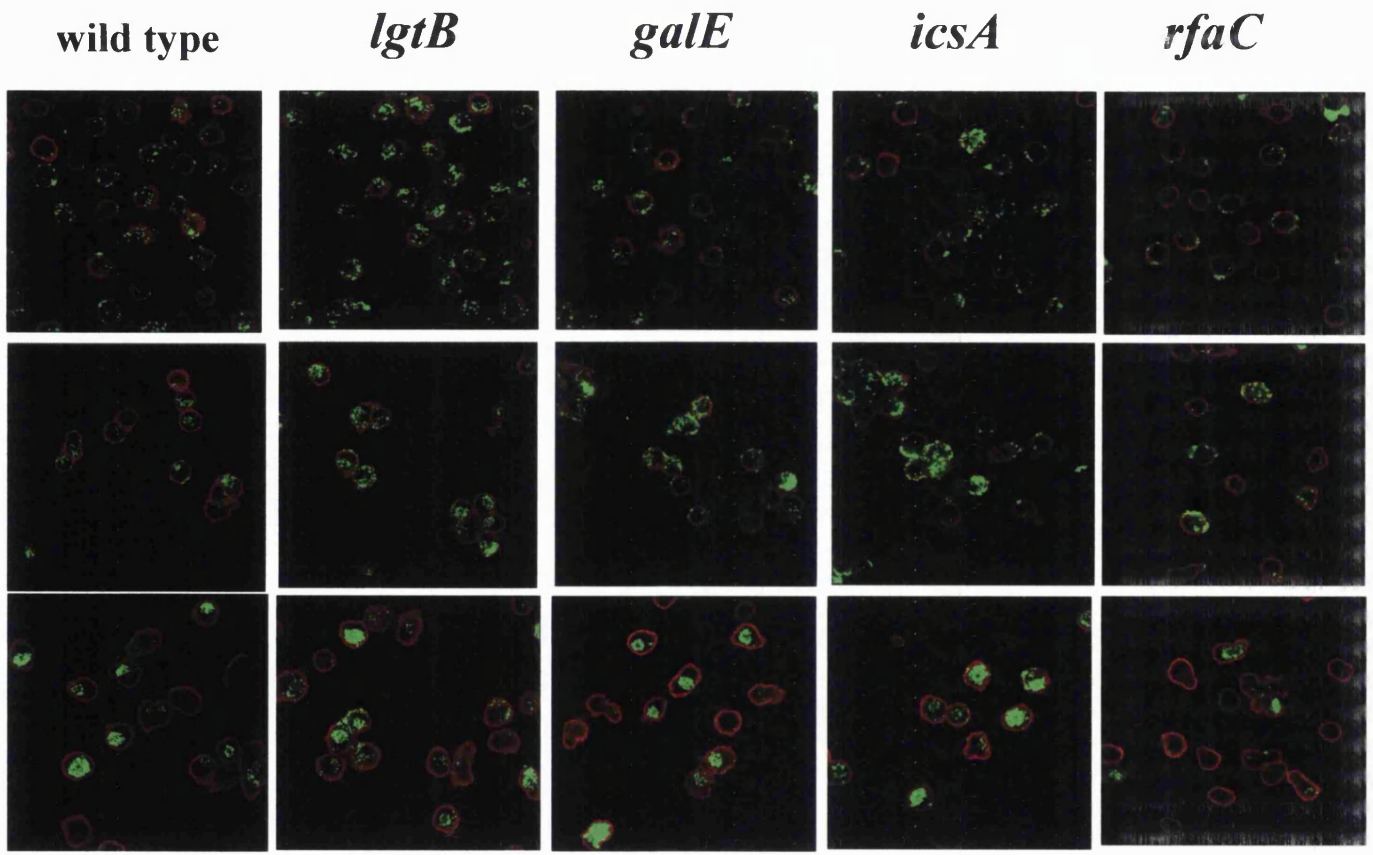

Figure 6.4 Binding and internalisation of *Neisseria meningitidis* oligosaccharide mutants by dendritic cells. DC were co-cultured with FITC labelled bacteria at 1:200 ratio. The cells were then fixed, washed, surface stained for MHC Class II and analysed by confocal micoscopy. Representative images from three separate experiments are shown.

6.3.2 Phagocytosis of *Neisseria meningitidis* lipid A mutant strains by dendritic cells

The role of lipid A fatty acyl number and distribution on DC phagocytosis of *N. meningitidis* was studied by using lipid A mutants *lpxL1, lpxL1/galE* and *lpxL2/galE*. DCs were incubated with the WT and mutant bacteria at DC/bacteria ratio of 1:50 and 1:200 (Figure 6.5). A time and dose dependent increase in bacterial association with the DC was found by FACS. Still, DC association of all lipid A mutants was less efficient as compared to the WT. Interestingly, differences in DC association were found between the lipid A mutants. At the low concentration, the *lpxL1* mutant associated more rapidly and at higher level with the DC than *lpxL2/galE*, whereas the *lpxL1/galE* mutant was found between these two. These differences in DC association between the lipid A mutants were even more marked at the high concentration. However, at this concentration differences were no longer seen in the level of DC association between the WT and the *lpxL1* and *lpxL1/galE* mutant after 4h of incubation, whereas DC association of the other mutants remained lower than the WT until 24h.

Figure 6.6 shows the internalisation of the lipid A mutants analysed by confocal microscopy. Lower association and less internalisation of the *lpxL1*, *lpxL1/galE* and *lpxL2/galE* mutant as compared to the WT was seen particularly at the early time points. After 4h of incubation, WT and the *lpxL1* mutant were internalised to similar extent, consistent with the FACS results. However, in contrast to the *lpxL1* mutant, the majority of the *lpxL1/galE* mutant was found to adhere to the cell surface without internalisation after 4h. Binding without internalisation was also observed with the *lpxL2/galE* mutant after 4h of incubation. By 24h, *lpxL1*,

lpxL1/galE and *lpxL2/galE* mutants were all internalised although by slightly fewer DCs than with the WT.

Taken together, all lipid A mutants showed reduced association and internalisation by DC compared to the WT and it seemed that the specific acylation pattern of the lipid A domain of LPS plays a role in DC association and internalisation of *N. meningitidis*.

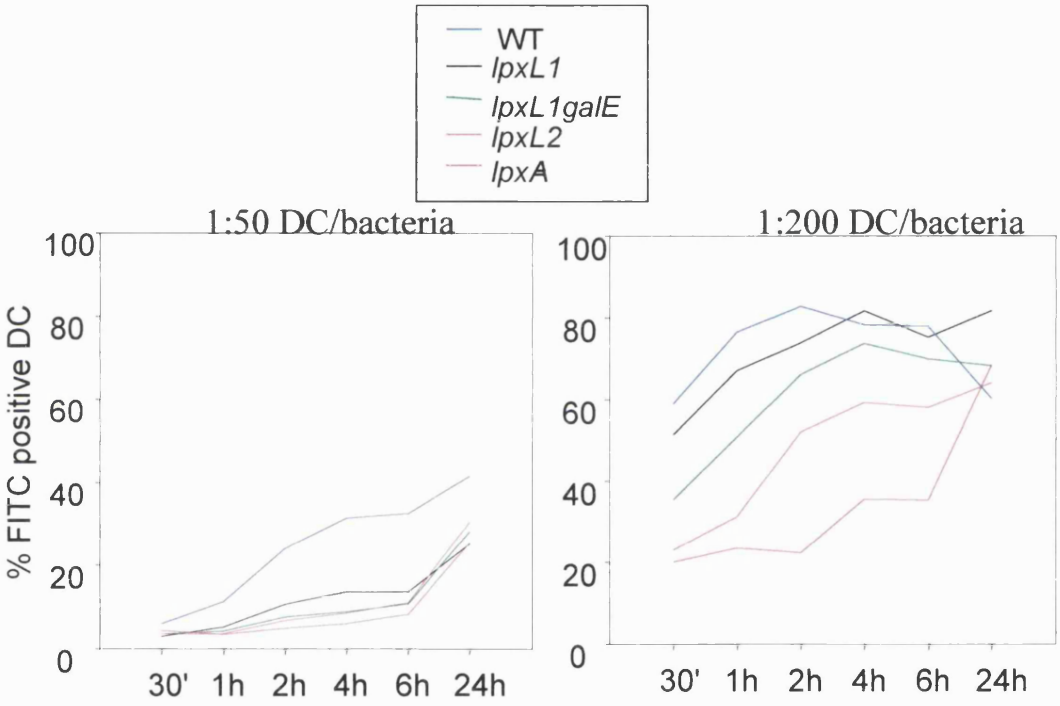

Figure 6.5 Association of *Neisseria meningitidis lpxL* mutants with dendritic cells. DC were co-cultured with FITC labeled WT *lpxL1*, *lpxL2, lpxL1galE* and *lpxA*- mutant bacteria at the ratio of 1:50 or 1:200 (DC/bacteria) for 30', 1h, 2h, 4h, 6h and 24h. WT and *lpxA*- are shown for comparison. The cells were then fixed, washed and analysed by flow cytometry. Representative experiment from three separate experiments with 3 different donors is shown.

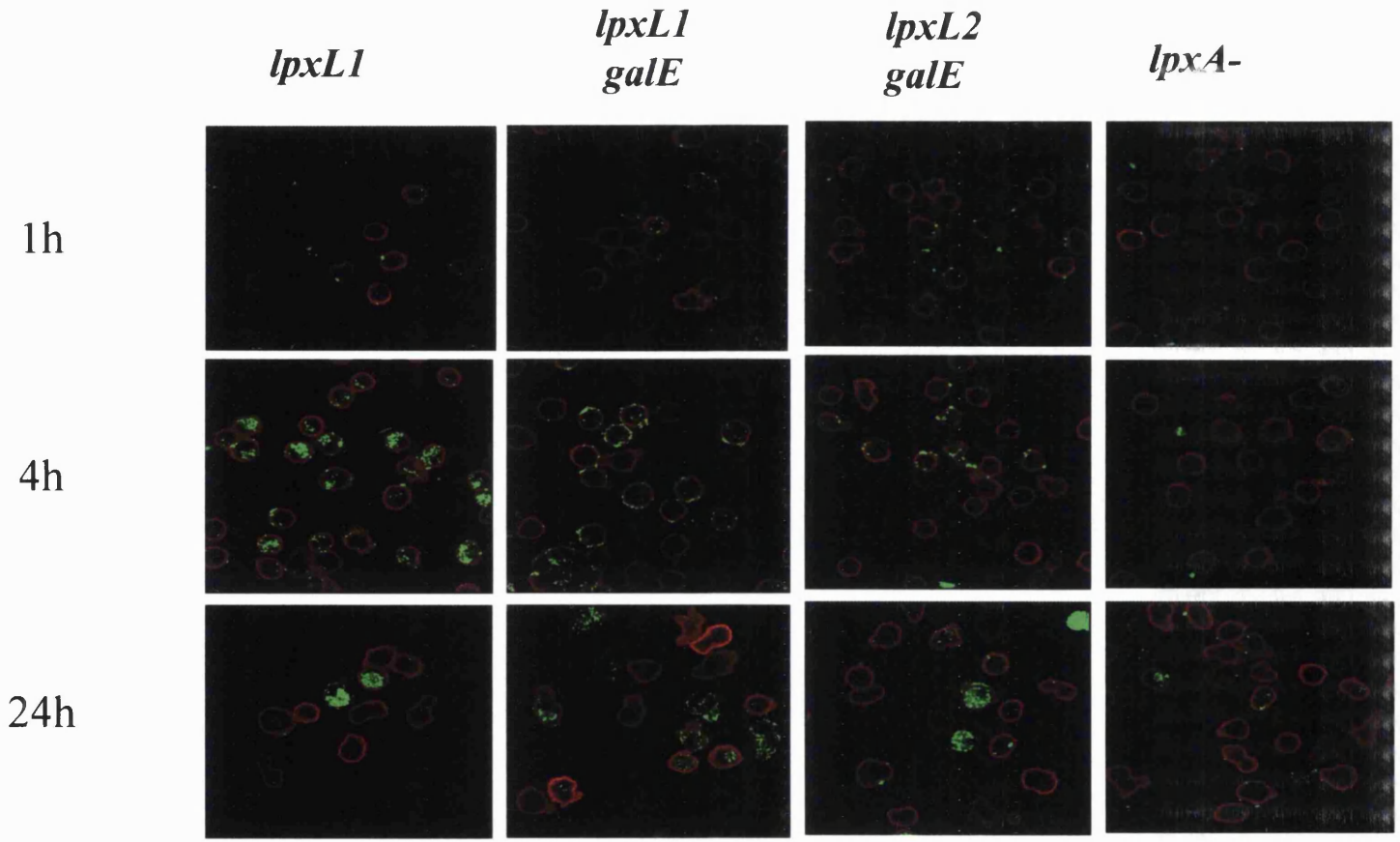

Figure 6.6 Internalisation of *Neisseria meningitidis lpxL* lipid A mutants with dendritic cells. DC were co-cultured with FITC labelled WT *lpxL1*, *lpxL2, lpxL1galE* and *lpxA-* mutant bacteria at the ratio of 1:200 (DC/bacteria) for 1h, 4h and 24h. WT and *lpxA-* are shown for comparison. Confocal image of DC after co-culture with FITC labelled WT *N. meningitidis* at 1:200 DC/bacteria ratio. After each time point, the cells were allowed to adhere to glass slides and then stained for MHC Class II (Texas Red). Representative experiment from three separate experiments with 3 different donors is shown.

6.3.3 Phagocytosis of lipid A mutants expressing foreign *lpxA* genes by dendritic cells

To study the effect of the fatty acyl length on phagocytosis by DCs, *N. meningitidis* strains expressing lipid A with either shorter (*lpxA* from *Pseudomonas aeruginosa*, HA25P) or longer (*lpxA* from *Escherichia coli*, HA01E) O-linked fatty acids were used. Figure 6.7A shows DC association with HA25P and HA01E mutants expressing foreign *lpxA* genes at time points between 30 minutes and 24h. At 1:200 ratio, both mutants were clearly less efficiently associated with the DC when compared to the WT. The HA25P mutant was more efficiently associated with the DC than the HA01E mutant bacteria. DC association with the HA01E, as well as the *lpxA*- mutant, remained very low until at 18h when higher levels were seen. Similar patterns were observed also at the lower concentration of 1:50 DC/bacteria ratio (not shown).

Internalisation of HA25P and HA01E mutant *N. meningitidis* by DC was analysed by confocal microscopy (Figure 6.7B). After 1h of stimulation, FITC labelled HA25P bacteria were found adherent to the cell surface and some had already been internalised by the DC. In contrast, very few HA01E were found adherent or internalised by the DC. By 4h, the HA25P mutant bacteria were clearly internalised by the DC, whereas the HA01E bacteria remained poorly attached to the DC. By 24h, both the HA25P and HA01E bacteria were found inside the DC, although the HA01E mutant bacteria to a lesser extent. Interestingly, internalisation of the HA01E bacteria by DC was similar to the *lpxA*- mutant. Taken together, all lipid A mutants showed reduced internalisation by DC compared to WT bacteria. These results suggest that the specific acylation pattern of the lipid A moiety of LPS, as well as the length of the fatty acyls play a major role in internalisation of *N. meningitidis* by DC.

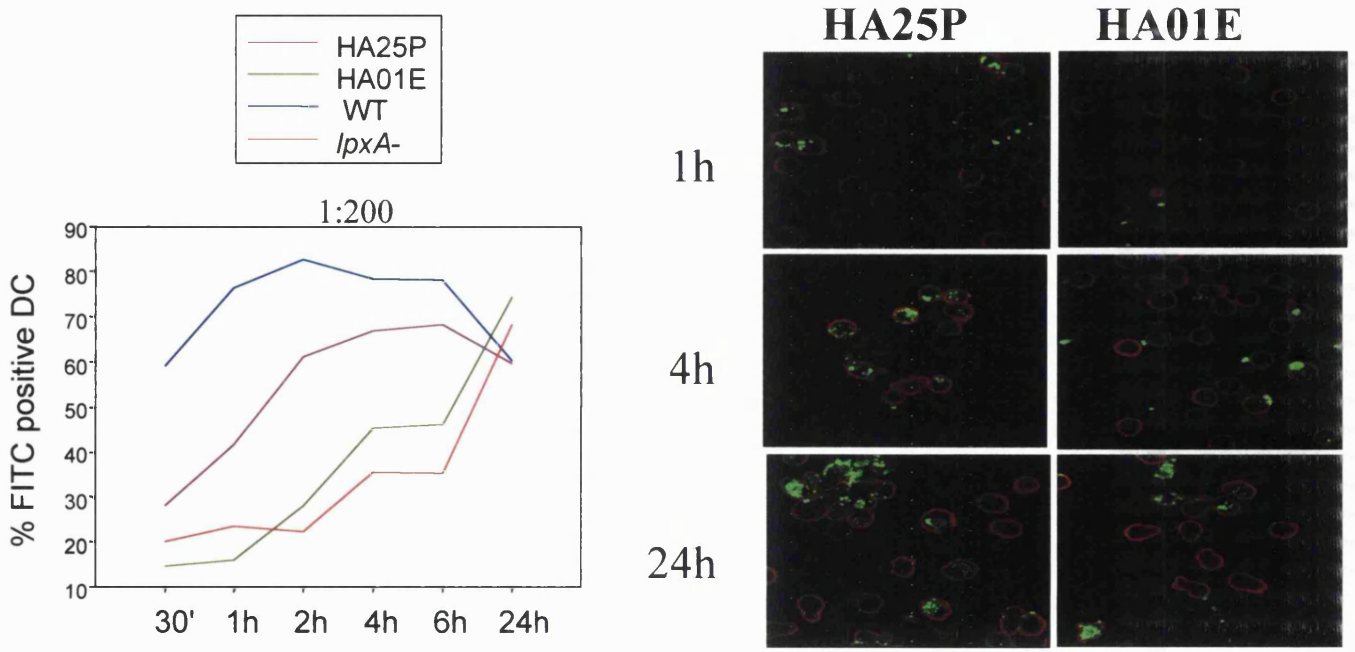

Figure 6.7 Dendritic cell internalisation of *Neisseria meningitidis* lipid A mutants expressing foreign *lpxA* genes. DC were co-cultured with FITC labelled HA25P and HA01E mutant bacteria at the ratio of 1:200 (DC/bacteria) for 30', 1h, 2h, 4h, 6h and 24h. The cells were then fixed, washed and analysed by flow cytometry **(A)**. WT and *lpxA-* are shown for comparison. Confocal image of DC after co-culture with FITC labelled WT *N. meningitidis* at 1:200 DC/bacteria ratio. After each time point, the cells were allowed to adhere to glass slides and then stained for MHC Class II (Texas Red) **(B)**. Representative experiment from three separate experiments with 3 different donors is shown.

6.3.4 Cytokine production by dendritic cells in response to oligosaccharide core and lipid A mutants

The results presented in the previous chapters have shown that maximal cytokine production by DCs in response to *N. meningitidis* requires LPS and bacterial internalisation. Clear differences in the internalisation of the lipid A and sugar mutants compared to the WT bacteria were observed in this chapter. Cytokine production in response to the mutant bacteria in the culture supernatants was therefore investigated.

DCs were co-cultured with the mutant and WT bacteria at 1:200 ratio for 18h and the supernatants collected for analysis of soluble IL12, IL10, TNF-α and IL6 by ELISA (Figure 6.8). The results are shown as the percentage of cytokine production compared to the WT (WT=100%) (Figure 6.8A). All sugar and lipid A mutants induced less IL12 by DCs compared to the WT bacteria. Interestingly, the shorter the oligosaccharide core expressed by the bacteria, the less they were able to induce IL12 by the DCs. All the oligosaccharide core mutants induced relatively more IL12 compared to the lipid A mutants with the exception of HA25P, which induced IL12 similar to *lgtB* and *galE*. The *lpxL1* mutant induced more than 50% higher levels of IL12 compared to the *lpxL1/galE* or the *lpxL2/galE*. Similar results to IL12 were obtained for IL10 (6.8A), TNF-α (6.8B) and IL6. In addition, similar pattern of cytokine production was observed with lower ratio (1:50) of DC/bacteria and by intracellular cytokine staining (not shown). The *lpxA*- induced the least amount of all cytokines confirming results shown in previous chapter of this thesis.

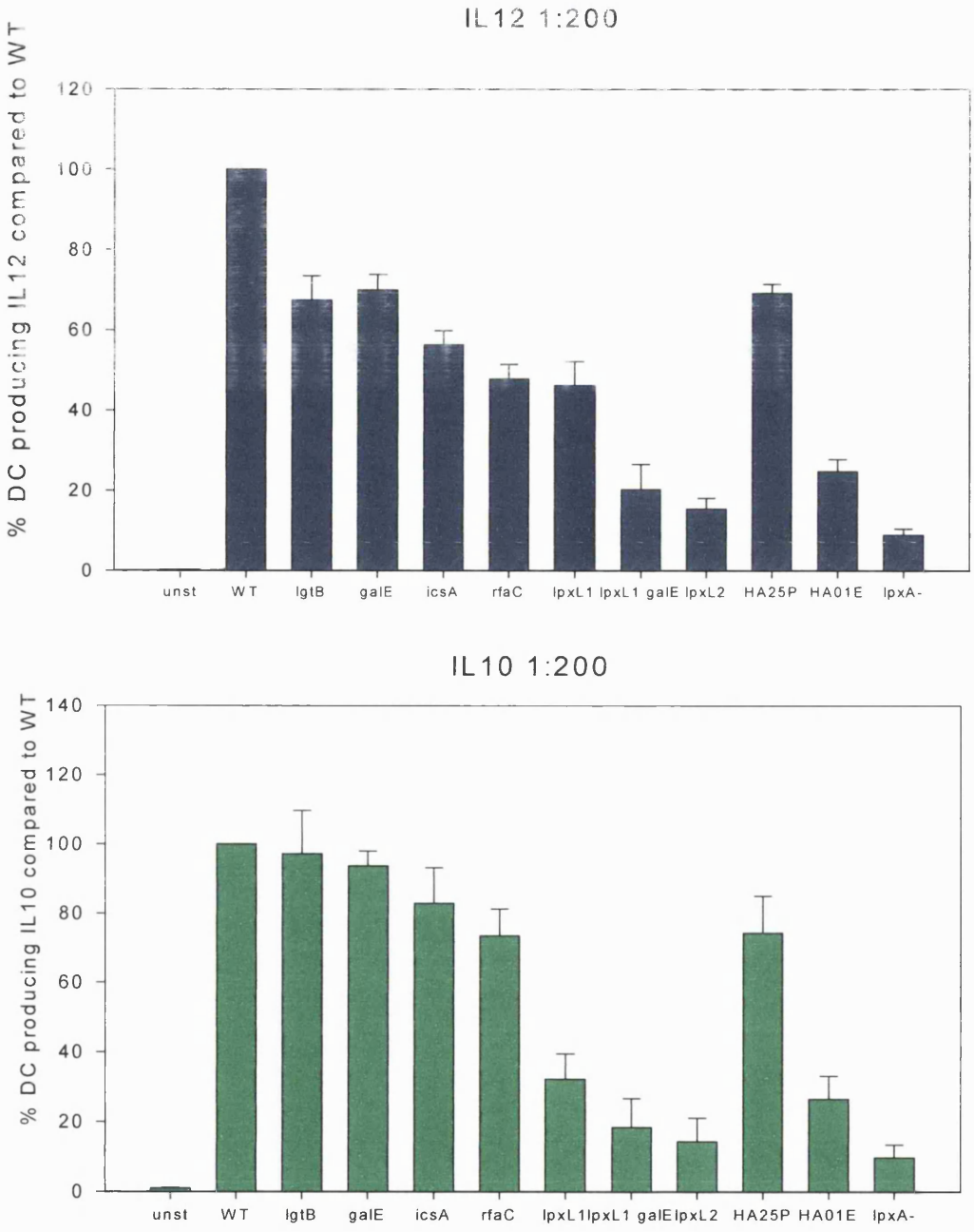

Figure 6.8A production of IL12 and IL10 by dendritic cells in response to oligosccharide core and lipid A mutant strains of *Neisseria meningitidis.* DCs were cultured for 18h in the presence of bacteria at 1:200 (DC/bacteria). The supernatants were then collected and analysed by ELISA. IL12 and IL10 production are shown. The data show the mean and SEM from three separate experiments performed amongst three different donors.

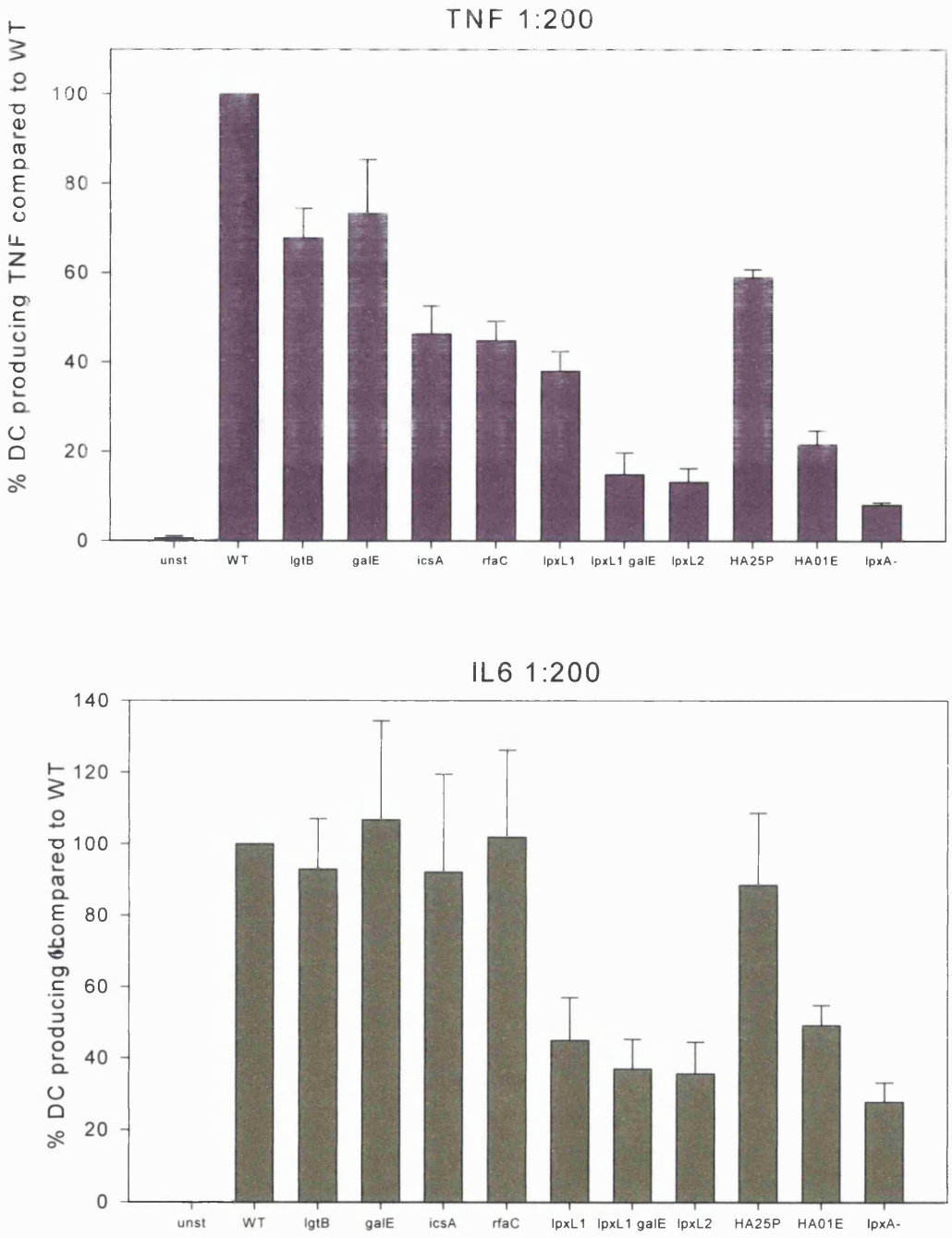

Figure 6.8B production of TNF-α and IL6 by dendritic cells in response to oligosccharide core and lipid A mutant strains of *Neisseria meningitidis.* DCs were cultured for 18h in the presence of bacteria at 1:200 ratio. The supernatants were then collected and analysed by ELISA. TNF-α and IL6 production are shown. The data show the mean and SEM from three separate experiments performed amongst three different donors.

6.3.5 Phagocytosis and cytokine production in response to *lgtB* and M992 strains of *N. meningitidis* by dendritic cells

Enhanced phagocytosis by DCs in response to the *lgtB* mutant was shown in the previous results (Figure 6.3 and 6.4). This mutant expressed N-acetyl glucosamine (GlcNac) as the terminal sugar of LPS, which may be the reason for its increased binding and internalisation by the DCs. Naturally occuring *N. meningitidis* strain M992, which expresses the L6 LPS immunotype with GlcNac as a terminal sugar, was therefore used to test this hypothesis. Both phagocytosis and cytokine production were analysed and compared with the WT and *lgtB* mutant bacteria. Figure 6.9 shows the phagocytosis time course of *lgtB*, M992 and WT bacteria after co-culture with DCs at 1:100 DC/bacteria ratio. Both *lgtB* and M992 were phagocytosed more efficiently by the DCs throughout the entire time course. These results were confirmed by confocal microscopy (not shown). Of note, in these experiments the bacteria were phagocytosed less efficiently than seen previously due to a possible deterioration in the quality of FCS batch in use.

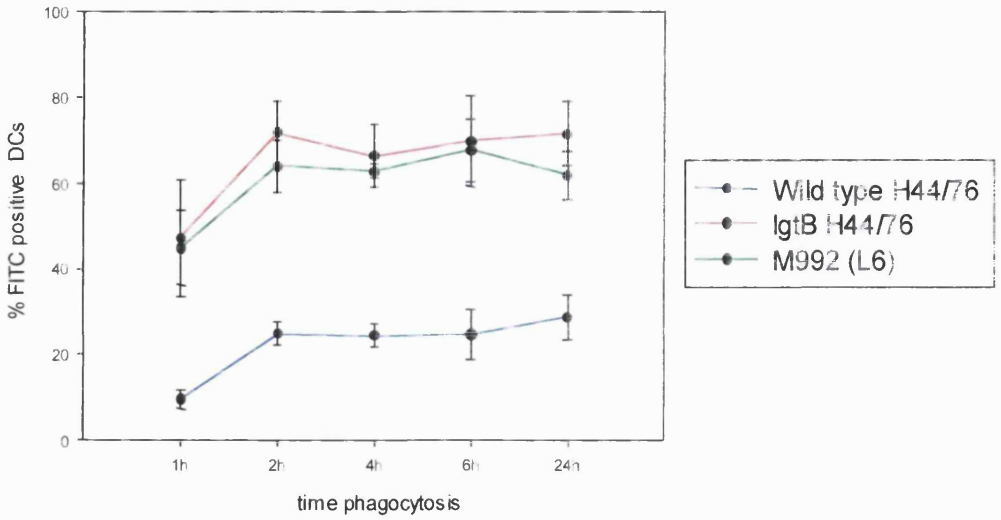

Figure 6.9 Phagocytosis of the *lgtB*, M992 and WT *Neisseria meningitidis* by dendritic cells. DCs were co-cultured with FITC labelled bacteria for 1h, 2h, 4h, 6h and 24h at 1:100 ratio, fixed, washed and then analysed by FACS. The data show the mean and SEM from three separate experiments performed with DCs from three different donors.

Next, IL12 and TNF-α production in response to the WT, M992 and *lgtB* was analysed. Interestingly, only minor differences were found in cytokine production by DCs in response to these strains (Fig 6.10). By intracellular staining, a tendency of increased IL12 and TNF-α production was seen in response to both M992 and lgtB compared to the WT bacteria but no difference was found by ELISA. The cytokine levels were typically lower than previously observed probably due to the reduced phagocytosis by DCs.

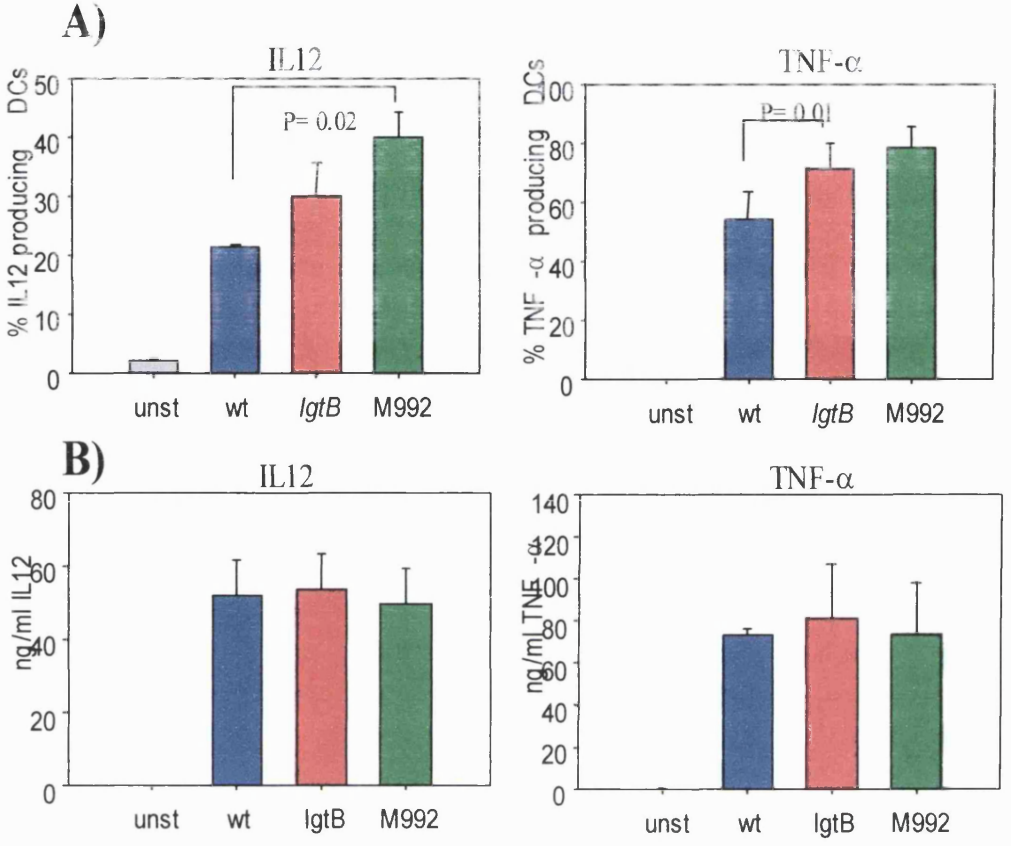

Figure 6.10 Cytokine production by dendritic cells in response to *lgtB*, M992 and WT *Neisseria meningitidis*. DCs were cultured for 18h with 1:100 ratio of DC/bacteria. The cells were then fixed, permeabilised and stained for IL12 and TNF-α intracellularly before analysis by FACS **(A)**. Alternatively, the supernatants were collected and analysed by ELISA **(B)**. The data show the mean and SEM from three separate experiments performed amongst three different donors.

6.3.6 Simultaneous analysis of internalisation and intracellular cytokine production by dendritic cells in response to mutant *Neisseria meningitidis*

ELISA analysis of cytokines measured the total amount of cytokines secreted by the DCs in response to the bacteria. With this method, it was not possible to distinguish cytokine production by DCs that had phagocytosed bacteria from the DCs that had not. In order to study the relationship between internalisation and cytokine production in further detail, simultaneous measurements of IL12 and TNF-α as well as phagocytosis by DCs in response to the WT, lipid A and sugar mutants were performed.

DCs were co-cultured with FITC labelled bacteria at a ratio of 1:200 for 18 hours and then stained for intracellular production of IL12 (Fig 6.11A) and TNF-α (Fig 6.11B). The quadrants were placed to separate DCs that were FITC positive from the DCs that were not. The percentages given are the relative amounts of cytokines produced by DCs that had not internalised bacteria (left side of quadrant) or that had internalised *N. meningitidis* (right side of quadrant). In this way, it was also possible to compare the effect of oligosaccharide and lipid A modifications on cytokine production by DCs once the bacteria were inside the cells. It was assumed that the bacteria were inside the DCs after 18h of stimulation based on confocal microscopy. The results for *lpxA*- mutant bacteria are shown together with the lipid A mutant strains, whereas the results for the WT bacteria are shown in conjunction with the sugar mutants.

For all the mutants, a higher percentage of DCs that had internalised bacteria were producing IL12 and TNF-α compared to the DCs that had not internalised bacteria (Figure 6.11). In this experiment, 42% of the DCs that had not internalised WT bacteria were producing IL12, whereas 69% of the DCs that had internalised did so. Although DCs that had not internalised bacteria were also producing TNF-α, virtually all of the DCs that had internalised *N. meningitidis* were producing high levels of TNF-α.

Next, cytokine production was studied in DCs that had phagocytosed bacteria (right side quadrant). Once phagocytosed (i.e DC FITC positive), all the sugar mutants, except the *rfaC,* were able to induce similar level of IL12 compared to the WT. In this experiment, 69% of the DCs that had internalised the WT bacteria were producing IL12, whereas 55% of the DCs that had internalised *rfaC* were producing IL12. These results suggested that the oligosaccharide chain of the bacteria once internalised did contribute to the IL12 response and had to contain at least the two heptoses and PEA in order to induce maximal IL12. This was not the case with TNF-α, which was induced by all the sugar mutants by over 90% of the DCs that had internalised the bacteria. Similar levels (%) of IL12 and TNF-α were produced by DCs that had phagocytosed *lpxL1* and HA25P lipid A mutants compared to the WT. In contrast, *lpxL1/galE*, *lpxL2/galE* and HA01E induced less IL12 and TNF-α than the WT after being phagocytosed, although the differences were more marked with IL12. No major differences were seen between the different strains in their ability to induce cytokines by DCs that had not phagocytosed bacteria. These results suggested that the structure of lipid A of LPS plays a role in DC cytokine production after internalisation of *N. meningitidis*, whereas the oligosaccharide chain did not seem to play a major role.

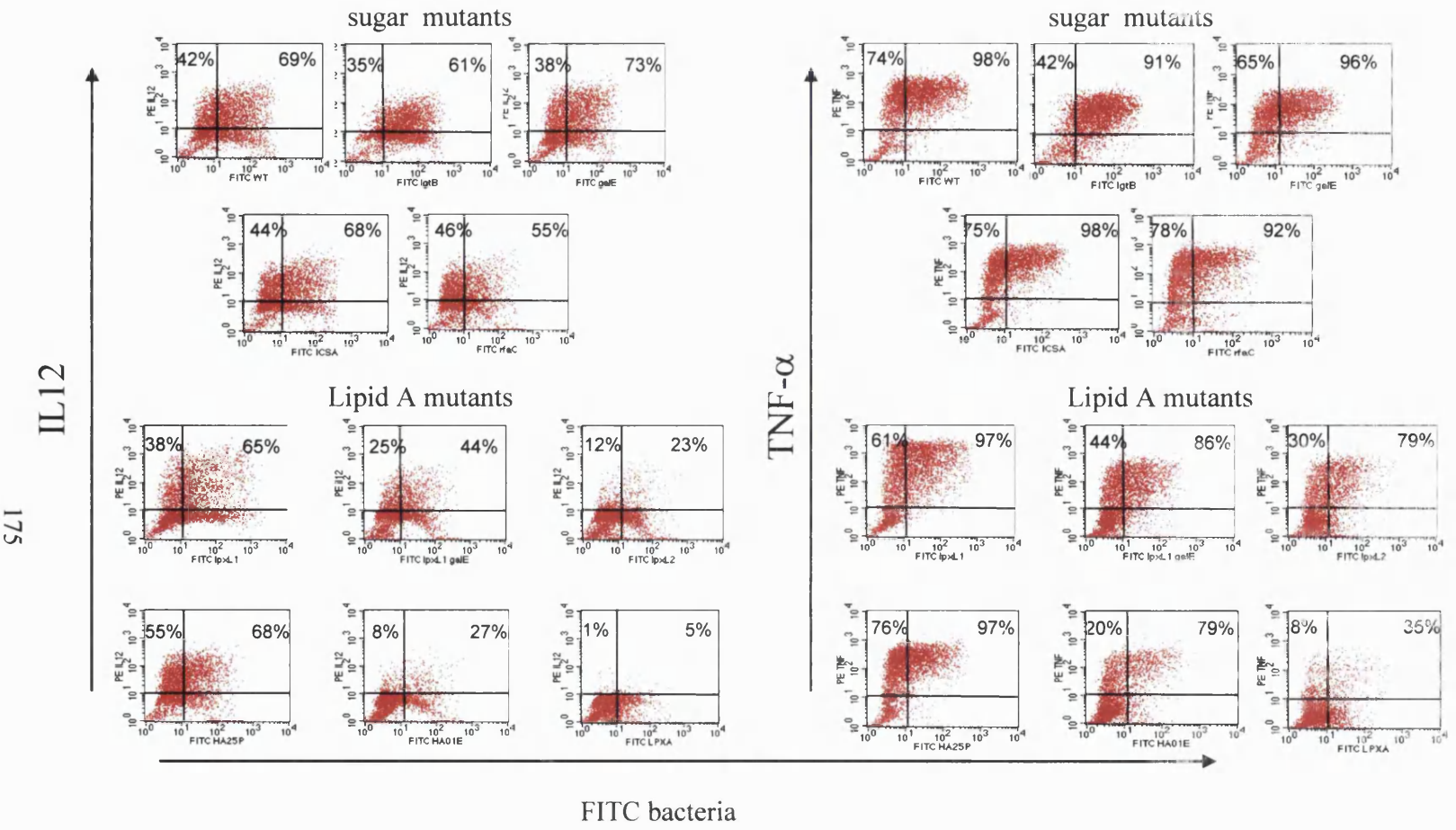

Figure 6.11 Internalisation and intracellular cytokine production in response to FITC labelled mutant *Neisseria meningitidis*. DCs were stimulated with FITC labelled bacteria at a DC/bacteria ratio of 1:200 for 18h in the presence of Brefeldin A. The cells were then permeabilised and stained for intracellular IL12 **(A)** or TNF-a **(B)**. The quadrants were placed to separate cells that had internalised FITC bacteria from the cells that had not. The percentages given are the relative amounts of cytokines produced by DCs that had not internalised bacteria (left) or that had internalised (right). A representative of 3 experiments is shown.

A summarising table of the results of this chapter is shown in table 6.1. The level of internalisation and binding is expressed based on the results obtained from confocal microscopy, whereas the cytokine results are based on intracellular and ELISA cytokine analyses.

Table 6.1. Summary of phagocytosis and cytokine production by DCs in response to oligosaccharide core and lipid A mutants of *N. meningitidis*. 'Total' equals cytokines measured by ELISA, 'phago' equals intracellular cytokine production by DCs that had phagocytosed bacteria.

+	**low**	+++	**high**
++	**moderate**	++++	**very high**

Strain	Surface binding	Internalisation	IL12 total	TNF-α total	IL12 phago	TNF-α phago
WT	+++	+++	+++	+++	+++	++++
lgtB	++++	++++	+++	+++	+++	++++
M992	++++	++++	+++	+++	+++	++++
galE	+++	++	++	++	+++	++++
icsA	+++	++	++	++	+++	++++
rfaC	+++	++	++	++	++	++++
lpxL1	++	++	++	++	+++	++++
lpxL1 galE	+++	+	+	+	++	+++
lpxL2 galE	+	+	+	+	+	++
HA25P	++	++	++	++	+++	++++
HA01E	+	+	+	+	+	++
lpxA-	+	+	+	+	-	-/+

6.4 Discussion

In this chapter, DC interaction and activation with oligosaccharide core and lipid A *N. meningitidis* mutants was investigated. The results showed that internalisation and cytokine production by DCs depended on the precise oligosaccharide and lipid A structure of *N. meningitidis*. Meningococcal LPS has received attention as a potential vaccine candidate and has in particular been recognised as a very strong adjuvant. The endotoxic properties of LPS are, however, the major drawbacks for using it in human vaccines. Truncated oligosaccharide chain mutants and lipid A mutants used in this thesis were used to study the interactions between DCs and bacteria with such altered LPS molecules. Some of the mutants clearly demonstrated altered activity in culture with human DCs. Such mutant *N. meningitidis* species with altered activities for DCs would of course be interesting candidates for future vaccines.

In general it is thought that the biological activity of the LPS molecule resides in its lipid A moiety, as purified lipid A preparations of LPS retain their biological activity, such as cytokine induction. Thus, the working hypothesis in this chapter was that the oligosaccharide chain, which is exposed to the bacterial surface, would play a role during initial interactions, such as binding to the DCs, whereas the lipid A portion could be important for cytokine signalling. It was therefore postulated that the binding of *N. meningitidis* by DCs and the subsequent signalling events for cytokines may be two closely related, but independent events.

In this chapter, the effect of modified lipid A on bacterial internalisation and cytokine production by DCs in response to *N. meningtidis* was tested. The results of the present study clearly demonstrate that the lipid A portion of LPS plays an important role in DC binding and internalisation of *N. meningitidis*. It was shown that bacteria expressing penta (*lpxL1*) or tetra (*lpxL2/galE*), instead of hexa, acylated WT lipid A were internalised less efficiently by DCs compared to the WT bacteria. Moreover, DC internalisation of mutants expressing shorter (HA25P) or longer (HA01E) O-linked fatty acyl chains was shown to be reduced.

The effect of lipid A alterations on internalisation is somewhat surprising. It is curious, how such changes in lipid A fatty acid chains might affect internalisation as these structures are buried deeply in the outer membrane of the bacteria and would not necessarily 'be seen' by the phagocytosing cells. One explanation is that the orientation of the LPS changes after mutations in the fatty acyl structure and distribution resulting in conformational changes that affect the recognition by cell surface receptors expressed by the DCs. In fact, the distribution and number of lipid A fatty acyl chains has been shown to affect the 'tilt angle' of lipid A in a membrane (Seydel *et al.*, 2000). The inclination of the disaccharide backbone, or the tilt angle, of lipid A was shown to be significantly larger the more fatty acyls were present and the more asymmetrically they were distributed on the sugar backbone of the lipid A.

Another explanation is that these mutants express quantitatively less LPS on their outer membrane, which in turn would result in less efficient internalisation by DCs. Steeghs *et al.* (personal communication) have shown that the lipid A

mutants express reduced levels of LPS compared to the WT. The LPS expression in the WT and *lpxL1*, *lpxL1/gale*, *lpxL2/galE* mutants was determined semi-quantitatively by TSDS-PAGE with serial dilutions of purified WT and lipid A mutant LPS as a standard. The *lpxL1* and *lpxL1/galE* mutants expressed similar amounts of LPS, which was approximately 30% less than the LPS expression by the WT. LPS expression of the *lpxL2* mutant was 50% reduced as compared to the WT. HA01E expressed severely reduced levels of LPS (Steeghs *et al.*, 2002). In addition, HA25P has mixed expression of LPS including some WT LPS in addition to the mutant LPS. The quantitative differences in LPS expression of these strains must be therefore taken into account when studying DC binding and internalisation. All the lipid A mutants showed reduced association and internalisation by DCs compared to the WT, which in part may be explained by the reduced LPS expression levels of these mutants. Since differences in DC association were also observed for lipid A mutants expressing almost similar amounts of LPS, such as the *lpxL1*/galE and *lpxL2* mutant, it may be concluded that the specific acylation pattern of the lipid A domain of LPS plays a role in DC association and internalisation of *N. meningitidis*. In the future, it would be interesting to study the lipid A mutants in conditions where the expression of LPS on the surface of the bacteria could be regulated and the amount standardised. One way of achieving this could be to create combination mutants that express mutated lipid A under IPTG-sensitive *lpxA* promoter, such as the HA3003 strain described in chapter 4.

A third possibility to explain the reduced phagocytosis of the lipid A mutants is that the interaction with the LPS binding protein (LBP) was altered in these

mutants. It was shown some 15 years ago that LBP binds to lipid A of several Gram-negative bacteria depending on the lipid A structure, whereas the oligosaccharide chain of the LPS had no effect on binding (Wright *et al.*, 1989). The results in chapter 4 showed that LBP was necessary for efficient phagocytosis of *N. meningitidis* by DCs. The WT bacteria have been shown to bind substantial amounts of LBP (Osman, M, personal communication). It will be therefore of great interest to study the binding of LBP also to the different lipid A mutants.

The results obtained with the sugar mutants showed that the structure of the oligosaccharide chain of LPS is an important determinant for internalisation of meningococci. The sugar mutants have been shown to express identical levels of LPS on the outer membrane and cannot therefore explain their reduced uptake by DCs (Liana Steeghs, personal communication). The *galE*, *rfaC* and *icsA* mutant bacteria, which express a truncated short sugar core, were all internalised by the DCs but initially a larger proportion remained attached to the cell surface compared to the WT or the other mutants. In addition, the *galE* mutation of the *lpxL1* mutant further reduced internalisation of the *lpxL1* mutant by the DCs indicating that the terminal tetrasaccharide (also called the lacto-N-neotetraose) of LPS are important for interaction with the DCs. Interestingly, the truncation of the single terminal galactose in the *lgtB* mutant resulted in consistently enhanced internalisation of the bacteria by DCs. These results were confirmed by using the naturally occurring M992 strain, which also expressed GlcNac as its terminal sugar. Our recent data suggest that both the *lgtB* mutant and the M992 strains, but not the WT, bind to Dendritic Cell Specific ICAM3 Grabbing Non-Integrin (DC-SIGN), expressed by DCs (manuscript in preparation). An interesting approach

for vaccine development would be to improve the delivery of antigens to DCs. In fact, DC targeting for meningococcal vaccine purposes has been suggested (Arigita *et al.*, 2003). In this study, PorA liposomes with mannosylated ligands for C-type lectins were used to target DCs. It is tempting to speculate that mutant LPS expressing GlcNac may prove ideal for use in vaccine preparations as more efficient DC responses could be obtained.

DCs activated with pathogens secrete several cytokines critically important for subsequent T cell responses. For example, IL12 produced by DCs is pivotal for the development of Th1 responses. The results in the present chapter showed that TNF-α as well as IL12 and IL10 production in response to the lipid A mutants was reduced compared to the WT organisms. The biological activity of the lipid A mutants was previously assessed by TNF-α production by a human macrophage cell line MM6 (Steeghs *et al.*, 2002). These results showed reduced TNF-α production in response to outer membrane complexes (OMC) obtained from HA25P or HA01E mutant strains compared to the WT OMC even after normalisation of the LPS content. It was therefore concluded that the reduction in the endotoxic activity was indeed due to the incorporation of longer or shorter fatty acids in meningococcal lipid A. Similarly, Both *lpxL1* and *lpxL2/galE* were shown previously to induce reduced levels of TNF-α in the macrophage cell line system (van der Ley *et al.*, 2001).

In the current chapter it was clearly shown that the more efficiently the bacteria were internalised, the higher the cytokine response by the DCs supporting the idea that bacterial internalisation is important for maximal cytokine production. The

results showed that the DCs were more likely to produce IL12 and TNF-α if they were also positive for *N. meningitidis* (Figure 6.11). Interestingly, no consistent increase in cytokine production was seen in response to the *lgtB* mutants or M992 strain, although both were internalised more efficiently by the DCs than the WT (Figure 6.8 and 9). In fact, in some experiments all sugar mutants, including the *lgtB,* induced less IL12 and TNF-α than the WT (Figure 6.8). These inconsistencies may have been at least partially due to different batches of FCS used. In general, the results in this thesis suggested that there was a maximum level of cytokines being produced by DCs after phagocytosis, which could not be exceeded even if more bacteria were internalised. For example, when the LPS regulatory strain HA3003 was grown in 250 μM IPTG it was more efficiently phagocytosed than the WT due to increased expression of LPS. The cytokine production in response to this strain in these conditions did not, however, exceed the levels obtained with the WT.

The results of this chapter showed that maximal cytokine production required internalisation of the bacteria regardless of the structure of LPS (Fig. 6.11). Attempts were made to compare cytokine production induced by the different mutants once they were phagocytosed by using simultaneous analysis of cytokines and phagocytosis. These data showed that the oligosaccharide mutants induced similar levels of IL12 and TNF-α compared to the WT after internalisation except for the *rfaC* mutant, which expressed a very short oligosaccharide chain and induced a slightly reduced IL12, but not TNF-α, production after internalisation. DCs that had internalised *lpxL1/galE, lpxL2/galE* and HA01E produced less IL12 than DCs that had internalised the WT or *lpxL1* or HA25P. This analysis was

based on the assumption that by 18h, any DCs positive for FITC would contain internalised bacteria. In addition, it did not take into account the differences in the amount of internalised bacteria. Thus, this analysis was a rather rough attempt to analyse cytokine production within the cells that contained phagocytosed bacteria. Taken together, these results may suggest that not only was internalisation needed for maximal cytokine production but also suggested that the mechanisms responsible for the increased cytokine response after phagocytosis were sensitive to modifications in the lipid A region of the LPS and to a lesser degree the sugar chain.

Human toll-like receptors (TLRs) are a recently discovered family of pattern recognition receptors and provide a mechanism by which phagocytosis and inflammatory responses can be linked (Aderem, 2003). TLRs are not generally thought be involved in the phagocytic process itself. Rather, they serve as signalling molecules that help to generate and define the nature of the effector response (Underhill, 2003). *N. meningitidis* is known to engage both TLR2 and TLR4 (Ingalls *et al.*, 2000;Pridmore *et al.*, 2001). The current study did not investigate whether the mutant forms of lipid A resulted in modified interactions with the TLRs expressed by DCs. It is likely, however, as other groups have previously shown that the acylation state of lipid A determines if LPS is recognised by human TLR4/MD2 complex (Beutler and Poltorak, ;Hajjar *et al.*, 2002;Lien *et al.*, 2000;Poltorak *et al.*, 2000). Poltorak *et al.* (2000) showed that a tetra acyl lipid A (similar to *lpxL2*) was unable to induce TLR4 signalling and failed to induce TNF-α production by macrophages. In the present study, alterations in the sugar core reduced phagocytosis by DCs as well as resulted in

diminished cytokine production. It is not known, if the putative interactions with TLRs remained unaffected as the current study was not designed to investigate this possibility. The *galE* mutated oligosaccharide chain of meningococcal LPS from B1940 strain did not affect signalling through TLR4 in HeLa or THP-1 cells (Pridmore *et al.*, 2003). A recent study showed, however that KDO_2 linked to meningococcal lipid A from strain NMB was required for maximal activation of the TLR4 pathway in macrophages, whereas other sugars of the oligosaccharide core had no effect on TLR4 signalling (Zughaier *et al.*, 2004). In this chapter, the 'shortest' oligosaccharide mutant was the *rfaC*, which expressed KDO_2 in addition to intact lipid A. It would be of great interest to study the interaction between DCs and a mutant expressing only the lipid A (so called deep rough mutant).

It is tempting to speculate that truncations of the oligosaccharide core may have affected binding of these mutants to phagocytic receptors expressed by DCs and therefore resulted in slightly reduced overall cytokine production as seen by ELISA but did not have an effect on the putative TLR signalling. In contrast, the reduced cytokine production in response to the lipid A mutants (with *lpxL1* and HA25P being possible exceptions) could be due to defective signalling via receptors such as TLRs. Future studies with cellular systems in which the role of TLRs during interactions with the mutant bacteria can be directly studied will be of great interest. Due to their central role in the recognition of bacteria and their components, the last result chapter in this thesis was designed to investigate the expression of TLR2 and TLR4 by monocyte derived DCs. In addition, their role in DCs during interactions with *N. meningitidis* was explored.

Chapter 7

Expression of Toll Like receptors 2 and 4 by dendritic cells

7.1 Introduction

Dendritic cells (DCs) express various pattern recognition receptors (PRRs) that recognise pathogen associated motifs and drive the DC maturation process. In particular, the mammalian toll like receptor (TLR) family play an important role in pathogen recognition in the innate immune response (Muzio and Mantovani, 2000). Of all ten TLRs that have been described in humans, TLR2 and TLR4 have been the most extensively studied. TLR4 together with MD-2 and CD14 form a signalling complex that responds to the LPS of many Gram-negative bacteria (Beutler, 2002). TLR2 responds to a number of bacterial products including components of Gram-positive bacterial cell walls, peptidoglycan, lipoproteins and lipoteichoic acid (Brightbill *et al.*, 1999;Hertz *et al.*, ;Hertz *et al.*, 2001;Hirschfeld *et al.*, 1999). TLR2 also responds to yeast (Underhill *et al.*, 1999).

N. meningitidis is known to engage both TLR2 and TLR4 and it is a potent inducer of TNF, IL12 and IL10 production by monocytes and DCs (Ingalls *et al.*, 2000;Ingalls *et al.*, 2001;Pridmore *et al.*, 2001). The bacteria must express LPS to stimulate optimal IL12 and TNF-α cytokine production by DCs (Dixon *et al.*, 2001). As was shown in chapters 4-6 in this thesis, phagocytosis of the bacteria depended on expression of LPS and was required for maximal IL12 and TNF-α production. These findings suggested that the signalling events required for cytokine production might not occur on the DC surface but rather in the phagosome or lysosome after bacterial internalisation and raised the possibility that TLR2 and/or 4 interactions with the bacteria may occur inside the cell. There

have been several reports that might support this view. Human TLR2 and TLR6 have been shown to be recruited to macrophage phagosomes where they signal the cell to make cytokines but do not appear to be involved in phagocytosis itself (Ozinsky *et al.*, 2000;Underhill *et al.*, 1999). Similarly, CpG DNA signals through TLR9 in DC lysosomes (Ahmad-Nejad *et al.*, 2002). In epithelial cells, LPS has been shown to co-localise with TLR4 in the Golgi complex, from where the signaling has also been shown to occur (Hornef *et al.*, 2002;Hornef *et al.*, 2003;Latz *et al.*, 2002).

In this chapter, the expression and cellular localisation of TLR2 and TLR4 by human DCs was explored with peptide specific antibodies.

7.2 Materials and Methods

7.2.1 Dendritic cell culture and activation

Monocytes were separated in a percoll discontinous density gradient, washed three times in RPMI 5% FCS and used as indicated. To generate DCs, monocytes were incubated for 5-6 days in RPMI supplemented with 5% FCS, 2.4mM L-glutamine, 100U/ml Penicillin-streptomycin, 100ng/ml of human recombinant GM-CSF and 50ng/ml of human recombinant IL-4. In some experiments, DCs were cultured with *N. meningitidis* H44/76 at a DC:bacteria ratio of 1:100 in RPMI 1640 supplemented with 5% FCS. To depolymerise microtubules, 100ng/ml of colcemid was added to cultures as indicated.

7.2.2 Immunostaining

DCs were stained for surface and intracellular expression of TLR2 and TLR4 with rabbit polyclonal antibodies raised against TLR2 and TLR4 peptides described in detail by: (Fenhalls *et al.*, 2002). For intracellular staining, DCs were fixed in 4% PFA for 15min and then permeabilised in HBSS-0.1% saponin. To prevent nonspecific binding, 10% human or goat serum was added for 30min prior to staining. Surface staining was carried out on live cells on ice. TLR antibodies (1^{st} layer), and rabbit IgG (control) antibodies were added at a final concentration of 10μg/ml for 1h on ice, washed carefully and detected with 1μg/ml of FITC or TRITC goat anti-rabbit IgG $F(ab)_2$ (2^{nd} layer). In some experiments, biotinylated goat anti-rabbit antibodies (2^{nd} layer) were used, after which PE-Cy5 (Cy-Chrome) conjugated streptavidin (3^{rd} layer) was added at 1μg/ml for 30 min. Lysosomes were identified with 10μg/ml mouse anti human LAMP-1 and the early endosomes with mouse anti human CD71 (transferrin receptor). Mouse monoclonal antibodies to human Golgin-97 were used at 10μg/ml to stain the Golgi complex. Microtubules were stained with mouse monoclonal TAT-1 antibody to α-tubulin (Woods *et al.*, 1989). Bound organelle antibodies were detected with Alexa 568 conjugated goat anti mouse IgG. Purified mouse IgG1 was used at 10μg/ml as an isotype control. Labelled cells were air dried on poly-L-lysine coated slides and analysed by confocal microscopy. All images were acquired in sequential scan mode to minimize fluorochrome emission overlap.

7.3 Results

7.3.1 Expression of TLR2 and TLR4 by dendritic cells and monocytes

Most of the studies so far have identified TLRs by analysis of mRNA expression due to unavailability of good commercial antibodies. In this chapter, DCs and monocytes were stained for surface and intracellular expression of TLR2 and TLR4 with peptide specific polyclonal antibodies (Figure 7.1A). By FACS analysis, abundant intracellular staining of both TLR2 and TLR4 was observed by DCs but no surface staining could be detected. Confocal microscopy confirmed the absence of TLR2 and TLR4 on the surface but clearly showed intracellular expression of both TLRs with a tubulovesicular staining pattern and a highly concentrated signal in the perinuclear region (white arrow). By comparison, strong cell surface and intracellular expression of TLR2 and TLR4 was found in monocytes (Figure 7.1B). These data clearly showed interesting differences in surface expression of TLRs between monocytes and DCs, which might reflect the different functions of the two cell types.

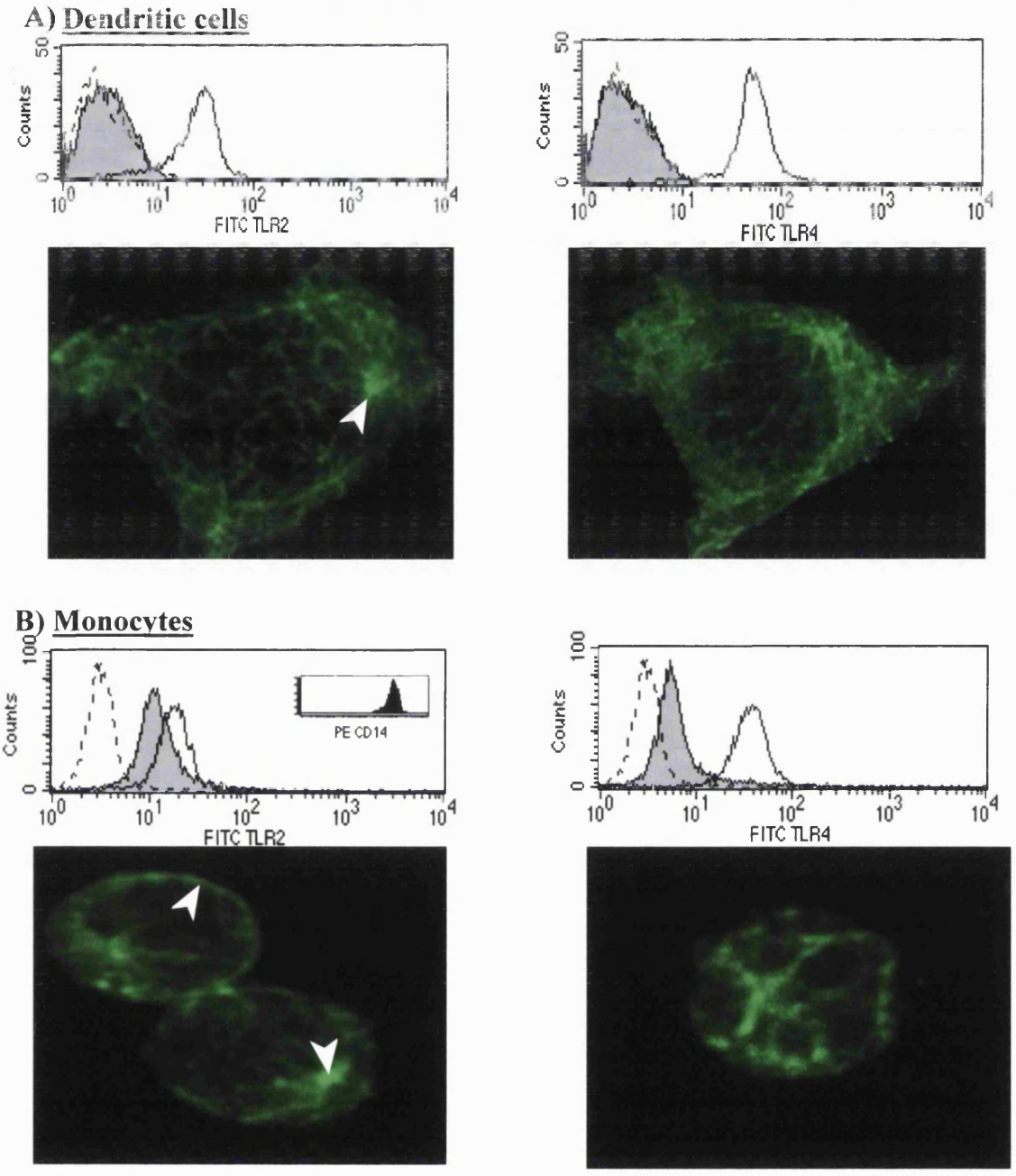

Figure 7.1 Expression of TLR2 and TLR4 by dendritic cells and monocytes. Surface and intracellular staining of TLR2 and TLR4 in DCs (**A**) and monocytes (**B**). For surface staining, cells were incubated on ice with TLR antibodies followed by FITC conjugated F(ab)2 goat anti rabbit IgG. For intracellular staining, DCs were fixed with 4% PFA and permeabilised in Saponin buffer and then stained. Representative FACS histograms of surface and intracellular staining and confocal images of intracellular staining from five experiments are shown. Arrows point to a highly concentrated signal in the perinuclear region (A and B), as well as surface staining in monocytes (B). **Dashed line= isotype control, filled grey= surface staining, black line= intracellular staining.**

7.3.2 Expression of TLR2 and TLR4 after activation of dendritic cells

No surface staining could be detected on immature DCs, although abundant intracellular TLR2 and TLR4 were present. In order to study whether activation of DCs with meningococci could increase the surface expression of the TLRs, DCs were cultured for 2h with WT *N. meningitidis* at DC/bacteria ratio of 1:100. In these experiments, a three step staining protocol with biotinylated secondary antibodies and Cy5 conjugated streptavidin were used to maximise the sensitivity of the staining. As shown in the histograms in Figure 7.2, no surface expression of either TLRs by DCs could be detected after 2h stimulation with the bacteria.

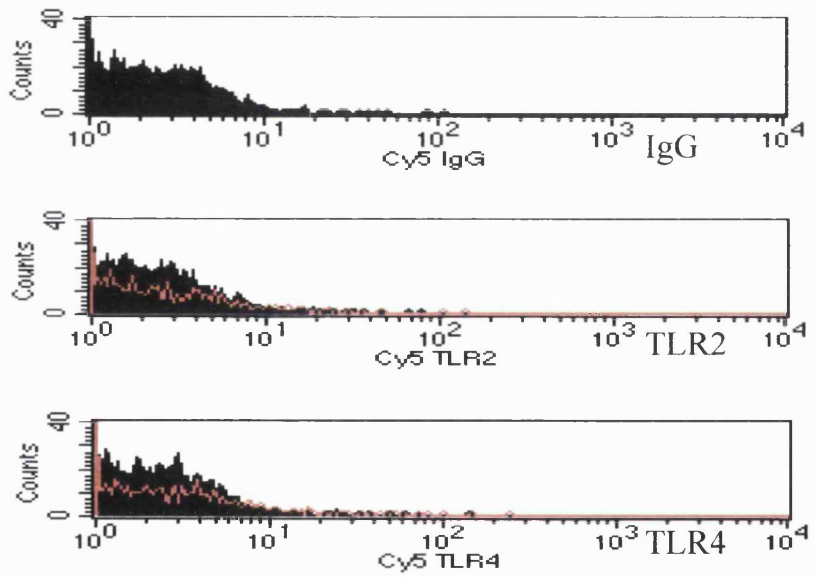

Figure 7.2 Surface expression of TLR2 and TLR4 by dendritic cells in the presence of *N. meningitidis*. DCs were cultured for 2h in the presence of *N. meningitidis*. The cells were then incubated on ice with TLR antibodies followed by biotinylated anti-rabbit antibodies and Cy5 conjugated streptavidin. Representative FACS histograms from two experiments are shown. Black fill= unstimulated, red= stimulated

In order to study whether the intracellular expression of TLR2 and TLR4 changed after activation, DCs were co-cultured with FITC labelled WT and *lpxA*- bacteria as well as Alexa 488 conjugated LPS from *E.coli*. The cells were then permeabilised and stained for TLR2 and TLR4. Dot plots allowed for a simultaneous study of FITC labelled bacteria or LPS and the level of TLR staining in the same cells. Quadrants were placed to separate DCs that had associated with the bacteria or LPS from the cells that had not. Figure 7.3 shows that the intracellular TLR2 and TLR4 staining was similar in the cells positive for WT, *lpxA*- or LPS and in the DCs that were not associated with the bacteria or the LPS. In addition, no consistent increase or decrease in the level of expression by the whole population of DCs (surface or intracellular) was seen after stimulation.

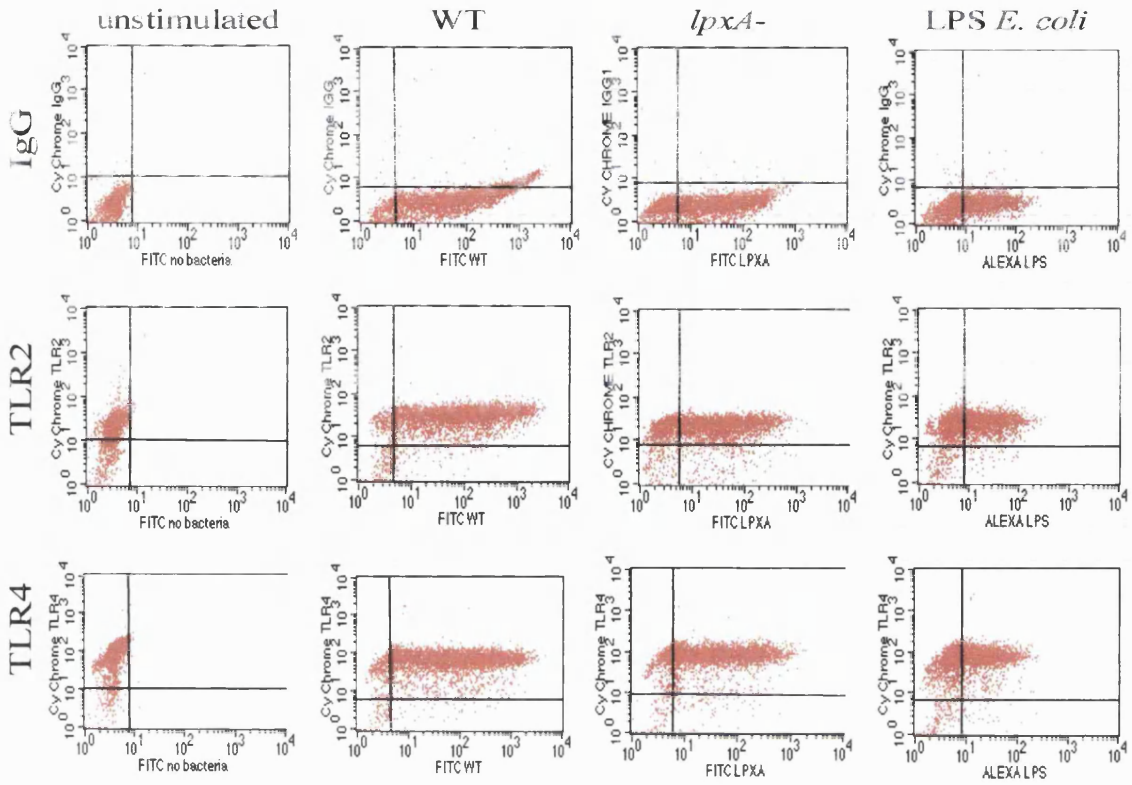

Figure 7.3 Expression of TLR2 and TLR4 by dendritic cells after activation. DCs were cultured for 5h in the presence of FITC labelled WT and *lpxA- N. meningitidis* (1:100 ratio) or Alexa 488 conjugated *E. coli* LPS (1000ng/ml). DCs were then fixed, permeablised in saponin buffer and incubated with TLR antibodies followed by biotinylated second-step antibodies and finally Cy5 conjugated streptavidin. Representative FACS histograms from three experiments are shown

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Although no changes in the levels of TLR2 and TLR4 were seen after stimulation, it was possible that spatial changes in the intracellular localisation of the TLRs did occur. Confocal microscopy was therefore employed to reveal the intracellular TLR staining patterns after stimulation with the WT bacteria and LPS. DCs were cultured with the bacteria and LPS and then stained for TLR2 and

TLR4. After staining, the cells were adhered on slides and the bacteria/LPS and TLR2/4 analysed simultaneously. Tubulovesicular staining pattern was observed for TLR4 as shown in figure 7.4 and for TLR2 (not shown). As expected, WT *N. meningitidis* were readily internalised after 5h of stimulation. In the merged image, both the TLR4 staining and bacteria were shown in order to reveal any co-localisation of the green and red staining, which would appear as yellow on the confocal images. Very little co-localisation was found between the TLRs and bacteria although some was occasionally seen. Most of the time, the bacteria seemed as if they were 'embedded' in the abundant TLR staining. In fact, the intracellular bacteria left clear 'holes' in the TLR staining as shown in figure 7.4.

Interestingly, DCs were able to internalise Alexa 488 conjugated LPS from *E. coli* as shown in the lower panel of images in figure 7.4. Internalised LPS appeared as diffuse staining in the cytoplasm as well as in clear vesicular structures. It was difficult, however, to judge if any co-localisation between TLR4 and LPS occurred in these experiments. Most of the time, the LPS containing vesicles were distinct from the TLR staining.

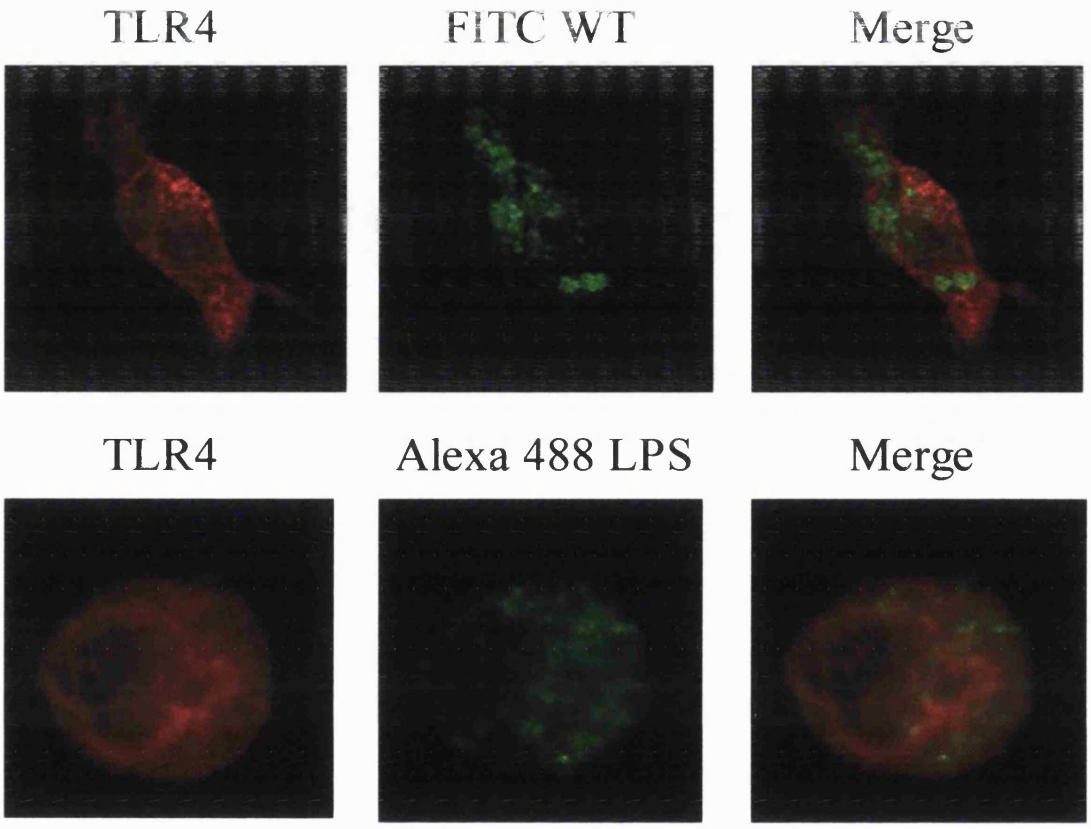


Figure 7.4 Expression of intracellular TLR4 by dendritic cells after stimulation with *Neisseria meningitidis* whole bacteria or LPS. DCs were cultured for 5h in the presence of FITC labelled WT *N. meningitidis* (1:100 ratio) or Alexa 488 conjugated *E. coli* LPS (1000ng/ml). DCs were then fixed, permeablised in saponin buffer and incubated with TLR antibodies followed by biotinylated second-step antibodies and finally Cy5 conjugated streptavidin. Representative FACS histograms from three experiments are shown.

7.3.3 Staining of cytoplasmic vesicles containing TLR2 and TLR4 with markers for early endosomes and lysosomes

In order to identify the cytoplasmic compartment in which the TLR2 and TLR4 containing vesicles resided, co-localisation experiments were carried out with confocal microscopy. It has been reported previously that TLR9 was localised in macrophage lysosomes where signalling in response to CpG DNA was shown to occur. It is possible therefore that bacteria, once internalised and processed within phagolysosomes could also signal from organelles in the endocytic/phagocytic pathway. For microscopy, DC lysosomes were identified by staining with LAMP-1 monoclonal antibodies (CD107a) and transferrin receptor CD71 was used as a marker of early endosomes. As can be seen in figure 7.5A, no-colocalisation of TLR2 with transferring receptor could be detected. Similar results were obtained for TLR4 (not shown). In addition, no co-localisation of TLR4 or TLR2 (not shown) was found with LAMP-1. In conclusion, these experiments indicated that TLR vesicles were distinct from the vesicles found in the endocytic pathway.

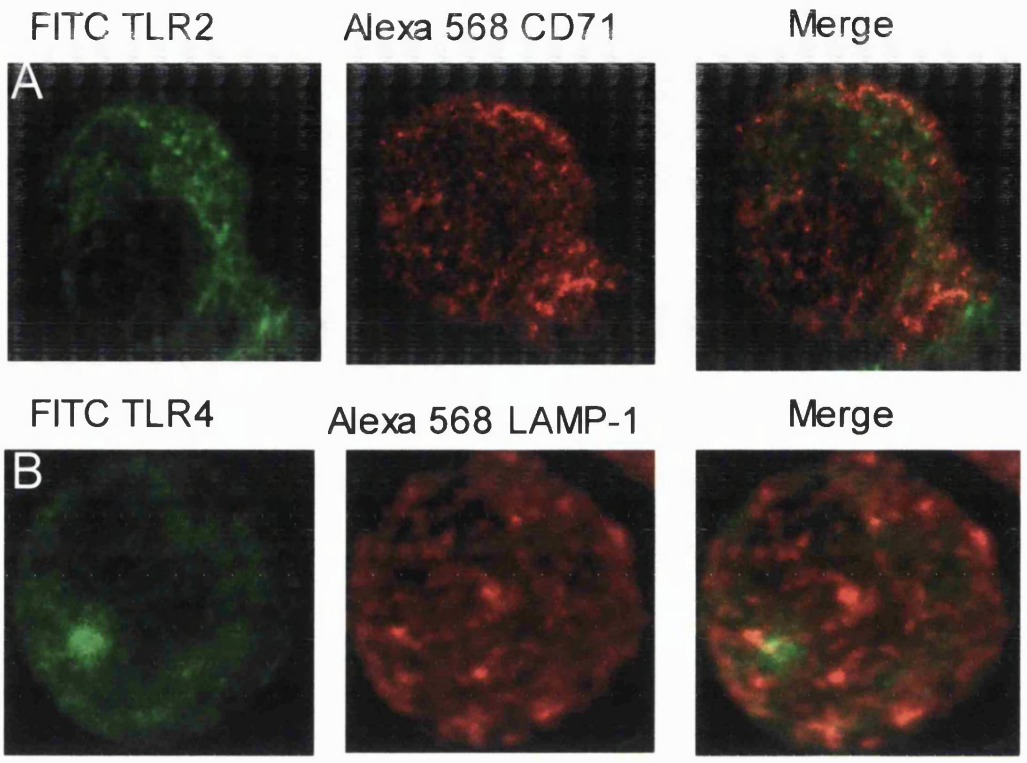


Figure 7.5 Cytoplasmic vesicles containing TLR2 and TLR4 are distinct from early endosomes and lysosomes. DCs were fixed and permeabilised with Saponin and TLR2 and TLR4 stained with TLR antibodies followed by FITC conjugated F(ab)2 goat anti rabbit IgG together with endosomal marker CD71 and lysosomal marker LAMP-1 antibodies. Representative confocal images from three experiments are shown. **A)** Expression of TLR2 and CD71 **B)** Expression of TLR4 and LAMP-1.

7.3.4 Staining of intracellular TLR2 and TLR4 and the Golgi complex

In addition to abundant tubulovesicular staining of intracellular TLR2 and TLR4, a highly concentrated perinuclear signal was observed (Figure 7.1). It was reported recently that TLR4 co-localised with the Golgi apparatus in epithelial cells and monocytes (Hornef *et al.*, 2002;Latz *et al.*, 2002). We therefore investigated the perinuclear region rich in TLR2 and TLR4 in DCs by staining TLRs together with golgin-97, a protein expressed in the trans-Golgi network. Confocal images revealed very close association, and some direct co-localisation, of the Golgi complex both with TLR2 (Figure 7.6A) and TLR4 (Figure 7.6B).

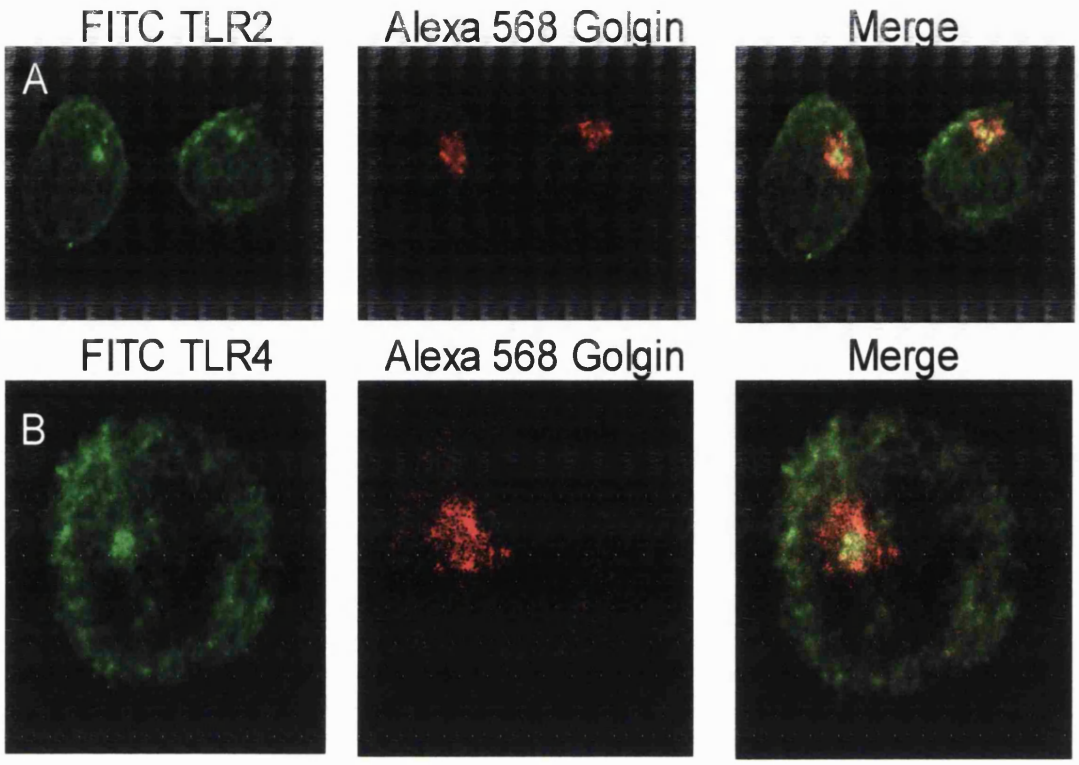


Figure 7.6 Association of TLR2 and TLR4 expressed by dendritic cells with the Golgi apparatus. DCs were fixed with 4% PFA, permeablised in Saponin buffer and stained with Alexa-568 Golgin-97 and TLR2 (**A**) and TLR4 (**B**) antibodies. Merged images show localisation of TLR and golgin at the centre of the Golgi apparatus. Representative images from three experiments are shown.

7.3.5 Staining of intracellular TLR2, TLR4 and microtubules

In mammalian cells, the Golgi complex is intimately associated with the cytoskeleton (Donaldson and Lippincott-Schwartz, 2000). It is centred at the microtubule organizing centre (MTOC), from which microtubules emanate and serve as tracks for intracellular vesicular transport. As the TLR2 and TLR4 were found associated very close to the Golgi complex, as well as in cytoplasmic tubulovesicular structures, DCs were stained for α-tubulin to reveal the

microtubules. As shown in figure 7.7, the perinuclear region rich in TLR2 (Figure 7.7A) and TLR4 (Figure 7.7B) co-localised with tubulin displaying high focal concentration at the MTOC as judged by intense yellow staining. Co-alignment of TLR vesicles with microtubules was observed suggesting that microtubules serve as transport tracks for TLR vesicles.

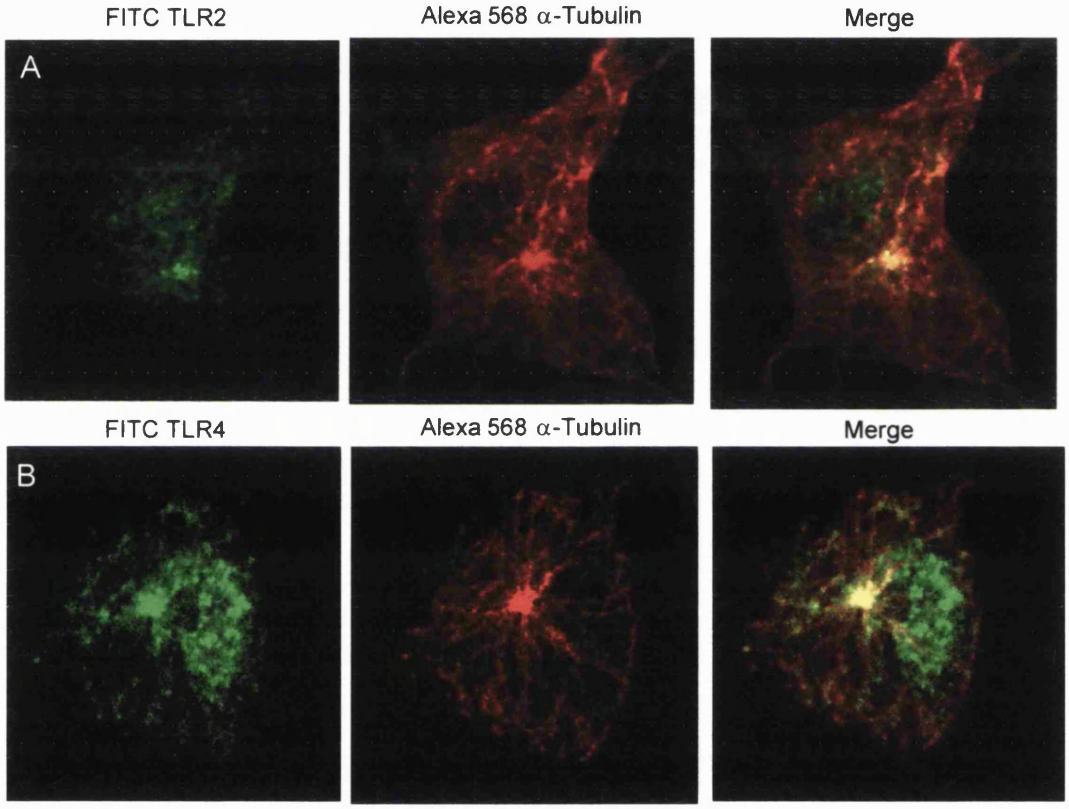


Figure 7.7 Co-localisation of TLR2 and TLR4 with α-tubulin: Fixed and permabilised DCs were stained for TLR2 **(A)** or TLR4 **(B)** and α-tubulin. Representative confocal images from three experiments are shown.

To provide further proof for the role of microtubules in association with TLR vesicles, colcemid (a close relative of colchicine) was used to depolymerise microtubules in DCs. This drug binds to tubulin subunits preventing polymerisation and leaving only the MTOC intact (Hagiwara and Takata, 2002). Figure 7.8 shows a confocal image of colcemid treated DCs stained for TLRs and microtubules. As can be seen, the microtubule network was disrupted by colcemid together with the TLR vesicles. A focus of TLR4 and TLR2 that co-localised with the MTOC was still visible. In fact, both the tubulin and TLRs were often found 'packed' on one side of the cell after using colcemid. This drug was used for a minimum of 5h in order to visibly depolymerise the microtubules.

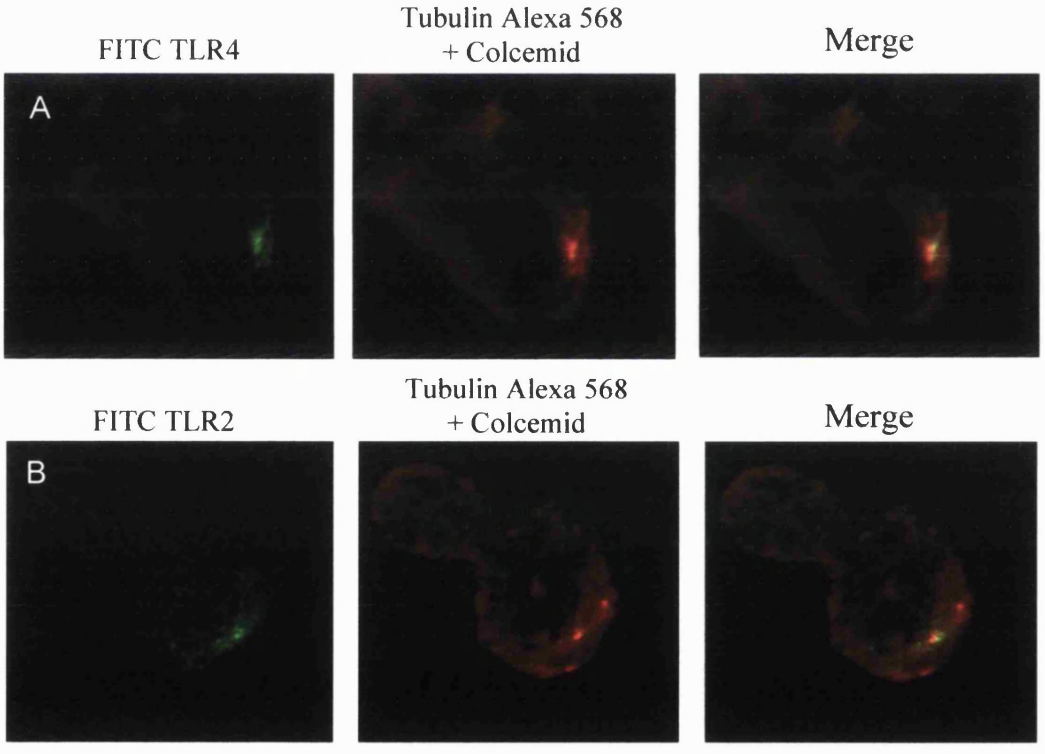
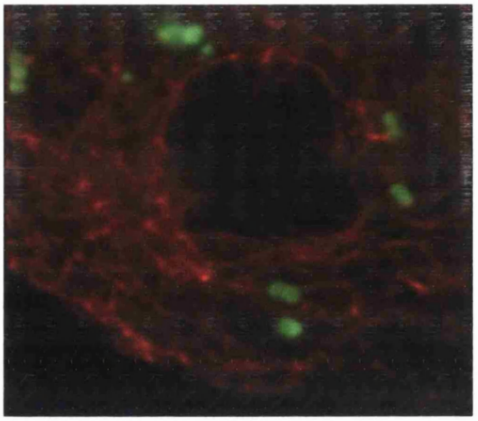


Figure 7.8 Co-localisation of TLR2 and TLR4 with α-tubulin after disruption of microtubules. Microtubules were depolymerised for 5h with 100ng/ml of colcemid and then stained for intracellular TLR4 **(A)** and TLR2 **(B)** together with α-tubulin. Representative confocal images from three experiments are shown.

7.3.6. Phagocytosis and cytokine production after disruption of microtubules

Evidence from the previous chapters in this thesis showed that internalisation of *N. meningitidis* by DCs was required for optimal cytokine production. To explore the role of intracellular TLR expression for DC activation and cytokine production by these bacteria, cytokine production was determined after disruption of the TLR microtubule association with colcemid. Importantly, colcemid did not prevent phagocytosis of the bacteria. As shown in confocal image in figure 7.9, treatment with colcemid disrupted the microtubules but had no effect on DCs capacity to internalise the bacteria. In contrast, disruption of microtubules with colcemid significantly ($p< 0.05$) reduced IL12 production but had only a marginal effect on TNF-α production (Figure 7.10). These results show that an intact microtubule network together with normal intracellular distribution of TLR2 and TLR4 is required for activation of DCs by the bacteria to produce IL12 but not for phagocytosis.

A) Tubulin + FITC bacteria



B) Tubulin + FITC bacteria after colcemid

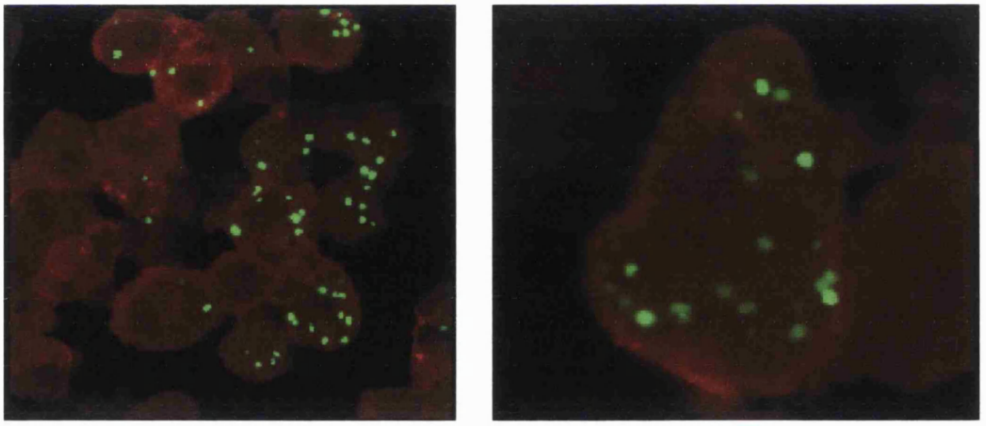


Figure 7.9 Phagocytosis of *N. meningitidis* by dendritic cells after disruption of microtubules. DCs were cultured in the absence **(A)** or presence **(B)** of 100ng/ml colcemid for 5h and then incubated with FITC labelled *N. meningitidis* for 14h at 1:100 DC/bacteria ratio. DCs were fixed and permeabilised and stained for α-tubulin (red). Representative images from 3 experiments are shown.

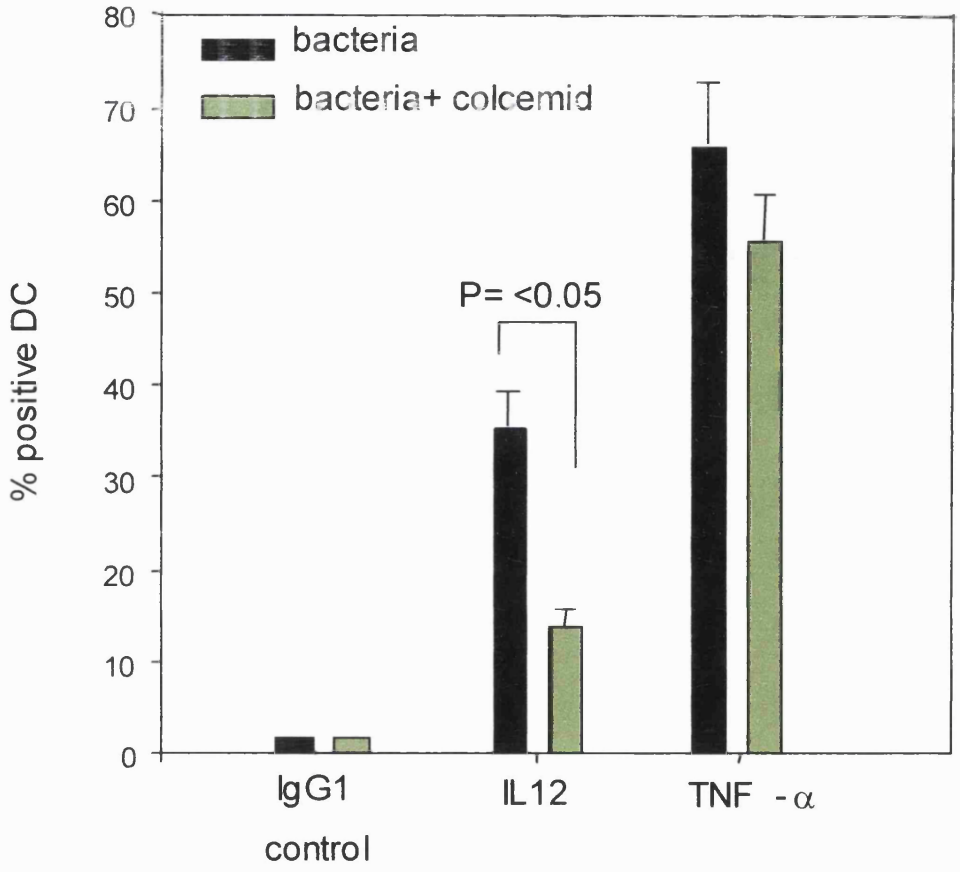


Figure 7.10 Intracellular cytokine production by dendritic cells in response to *Neisseria meningitidis* after disruption of microtubules. DCs were cultured in the absence or presence of 100ng/ml colcemid for 5h and then incubated with *N. meningitidis* at 1:100 ratio for 14h. DCs were then fixed, permeabilised and stained for IL12, TNF-α or with IgG1 control antibodies. The mean and SEM of 3 individual experiments are shown.

7.4 Discussion

Bacteria and other microbes binding to PRRs on DCs are internalised and processed for presentation to T cells (Guermonprez *et al.*, 2002). The activated DCs also release cytokines and chemokines to alert other immune cells to the site of infection and provide a second signal to the responding T cells. As immature DCs constantly internalise antigens from their microenvironment, activation of the cells via PRRs must be tightly regulated to prevent DCs migration and cytokine production in the absence of infection.

Data presented in this chapter show that immature DCs expressed substantial amounts of intracellular TLR2 and TLR4 whereas cell surface expression could not be detected by either flow cytometry or confocal microscopy. In most reports, TLR expression by DCs has been detected by mRNA analysis and not by direct staining (Muzio *et al.*, 2000;Visintin *et al.*, 2001;Zarember and Godowski, 2002). In one study however, TLR4 was readily detected on the surface of monocytes but expression on immature DCs was estimated as less than 100 molecules per cell, which did not increase on maturation (Visintin *et al.*, 2001). Similarly, no increase upon stimulation, of either surface or intracellular TLR2 and TLR4, with *N. meningitidis* bacteria or *E. coli* LPS was found in the present study. Although the presence of very few TLR molecules on the surface of DCs cannot be excluded by our experiments, it is clear that expression is very much less than the levels found on other cells such as monocytes, granulocytes and cell lines and may be absent all together (Latz *et al.*, 2002;Sabroe *et al.*, 2002;Visintin *et al.*, ;Visintin *et al.*, 2001). It has been shown that mRNA levels of TLR2 and TLR4

decrease markedly during DC generation from monocytes indicating different expression of TLRs by these cells (Visintin *et al.*, 2001). Interestingly, no surface expression of TLR2 or TLR4 was found in myeloid DCs purified from blood, whereas monocytes were found to express abundant surface TLR2 and TLR4 in a similar pattern described in this chapter (Jarrossay *et al.*, 2001).

Double staining and co-localisation studies by confocal microscopy showed that intracellular TLR2 and TLR4 in DCs and monocytes are expressed in close association with the Golgi and microtubules suggesting that microtubules may serve as a transport network for the TLRs. TLR4 has been reported to traffic between the Golgi apparatus and the cell surface in TLR4 transfected epithelial cells consistent with this suggestion but in DCs they seem to remain intracellular (Latz *et al.*, 2002). Although TLR2 and TLR4 were associated with microtubules in DCs, it was not clear whether they were indeed moving and if so in which direction. In macrophages, TLR2 and TLR6 have been found to localise around phagosomes containing yeast (Ozinsky *et al.*, 2000;Underhill *et al.*, 1999). Similarly, intracellular TLR9 has been found to form complexes with MyD88 around macrophage lysosomes following the uptake of CpG DNA (Ahmad-Nejad *et al.*, 2002). No co-localisation with the lysosomes or endosomes was found (figure 7.5) suggesting the intracellular localisation of TLR2 and TLR4 was distinct from TLR9. A recent study showed that TLR3 is only expressed intracellularly by DCs, but not monocytes (Matsumoto *et al.*, 2003). Interestingly, Matsumoto *et al.* also found a spot-like signal near the nucleus in DCs, but not monocytes, which closely localised to the MTOC. The authors concluded that their TLR3 antibody must therefore cross-react with a protein in the MTOC!

Transport of bacterial LPS to the golgi apparatus has been shown (Thieblemont and Wright, 1997;Thieblemont and Wright, 1999). Interestingly, DCs were able to internalise large amounts of Alexa 488 labelled *E.coli* LPS (Figure 7.4). However, no transport to the Golgi was observed as LPS remained either diffuse or concentrated in small vesicles in the cytoplasm of the DCs. In addition, no clear co-localisation with the TLR2 and TLR4 was observed. In epithelial cells, LPS together with TLR4 has been found to reside around the Golgi complex in a para-nuclear location (Hornef *et al.*, 2002) where the signalling has been shown to occur (Hornef *et al.*, 2003). Rapid recycling of a TLR4/MD-2/CD14 complex between the Golgi and plasma membrane has been described (Latz *et al.*, 2002). In this system, monocytes and HEK293 cell lines were used, both of which clearly express surface TLR4 and it was concluded that the signalling events were initiated at the cell membrane. In contrast, the absence of detectable TLR2 or TLR4 on the DC surface described here suggested that DC activation by LPS expressed by bacteria occurs either inside the cell after phagocytosis or through some other receptor. Disruption of the intracellular expression of TLR2 and TLR4 by depolymerising microtubules was shown here to inhibit IL12 production but not phagocytosis by DCs activated by *N. meningitidis* and may suggest that these bacteria can activate DCs through TLR2 and TLR4 inside the cell. As TLRs other than TLR2 and TLR4 such as TLR3 have been shown to be expressed intracellularly, the possibility that they also participate in this response cannot be excluded (Matsumoto *et al.*, 2003). Interestingly, the intracellular NOD molecules have been shown to be involved in Gram-negative bacterial sensing and may therefore be involved in the DC response to *N. meningitidis* (Girardin et

al., 2003;Inohara et al., 2001). Intracellular staining by confocal microscopy of the NOD proteins and *N. meningitidis* would be an exciting area for future research.

It was curious that only IL12, and not TNF-α, production was affected by microtubule depolymerisation. In chapter 5, a role for phagocytosis in the production of both of these cytokines was established, although a more prominent effect was seen with IL12. In general, the kinetics and regulation of IL12 by DCs differ from other cytokines, such as IL10 and TNF (Kalinski *et al.*, 1999;Laderach *et al.*, 2003;Langenkamp *et al.*, 2000;Snijders *et al.*, 1998;Trinchieri, 2003). The bioactive form of IL12 is a heterodimer composed of a 35-kDa light chain (p35) and 40-kDa heavy chain (p40), which are regulated independently (Trinchieri, 2003). The biological effects of IL12 are numerous and distinct from cytokines such as TNF-α. After activation of DCs in the periphery, migrating DCs arrive at the lymph nodes to interact with T cells. Together with costimulatory molecules, the presence of IL12 produced by DCs has profound effects on T cell differentiation and activation. Tight regulation of IL12 production has therefore an important effect on the development of the adaptive immunity. DCs are often referred to as being the 'link between innate and adaptive immunity'. This is due to their ability to produce large amounts of bioactive IL12, which in turn can regulated by different TLRs.

Although internalisation of the bacteria seemed normal after disruption of the microtubules, it is possible that phagosome movement and 'maturation' inside the cells was affected and could account for the reduced IL12. Interestingly, it has

been reported that phagosomes themselves move along microtubules and appear clustered near the MTOC in the perinuclear region (Blocker *et al.*, 1996;Blocker *et al.*, 1998). It was tempting to speculate that this could provide the meeting point between phagosomes and TLRs. Co-localisation studies by confcoal microscopy were employed in order to reveal any close proximity between bacteria or LPS with the TLRs inside the cells but no consistent pattern was found that would suggest co-localisation. It is possible that the changes in the expression of TLRs were transient and not detected over the time period studied. Indeed, the expression of TLR2 in macrophage phagosomes after ingestion of yeast zymosan was found to be very transient and lasted only a few minutes (Underhill *et al.*, 1999).

Taken together, the results in this chapter suggested that the intracellular expression of TLRs in DCs may have some functional importance in response to *N. meningitidis*. It is possible that the reason for the increased cytokine production by DCs after phagocytosis of *N. meningitidis* described in this thesis could have been due to the intracellular localisation of TLRs in these cells. DCs may therefore require internalisation of antigen for processing and presentation to T cells before activation and migration. This would ensure that DCs activated by microbial products do not arrive in the draining lymph nodes empty handed.

Chapter 8

General discussion

General discussion

The aim of this thesis was to shed some light on the extremely complex interactions that may occur during DC interactions with pathogens such as *N. meningitidis*. The findings by Dixon *et al.* (2001) created the conceptual basis to this work that these interactions depend on the bacterial structure, and pointed towards a particularly important role of the membrane bound LPS. The data showed early on that DC responses to purified LPS differed substantially from the responses to whole bacteria. As DCs are highly phagocytic cells, this suggested that bacterial internalisation by DCs may be a critical event during interactions with *N. meningitidis*. Hence, any bacterial property that interferes with internalisation may have fundamental effects on DC maturation and activation. The major findings of this thesis support this view. Firstly, internalisation of the bacteria was found to depend on the structure of LPS. Secondly, uptake of bacteria by DCs was a prerequisite to cytokine production, particularly IL12.

DCs are highly specialised antigen-presenting cells that form a gateway between the innate and adaptive immune systems. The primary role of DCs *in vivo* is to capture antigen from their microenvironment and to alert other immune cells to a potential danger. Some ten years ago, DCs were almost impossible to study *in vitro* as they were extremely difficult to isolate and were present in very low numbers *in vivo*. The method developed by Sallusto and colleagues (1994) made it possible to culture high numbers of DCs from peripheral blood monocytes that display phenotypic characteristics of immature DCs, such as high capacity for phagocytosis.

N. meningitidis is exclusively a human pathogen and *in vivo* studies of immune responses to this organism have been hampered by the lack of a suitable animal model. The main aim of this thesis was to explore the critical determinants for DC activation during interactions with *N. meningitidis*. The use of monocyte derived DCs as a model for immunogenicity of *N. meningitidis* was a particularly attractive alternative for a study of bacterial interactions with human immune cells. By culturing DCs *in vitro*, homogenous populations of cells were obtained that allowed us to study the interactions with the bacteria at a single cell level in a controlled environment. As with any *in vitro* culture system, this approach had its limitations. The interactions with other cell types were lost in the simplified *in vitro* conditions and may not have given a full picture of the complex interactions that occur *in vivo*. As several subtypes of DCs have been discovered *in vivo*, monocyte derived DCs may represent only a minor population of DCs that would interact with *N. meningitidis* during invasive disease. In addition, the choice of bacterial dose and LPS concentration and whether they correspond to the situation found *in vivo* are difficult to judge.

Internalisation of the bacteria was found to be linked to the subsequent cytokine production, in particular IL12. The recent discovery of Toll-like receptors (TLRs) may provide an explanation of how phagocytosis and cytokine responses are linked (Underhill and Ozinsky, 2002). In Chapter 7, the expression of TLR2 and TLR4 by DCs was studied. Surprisingly, no surface expression of the TLRs was found but high levels of both TLR2 and TLR4 were found intracellularly in tubulovesicular pattern that co-localised with the microtubules near the Golgi apparatus. It was therefore

postulated that interaction with TLR2 and TLR4 and DC activation occurs after the bacteria have been internalised. This would make functional sense as it would ensure that DCs take up the bacteria for antigen processing before they migrate to draining lymph nodes, where they interact with T cells. It could also at least partially explain why internalisation of the bacteria was required for maximal IL12 production. To test this hypothesis, experiments were designed where microtubules were depolymerised and the cytokines measured after disruption of intracellular TLR2 and TLR4 content. This data proved however inconclusive and provides at best, indirect evidence and does not prove that signalling initiates within the cell. A more convincing approach would be to directly 'silence' the different TLRs by using methods such as RNA interference (RNAi). The first successful RNAi inhibition on human DCs was recently reported and will most certainly prove useful in the future (Laderach *et al.*, 2003). Another exciting approach would be to use fluorochrome tagged transfected TLRs, which would allow the precise cellular localisation to be monitored in live DCs during bacterial interactions by multiphoton confocal microscopy.

Interestingly, changes in both lipid A structure and oligosaccharide chain of LPS in isogenic mutants resulted in modified internalisation by the DCs. It is likely that such changes in LPS structure may have interfered with the binding of the bacteria to receptors expressed by DCs. The experiments in this thesis were not designed to address the question of which receptors were involved in the initial binding of *N. meningitidis*. However, Dr Mohamed Osman in our laboratory has recently found that WT *N. meningitidis* binds to DCs via CD18/CD11b in an LBP dependent manner and this triggers phagocytosis (manuscript submitted). Previous reports have also shown

that LPS binds to CD18/CD11b (CR3) and can mediate phagocytosis (Wright *et al.*, 1989) LBP has been shown to increase phagocytosis of Gram-negative bacteria (Klein *et al.*, 2000) and binds to WT *N. meningitidis* (M. Osman, personal communication). It is likely that the LBP binding depends on the interactions between the different mutant LPS structures. These experiments would of course provide useful information on the mechanisms of bacterial recognition by DCs and are currently underway in our laboratory.

A model how *N. meningitidis* may interact with human DCs is suggested in figure 8.1.

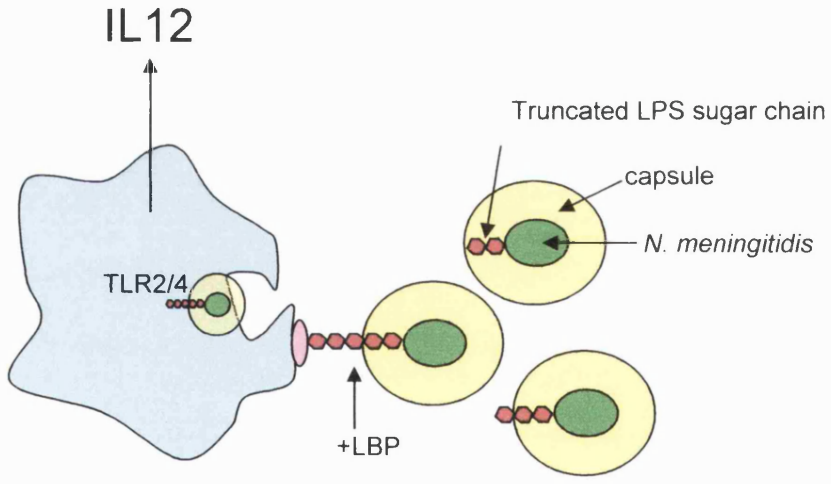


Figure 8.1 Model of DC interaction with *Neisseria meningitidis*. The length of the oligosaccharide chain determines the efficiency of initial binding of the bacteria to DCs. Once bound to the DC via yet unknown receptor in the presence of LPS binding protein (LBP), the bacterium will be internalised. Inside the DC, the lipid A portion of the LPS is exposed and may interact with toll-like receptors, which increases the production of IL12.

Immune responses to bacteria are initiated by DCs, which phagocytose and process bacterial antigens for presentation to T cells. After bacterial ingestion, DCs start to secrete cytokines and express co-stimulatory molecules that influence the subsequent T cell response. In this thesis, internalisation of the bacteria was found to be of major importance for the subsequent cytokine production by the DCs. In particular, maximal production of IL12 was induced by bacteria only after their phagocytosis by the DCs. The production of IL12 is a hallmark for the generation of adaptive immune responses during bacterial infections. The polarisation of T helper (Th) cell responses is mediated by cytokines and IL12 has been shown to be critical in the development of Th1 response. It was therefore of great interest to investigate whether DCs activated with the WT or the LPS deficient bacteria induced differential priming of naive T cells. The results obtained by a fellow PhD student Jennifer Allen illustrated that the Th cell response could indeed be modulated depending on the activation stimulus of DCs. It was shown that Th cell polarisation into IFN-γ secreting phenotype was increased in the presence of DCs that had been activated with the WT bacteria compared to the *lpxA-* mutant. Interestingly, a higher ratio of IL4/IL13 secreting Th cells compared to IFN-γ secreting cells was observed after co-culture with DCs stimulated with the LPS free organisms (Jennifer Allen, PhD thesis 2004). In addition to Th1 polarisation, the presence of LPS in the bacterial outer membrane was necessary for up-regulation of CCR7 and migration across endothelium. These results highlighted the significance of DCs activation status in the functional development of the following adaptive immune response.

The role of LPS in phagocytosis and cytokine production by DCs has major implications for immune responses and vaccine design to *N. meningitidis*. The polysaccharide capsule of group B *N. meningitidis* is poorly immunogenic and recent efforts have concentrated on the development of OMV vaccines (Cartwright *et al.*, 1999). Meningococci are naturally able to release small membrane vesicles or blebs, which contain all the constituents of the OM, including LPS. The natural presence of several immunogenic proteins makes these OMVs an ideal vaccine candidate. However, the endotoxic properties and therefore the adverse reactions caused by LPS present in the OMVs limits their use.. The immunogenicity of LPS-free 'detoxified' OMVs has been studied, but the results have been discouraging. Unfortunately, the poor immunogenicity of these OMVs in children under 4 and the requirement for repetitive boosters were major drawbacks (Cartwright *et al.*, 1999). An effective vaccine against group B organisms should ideally generate high levels of bactericidal antibody and generate long-term memory.

Addition of adjuvants can be expected to improve the immunogenicity of OMV vaccines. LPS, being a potent adjuvant, has obtained serious attention as a potential component in future vaccines. Inclusion of LPS would not be possible however without structural modifications of the LPS molecule that would reduce its endotoxicity with retained adjuvant effect. Separating the adjuvant effect from toxicity is a major task and of considerable importance if LPS was to be included in a vaccine preparation. DCs are in the key position when selecting vaccine components as they are responsible for antigen internalisation and subsequently produce cytokines and co-stimulatory molecules required for an appropriate Th differentiation, which in

turn is required for antibody production by B cells. As the membrane bound LPS was found to be essential for optimal IL12 production by DCs, whole bacteria may be required to generate effective immune responses in a vaccine setting. Genetic engineering of the LPS in the whole bacteria could prove ideal and necessary for future design of group B meningococcal vaccine. In this thesis, DC responses to mutant bacteria expressing modified LPS were studied in order to identify the LPS structures able to activate DCs in the most effective way likely to generate protective immunity without toxicity. The results of this thesis showed that the structure of LPS has major consequences in the way the DCs respond to whole *N. meningitidis*. Toxicity is often measured as TNF-α production and the lipid A mutant bacteria induced clearly reduced TNF production by DCs. Interestingly, the *lpxL1* mutant was still able to induce good levels of IL12 despite the reduced TNF-α production and has therefore great potential for future vaccine development.

Of major interest was the finding that the *lgtB* oligosaccharide core mutant showed consistently increased phagocytosis by the DCs. This mutant lacks the terminal galactose of the LPS and expressed N-acetyl glucosamine (GlcNac) as its terminal sugar residue. Interestingly, our recent data generated in collaboration with Dr Liana Steeghs (Utrecht, the Netherlands) shows that this mutant binds selectively to the newly discovered DC specific lectin DC-SIGN (manuscript in preparation). One approach for vaccine development is to improve the delivery of antigens to DCs. It is tempting to speculate that mutant LPS expressing GlcNac may prove ideal for use in vaccine preparations as enhanced targeting of DCs could be obtained. In fact, DC

targeting for meningococcal vaccine purposes has been suggested (Arigita *et al.*, 2003). PorA liposomes with mannosylated ligands for C-type lectins were used to target DCs. Interestingly, in this study only the uptake of DC targeted PorA liposomes induced IL12 production, whereas non-targeted liposomes failed to induce IL12. We are currently evaluating the subsequent T cell responses in response to DC activation by the *lgtB* mutant as it has been reported that triggering of DC-SIGN signalling cascades may result in the generation of a Th2 response (Geijtenbeek *et al.*, 2003). Generation of oligosaccharide core and lipid A combination mutants such as *lpxL1* and *lgtB* and their interaction with human DCs will be of major interest for future research.

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Publications

The following publications have arisen from the work described in the result sections of this thesis:

PAPERS

Uronen-Hansson H, Allen J, Osman M, Squires G, Klein N and Callard R: 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 IL12 production in response to internalised bacteria. Immunology 2004. 111:173-178.

Uronen-Hansson H, Steeghs L, Allen J, Dixon G, Osman M, van der Ley P, Wong S, Callard R, and Klein N: Human dendritic cell activation by *Neisseria meningitidis*: Phagocytosis depends on expression of lipopolysaccharide (LPS) by the bacteria and is required for optimal cytokine production. Cellular microbiology, *in press*

Uronen-Hansson H, van der Ley P, van Mourik A, Klein N, Callard R, van de Winkel J and Steeghs L. Internalisation and cytokine production by human dendritic cells in response to *Neisseria meningitidis* depend on the structure of the oligosaccharide chain and lipid A of LPS. Manuscript in preparation

ABSTRACTS

Uronen-Hansson H, Steeghs L, Allen J, Dixon GLJ, Osman M, van der Ley P, Wong SYC, Callard R and Klein N. Human dendritic cell activation by *Neisseria meningitidis*: Phagocytosis depends on expression of lipopolysaccharide (LPS) by the bacteria and is required for optimal cytokine production. Poster in Molecular approaches to Vaccine Design –meeting, Cold Spring Harbor, USA 2003.

Uronen-Hansson H, Allen J, Osman M, Squires G, Klein N and Callard R: Intracellular expression of TLR2 and TLR4 by human monocyte derived dendritic cells. Poster in Toll-like receptors, Biochemical Society Meeting, Novartis Horsham UK, 2003.

Uronen-Hansson H, Allen J, Dixon G, Andersen SR , Wong S, Callard R, and Klein N: Human dendritic cell activation by *Neisseria meningitidis:* Phagocytosis depends on expression of lipopolysaccharide (LPS) and is required for optimal cytokine production. Poster in Neisseria 2002 meeting, Oslo Norway, 2002.

Uronen H, Allen J, Dixon G, Andersen SR, Wong S, Callard R and Klein N. Neisseria meningitidis activated human dendritic cells: Role of bacterial structure and phagocytosis. Poster in Dendritic cells at the host pathogen interface, Airlie Warrenton VA, USA, 2002.

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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

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SUMMARY

The activation of dendritic cells (DCs) by microbes is mediated by pattern recognition receptors including the Toll-like receptors (TLR). Bacterial lipopolysaccharide acts via TLR4 whereas peptidoglycan and lipoprotein responses are mediated by TLR2. It is generally accepted that TLR binding to microbes occurs at the cell surface but this has not been directly demonstrated for human DCs. We show here that TLR2 and TLR4 are expressed inside DCs in an abundant tubulovesicular pattern with a focus of intense staining adjacent to the nucleus. In contrast, there was no detectable expression on the cell surface. TLR2 and TLR4 were readily found both intracellularly and on the surface of monocytes. They were shown to be closely associated with the Golgi complex and colocalized with α-tubulin, displaying a high focal concentration at the microtubule organizing centre. Alignment of TLR2 and TLR4 with microtubules was observed, suggesting that microtubules serve as transport tracks for TLR vesicles. Depolymerization of the microtubule network disrupted the intracellular expression of TLR2 and TLR4 and profoundly inhibited interleukin-12 (IL-12) production in response to *Neisseria meningitidis* but did not prevent phagocytosis. These data are consistent with the bacterial signalling through TLR2 and TLR4 required for IL-12 production occurring inside DCs after phagocytosis.

INTRODUCTION

Dendritic cells (DCs) are highly specialized antigen-presenting cells that form a gateway between the innate and adaptive immune systems.[1] Immature DCs express surface pattern recognition receptors that bind to microbes or microbial products, which are then internalized and processed by the DCs.[1] Whole bacteria, yeasts, protozoa and microbial products have all been found to induce DCs to express surface costimulatory molecules and secrete cytokines required for initiating the T-cell immune response.[2] Production of interleukin-12 (IL-12) helps to polarize T helper cells towards the T helper type 1 (Th1) phenotype, which has been shown to be critical in the effector response against many pathogens. Various pattern recognition receptors that recognize pathogen-associated motifs are expressed by immature DCs and drive the maturation process. In particular, the mammalian Toll-like receptor (TLR) family play an important role in pathogen recognition in the innate immune response.[3] Of the 10 TLRs that have been described in humans, TLR2 and TLR4 have been the most extensively studied. TLR4, together with MD-2 and CD14, form a signalling complex that responds to the lipopolysaccharide (LPS) of many Gram-negative bacteria.[4,5] TLR2 responds to a number of bacterial products including components of Gram-positive bacterial cell walls, peptidoglycan, lipoproteins and lipoteichoic acid.[6–8] TLR2 also responds to yeast.[9]

The Gram-negative bacteria *Neisseria meningitidis* is a major cause of bacterial meningitis and septicaemia. It is known to engage both TLR2 and TLR4 and it is a potent inducer of tumour necrosis factor (TNF), IL-12 and IL-10 production by monocytes and DCs.[10,11] We have recently shown that bacteria must express LPS to stimulate optimal IL-12 and TNF-α cytokine production by DCs.[12] Moreover, phagocytosis of the

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bacteria depended on the expression of LPS and was required for cytokine production, particularly that of IL-12 (Uronen-Hansson *et al.* submitted for publication). These findings suggested to us that the signalling events required for cytokine production might not occur on the DC surface but rather in the phagosome or lysosome after bacterial internalization and they raised the possibility that TLR2 and/or TLR4 interactions with the bacteria may occur inside the cell. There have been several reports that might support this view. Human TLR2 and TLR6 have been shown to be recruited to macrophage phagosomes where they signal the cell to make cytokines but do not appear to be involved in phagocytosis itself.[9,13] Similarly, CpG DNA signals through TLR9 in DC phagosomes[14] and LPS has been shown to colocalize with TLR4 in the Golgi complex of epithelial cells.[15,16]

In the present study, the expression and localization of TLR2 and TLR4 in human DCs was investigated using specific antibodies raised against TLR peptides. TLR2 and TLR4 were not present on the surface of DCs but were readily detected inside the cell, associated with tubulovesicular structures close to the Golgi complex. Colocalization of TLR2 and TLR4 with DC microtubules was observed, suggesting that TLR vesicles move along these structures. Depolymerization of the microtubule network disrupted intracellular TLR2 and TLR4 and inhibited IL-12 production in response to *N. meningitidis* but did not prevent phagocytosis. These results suggest that the TLR activation by *N. meningitidis* required for IL-12 production occurs inside DCs and not on the cell surface.

MATERIALS AND METHODS

DC culture and activation

DCs were generated from peripheral blood mononuclear cells as described previously.[17] In brief, monocytes were prepared from peripheral blood mononuclear cells by centrifugation over multistep Percoll gradients. The monocyte fraction was >95% $CD14^+$ $CD3^-$ $CD19^-$. To generate DCs, monocytes were incubated for 5–6 days in RPMI-1640 supplemented with heat-inactivated 5% fetal calf serum, 2·4 mM L-glutamine, 100 U/ml penicillin–streptomycin (all from Gibco, Paisley UK), 100 ng/ml human recombinant granulocyte–macrophage colony-stimulating factor and 50 ng/ml human recombinant IL-4 (Schering-Plough, Welwyn Garden City, Herts UK). Immature DCs prepared in this way were $CD14^{low}$, $CD83^{-ve}$, $CD86^{low}$, $CD25^{-ve}$. They also expressed human leucocyte antigen (HLA) DR, HLA DQ, HLA Class I, CD40 and CD1a, and were negative for both CD19 and CD3 as described previously.[12]

In some experiments, DCs were cultured with *N. meningitidis* H44/76 at a DC to bacteria ratio of 1 : 100 in RPMI-1640 supplemented with 5% heat-inactivated fetal calf serum. When intracellular cytokines were to be measured, the protein transport inhibitor Brefeldin A (Sigma, Poole, UK) was added at 10 μg/ml. To depolymerize microtubules, 100 ng/ml of colcemid (Gibco) was added to cultures as indicated.

Bacteria and phagocytosis assay

Group B *N. meningitidis* H44/76 were grown on gonococcal agar (Difco, Basingstoke, UK) supplemented with Vitox (Oxoid Ltd, Basingstoke, UK) in an atmosphere of 6% CO_2 in air at 36°. The bacteria were used in stationary phase after culture for 18 hr. Suspensions of bacteria were prepared in RPMI-1640 medium without phenol red (Gibco), and their optical density was measured at 540 nm. Bacteria were fixed in 0·5% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min and washed thoroughly in RPMI-1640 medium. Fluorescein isothiocyanate (FITC) -labelled bacteria were prepared by incubation with 0·5 mg/ml FITC (Sigma) for 20 min at 37° followed by extensive washing.

Immunostaining

DCs were stained for surface and intracellular expression of TLR2 and TLR4 with rabbit polyclonal antibodies raised against TLR2 and TLR4 peptides.[18] For intracellular staining, DCs were fixed in 4% PFA for 15 min and then permeabilized in Hanks' balanced salt solution with 0·1% saponin (Sigma). To prevent non-specific binding, 10% human serum was added for 30 min. Surface staining was carried out on live cells on ice. TLR antibodies and rabbit immunoglobuin G (IgG) control antibodies (Caltag Medsystems, Silvestone, UK) were added at a final concentration of 10 μg/ml for 1 hr on ice, washed carefully and detected with 1 μg/ml of FITC goat anti-rabbit IgG $F(ab)_2$ (Caltag). Mouse monoclonal antibodies to human Golgin-97 (Molecular Probes, Cambridge Biosciences, Cambridge, UK) were used at 10 μg/ml to stain the Golgi complex. Microtubules were stained with mouse monoclonal TAT-1 antibody to α-tubulin.[19] Bound antibodies were detected with Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes). Purified mouse IgG1 was used at 10 μg/ml as an isotype control (Pharmingen). To detect intracellular cytokines, DCs were fixed with 4% PFA, washed in PBS containing 0·1% sodium azide and 0·5% bovine serum albumin (all Sigma) and permeabilized in 50 μl of Permeabilisation solution (Caltag Medsystems). Cells were then incubated with phycoerythrin-conjugated monoclonal antibodies to TNF-α (Becton Dickinson, Oxford UK), IL-12 p40/70 (Pharmingen) or isotype-matched controls for 30 min at room temperature in the dark. The cells were then washed twice in PBS, fixed in CellFix (Becton Dickinson) and analysed on a FACScalibur using CELL QUEST software (Becton Dickinson).

Confocal microscopy

Labelled cells were air-dried on poly L-lysine-coated slides and mounted with Citifluor (Citifluor Ltd, Leicester, UK). Confocal images were obtained using a Leica SP2 confocal laser scanning microscope system (Leica, Milton Keynes, UK) fitted with appropriate filter sets. Between 15 and 20 optical sections (0·2–0·5 μm) spanning the entire DC were projected and superimposed with Leica confocal imaging software. All images were acquired in sequential scan mode with a Gain typically no more than 650 V, as recommended by Leica Microsystems, to minimize fluorochrome emission overlap.

RESULTS

Human DCs express TLR2 and TLR4 intracellularly but not on the cell surface

DCs were stained for surface and intracellular expression of TLR2 and TLR4 (Fig. 1a). By fluorescence-activated cell sorter

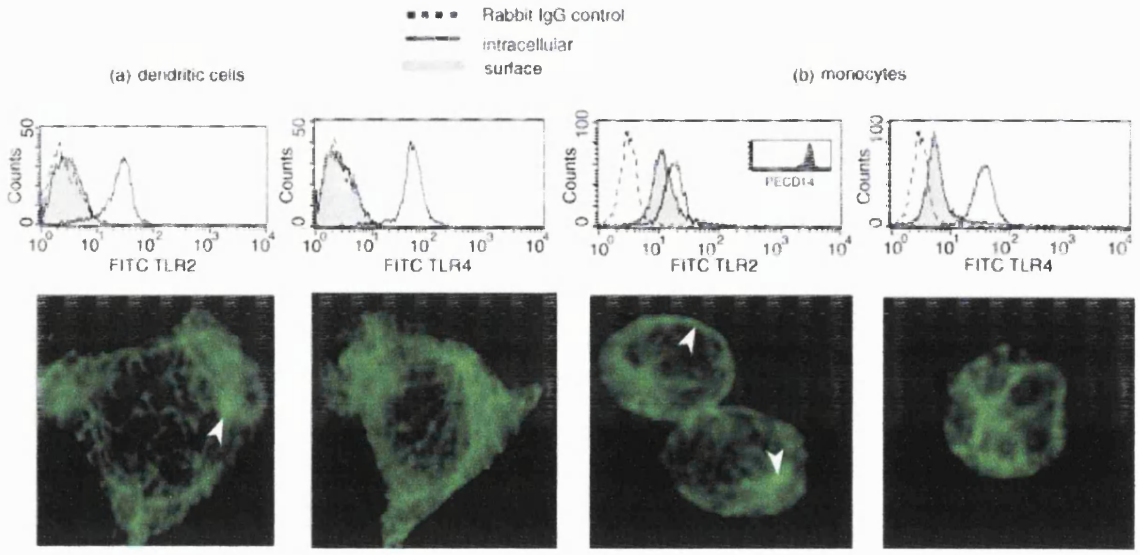

Figure 1. Surface and intracellular staining of TLR2 and TLR4 in DCs (a) and monocytes (b). For surface staining cells were incubated on ice with TLR antibodies followed by FITC-conjugated $F(ab)_2$ goat anti-rabbit IgG. For intracellullar staining DCs were fixed with 4% PFA, permeabilized in Saponin buffer and then stained. Representative FACS histograms of surface and intracellular staining and confocal images of intracellular staining from five experiments are shown. Arrows point to a highly concentrated signal in the perinuclear region (a,b) as well as surface staining on monocytes (b).

analysis, abundant intracellular staining of both TLR2 and TLR4 was observed but no surface staining could be detected. Confocal microscopy confirmed the absence of TLR2 and TLR4 on the surface but clearly showed intracellular expression of both TLRs with a tubulovesicular staining pattern and a highly concentrated signal in the perinuclear region. Activation of the DCs with *N. meningitidis* bacteria or with LPS did not induce surface expression of either TLR (data not shown). By comparison, strong cell surface and intracellular expression of TLR2 and TLR4 was found in monocytes (Fig. 1b).

TLR2 and TLR4 are highly expressed in a perinuclear region close to the Golgi complex associated with microtubules

It was reported recently that TLR4 colocalizes with the Golgi apparatus in epithelial cells and monocytes.[15,16] Therefore the perinuclear region in DCs that is rich in TLR2 and TLR4 was investigated for colocalization with Golgin-97, a protein expressed in the *trans*-Golgi network. Confocal images revealed very close association of the Golgi complex with TLR2 (Fig. 2a) and TLR4 (Fig. 2b).

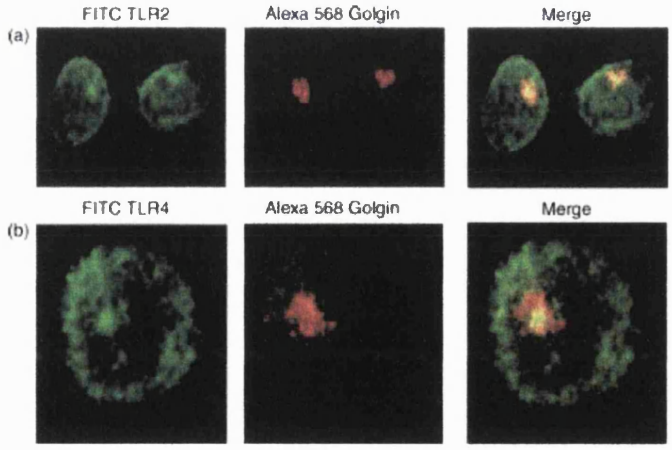

Figure 2. Association of TLR2 and TLR4 with Golgi apparatus. DCs were fixed with 4% PFA, permeabilized in Saponin buffer and stained with Alexa-568 Golgin-97 and TLR2 (a) and TLR4 (b) antbodies. Merged images show localization of TLR and Golgin at the centre of the Golgi apparatus. Representative images from three experiments are shown.

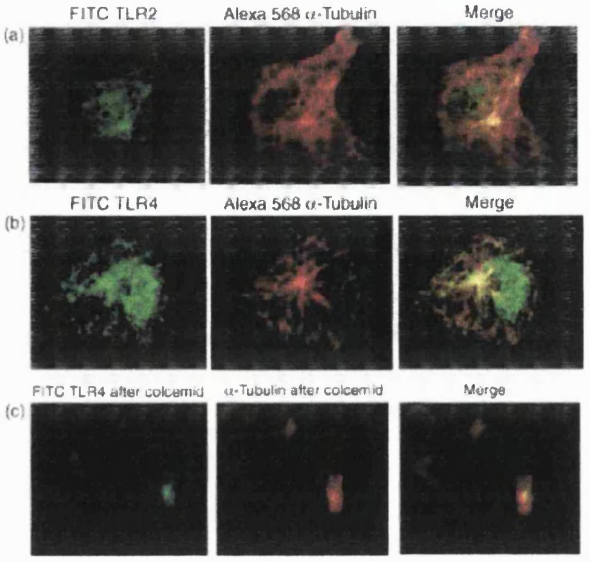

Figure 3. Co-localization of TLR2 and TLR4 with α-tubulin. Fixed and permeabilized DCs were stained for TLR2 (a) or TLR4 (b) and α-tubulin. Microtubules were depolymerized for 5 hr with 100 ng/ml of colcemid and then stained for intracellular TLR2 and TLR4 together with α-tubulin (c). Depolymerization of microtubules disrupted the intracellular TLR2 and TLR4 vesicles. Representative confocal images from three experiments are shown.

In mammalian cells, the Golgi complex is intimately associated with the cytoskeleton.[20] It is centred at the microtubule-organizing centre (MTOC), from which microtubules emanate and serve as tracks for intracellular vesicular transport. As the TLR2 and TLR4 were found very close to the Golgi complex, as well as in cytoplasmic tubulovesicular structures, DCs were stained for α-tubulin to reveal the microtubules. As shown in Fig. 3, the perinuclear region rich in TLR2 (Fig. 3a) and TLR4 (Fig. 3b) colocalized with tubulin, displaying a high focal concentration at the MTOC. Co-alignment of TLR vesicles with microtubules was observed, suggesting that microtubules serve as transport tracks for TLR vesicles.

To examine further the role of microtubules in the transport of TLR vesicles, colcemid was used to depolymerize microtubules in DCs. This drug binds to tubulin leaving only the MTOC intact.[21] Figure 3(c) shows a confocal image of colcemid-treated DCs stained for TLR4 and microtubules. As can be seen, the microtubule network has been disrupted together with the TLR vesicles. A focus of TLR4 that colocalizes with the MTOC is still visible. Similar results were obtained with TLR2 (not shown).

Depolymerization of microtubules disrupts IL-12 production by DCs in response *N. meningitidis*

It was shown recently that internalization of the Gram-negative bacterium *N. meningitidis* by DCs is required for optimal cytokine production, particularly IL-12 (submitted for publication). To explore the role of intracellular TLR expression for DC activation and cytokine production by these bacteria, IL-12 production was determined after disruption of the TLR microtubule association with colcemid. As shown in Fig. 4, treatment with colcemid significantly reduced IL-12 production but had only a marginal effect on TNF-α production. Importantly, colcemid did not prevent phagocytosis of the bacteria (Fig. 4b). These results show that an intact microtubule network together with intracellular expression of TLR2 and TLR4 is required for activation of DCs by the bacteria to produce IL-12 but not for phagocytosis.

DISCUSSION

Bacteria and other microbes that bind to pattern recognition receptors on DCs are internalized and processed for presentation to T cells. The activated DCs also release cytokines and chemokines to alert other immune cells to the site of infection and to provide a second signal to the responding T cells. As immature DCs constantly internalize antigens from their microenvironment, activation of the cells via pattern recognition receptors must be tightly regulated to prevent the migration of DCs and subsequent cytokine production in the absence of infection. We show here that immature DCs express substantial amounts of intracellular TLR2 and TLR4 whereas cell surface

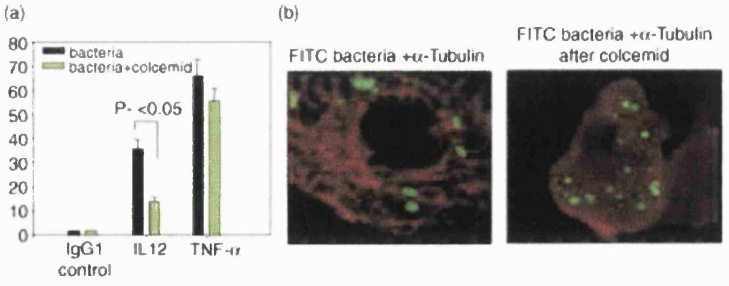

Figure 4. Depolymerization of microtubules disrupts IL-12 production in response to *Neisseria meningitidis*. DCs were pre-incuabated for 5 hr in the presence or absence of 100 ng/ml colcemid and then stimulated for 14 hr with *N. meningitidis* bacteria at a ratio of 1 : 100 in the presence of brefeldin A. DCs producing IL-12 and TNF-α were determined by intracellular staining. Mean and SEM of three different experiments with three different donors are shown (a). Phagocytosis after 14 hr with the bacteria is shown by confocal imaging (b).

expression could not be detected by either flow cytometry or confocal microscopy. In most reports, TLR expression by DCs has been detected by messenger RNA analysis and not direct staining.[22–24] In one study however, TLR4 was readily detected on the surface of monocytes but its expression on immature DCs was estimated as less than 100 molecules per cell, which did not increase on maturation.[23] Surface expression of TLR4 on human DCs has not been described. Although the presence of very few TLR molecules on DCs cannot be excluded by our experiments, it is clear that expression is very much less than the levels found on other cells such as monocytes, granulocytes and cell lines and may be absent all together.[16,23,25] Interestingly, messenger RNA levels of TLR2 and TLR4 decrease markedly during DC generation from monocytes indicating different requirements for TLRs by these cells.[23]

Double staining and colocalization studies by confocal microscopy showed that intracellular TLR2 and TLR4 in DCs and monocytes are expressed in close association with microtubules (Fig. 3) suggesting that microtubules may serve as a transport network for the TLRs. TLR4 has been reported to traffic between the Golgi apparatus and the cell surface in TLR4-transfected epithelial cells, consistent with this suggestion but in DCs they remain intracellular. Although TLR2 and TLR4 were associated with microtubules in DCs (Fig. 3) it was not clear whether they were indeed moving and if so in which direction. In macrophages, TLR2 and TLR6 have been found to localize around phagosomes containing yeast.[9,13] Similarly, intracellular TLR9 has been found to form complexes with MyD88 around macrophage lysosomes following the uptake of CpG DNA.[14] Transport of bacterial LPS to the Golgi apparatus has been shown.[26,27] In epithelial cells, LPS together with TLR4 has been found to reside around the Golgi complex in a paranuclear location[15] and rapid recycling of a TLR4/MD-2/CD14 complex between the Golgi and plasma membrane has been described.[16] In this system, monocytes and HEK293 cell lines were used, both of which clearly express surface TLR4 and it was concluded that the signalling events were initiated at the cell membrane. In contrast, the absence of detectable TLR2 or TLR4 on the DC surface may suggest that DC activation by LPS expressed by bacteria occurs either inside the cell after phagocytosis or through some other receptor. Other studies have shown that pro-inflammatory cytokine production in response to *N. meningitidis* depends on TLR2 and TLR4.[10] In addition, phagocytosis of *N. meningitidis* requires the expression of LPS on the bacterial outer membrane and is necessary for maximal IL-12 production (Uronen-Hansson *et al.* submitted for publication). In this report we showed that that wild-type *N. meningitidis* were readily and rapidly phagocytosed by the DCs, whereas isogenic LPS-deficient mutant bacteria were not. This mutant completely lacks LPS, whereas other surface antigens are unaltered.[28,29,29] Disruption of microtubules, and therefore the intracellular expression of TLR2 and TLR4, was shown in the current paper to inhibit IL-12 production, but not phagocytosis, by DCs activated with *N. meningitidis* (Fig. 4).

In view of these findings, we suggest that DC binding to *N. meningitidis* via LPS and the subsequent signalling for IL-12 production are two independent, but closely related, events. Recent data from our laboratory suggest that phagocytosis of wild-type bacteria requires LPS binding protein (LBP) and can occur by binding CR3 (CD11b/CD18) (Osman *et al.* manuscript in preparation). This is consistent with previous findings that CR3 binds LPS.[30] Interestingly, TNF-α production by DCs was only marginally affected after disruption of microtubules. Experiments with cytochalasin D as well as with transwells (Uronen-Hansson *et al.* submitted for publication), in which phagocytosis of bacteria was inhibited or the bacteria were physically separated from DCs by a porous membrane, showed a more significant reduction in IL-12 production compared to TNF-α. These findings suggested that production of IL-12 depends on internalization of whole bacteria whereas TNF-α production can be more easily induced by soluble bacterial components, such as LPS, released by the bacteria. Some signalling from the cell surface is likely to occur as our previously published data showed that purified meningococcal LPS can induce some TNF-α production, but not IL-12, by DCs. Only whole bacteria induced significant levels of IL-12 production.[12] Interestingly, TNF-α production in response to fungal particles required recognition by Dectin-1 and TLR2, but could occur in the absence of phagocytosis.[31,32] As TLRs other than TLR2 and TLR4, such as TLR9, are expressed intracellularly, the possibility that they also participate in the response cannot be excluded. It is suggested that the intracellular expression of TLRs in DCs may ensure internalization of antigen for processing and presentation to T cells before activation and migration. This would ensure that DCs activated by surface binding of microbial products to pattern recognition receptors do not arrive in the draining lymph nodes empty handed.

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Human dendritic cell activation by *Neisseria meningitidis*: Phagocytosis depends on expression of lipooligosaccharide (LOS) by the bacteria and is required for optimal cytokine production

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Summary

Group B *Neisseria meningitidis* is a human pathogen, for which a universally effective vaccine is still not available. Immune responses to bacteria are initiated by dendritic cells (DC), which internalize and process bacterial antigens for presentation to T cells. We show here that optimal IL12 and TNF-α production by human monocyte derived DC in response to killed serogroup B *N. meningitidis* depends on physical contact and internalization of the bacteria by DC. The majority of DC producing cytokines had internalized *N. meningitidis* while inhibition of bacterial internalization markedly impaired IL12 and TNF-α, but not IL6 production. Internalization of *N. meningitidis* was shown to depend on lipooligosaccharide (LOS) expressed by the bacteria with poor internalization of LOS deficient bacteria compared to WT bacteria. Restoration of LOS biosynthesis in a LOS regulatory strain also restored both internalization and cytokine production and was enhanced in the presence of LPS binding protein (LBP). These results suggest that DC phagocytosis depends on expression of LOS within the bacteria and that optimal cytokine production, particularly IL12, requires internalization of the bacteria. These findings have important implications for designing vaccines that will induce protective immune responses to group B *N. meningitidis.*

Introduction

Dendritic cells (DC) are highly specialized antigen-presenting cells that form a gateway between the innate and adaptive immune systems. Immature DC express surface pattern recognition receptors that bind to microbes and microbial products, which are then internalized and processed for presentation to T cells (Guermonprez *et al.*, 2002; Moll, 2003; Palucka and Banchereau, 1999). Whole bacteria, yeasts, protozoa and microbial products have all been found to induce surface costimulatory molecules and cytokine production by DC (d'Ostiani *et al.*, 2000; Gorak *et al.*, 1998; Henderson *et al.*, 1997; Paschen *et al.*, 2000; Rescigno *et al.*, 1999). Whether these DC responses are optimal for generating effective immune responses to the microorganism is unclear. The subsequent DC response is crucial both for initiating the immune response and for directing the type of response to a particular pathogen (Moll, 2003). The importance of DC function is exemplified by Th1 differentiation in response to IL12 production by DC activated with bacterial products, such as LPS (Macatonia *et al.*, 1995). This is an important consideration for vaccine design to organisms such as *N. meningitidis,* which remains a significant cause of mortality and morbidity worldwide (Brandtzaeg and van Deuren, 2002). Effective subunit vaccines have been developed against serogroups A and C, but a safe vaccine has not yet been developed against serogroup B.

We and others have previously shown that human monocytes and DC activated with serogroup B *N. meningitidis* increase expression of surface MHC II, CD40 and CD83 and secrete IL-1, IL-6, TNF-α and IL12 (Dixon *et al.*, 2001; Kolb-Maurer *et al.*, 2001; Uronen *et al.*, 2000). In contrast, DC activated with an LOS deficient, *lpxA*- isogenic

mutant of *N. meningitidis* fail to make significant amounts of IL12 and production of the cytokines other than IL6 is significantly reduced, whereas expression of surface activation molecules was not different to DC activated with the wild type (WT) bacteria (Dixon *et al.*, 2001). Addition of purified LOS from *N. meningitidis* did not reconstitute the cytokine response. These studies highlight the importance of LOS expressed within the bacterial outer membrane for DC cytokine production but not expression of surface costimulatory molecules. Although LOS is known as a potent activator of DC (Verhasselt *et al.*, 1997; Brandtzaeg *et al.*, 2001), its role in phagocytosis and antigen processing of bacteria has not been elucidated. In the study reported here, a link between phagocytosis and cytokine production by DC activated with *N. meningitidis* H44/76 bacteria was investigated. The results show that phagocytosis of *N. meningitidis* by human DC depends on the expression of LOS by the bacteria and that phagocytosis is necessary for optimal cytokine production, especially IL12. These findings have important implications for designing vaccines that will induce protective immune responses to group B *N. meningitidis*.

Results

Contact between *N. meningitidis* and DC is required for maximal cytokine production

We have shown previously that co-culture of whole *N. meningitidis* with human DC results in IL-1, TNF-α and IL12 production as well as enhanced expression of surface activation markers (Dixon *et al.*, 2001). In contrast, purified LOS was found to be a poor inducer of cytokines, in particular IL12. One hypothesis to explain this observation is that direct contact between DC and bacteria is required for maximal cytokine production. To explore this possibility, we used a transwell system, in which DC were physically separated from the bacteria by 0.1μm porous membranes. Cytokines were then measured by intracellular staining in the presence of Brefeldin A. Activation of DC by PFA inactivated whole bacteria under these conditions resulted in a significant reduction ($p<0.05$) in IL12 and TNF-α (Figure 1A), but not in IL6 production. IL12 and TNF-α production in response to 100ng/ml of purified meningococcal LOS was significantly (< 0.05) reduced compared to the whole bacteria. In contrast, IL6 production was similar in response to whole bacteria and the purified LOS. The transwell had no effect on LOS induced cytokine production. Separation of DC and bacteria by transwells did not affect expression of the surface markers MHC Class II and CD86 (Figure 1B). These results suggest that physical contact between whole bacteria and DC is necessary for maximal IL12 and TNF-α production but not necessary for expression of surface costimulatory molecules or IL6 production.

Cytokines are produced mainly by DC that are associated with *N. meningitidis*

To further investigate the relationship between bacterial contact and cytokine production, a flow cytometric technique was used to analyze bacterial association and intracellular cytokine production by DC simultaneously. DC were co-cultured with FITC labeled bacteria for 18 hours and then stained for intracellular cytokine production (Figure 2). By 18h, 76% of the DC were associated with the bacteria (i.e. were FITC positive). In this experiment, 71% (57/80) of DC which were associated with bacteria were also producing TNF-α and 42% (33/78) were producing IL12. In contrast, only 25% of DC not in contact with bacteria produced TNF-α and only 18% produced IL12. These results are typical of three separate experiments showing that DC in contact with bacteria had a 2-3 fold increase in TNF-α and IL12 production.

The flow cytometric technique used in these experiments did not distinguish between cell surface bound and internalized bacteria. Optical sectioning by confocal microscopy was therefore used to ascertain the location of DC associated bacteria. Figure 3A clearly shows that the FITC bacteria were indeed internalized after 18h of co-culture. The role of internalization in DC cytokine production was then examined by inhibiting bacterial uptake with cytochalasin D and toxin B. As shown by confocal microscopy, cytochalasin D completely inhibited internalization but did not influence the binding of the bacteria to DC (Figure 3B). Cytochalasin D also significantly inhibited production of both TNF-α and IL12, but not IL6, in response to *N. meningitidis* (Figure 3B). Similar results were obtained when phagocytosis was inhibited by toxin B and when cytokines were assayed

by intracellular staining rather than ELISA (data not shown). As shown in figure 3C, cytochalasin D did not have an effect on DC surface activation markers MHC Class II and CD86 in response to *N. meningitidis*. These results show that internalization of the bacteria is required for IL12 and to a lesser extent TNF-α production. Binding without internalization was, however, sufficient for IL6 production and DC maturation, as assessed by surface markers expression.

Expression of LOS by *N. meningitidis* is needed for rapid internalization by DC

We have shown previously that an LOS deficient mutant *lpxA-* of *N. meningitidis* is much less potent at inducing IL12 and TNF-α production by DC than the WT bacteria, whereas IL6 production remained high (Dixon *et al.*, 2001). Our findings, presented here, that bacterial internalization is required for optimal IL12 and TNF-α production raised the possibility that the reason for the inferior IL12 and TNF-α response to the *lpxA-* bacteria may be related to their physical association with DC. To examine this possibility, DC cytokine production and internalization of WT and *lpxA-* mutant bacteria were compared. Immature DC were incubated with FITC labeled bacteria at a ratio of 1:100 (DC/bacteria) and then analyzed by confocal microscopy. By taking optical sections of DC with the confocal microscope it is possible to distinguish between bacteria that are present inside the cell and those that have adhered to the surface. Typically, 15-25 optical sections (0.2-0.5μm) spanning the entire DC from top to bottom were projected and superimposed. Figure 4A shows that the majority of DC had internalized the WT bacteria within 1 hour, whereas the *lpxA-* mutant bacteria were very poorly adhered/internalized even after 18 hours of co-culture. Further confirmation that the bacteria had been internalized was

obtained by a differential labeling technique (Figure 4B). DC were incubated with WT or *lpxA- N. meningitidis* for 18 hours and then stained with To-Pro3, which labels all DNA including DC nuclei and the bacteria in non-permeabilized cells. A FITC conjugated monoclonal antibody (Mab p1.7) to *N. meningitidis* porin was used to identify non-internalized bacteria as only surface bound bacteria would be labeled in non-permebilized cells. It is clear from figure 4B that virtually all WT bacteria associated with the DC had been internalized with few left on the surface. In contrast, when *lpxA-* bacteria were used, most were seen on the DC surface and had not been internalized as the bacteria were stained both blue and green. Similar results were obtained when DC association with the WT and *lpxA-* mutant bacteria was examined by flow cytometry. A summary of ten such experiments is shown in figure 4C. The majority of DC were associated with the WT bacteria within 1 hour. In contrast, only about 15% of DC were associated with the *lpxA-* mutant bacteria. Although this proportion rose slowly over time, it remained significantly less than the proportion of DC associated with WT bacteria over the entire time course. Furthermore, addition of purified meningococcal LOS did not increase the internalization of the *lpxA-* bacteria (data not shown) indicating that internalization depends on LOS being expressed by the bacteria.

Induction of LOS biosynthesis restores both internalization and cytokine production

To directly test the role of LOS in internalization and to further examine the relationship between neisserial LOS content and DC cytokine production, the *lpxA* regulatory strain HA3003 derived from the *N. meningitidis* strain H44/76 was used (Steeghs *et al.*, 2001). To test for *lacI*^{q}*-tac* control of *lpxA* in HA3003, LOS was extracted from whole cells

which were grown in the absence or presence of increasing concentrations of IPTG and analyzed by Tricine SDS-PAGE and silver staining (Figure 5A). As expected, when HA3003 was grown in the absence of IPTG, LOS was barely detectable. In contrast, an IPTG dose-dependent increase in LOS expression was found when IPTG concentrations up to 10μM were used. At 10μM IPTG or more, LOS biosynthesis in strain HA3003 was restored to the WT level.

Figure 5B shows DC association with FITC bacteria after 2h of co-culture with HA3003 strain grown in the absence or presence of 2μM or 10μM of IPTG. Similarly to *lpxA*-, HA3003 grown in the absence of IPTG (expressing very low levels of LOS) was poorly phagocytosed. However, restoration of LOS biosynthesis by IPTG restored DC association of HA3003 *N. meningitidis* to levels comparable with the WT bacteria. Moreover, the LOS dependent increase in association of HA3003 with DC also restored internalization of the bacteria as studied by confocal microscopy (data not shown) and resulted in a concomitant increase in cytokine production (Figure 5C). Similar levels of IL12 and IL10 were produced by DC in response to either the HA3003 strain grown in the presence of IPTG as the WT bacteria after 18h of co-culture as measured by ELISA. In contrast, *lpxA*- and HA3003 grown in the absence of IPTG induced significantly less IL12 and IL10 compared to the WT or the LOS-expressing HA3003 strain. Similar results were obtained by intracellular staining of cytokines (not shown). In conclusion, these data confirm the requirement of LOS for both the internalization of *N. meningitidis* and subsequent cytokine response.

LBP is required for binding and phagocytosis of *N. meningitidis* to DC

Further proof of the requirement for LOS was obtained by examining internalization and cytokine production in the absence of LOS binding protein (LBP). Binding of LOS to LBP greatly enhances LOS internalization and cell activation by molecules, such as CD14, TLR4 and CD11b/CD18 (Tobias *et al.*, 1999; Ulevitch and Tobias, 1999). Internalization of *N. meningitidis* by DC was compared in serum free medium, serum free medium with added recombinant LBP and medium with FCS, which contains free LBP (Figure 6A). As before, most DC cultured in FCS rapidly internalized the bacteria. In serum free conditions however, very few bacteria were either bound or internalized by the DC. When the cells were cultured in serum free conditions but with added recombinant LBP, a pronounced increase in binding and internalization was observed by flow cytometry and confocal microscopy (Figure 6A and 6B). A similar picture was obtained for cytokine production (Figure 6C). In serum free conditions, the proportion of DC producing TNF-α and IL12 was much lower than DC cultured in medium with FCS. Addition of recombinant LBP to the serum free cultures increased both TNF-α and IL12 production. LBP alone did not activate DC and did not induce any detectable cytokine production (not shown). In these experiments, DC were cultured for a maximum of 7 hours because longer culture in serum free conditions resulted in lower viability. The levels of IL12 production were therefore less than typically obtained after 18 hours culture. Nevertheless, the results clearly demonstrate a crucial role for LBP in the internalization and cytokine production of *N. meningitidis* by DC.

Discussion

Group B *N. meningitidis* bacteria are potent activators of human DC inducing expression of surface activation markers and production of proinflammatory cytokines (Dixon *et al.*, 2001; Kolb-Maurer *et al.*, 2001; Unkmeir *et al.*, 2002). In addition, DC have been shown to phagocytose and kill *N. meningitidis* (Kolb-Maurer *et al.*, 2001; Unkmeir *et al.*, 2002). Various bacterial components have been implicated in DC activation but LOS is generally thought to be the most potent (Kadowaki *et al.*, 2001; Riva *et al.*, 1996; Verhasselt *et al.*, 1997). However, purified LOS from *N. meningitidis* is unable to stimulate optimal cytokine production in DC, particularly IL12 (Dixon *et al.*, 2001). Intact Gram-negative bacteria are more potent inducers of NF-κB translocation than purified LOS suggesting that phagocytosis of whole bacteria leads to increased cell signaling compared to purified bacterial components (Hofer *et al.*, 2001). Here we demonstrate that membrane-bound LOS is required for association and subsequent internalization of the bacteria by DC, which is a prerequisite for maximal cytokine (IL12 and TNF-α) production but not costimulatory molecule (MHC Class II and CD86) expression or IL6 production.

The requirement of membrane-bound LOS for maximal cytokine production by DC was demonstrated with a transwell membrane culture system used to separate bacteria from DC. Under these conditions, bacterial components such as DNA, outer membrane particles and LOS released during storage of the bacteria will pass through the membrane and make contact with the DC whereas the intact bacteria cannot. In these experiments, TNF-α and IL12 production was reduced to less than half compared to co-culture of

bacteria and DC allowing contact and internalization. In contrast, separation of intact bacteria and DC in the transwell cultures did not have any effect on the increase in expression of surface activation markers or IL6 production.

We found that DC were very effective at internalizing killed wild type *N. meningitidis* raising the possibility that uptake of the bacteria may be a critical event for DC cytokine production. To address this, intracellular cytokine staining of DC co-cultured with *N. meningitidis* was performed. Indeed, IL12 and TNF production was found to be confined mainly to DC that had internalized the bacteria. Although the flow cytometric technique used in these experiments did not distinguish between bacteria on the cell surface and bacteria inside the cell, confocal microscopy and double staining with To-pro3 and p1.7 Mab showed that the majority of bacteria after co-culture with DC had been phagocytosed. Furthermore, toxin B or cytochalasin D blocking of phagocytosis of *N. meningitidis* by DC profoundly inhibited TNF-α and IL12 production without an effect on DC viability or expression of surface activation molecules. Interestingly, cytochalasin D a fungal toxin from *Zygosporium mansonii* that inhibits actin polymerisation did not inhibit IL6 production showing that integrity of the cytoskeleton is not required for all cytokine production or expression of maturation markers. Moreover, the results from the transwell studies demonstrate that IL6 production by DC, in contrast to IL12 and TNF-α, is more easily induced by diffusible bacterial products, such as LPS. These results strongly support an important role of bacterial internalization for production of IL12 and TNF-α, but not IL6, by DC. Internalization of bacteria for antigen processing before

activation and migration could ensure that DC do not arrive in the draining lymph nodes without antigen.

We reported previously that the LOS deficient *lpxA-* mutant of *N. meningitidis* induced DC activation markers CD40, CD80, CD83 and CD86 consistent with maturation from immature DC but was unable to induce high levels of TNF-α and IL-1 and in particular IL12 (Dixon *et al.*, 2001). The levels of IL6 were comparable to the WT bacteria. Most importantly, addition of purified meningococcal LOS was unable to restore the response to the *lpxA-* bacteria. Here, we demonstrate that the reason for the poor cytokine production in response to the *lpxA-* bacteria was related to defective binding and/or internalization by DC. The results from many experiments showed that there was very little association and internalization of the *lpxA-* up to 18 hours and even at this later time point only a few DC had internalized *lpxA-* compared to wild type bacteria. In our experiments, PFA inactivated organisms were used. Similar phagocytosis and cytokine production was obtained when heat or ethanol killed bacteria were used demonstrating that the inactivation method of the bacteria does not affect the interaction with the DC (data not shown). Since the constituents other than LOS of the membrane are shared between the WT and the *lpxA- N. meningitidis* (Steeghs *et al.*, 2001), the reduced IL12 and TNF-α production by DC must be linked to their inability to bind or take up the mutant bacteria due to the lack of LOS. This was confirmed by using the *lpxA* regulatory strain HA3003. Restoration of LOS expression in HA3003 restored internalization and cytokine production by DC in response to *N. meningitidis*. In accordance with this important role of LPS in bacterial internalization by DCs, Jones et al. recently

demonstrated that sialic acids of *N. meningitidis* LOS can interact with phagocytic receptors on macrophages and enhance uptake of live bacteria (Jones *et al.*, 2003).

Internalization of LPS has been shown previously to depend on LPS complex formation with LPS binding protein (LBP), which then catalyzes its transfer to either membrane bound or soluble CD14 (Tobias *et al.*, 1999). The reduction in internalization and cytokine production by DC in media without LBP and its restoration by addition of recombinant LBP (Figure 6) provides direct evidence of the role of LPS in DC internalisation of *N. meningitidis*. This is interesting in view of the fact that DC express only low levels of CD14, which is lost on activation by the bacteria. It is possible that the low amount of CD14 expressed by the immature DC, or soluble CD14, is sufficient for the response. Alternatively, it may be operating through a CD14-independent pathway as indicated by studies showing that LBP can potentate LOS responses in the absence of CD14, especially in response to whole bacteria (Klein *et al.*, 2000; Moore *et al.*, 2000). In this respect, CR3 (CD11b/CD18) was previously shown to bind LOS and may provide such a pathway (Wright *et al.*, 1989). The role of CR3 in DC interaction with *N. meningitidis* is currently under investigation.

Other groups have studied the interactions of group B *N. meningitidis* with human DC (Kolb-Maurer *et al.*, 2001; Unkmeir *et al.*, 2002). In these studies, adhesion and phagocytosis of live encapsulated bacteria and a mutant unencapsulated *N. meningitidis* were compared. Expression of the capsule significantly impaired adherence of bacteria and phagocytosis by DC and resulted in reduced cytokine production. In these

experiments, DC were shown to phagocytose and kill live *N. meningitidis*, although the amount of bacterial internalization was typically less than observed in the current study, which used inactivated organisms. Our experiments have also shown that live encapsulated WT bacteria are not as readily phagocytosed by DC as the killed organisms (unpublished observations). The mechanisms of how live *N. meningitidis* avoid phagocytosis are not yet completely understood and need further investigation. It is likely that DC would encounter both live and dead bacteria *in vivo* during natural infection as innate immune mechanisms, such as the complement activation, and/or treatment with antibiotic kill bacteria at an early stage of infection. Interestingly, a recent study showed that sialic acids of *N. meningitidis* LOS can interact with phagocytic receptors on macrophages and enhance the bacterial uptake (Jones *et al.*, 2003).

This is the first report that shows an important link between internalization of *N. meningitidis* and cytokine production by human DC. Cytokine production by murine macrophages has been shown to be uncoupled from phagocytosis (Peiser *et al.*, 2002). Phagocytosis of *N. meningitidis* by macrophages from SR-A knockout mice was completely abolished showing that SR-A acts as the major receptor for *N. meningitidis* in mice. Phagocytosis by murine macrophages was however LOS-independent since the *lpxA*- bacteria were equally taken up when compared to the WT bacteria. These results highlight the differences between species and the different functions of macrophages and DC. Interestingly, a recent report showed that production of IL12 by human DC in response to a Gram-positive bacterium *Streptococcus pneumoniae* also required internalization of the bacteria (Colino and Snapper, 2003). Inhibition of DC phagocytosis

of *S. pneumoniae* by cytochalasin D resulted in more than 80% reduction in IL12 production by DC, whereas IL6 was unaffected. In addition, surface marker expression was unaffected by cytochalasin treatment. These results are consistent with our findings.

Overall, our experiments provide convincing evidence that LOS expressed by the bacteria is required for rapid phagocytosis of *N. meningitidis* by DC and is essential for optimal cytokine production, particularly IL12. The requirement for internalization of the bacteria is consistent with the report that human toll-like receptors (TLRs) may be expressed inside the cell. For example, TLR2 is not required for phagocytosis but is recruited specifically to macrophage phagosomes where it is thought to signal the cell for cytokine production by sampling the content of phagosomes (Gantner *et al.*, 2003; Underhill *et al.*, 1999). Similarly, it has been shown recently that TLR9 is expressed in lysosomes, where it signals in response to CpG DNA (Ahmad-Nejad *et al.*, 2002) and LOS has been shown to co-localise with TLR4 within the Golgi complex of epithelial cells (Hornef *et al.*, 2002). With this in mind, it is interesting that we have been able to detect intracellular but not cell surface expression of TLR2 and TLR4 in DC with TLR peptide specific antibodies (Uronen-Hansson et al, *in press*). This suggests that the signaling events might not occur on the cell surface, but rather inside the cell after bacterial internalization. In view of these findings, we suggest that DC binding to *N. meningitidis* via LOS and the subsequent signaling for IL12 production are two independent, but closely related, events. Interestingly, the intracellular Nod molecules have been shown to be involved in Gram-negative bacterial sensing and may therefore be involved in the DC response to *N. meningitidis* (Girardin et al., 2003; Inohara et al., 2001).

The results presented in this study may be of particular importance in the design of future *N. meningitidis* group B vaccines. Current strategies are predominantly directed at identifying immunogenic bacterial subunits. It is hoped that once discovered, these immunogens can be used alone or as outer membrane vesicle vaccines. However, the processing of such vaccines by DC will be critical in generating an effective and long lasting immune response. Consideration of how and what to deliver to DC to achieve this aim will be crucial to the development of a successful *N. meningitidis* group B vaccine.

Experimental procedures

Bacteria

N. meningitidis serogroup B (strain H44/76) used in this study is a piliated and encapsulated Norwegian isolate from a case of fatal septicemia (Holten, 1979). Construction of the viable LOS deficient, isogenic mutant *lpxA-* by insertional inactivation of the *lpxA* gene with a kanamycin resistance cassette has been described previously (Steeghs *et al.*, 1998). Complete absence of LOS activity in the mutant was determined by Limulus amoebocyte assay, whole cell ELISA with LOS specific monoclonal antibody and by gas chromatography/mass spectrometry analysis. In addition, the expression and assembly of integral outer membrane proteins appeared largely unaffected in the *lpxA-* mutant (Steeghs *et al.*, 2001). The *lpxA* regulatory strain HA3003 has been described previously (Steeghs *et al.*, 2001). This strain carries the *lpxA* gene under control of an isopropyl-BD-Thiogalactopyranoside (IPTG)-inducible promoter allowing for a controlled biosynthesis of LOS by the bacteria. To induce the promoter, HA3003 strain was grown in the presence of 2-250 μM IPTG (Roche). All bacteria were grown on meningococcal agar (Difco, Basingstoke, UK) supplemented with Vitox (Oxoid Ltd., Basingstoke, UK) and cultured in 6% CO_2 in air at 36^0C. The bacteria were used in stationary phase after culture for 18 hours. Suspensions of bacteria were prepared in RPMI 1640 medium without phenol red (Gibco, Paisley, UK), and their optical density measured at 540nm. Paraformaldehyde (PFA) inactivated bacteria were used in this study. To kill bacteria, 0.5% PFA in PBS was used for 15 minutes and washed thoroughly in RPMI medium. After this, bacteria were non-viable as judged by viability counts and propidium iodide staining. FITC labeled (PFA inactivated) bacteria

were prepared by incubation with 0.5mg/ml of FITC (Sigma, Poole, UK) for 20 min at 37°C. Meningococcal LOS from *N. meningitidis* serogroup B (strain H44/76) was prepared by hot aqueous phenol extraction, ultra-centrifugation, gel filtration and cold ethanol-NaCl precipitation, as described previously (Andersen *et al.*, 1995).

LOS analysis

Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 4% stacking and 16% separating gels as described (Lesse *et al.*, 1990). Proteinase K-treated, boiled bacterial cells were used as samples (Apicella *et al.*, 1994). The gels were run for 3h at a constant current of 20mA and silver stained (Tsai and Frasch, 1982).

Dendritic cell culture and activation

DC were generated from human peripheral blood mononuclear cells (PBMC) as described previously (Dixon *et al.*, 2001; Sallusto and Lanzavecchia, 1994). In brief, monocytes prepared from PBMC by centrifugation over Percoll were incubated for 7 days in RPMI supplemented with 10% FCS, 2.4mM L-glutamine, 100U/ml penicillin-streptomycin (all from Gibco, Paisley UK), 100ng/ml of human recombinant GM-CSF and 50ng/ml of human recombinant IL-4 (Schering-Plough, Welwyn Garden City, Herts UK). Unstimulated DC at day 7 were $CD14^{low}$, $CD83^{-ve}$, $CD86^{low}$, $CD25^{-ve}$. They also expressed HLA DR, HLA DQ, HLA Class I, CD40 and CD1a, and were negative for both CD19 and CD3 as described previously (Dixon *et al.*, 2001).

DC were cultured at a concentration of 10^6/ml with *N. meningitidis* H44/76 or LOS deficient *lpxA*- mutant bacteria at 10^8/ml (E.g. DC/bacteria ratio of 1:100) and 100ng/ml purified meningococcal LOS in RPMI 1640 supplemented with 10% FCS. In some experiments, transwells™ with 0.1μm pores were used (Costar) with DC in the lower chamber and bacteria or LOS added to the top chamber. When intracellular cytokines were to be measured, the protein transport inhibitor Brefeldin A (Sigma, Poole, UK) was added at 10μg/mL. In our hands, inclusion of Brefeldin A for 18h gave the best results, particularly for IL12 and was not toxic to the DC. In some experiments, 500ng/ml of cytochalasin D (Sigma) or 10ng/ml toxin B (kind gift from Anne Ridley, Ludwig Institute for Cancer Research, London) were added 30 min prior to the addition of the bacteria. In serum free experiments, DC were cultured in RPMI (Gibco, Paisley, UK) and 2.5% BSA (LOS free, Sigma). Recombinant human LOS binding protein (LBP) (Biometec, Greifswald, Germany) was added at 1μg/ml as indicated.

Surface marker and cytokine measurements

Surface expression of activation antigens (CD3, CD14, CD19, CD40, CD83, CD86, CD25, HLA-DR, HLA-DQ, HLA Class I, and CD1a) was detected by staining with 5μl of the appropriate FITC conjugated monoclonal antibody (Caltag Medsystems, Silvestone, UK) for 30min on ice followed by washing in PBS. To measure intracellular cytokine production, DC were fixed with 4% PFA in PBS for 15 minutes, washed in PBS containing 0.1% sodium azide and 0.5% BSA (all from Sigma) and permeabilized in 50μl Permeabilization solution (Caltag). Cells were then incubated with monoclonal antibodies to TNF-α (Becton Dickinson BD, Oxford UK), IL12 p40/70 (Pharmingen) or isotype

matched controls for 30 minutes at room temperature in the dark. The cells were then washed twice in PBS, fixed in CellFix (Becton Dickinson) and analyzed by flow cytometry on a FACScalibur using Cell Quest software (Becton Dickinson). DC made up a distinct population identified by forward and right angle scattering. At least 95% of cells in this population were DC as defined by their expression of MHC II, CD1a, CD25, CD80, CD83 and CD86. A minimum of 5000 events within the gates corresponding to DC was collected for analysis. For ELISA assays of soluble cytokines, CytoSets™ ELISA kits (Biosource Europe S.A, Nivelles Belgium) for human IL12, TNF-α, IL6 and IL-10 were used according to manufacturer's instructions.

Phagocytosis assay

DC binding and phagocytosis of bacteria was determined by a combination of flow cytometry and confocal microscopy. For detection by flow cytometry, DC were incubated with FITC labeled bacteria for periods of time between 1 and 18 hours, fixed in FACSFix (BD), washed and analyzed on a FACSCalibur. DC associated with bacteria were easily identified by fluorescence within the gated DC population. DC phagocytosis and activation by FITC conjugated bacteria was indistinguishable from unlabelled bacteria. For confocal microscopy, DC stimulated with FITC labeled *N. meningitidis* at a ratio of 1:100 were allowed to adhere for 10 min to an adhesion slide (Bio-Rad Laboratories Ltd., Herts, UK). To stop phagocytosis, DC were fixed with 4% PFA for 10 min. In some experiments, intracellular bacteria and the nuclei of DC were stained with the cell permeable DNA stain To-Pro3 (Molecular Probes, Cambridge Biosciences, Cambridge, UK) for 10 min. Extracellular bacteria were identified by staining with

10μg/ml of FITC conjugated P1.7 antibodies specific for *N. meningitidis* (NIBSC, South Mimms. UK) for 30 min. Visualization of DC was enhanced in some experiments by staining with 5μg/ml of anti MHC Class II monoclonal antibodies (Dako, Glostrup, Denmark). After washing, bound antibody was detected with 5μg/ml of Texas Red - conjugated goat anti-mouse antibody (Molecular probes) for 1h. The slides were washed and mounted in Citifluor (Citifluor Ltd, UK). Confocal images were obtained using a Leica SP2 confocal laser scanning microscope system (Leica, Milton Keynes, UK) fitted with appropriate filter sets. To identify intracellular bacteria, 15-20 optical sections (0.3-0.5μm) spanning the entire DC were projected and superimposed with Leica confocal imaging software.

Statistics

The results were analyzed by paired *t*-test using Sigmaplot 2000 analysis package. *P*-values < 0.05 were considered to be statistically significant

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Figure legends

Figure 1. Intracellular cytokine production and surface marker expression by DC stimulated with *N. meningitidis* H44/76 in transwell™ cultures. DC cultured in the lower chamber of a transwell were separated from the bacteria added to the upper chamber by a membrane with 0.1μm pores. Purified LOS was added at 100ng/ml to the upper chamber. **A)** Intracellular IL12, TNF-α and IL6 were measured after 18h of incubation in the presence of brefeldin A. The cells were permeabilized and analyzed by flow cytometry for intracellular cytokines and the results expressed as % positive DC. **B)** MHC Class II and CD86 expression after 18h as indicated by median fluorescence intensity (MFI). DC were gated according to forward and side scatter. Data are expressed as the mean and SEM of three separate experiments from three different donors. WT= wild type *N. meningitidis*, TW=transwell

Figure 2. Cytokines are produced mainly by DC that are associated with *N. meningitidis*. DC were stimulated with FITC labeled bacteria for 18h in the presence of brefeldin A and then stained for intracellular TNF-α and IL12. The dot plots show percentage of DC associated with FITC-labeled bacteria and intracellular cytokines. A representative of three experiments is shown.

Figure 3. Inhibition of phagocytosis by cytochalasin D. A) DC were incubated with cytochalasin D at a concentration of 500ng/ml for 30 min and then cultured with FITC labeled bacteria at a ratio of 1:100 for 18h. The cells were then adhered to slides, stained for MHC Class II (Texas Red) and examined by confocal microscopy. Bacteria appear as green (FITC) particles. **B)** Cytokine production after inhibition of phagocytosis with cytochalasin D. DC were incubated with cytochalasin D for 30 min and then cultured with *N. meningitidis* at a ratio of 1:100 for 18h. Supernatants were then analyzed by ELISA for TNF-α, IL12 and IL6. Results are expressed as the mean and SEM of four experiments. **C)** Effect of cytochalasin D treatment on surface marker expression. DC were first incubated with cytochalasin D and then stimulated with *N. meningitidis* for 18h and the expression of MHC Class II and CD86 analyzed by FACS. DC stimulated with *N. meningitidis* (black line), DC treated with cytochalasin D and then stimulated with *N. meningitidis* (red line), unstimulated DC (dotted line). The histograms shown are representative of five experiments.

Figure 4. Phagocytosis of wild type and *lpxA*- mutant *N. meningitidis*. A) DC were cultured with FITC labeled bacteria at a ratio of 1:100 for 1, 5 and 18h then adhered to glass slides, stained for MHC Class II (Texas Red) and examined by confocal microscopy. **B)** Confocal images of phagocytosis after 18h using differential staining with To-Pro3 (blue) and FITC conjugated P1.7 antibodies (green) specific for *N. meningitidis* to distinguish between internalized (blue) and externally bound (blue and green) bacteria. To-Pro3 labels all DNA including DC nuclei and the bacteria without

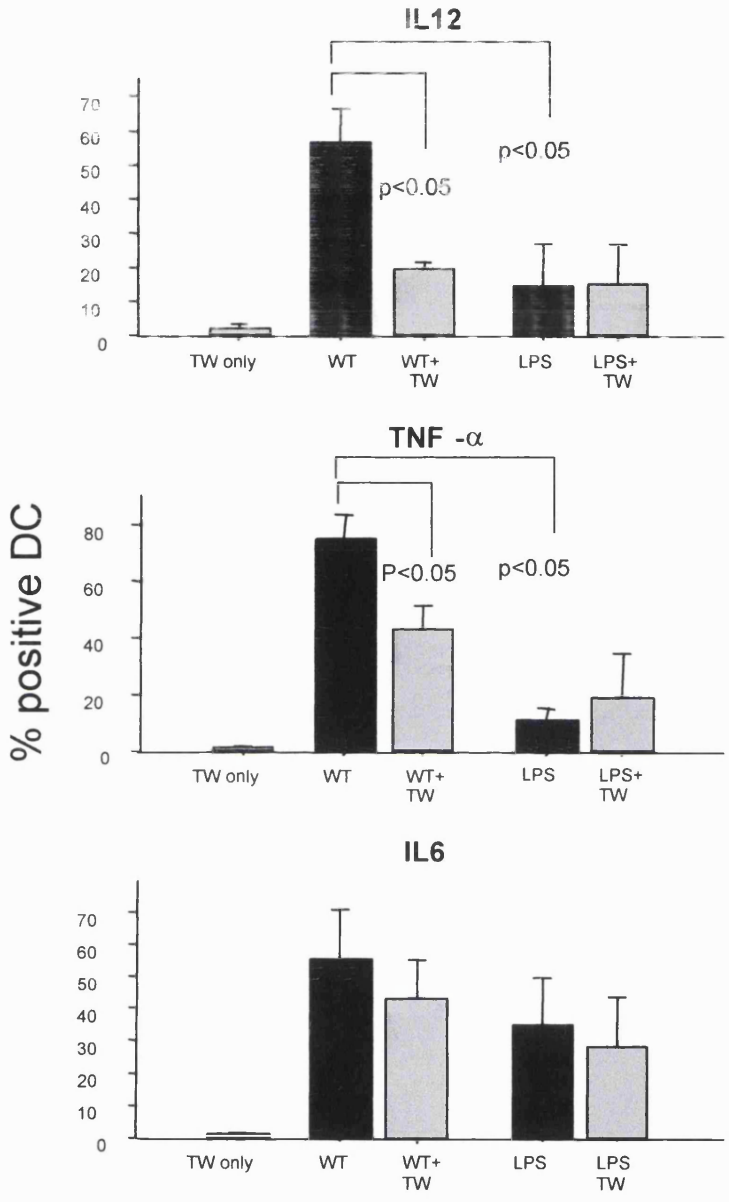
permebilization. FITC Mab p1.7 stains only surface bound bacteria in non-permebilized DC. Arrows show internalized meningococci. Confocal images shown are representative of at least three separate experiments. **C)** Time course of phagocytosis determined by FACS analysis of DC in response to FITC labeled WT and *lpxA*- bacteria. Results are expressed as the mean and SEM of ten separate experiments.

Figure 5. Phagocytosis and cytokine production by DC in response to *lpxA* regulatory strain HA3003. **A)** Strain HA3003 bacteria were grown in the absence or presence of 2μM, 10μM or 50μM μM IPTG. The LOS content was analyzed in whole bacterial cell lysates by Tricine SDS-PAGE and silver staining. **(B)** DC and FITC labeled bacteria were cultured at 1:100 ratio (DC:bacteria). Phagocytosis by DC was analyzed by FACS after 2h. **C)** IL12 and IL10 production by DC in response to WT, *lpxA*- and HA3003 strain were measured in culture supernatants by ELISA after 18h of co-culture. Mean and SEM of three separate experiments is shown.

Figure 6. LBP is required for phagocytosis and optimal cytokine production. DC were cultured with *N. meningitidis* at a ratio of 1:100 in RPMI 1640/10% FCS, RPMI/2.5% BSA or RPMI/2.5% BSA with 1μg/ml of recombinant human LBP. **A)** Phagocytosis was measured by FACS and **B)** confocal microscopy after 7h. **C)** Intracellular production of TNF-α and IL12 was measured after 7h of stimulation. In control experiments, LBP alone did not activate DC or induce detectable cytokine production (not shown). Results shown are representative of three separate experiments.

Figure 1

A)


IL12
70
60
50
40
30
20
10
0
p<0.05
p<0.05
TW only
WT
WT+ TW
LPS
LPS+ TW
TNF -α
% positive DC
80
60
40
20
0
P<0.05
p<0.05
TW only
WT
WT+ TW
LPS
LPS+ TW
IL6
70
60
50
40
30
20
10
0
TW only
WT
WT+ TW
LPS
LPS TW

B)

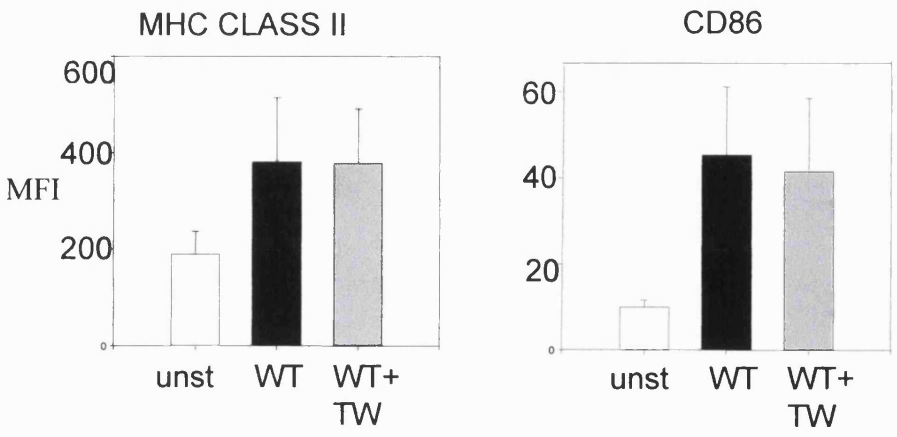
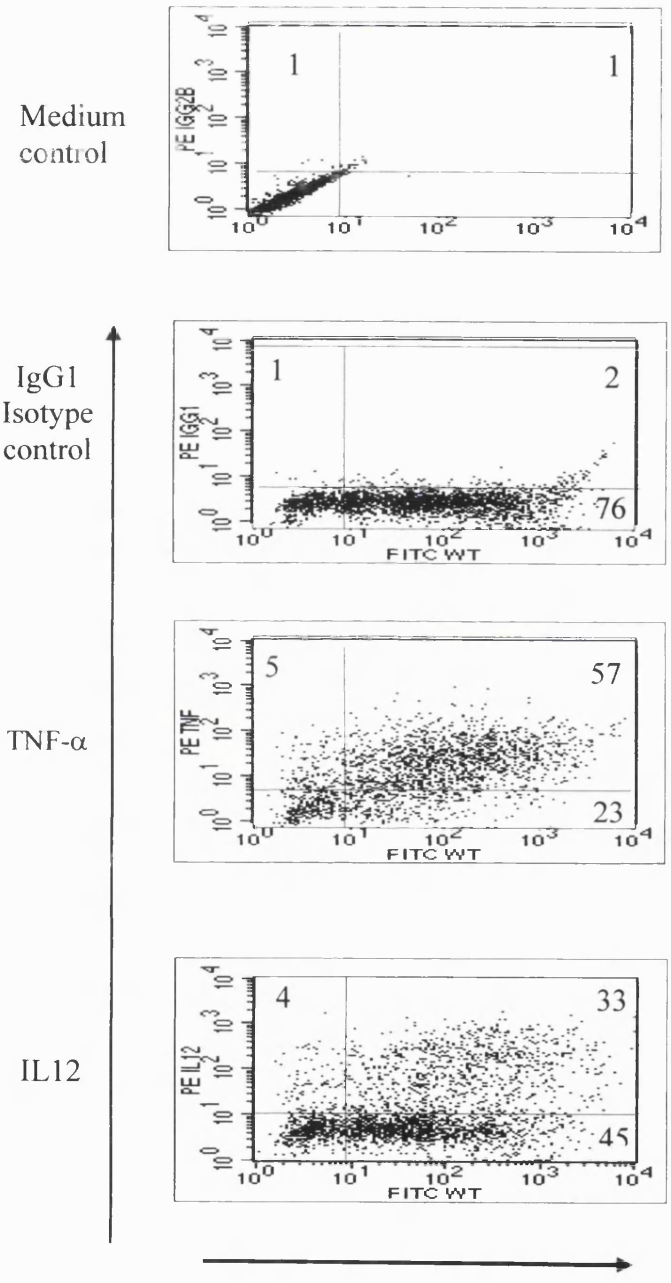

MHC CLASS II
600
400
MFI
200
0
unst
WT
WT+ TW
CD86
60
40
20
0
unst
WT
WT+ TW

Figure 2



FITC labeled *N. meningitidis*

Figure 3

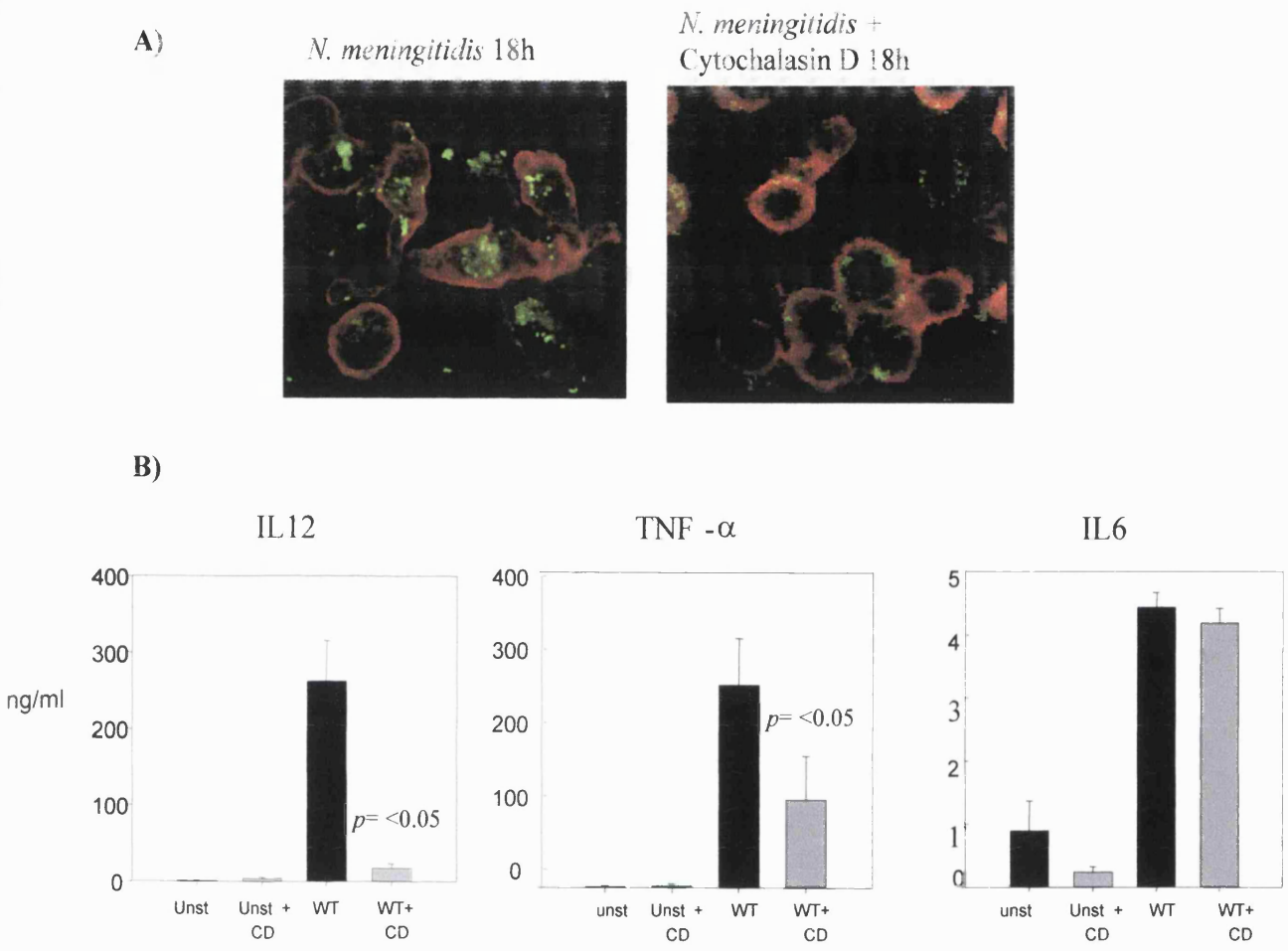
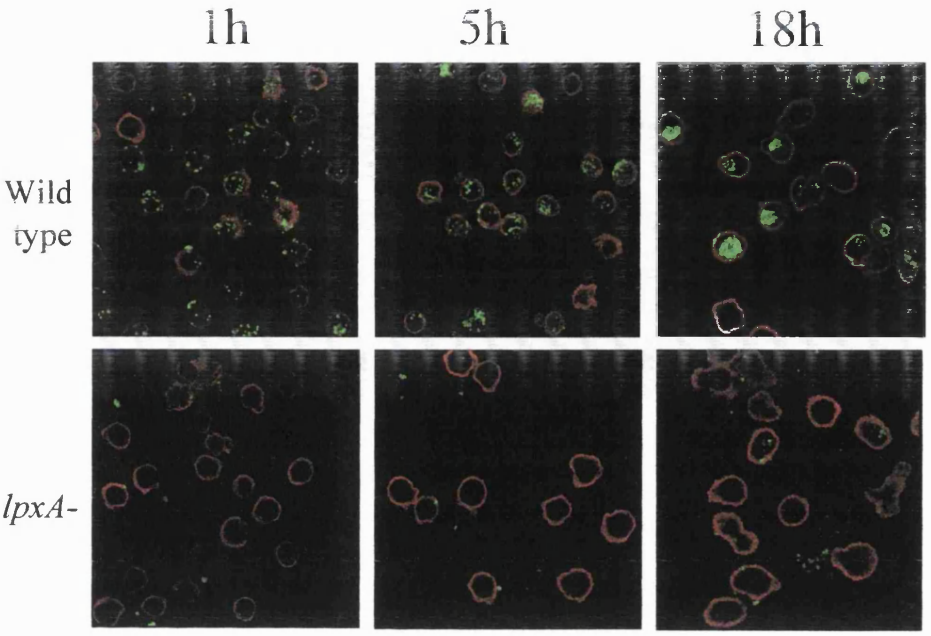
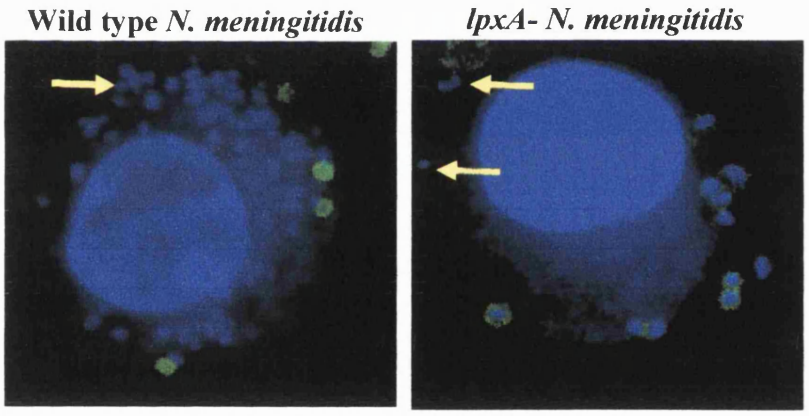

A)
N. meningitidis 18h
N. meningitidis + Cytochalasin D 18h
B)
IL12
TNF -α
IL6
ng/ml
400
300
200
100
0
p= <0.05
Unst
Unst + CD
WT
WT+ CD
unst
5
4
3
2
1
C)
Counts
50
0
10^0
10^1
10^2
10^3
10^4
MHC Class II
CD86
Unstimulated
N. meningitidis
N. meningitidis + cytochalasin D

Figure 4

A)


1h
5h
18h
Wild type
lpxA-

B)


Wild type N. meningitidis
lpxA- N. meningitidis

C)

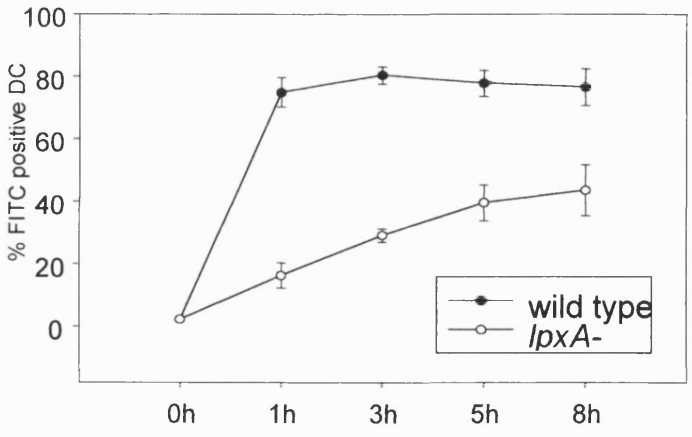
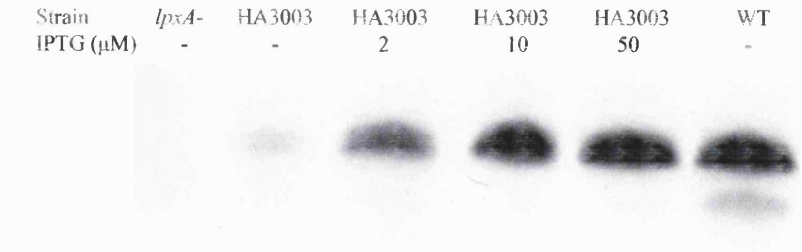
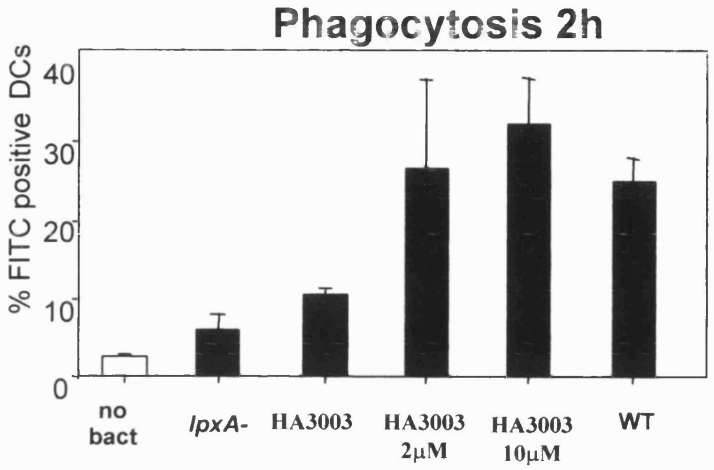

100
80
60
40
20
0
% FITC positive DC
0h
1h
3h
5h
8h
wild type
lpxA-

Figure 5

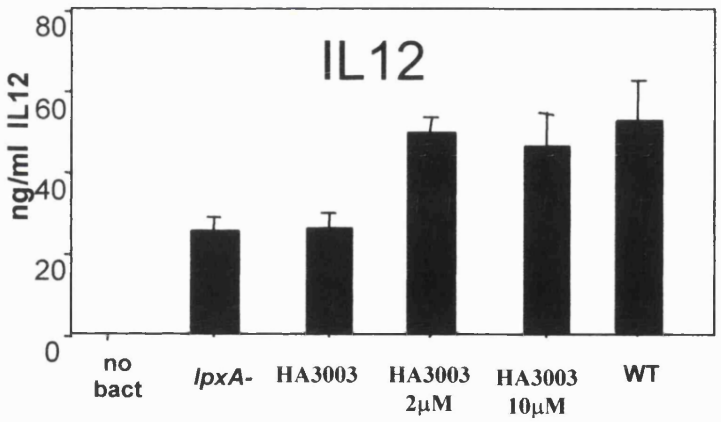
A)


Strain
IPTG (μM)
lpxA-
-
HA3003
-
HA3003
2
HA3003
10
HA3003
50
WT
-

B)


Phagocytosis 2h
% FITC positive DCs
0
10
20
30
40
no bact
lpxA-
HA3003
HA3003 2μM
HA3003 10μM
WT

C)


IL12
ng/ml IL12
0
20
40
60
80
no bact
lpxA-
HA3003
HA3003 2μM
HA3003 10μM
WT

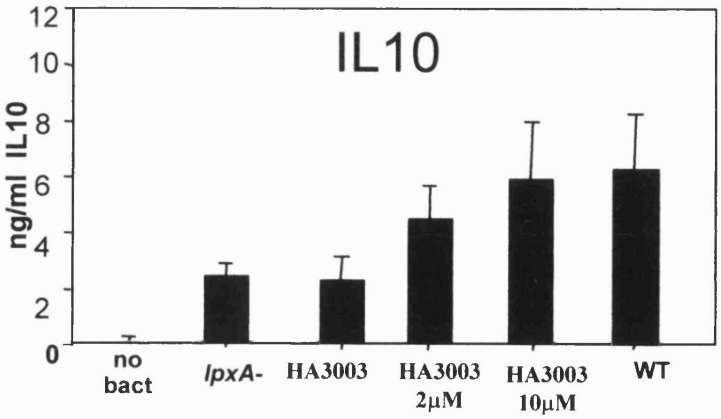

IL10
ng/ml IL10
0
2
4
6
8
10
12
no bact
lpxA-
HA3003
HA3003 2μM
HA3003 10μM
WT

Figure 6