Dendritic Cells and the Immune

Response to *Neisseria meningitidis*

Jennifer Sarah Allen
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Immunobiology Unit

Institute of Child Health

University College London

WC1N 1EH

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Abstract

The human specific pathogen *Neisseria meningitidis* is a major cause of bacterial sepsis and meningitis. Effective vaccines have been developed against group A and C strains, but as yet there is no suitable vaccine available for group B *N. meningitidis*. The activation of Dendritic Cells (DCs) is fundamental to the initiation of an immune response. Microorganisms and microbial products induce DC maturation, including expression of co-stimulatory molecules, HLA molecules, chemokine receptors and cytokines. Maturation and migration of DCs to secondary lymphoid organs is required for the optimal induction of T-lymphocyte responses. This study investigated the importance of lipopolysaccharide (LPS) in the human pathogen *N. meningitidis* for DC maturation, migration and activation of T cells. DCs activated with wild type *N. meningitidis* increased expression of co-stimulatory molecules and HLA-DR, and produced high levels of cytokines (TNFα and IL-12). Expression of the chemokine receptor CCR7 was increased and DCs migrated in response to the chemokine MIP3β and showed enhanced migration through an endothelial monolayer. In co-culture experiments, T cell proliferation was enhanced by DCs that had been activated with wild type bacteria. In addition, T cell IFNγ production was increased and IL-4/IL-13 decreased compared to controls, this is consistent with Th1 differentiation. In contrast, although DCs activated by the LPS deficient isogenic mutant *lpxA*- increased expression of co-stimulatory molecules and HLA-DR, they produced less cytokines (TNFα and IL-1) than DCs stimulated with wild type bacteria and little or no IL-12. In addition, they did not increase expression of CCR7 or migrate in the presence of MIP3β. DCs stimulated with *lpxA*- were able to activate T cells to proliferate but did not induce Th1 differentiation. TLR4 has been shown to be important for responses to LPS but the Asp299Gly polymorphism described in humans was found not to affect monocyte responses to wild type *N. meningitidis*. These findings have important implications for vaccine design to group B *N. meningitidis* and suggest that in vitro responses to DCs may be used as a surrogate for in vivo immunisation experiments to test vaccine efficacy.

Acknowledgements

I'd like to dedicate this thesis to the loving memory of my father

~ Brian William Allen ~

1949-2000

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Abbreviations

APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CD	Cluster Differentiation
CFSE	CarboxyFluorescein Succinimidyl Ester
CPM	Counts Per Minute
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Associated Molecule 4
DC	Dendritic Cell
DC_{medium}	Day 6 DCs stimulated for 18hours in medium alone
DC_{LPS}	Day 6 DCs stimulated for 18hours with 10ng/ml LPS
DC_{LPXA}	Day 6 DCs stimulated for 18hours with 10^7organisms/ml *lpxA*-
DC_{WT}	Day 6 DCs stimulated for 18hours with 10^7organisms/ml WT
DC SIGN	Dendritic Cell Specific ICAM3 Grabbing Non-integrin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleotide Triphosphates
EDTA	Ethenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EVOM	Electrical Voltage Ohm Meter
FACS	Flourescent Activator Cell sorter
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate isomer
GMCSF	Granulocyte-Monocyte Colony Stimulating Factor

GPI	glycosylphosphatidylinositol
HBSS	Hanks Balanced Salt Solution
HEPES	Hydroxyethyl]piperazine-N'-[2-ethansulfonic acid]
HSP60	Heat Shock Protein 60
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM	Inter Cellular Adhesion Molecule
ICOS	Inducible co-stimulator
IL	Interleukin
IκB	Inhibitory κB
IMS	Industrial Methylated Spirits
IRAK	Interleukin-1 Receptor-Associated Kinase
LBP	LPS Binding Protein
LFA	Lymphocyte Function-Associated Antigen
LPS	Lipopolysaccharide
MCP	monocyte chemotactic protein
MFI	Median Flourescent Intensitiy
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MLR	Mixed Lymphocyte Reaction
MYD88	Myeloid Differentiation Factor 88
NFκB	Nuclear Factor κB
OMP	Outer Membrane Protein
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PECAM	Platelet/Endothelial Cell Adhesion Molecule
PHA	Phytohemagglutinin
PGE2	Prostaglandin E2
PMA	phorbal 12-myristate 13-acetate
PRR	Pattern Recognition Receptor
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SEB	Staphylococcus Enterotoxin B
TBE	Tris Borate EDTA
TCR	T cell receptor
Th Cell	T helper Cell
Th1 Cell	T helper type 1 Cell
Th2 Cell	T helper type 2 Cell
TIR Domain	Toll IL-1 Receptor Domain
TLR	Toll-Like Receptor
TRAF	TNF-Receptor Associated Protein
UV	Ultra Violet
VLA	Very Late Antigen

Chapter 1

Introduction

1.1 Host defence

All multi-cellular organisms have some form of defence system to protect the host from invading pathogens. In vertebrates host defence has traditionally been divided into two components, the innate and the adaptive immune systems. The adaptive immune system is exclusive to vertebrates and is composed of two major cell types, T and B lymphocytes. The receptors for both T and B lymphocytes are generated somatically during development by gene rearrangement, resulting in a large and diverse repertoire of antigen receptors. Following recognition of specific antigen by a receptor clonal expansion of lymphocytes occurs. This is essential in generating an efficient immune response. Clonal expansion is the proliferation and differentiation of lymphocytes into effector cells. The persistence of long lived memory lymphocytes after an initial infection results in future protection by rapid responses to subsequent infections by the same pathogen.

Adaptive immunity is restricted to vertebrates but the ability to recognise and defend against invading pathogens is common to all multi-cellular organisms and is known as innate immunity. The innate immune system is dependent on a limited number of germ line encoded receptors. As a result of this limitation, receptors of the innate immune system have evolved to recognise molecular structures on pathogens that are common to a large number of micro-organisms. These molecular structures are conserved products of microbial metabolism that are essential for the survival or pathogenicity of the micro-organism and thus are not subject to change. The molecular structures recognised by the innate immune system have been designated pathogen associated molecular patterns (PAMPs) (Janeway-CA J., 1998)(Medzhitov R. et al., 1997). Examples of PAMPs include the lipopolysaccharides of Gram-

negative bacteria, the teichoic acids of Gram-positive bacteria, unmethylated CpG

motifs characteristic of bacterial DNA, double stranded RNA found in viruses, and

mannans of yeast cell walls (reviewed in (Medzhitov R. et al., 1997)). The receptors

of the innate immune system, which recognise the conserved patterns on microbes

are called pattern recognition receptors (PRRs) (Janeway-CA J., 1998)(Medzhitov R.

et al., 1997). Examples include mannose binding lectin (MBL), mannose receptor,

DEC-205, CD14 and the toll-like receptor family (TLR).

The innate and adaptive branches of the vertebrate immune system have traditionally

been dealt with as two separate entities. However dendritic cells (DCs) represent a

unique link between these two systems. As a component of the innate immune

system, DCs have the ability to recognise and respond to invading micro-organisms.

Adaptive immune responses are then initiated when T cells recognise antigenic

peptides bound to MHC molecules expressed on DCs.

1.2 Dendritic cells

As professional antigen presenting cells (APCs), the main role for DCs in an immune

response is not to clear invading pathogens, but to alert the immune system to the

potential danger. Following recognition and uptake of a pathogen, DCs release pro-

inflammatory cytokines and chemokines that recruit other cells such as macrophages,

neutrophils and NK cells to the site of infection. DCs then migrate to the secondary

lymphoid organs where they present processed antigen to T lymphocytes.

1.2.1 Dendritic cell lineages and origins

Dendritic cells are derived from $CD34^+$ haematopoietic bone marrow stem cells (Fig

1.1). In humans there are many different populations of DCs that reside in different

tissues. These different DC populations may be derived from different lineages.

Though DCs have been identified in tissue sections *ex vivo*, until recently *in vitro*

culture of immature DCs has not been possible. The ability to culture DCs from

monocytes *in vitro* in the presence of GMCSF and IL-4 has enabled more extensive

investigations into all aspects DC biology (Sallusto F. et al., 1994). Monocytes also

differentiate into DCs following transendothelial migration and phagocytosis in the

absence of additional cytokines (Randolph G.J. et al., 1998a)(Randolph G.J. et al.,

1999).

Another DC type can be derived from plasmacytoid cells, which are circulating

$CD11c^-$ cells (Fig 1.1). Plasmacytoid cells cultured *in vitro* with IL-3 acquire a DC

phenotype (Grouard G. et al., 1997). They are thought to be of lymphoid lineage as

they are IL-3 dependent and express $pT\alpha$, a molecule that assembles with the T cell

receptor (TCR) β chain to form a pre TCR (Res P.C. et al., 1999). Plasmacytoid DCs

have been identified as the major type 1 interferon ($IFN\alpha/\beta$) producing cells

(Kadowaki N. et al., 2000) (Cella M. et al., 1999a). They respond to viral antigens

and express only TLRs 7 and 9 (Kadowaki N. et al., 2001).

Langerhans cells (LCs) are a type of DC that populate squamous epithelia including

skin, conjunctiva and respiratory and genital mucosal surfaces (Girolomoni G. et al.,

2002). LCs contain Birbeck granules, unique intracellular organelles that are

involved in endocytosis. They also express CD1a and E-cadherin. The adhesion

molecule E-cadherin retains LCs in the epidermis by homotypic interactions with keratinocytes. LCs differentiate in the skin from a circulating myeloid $CD34^+$ and $CD1a^+$ precursor, probably under the influence of TGF-β and IL-15 (Girolomoni G. et al., 2002)(Liu Y.J., 2001). LCs can be derived *in vitro* by culturing $CD34^+$ cells in GM-CSF, TNFα, TGF-β, and flt-3 ligand (Gatti E. et al., 2000).

Another DC subset referred to as interstitial DCs are also derived from $CD34^+$ haematopoietic stem cells. These cells differentiate in steady state conditions and have been identified in the human foetus and new born rats (Fossum S., 1989). Interstitial DCs express CD2, CD9, CD68 and Factor XIIIa (Liu Y.J., 2001), and reside in many tissues including the dermis layer of skin and mucosal surfaces.

Immature LCs and interstitial DCs are already committed to a DC phenotype. They have dendrites or veils and express moderate levels of co-stimulatory molecules. Monocytes and plasmacytoid cells only differentiate into DCs after appropriate stimulation (Fig 1.1).

Mice have a similarly complex number of DC subsets that include $CD8\alpha^+$ 'lymphoid' DCs, $CD8\alpha^-$ 'myeloid' DCs, $CD8\alpha^-CD11b^-$ DCs and LCs, as well as other subsets identified in specific tissues and organs (reviewed in (Pulendran B. et al., 2001)).
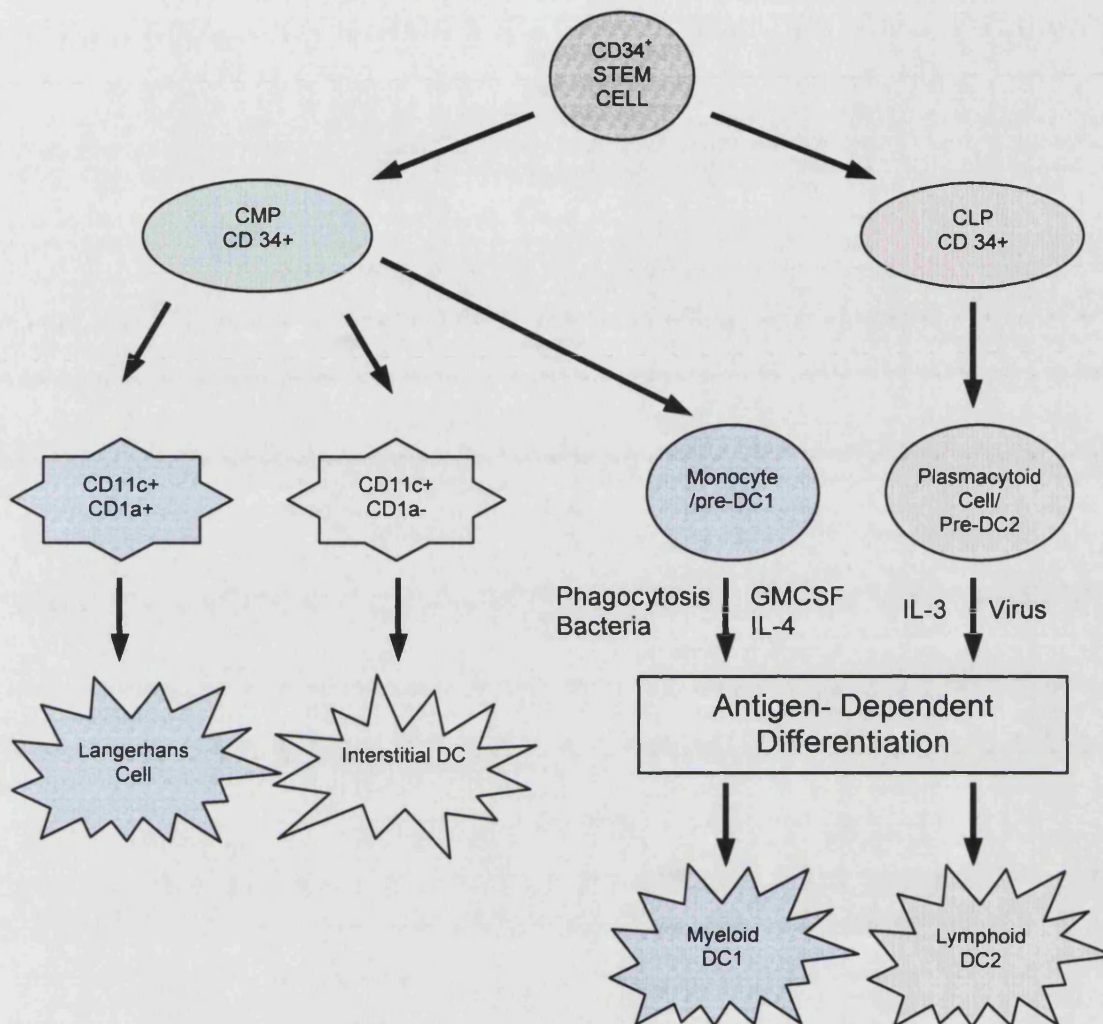
Figure 1.1 Dendritic cell lineages

Haematopoietic stem cells (CD34^{+}) differentiate into common lymphoid precursor (CLP) and common myeloid precursor (CMP). The CMPs differentiate into Langerhans cell precursors and interstitial DC precursors in blood. The Langerhans cell precursors (CD11c^{+}CD1a^{+}) migrate from blood to the skin epidermis and become Langerhans cells. The CD11c^{+}CD1a^{-} cells migrate to other tissues and become interstitial DCs. Both of these processes of DC differentiation are independent of antigen. CMP cells also give rise to monocytes (pre-DC1), which can differentiate into DCs following phagocytosis of bacteria. The CLP cells are thought to differentiate into plasmacytoid cells (pre-DC2), which produce large amounts of IFNα/β and differentiate into plasmacytoid DCs during viral infection.

Adapted from (Liu Y.J., 2001).

Many of the notions of DC lineage and precursors are still controversial. There are

arguments that suggest that the $CD8\alpha^+$ DC in mouse and the plasmacytoid DC in

humans are not of lymphoid origin and can be derived from myeloid precursors. For

example the mouse $DC8\alpha^-$ myeloid DC can be induced to express $CD8\alpha$ *in vitro*

(Brasel K. et al., 2000). It is also not clear why so many different DC subsets are

required. It may be related to specific locations within the body. Five different types

of DC have been identified in human tonsils, which may have different functions in

the T and B cell local micro- environments (Summers K.L. et al., 2001). It is also

possible that the different DC subsets have evolved to respond to different pathogens.

For example the plasmacytoid DCs express TLR7 and TLR9 and play an important

role in the defence against viral infections (Kadowaki N. et al., 2001).

1.2.2 Antigen capture and processing by dendritic cells

DCs constantly sample their environment using three main mechanisms of

endocytosis. Small molecules and solutes are taken up constitutively by non-specific

macropinocytosis. This is a cytoskeletal dependent type of fluid phase endocytosis,

mediated by membrane ruffling and the formation of large vesicles (1-3μm).

Immature DCs are able to take up extracellular fluid equivalent to one cell's volume

every hour (Sallusto F. et al., 1995). DCs take up large volumes of fluid by this

mechanism and concentrate the soluble antigens in protease containing endocytic

compartments by the rapid exchange of water across their cell membrane via

aquaporins (de Baey A. et al., 2000).

Larger molecules binding to PRRs are internalised by receptor mediated endocytosis.

Membrane receptors cluster in clatharin coated pits together with the bound ligand.

These pits pinch off as coated vesicles inside the cell and then traffic through the

endosomal/lysosomal pathway (Steinman R.M. et al., 1995).

Large insoluble particles up to 6 μm in size are captured by phagocytosis. Receptors

cluster at the antigen binding site and generate a phagocytic signal. This results in

local polymerisation of actin filaments underneath the phagocytic target and

remodelling of the plasma membrane to form extensions (pseudopods), which engulf

the captured particle. The reorganisation of the actin cytoskeleton is controlled by

small GTPases of the Rho family (reviewed in (Castellano F. et al., 2001)).

Antigens taken up by macropinocytosis or receptor mediated endocytosis are

directed to the endosomal/lysosomal pathway where they are processed and loaded

on to MHC class II molecules for presentation. In comparison, particles taken up by

phagocytosis do not traffic to the endosomal/lysosomal pathway but are degraded

and loaded onto MHC class II molecules within the phagosome (Ramachandra L. et

al., 1999)(Ramachandra L. et al., 2000).

DCs express several receptors that mediate endocytosis and/or phagocytosis. For

example, the mannose receptor is a C-type lectin containing multiple carbohydrate-

binding domains, which bind to a variety of antigens expressing mannose or fucose

residues (Sallusto F. et al., 1995). The mannose receptor traffics to early endosomes

where it disassociates from its ligands at endosomal pH and recycles to the cell

surface to engage in further antigen uptake (reviewed in (Stahl P.D., 1992). DEC

205, a homologue of the mannose receptor, is expressed by DCs and binds

carbohydrate conjugated antigen (Kato M. et al., 2000). Following internalisation

DEC-205 traffics directly to late endosomes and lysosomes before recycling to the cell surface (Mahnke K. et al., 2000). Fc receptors FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), expressed on DCs, capture and internalise IgG bound immune complexes (Fanger N.A. et al., 1996)(Sallusto F. et al., 1994). These receptors are not recycled following internalisation, but are degraded in endosomes along with the bound antigen. The adhesion molecule DC-SIGN also plays a role in DC antigen capture, internalisation and delivery to the late endosome and lysosome compartments (Engering A. et al., 2002).

After internalisation by DCs, most protein antigens are digested to small peptide chains, which associate with MHC class II molecules in endocytic compartments. Endosomes are rich in enzymes, especially the cystein proteases of the cathepsin family. These proteases act optimally at acidic pH and have broad substrate specificity. They digest internalised antigen to small peptide fragments for loading onto MHC class II molecules (reviewed in (Watts C., 2001a)). The enzyme gamma-interferon inducible lysosomal thiol reductase (GILT) reduces disulphide bonds optimally at acidic pH and is important in the reduction and unfolding of antigenic proteins (Watts C., 2001b). Unfolded peptides of around 25 residues bind to the MHC class II peptide-binding groove and avoid further digestion.

Newly synthesised MHC class II molecules traffic to the endosomal-lysosomal pathway. It is still unclear at what point the MHC II molecules fuse with the endosomal-lysosomal pathway but evidence suggests that at least some MHC II molecules are trafficked to the early endosomes (Pond L. et al., 1999). Newly synthesised class II molecules are engaged by an invariant chain protein (Ii), which

encourages export from the ER and targets to the endosomal pathway. Ii also acts as

a surrogate peptide in the MHC binding groove, stabilising the α/β dimmer. The Ii is

removed from the binding groove and replaced by antigenic peptide, this is

facilitated by endosomal proteases and the HLA-DM protein in a process known as

DM-peptide exchange (reviewed in (Watts C. et al., 2000)(Watts C., 2001a)).

Dendritic cells are also able to present on MHC class I complexes. MHC class I

presentation is usually implemented for endogenous antigens in virally infected cells

or tumour cells. Loading of MHC class I molecules takes place in the ER using

peptides generated in the cytosol. However DCs are able to initiate CD8 T-cell

responses to exogenous antigens in a process known as cross-presentation, where

material taken up by endocytosis can translocate from the endosomes to the cytosol.

This process is dependent on transporter associated with antigen processing (TAP)-

dependent (Albert M.L. et al., 1998)(Rescigno M. et al., 1998b)(Sigal L.J. et al.,

1999). The ability of DCs to cross-present antigen from tissues has important

implications for DC induction of peripheral tolerance involving CD8^{+} T cells (Albert

M.L. et al., 2001). Recent evidence has shown that DCs can also present lipid antigen

to T cells via the CD1 pathway (Prigozy T.I. et al., 2001). The CD1 family of

molecules are structurally similar to the classical MHC class I molecules.

1.2.3 Dendritic cell maturation

After exposure to microbial products or inflammatory stimuli, DCs undergo a series

of coordinated maturation events. Micro-array techniques have established at least

255 genes are regulated during DC differentiation and maturation including secreted

proteins, adhesion molecules, signalling molecules and genes involved in lipid

metabolism (Le Naour F. et al., 2001). Maturation is associated with a decrease in endocytosis and endocytic receptor expression in conjunction with transport of MHC/peptide complexes to the cell surface and an increase in expression of surface co-stimulatory molecules (Chow A. et al., 2002)(Sallusto F. et al., 1994)(Sallusto F. et al., 1995) (table 1.1).

Immature DCs	Mature DCs
High endocytosis	Low endocytosis
High phagocytosis	Low Phagocytosis
High intracellular MHCII	High surface MHCII
High CCR1, CCR5, CCR6	Low CCR1, CCR5, CCR6
Low CCR7	High CCR7
Low CD54, CD80, CD86	High CD54, CD80, CD86
Low CD40	High CD40
Low CD83	High CD83

Table 1.1 Properties of immature and mature dendritic cells

On receiving a maturation signal, DCs transport MHC-antigen complexes that are arrested at the peptide loading stage to the cell surface (Inaba K. et al., 2000)(Lutz M.B. et al., 1997). A change in pH of the MHC II containing lysosomal compartments during DC maturation results in the activation of enzymes and the loading of peptide onto MHC molecules followed by presentation on the surface of the DC (Trombetta E.S. et al., 2003). This phenomenon explains why DCs can present peptides from antigens previously internalised hours before maturation

occurs. Interestingly, co-stimulatory molecules and MHC class I molecules have been identified in the MHC class II vesicles that migrate to the cell surface. This results in clustering of MHC molecules and co-stimulatory molecules on the surface of DCs ready for T cell activation (Turley S.J. et al., 2000).

Following stimulation, DCs increase their expression of the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) (Caux C. et al., 1994)(Inaba K. et al., 1994). Increased expression of these molecules is important for generating an optimal T cell response. CD80 and CD86 both bind to CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA4) on T cells. CD28 ligation enhances the magnitude and duration of T-cell responses, inhibits apoptosis, increases cytokine production and increases expression of cell adhesion molecules (reviewed in (Chambers C.A., 2001)(Lenschow D.J. et al., 1996)). The engagement of CD80 or CD86 with CTLA-4 results in an inhibitory effect. CTLA-4 has a higher affinity for CD80 and CD86 than CD28; it is not expressed constitutively on T cells but is expressed following TCR and CD28 ligation (Chambers C.A., 2001).

A combination of cytokines including TNFα, IL-1α, IL-1β, IL-6, IL-8, IL-12 and IL-18 are released by DCs after receiving a maturation signal including micro-organisms or microbial products such as LPS, or by CD40 ligation (Morelli A.E. et al., 2001)(Saint-Vis B. et al., 1998)(Verhasselt V. et al., 1997). The release of pro-inflammatory cytokines TNFα, IL-1 and IL-6 aids the local inflammatory response at the site of infection. TNFα induces activation of endothelial cells, increasing the infiltration of leukocytes to the site of infection. IL-6 is a potent growth and differentiation factor for B and T cells, and along with IL-1 systemically induces

fever. IL-1 and TNFα also activate other DCs and macrophages arriving at the site of

infection. The chemokine IL-8 released by DCs upon activation attracts other

leukocytes, including neutrophils and basophils, to the site of infection. The

cytokines IL-12 and IL-18 are both important in the activation of naïve T helper cells

to a Th1 phenotype, as discussed in detail below.

1.2.4 Dendritic cell migration

DC maturation is closely linked to migration from the site of inflammation to the

secondary lymphoid tissues (Roake J.A. et al., 1995). This movement of DCs is well

known, but the precise mechanisms of migration *in vivo* are not fully understood

although several important factors have been identified.

Chemokines are small soluble molecules that have been shown to play an important

role in DC migration. There are two families of chemokines, the CXC and the CC

chemokines, which are distinguished according to the position of their first two

cystine residues (Sozzani S. et al., 1999). The receptors for chemokines are also

classified CXCR or CCR according to their ligands. The chemokine MIP3α (CCL20)

is expressed by activated venous endothelial cells and is involved in the recruitment

of DC precursors. Endothelial cells of the lymphatic system constitutively express

chemokines MIP3β (CCL19) and 6Ckine (CCL21), which attract activated DCs and

naïve T cells. Other chemokines are not constitutively expressed by tissues but are

induced as part of the inflammatory response. Following recognition of an invading

pathogen by receptors such as TLRs, tissue macrophages release chemokines to

attract leukocytes, including immature DCs, to the site of infection. Inducible

chemokines include IL-8 (CXCL8), MIP-1α (CCL3), MIP-1β (CCL4), RANTES

(CCL5) and IP-10 (CXCL10) (reviewed in (Luster A.D., 2002)).

Immature DCs express CCR1, CCR2, CCR5, CCR6 and CXCR1 and they respond to

MIP-1α, MIP-1β, RANTES, MCP-3 (CCL7), MCP-4 (CCL13) and MIP-3α

(CCL20) (Sozzani S. et al., 1997). Following stimulation, DCs decrease their

expression of CCR1 and CCR5 (Sallusto F. et al., 1998)(Sozzani S. et al., 1998), and

become unresponsive to their ligands (MIP-1α, MIP-1β, RANTES and MCP-3). At

the same time as the decrease in CCR1 and CCR5 expression there is an increase in

CCR7 expression and DCs become responsive to chemokines MIP-3β (CCL19) and

6Ckine (CCL21) (Sallusto F. et al., 1998)(Sozzani S. et al., 1998). This change in

expression of chemokine receptors by DCs following stimulation contributes to their

migration from the peripheral tissues to the draining lymph nodes. Chemokines

MIP-3β and 6Ckine are expressed in T cell zones of lymph nodes, by high

endothelial venules of lymphatic tissues and by lymphatic endothelial cells

(Kellermann S.A. et al., 1999).

In order to reach the secondary lymphoid tissues DCs must first pass through the

extra-cellular matrix of the tissues at the site of inflammation and cross endothelial

barriers. This process involves binding of adhesion molecules. For example

Langerhans cells (LCs) are retained in the skin by E-cadherin binding to

keratinocytes. Following stimulation expression of E-cadherin on LCs is decreased

and they emigrate from the skin (Jakob T. et al., 1998)(Tang A. et al., 1993). The

adhesion molecule ICAM-1 is also important for DC migration from the skin to

lymph nodes. ICAM-1 knock out mice have a reduced number of DCs in their

regional lymph nodes but no change in the number of DCs in the skin compared to wild type controls. It was also shown that ICAM-1 expression on regional lymphatics is essential, rather than ICAM-1 expression by DCs (Xu H. et al., 2001).

Antibody inhibition studies *in vitro* have shown that adhesion molecules CD11a, CD11b, CD18 and VLA-4 are important for DC binding and migration across vascular endothelium (Brown K.A. et al., 1997)(D'amico G. et al., 1998). CD18 and PECAM-1 are especially important for transmigration through endothelial layers, and the integrins VLA-4 and VLA-5 are important for DC binding to the extra cellular matrix (D'amico G. et al., 1998). It is unclear however if these *in vitro* observations mean that these adhesion molecules are important for DC migration *in vivo*. The DC specific C-type lectin DC-SIGN, binds to ICAM-2 on endothelial cells and mediates DC tethering and rolling under physiological flow conditions. Both vascular and lymphatic endothelium in lymphoid tissue abundantly express the DC-SIGN ligand ICAM-2 in steady state conditions, indicating a possible role for ICAM-2 and DC-SIGN interactions in DC migration (Geijtenbeek T.B. et al., 2000).

The transporter protein multi-drug resistant-1 p-glycoprotein (MDR1 p-glycoprotein) and multi-drug resistant protein 1 (MRP1) have both been found to play an important role in DC migration to lymph nodes. Blocking of either of these lipid transporters with monoclonal antibodies results in inhibition of migration. It is thought that these molecules facilitate DC migration in response to the MIP3β (Randolph G.J. et al., 1998b)(Robbiani D.F. et al., 2000).

The migration of DCs from the periphery to the lymphatic system has always been closely associated with DC maturation, but recent studies have indicated that maturation and migration may not be as closely associated as initially thought. For example DC stimulation with prostaglandin E2 (PGE2) in combination with other stimuli results in migration. However in the absence of PGE2, DCs were found to mature but not migrate (Luft T. et al., 2002)(Scandella E. et al., 2002). Furthermore PGE2 given in combination with other stimuli resulted in an increase in CCR7 expression and increased sensitivity to CCR7 ligands MIP3β and 6Ckine (Scandella E. et al., 2002).

There is also evidence of migration in the absence of maturation. TNFα and other inflammatory stimuli were found to induce CCR7 expression and migration of LCs from inflamed skin to lymph nodes without increased CD83 or CD86 expression (Geissmann F. et al., 2002)(Randolph G.J., 2002). This has implications for the role of DCs in priming regulatory T cells and controlling peripheral tolerance as discussed later (section 1.2.7).

1.2.5 Dendritic cell interactions with T cells

Dendritic Cells migrate to the T cell areas of secondary lymphoid organs where they interact with and stimulate T cells (Stoll S. et al., 2002). Adhesion molecules play an important part in the initial interactions of DCs and T cells. Naïve resting T lymphocytes express high levels of ICAM-3. Initial contact between DCs and T cells is mediated by ICAM-3 and DC-SIGN, followed by ICAM-1 and LFA-1 interactions (Bleijs D.A. et al., 2001)(Montoya M.C. et al., 2002). Although these initial adhesion molecule contacts are antigen independent, following TCR binding to MHC

activated LFA-1 binds ICAM-1 with higher avidity, thus stabilising the complex (Figure 1.2a).

The point of contact between the DC and T cell is known as the 'immunological synapse', where TCRs and MHC molecules cluster together in a central area with co-stimulatory molecules. These are surrounded by a ring of adhesion molecules forming a stable complex ((Dustin M.L. et al., 2000) (Grakoui A. et al., 1999) and reviewed in (Davis D.M., 2002)(van der Merwe P.A., 2002)) (Figure 1.2b). The stability of the synapse and the length of time it is formed controls T cell stimulation.

Both the number of MHC-TCR interactions and their duration effect T cell stimulation. Co-stimulatory molecules and their ligands such as CD28-CD80/CD86, CD40-CD40 ligand (CD40L), OX40–OX40 ligand (OX40L), CD27-CD70 enhance T cell receptor signalling and provide additional signals that aid the proliferation of T cells. Interestingly, different co-stimulatory molecules are required for $CD4^+$ cell priming than for $CD8^+$ cell priming. CD28, CD40L and OX40L expression are all essential for $CD4^+$ cell priming, whereas $CD8^+$ cells require 4-1BBL and to a certain extent CD28 (Chen A.I. et al., 1999)(Howland K.C. et al., 2000)(Kaech S.M. et al., 2002)(Whitmire J.K. et al., 2000). The requirement for co-stimulation of $CD4^+$ T cells can be overcome by higher concentrations of antigen and longer duration of TCR-MHC signalling (Iezzi G. et al., 1999).

Figure 1.2 Contact between dendritic cells and T cells and the formation of the immunological synapse

(a) Mature DCs adhere to naïve T cells upon arrival in the lymph node through DC-SIGN/ICAM-3 interactions, thus allowing the formation of low avidity LFA-1/ICAM-1 bonds. Following ligation of the TCR by peptide loaded MHC, LFA-1 is activated and stabilises the formation of the immunological synapse through high avidity LFA-1/ICAM-1 and CD2/LFA-3 interactions. Adapted from (Bleijs D.A. et al., 2001).

(b) The immunological synapse involves the supramolecular organisation of T cell receptor, co-stimulatory and adhesion molecules. The signalling complexes of MHC/TCR and co-stimulatory molecules cluster at the centre of the synapse; these are surrounded by a ring of adhesion molecules including LFA-1/ICAM-1 and CD2/LFA-3 associations. Adapted from (Davis D.M., 2002).

(a) Initial contact between a dendritic cell and T cell
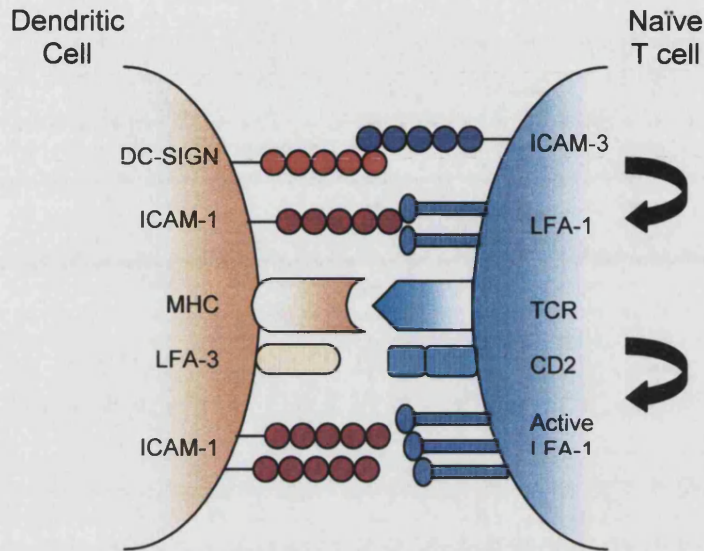
(b) Formation of the immunological synapse
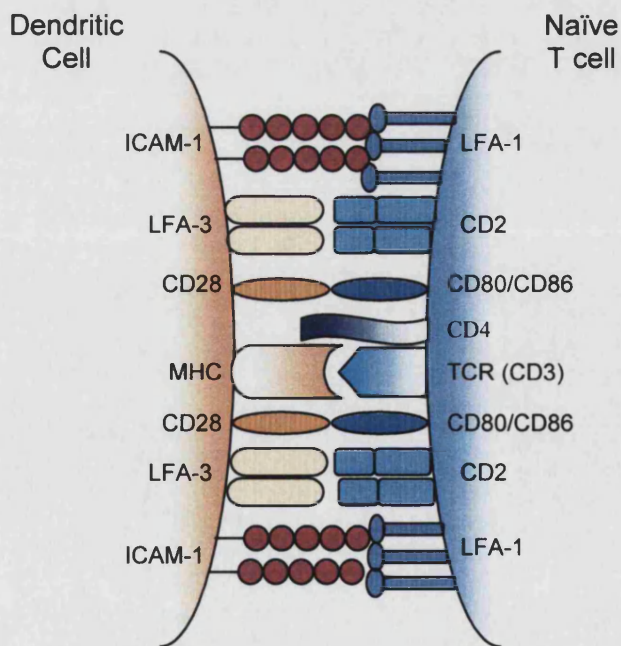
Other molecules with co-stimulatory roles in T cell activation are constantly being

identified. New members of the B7 (CD80/CD86) family expressed by DCs include

B7-H3 (Chapoval A.I. et al., 2001) and B7-DC (Tseng S.Y. et al., 2001). The human

inducible co stimulator protein (ICOS) expressed on T cells is a member of the CD28

family and plays a role in T cell stimulation and differentiation (Aicher A. et al.,

2000)(Greenwald R.J. et al., 2002).

1.2.6 Differentiation of T helper cells to type 1 (Th1) or type 2 (Th2) phenotypes

Naïve T cells are fastidious in their requirements for priming. They require high

levels of antigen loaded MHC molecules and co-stimulatory molecules, which are

expressed on activated DCs. Naïve $CD4^+$ T cells have the potential to develop into

IFNγ producing Th1 effector cells or IL-4/IL-5/IL-13 producing Th2 effector cells.

Th1 cells are important in the activation of macrophages, the induction of IgG

antibodies to mediate opsinisation and phagocytosis and in the support of activated

$CD8^+$ effector cells. Th1 responses confer resistance to intracellular pathogens. Th2

cells stimulate the growth and differentiation of mast cells and eosinophils as well as

the production of antibody isotypes including IgE. Th2 cells are involved in the

response against extra cellular pathogens. DCs are fundamental in determining the

balance between Th1 and Th2 effector cells (reviewed in (Jankovic D. et al.,

2001)(Kalinski P. et al., 1999)(Maldonado-Lopez R. et al., 2001))

Initially different DC subsets were thought to promote the differentiation of Th1 and

Th2 cells. *In vitro*, myeloid DCs were found to promote a Th1 response and

lymphoid/plasmacytoid DCs induced a Th2 response (Rissoan M.C. et al., 1999). It

has since been demonstrated however that myeloid DCs can induce a Th2 response

following stimulation with a glycoprotein (ES62) secreted by a filarial nematode

(Whelan M. et al., 2000) or by stimulation with toxin from the extra-cellular

bacterium *Vibrio cholerae* (de Jong E.C. et al., 2002). It has also been shown that

plasmacytoid DCs can produce type 1 interferons (IFNα/β) and induce a Th1

response following stimulation with influenza virus and CD40 ligation (Cella M. et

al., 2000).

Three signals are required for the optimal priming and activation of naïve Th cells.

Signal 1 is the antigen specific recognition of MHC peptide by TCR; signal 2 is co-

stimulation via CD80/CD86 interactions with CD28 and other co-stimulatory

molecule interactions such as CD40-CD40L and OX40-OX40L; and signal 3 is

mediated by soluble factors (cytokines). IL-12 is the best characterised signal 3 and

is essential for the development of a Th1 response and the defence against

intracellular bacteria (Heufler C. et al., 1996). Individuals found lacking a functional

IL-12 receptor are unable to mount a Th1 response and are susceptible to

intracellular infections with mycobacteria and salmonella (de Jong R. et al., 1998).

IL-12 is a heterodymic cytokine composed of p40 and p35 subunits that together

form the biologically active p70 molecule. The p35 subunit is constitutively

transcribed and is regulated post translationally, whereas transcription of the p40

subunit is induced following stimulation (reviewed in (Abdi K., 2002)). The IL-

12p40 subunit also combines with an IL-12p35 related molecule, p19, to form the

biologically active IL-12 related cytokine IL-23 (Oppmann B. et al., 2000). DCs

produce IL-12 following stimulation with certain pathogens including

Staphylococcus aureus (Heufler C. et al., 1996), *Mycobacteria tuberculosis*

(Giacomini E. et al., 2001) and *Toxoplasma gondii* (Sousa C. et al., 1999b)(Sousa

C.R. et al., 1997). Other factors such as IFNγ and CD40 ligation have been found to

increase DC IL-12 production (Sousa C. et al., 1999b). CD40 ligation alone does not

lead to IL-12 production, but together with a microbial stimulus enhances IL-12

production (Schulz O. et al., 2000). Memory T cells can induce DC IL-12 production

by CD40L binding of CD40 along with IFNγ stimulation, thus enhancing the

immune response, whereas naïve T cells are unable to induce IL-12 production in

DCs (Snijders A. et al., 1998).

IL-18 and type 1 interferons produced by DCs have also been implicated in inducing

Th1 responses. Naïve T cells do not make IFNγ in response to IL-18 alone because

they lack a functional IL-18 receptor. However a positive feedback mechanism

occurs as IL-12 stimulation of naïve T cells results in up regulation of IL-18 receptor

expression. IL-18 signalling then increases IL-12 receptor expression (reviewed in

(Akira S., 2000)).

Thus far no DC derived soluble factors have been identified that induce a Th2

response. IL-4 production is essential, but produced by T cells and not DCs

(Banchereau J. et al., 2001). DCs generated in the presence of prostaglandin E2

(PGE2) express co-stimulatory molecules and induce naïve Th cells to proliferate

and differentiate into Th2 cells. PGE2 treated DCs were unable to produce IL-12

upon stimulation with LPS or other microbial products (Kalinski P. et al., 1997). Co-

stimulatory molecules can play an influential role in Th2 development depending on

the activating stimulus. The Th2 response induced by DCs stimulated with a protein

extract from the helminth *Schistosoma mansoni* is dependent on OX40-OX40L

interactions whereas the Th2 response induced by DCs stimulated with cholera toxin

is not (de Jong E.C. et al., 2002). The lack of soluble Th2 inducing factors could be explained by the theory that a Th2 response is the default pathway, which occurs when DCs are activated and express a high concentration of co-stimulatory molecules but do not secrete Th1 inducing cytokines such as IL-12.

There are other factors that influence Th1/Th2 differentiation including the kinetics of DC-T cell interactions and cell ratio numbers. A relatively short TCR stimulation time promotes Th1 polarisation whereas prolonged TCR stimulation is required for the development of a Th2 response even in the presence of IL-4 (Iezzi G. et al., 1999). Delay between DC activation and interaction with T cells may also favour a Th2 response. For example, DCs stimulated with the Th1 inducing stimulus LPS were initially found to induce a Th1 response but when the same DCs were used to stimulate naïve T cells at later time points (after 48 hours), T cells differentiated to a Th2 phenotype (Langenkamp A. et al., 2000). The ratio of DCs to T cells may also influence polarisation. Low ratios of 1 DC to 300 T cells resulted in a Th2 response, whereas a ratio of 1 DC to 4 T cells gave a Th1 response. These observations of kinetics and ratios influencing polarisation are interesting, but the *in vivo* significance of these observations has yet to be explored.

1.2.7 The role of DCs in inducing regulatory T cells and tolerance

The role of DCs in inducing and maintaining regulatory T cells and tolerance is currently an area of major interest. There is increasing evidence that certain subsets of T cells exert regulatory control limiting immune responses to self-antigen and preventing autoimmune disease. In mice, T regulatory cells make up 5-10% of peripheral $CD4^+$ T cells. They express CD25 and CTLA-4, but do not proliferate

after activation and have a suppressor effect on other T cells (Sakaguchi S., 2000)(Thornton A.M. et al., 2000). Stimulation of naïve CD4$^+$ T cells with immature DCs resulted in CTLA-4$^+$, non-proliferating T cells that did not produce IFNγ, IL-2 or IL-4 but did produce IL-10. These IL-10 producing T cells inhibited the proliferation of Th1 cells in co-culture in a contact dependent but antigen independent manner (Jonuleit H. et al., 2000). Another study found that injecting immature DCs pulsed with influenza matrix protein or keyhole limpet hemocyanin resulted in antigen specific inhibitory IL-10 producing T cells (Dhodapkar M.V. et al., 2001). This regulation induced only by immature DCs means that it is unlikely that pathogen stimulated mature DCs will result in a tolerogenic response. The possibility of immune responses to self-antigens is also limited, as immature DCs can not induce an effective immune response.

1.3 *Neisseria meningitidis*

N. meningitidis is an encapsulated Gram-negative diplococcus, which is exclusively a human pathogen. To this day *N. meningitidis* remains a leading cause of bacterial meningitis and sepsis. Reports of illness resembling meningococcal disease can be dated back to the 16th century, though the organism was first isolated from a patient with meningococcal disease in 1887 (reviewed in (Rosenstein N.E. et al., 2001)).

The mortality rate among patients with meningococcal sepsis is still around 40%, and 11-19% of survivors have sequelae that can include hearing loss, mental retardation or limb amputation (Rosenstein N.E. et al., 1999). During infection *N. meningitidis* often crosses the endothelium of the blood-brain barrier and enters the subarachnoid

space. This infection of the meninges occurs in ~50% of cases, resulting in meningitis (Rosenstein N.E. et al., 1999).

1.3.1 Classification of bacteria and epidemiology

N. meningitidis is classified into serogroups according to the chemical composition of the polysaccharide capsule. Thirteen different serogroups have been identified of which only 5 have been associated with disease causing isolates. These are the serogroups A, B, C, Y and W-135 (Rosenstein N.E. et al., 2001). Serogroups A, B and C account for most cases of meningococcal disease world wide with serogroups B and C responsible for the majority of cases in industrialised countries. Serogroup A strains and to a lesser extent serogroup C strains dominate in third world countries (Schwartz B. et al., 1989). There is an area of sub-Saharan Africa referred to as the 'meningitis belt', which extends from Ethiopia in the east to Senegal in the west. Recurrent outbreaks of serogroup A disease occur in this region. In 1996 the largest outbreak ever recorded occurred here, with a total number of 152,813 cases reported, and 15,783 deaths, though this is likely to be an underestimate.

N. meningitidis are classified further into serosubtypes on the basis of their class 1 outer membrane proteins (OMPs), then into serotypes on class 2 or class 3 OMPs and finally into immunotypes based on their lipopolysaccharide (Fig 1.3)

1.3.2 The surface structure and composition of *N. meningitidis*

The polysaccharide capsule protects the bacteria by reducing phagocytosis and complement mediated bacteriolysis (Fig 1.3)(Vogel U. et al., 1999). Strains that are capsule deficient are more readily phagocytosed by DCs and other phagocytes (Kolb-

Maurer A. et al., 2001)(Unkmeir A. et al., 2002). Pili, composed of protein subunits

called pillins, protrude through the capsule from the inner membrane (Fig 1.3). They

are adhesive molecules that orchestrate the initial adhesion of bacteria to host

epithelial and endothelial cells by binding to the complement membrane co-factor

protein, CD46, expressed by most cells (Nassif X. et al., 1994).

The outer membrane of *N. meningitidis* is composed of an asymmetric lipid bi-layer

into which are embedded many outer membrane proteins (OMPs) (Fig 1.3). The

OMPs are essential for bacterial nutrient acquisition and protein secretion. The major

OMPs of *N. meningitidis* are divided into 5 classes based on their molecular

weights(Tsai C.M. et al., 1981). The class 1 OMP is a porin (PorA), which forms

cation secretive pores in the outer membrane (Tommassen J. et al., 1990). Class 2

and class 3 OMPS are PorB1 and PorB2. These are mutually exclusive between *N.*

meningitidis serotypes and form anion selective pores in the outer membrane.

Porins are expressed as trimers, where each monomer is a 6-stranded β barrel with 8

surface exposed loops. The high variability in these surface exposed loops forms the

basis for serosubtyping and serotyping (reviewed in (Massari P. et al., 2003b)).

Porins interact directly with the membranes of target cells, forming aqueous

transmembrane channel. PorB also aids the survival of *N. meningitidis* by protecting

host cells from apoptosis by reducing mitochondrial depolarisation and release of

cytochrome C (Massari P. et al., 2003a)(Massari P. et al., 2003b).
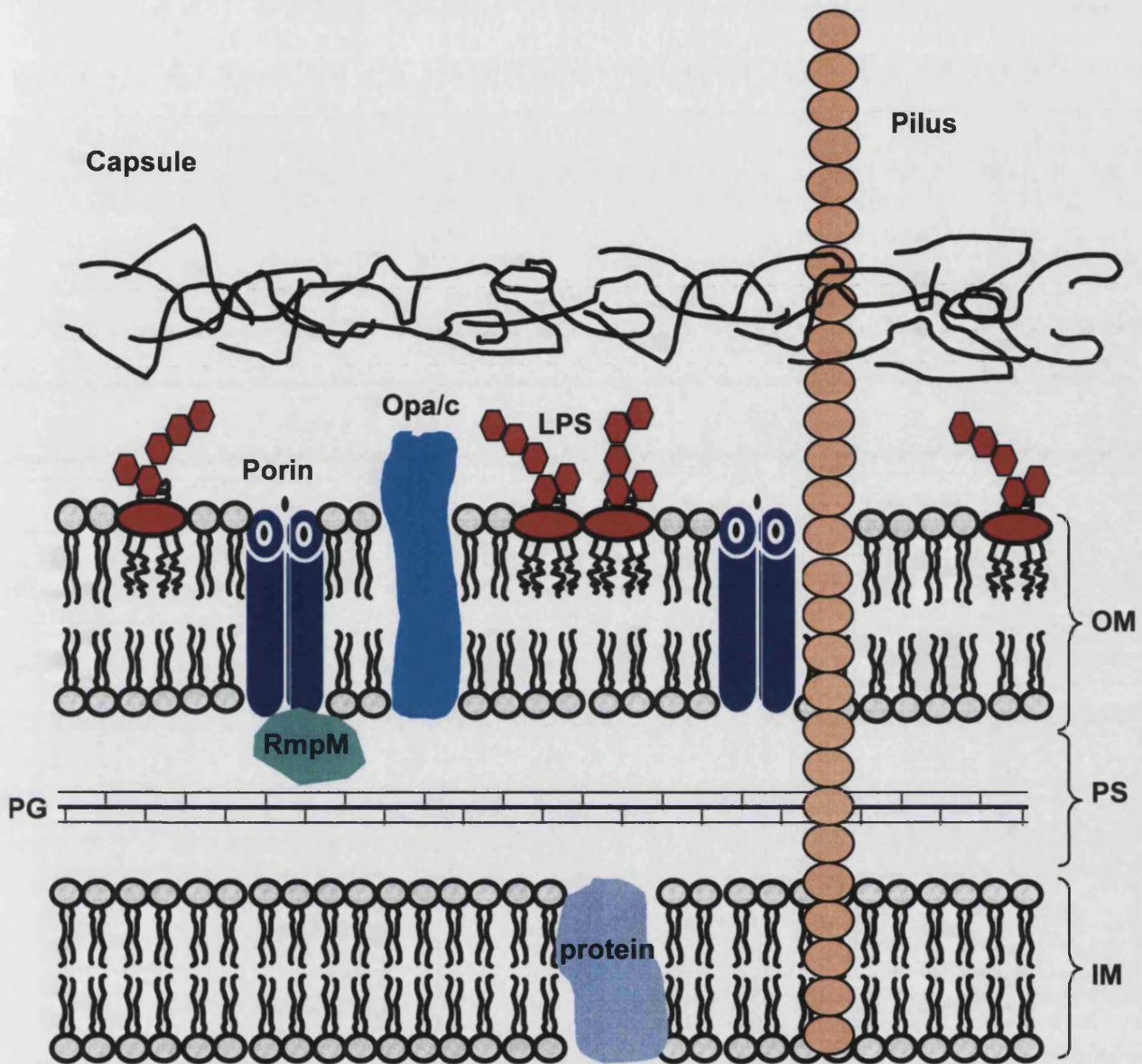
Figure 1.3 Surface structure of *Neisseria meningitidis*

As with all Gram-negative bacteria, *N. meningitidis* have an outer membrane (OM) and an inner membrane (IM) separated by a periplasmic space (PS), which contains a peptidoglycan layer (PG). The OM is an asymmetric lipid bilayer with the outer leaflet composed of phospholipids and lipopolysaccharide (LPS) and the inner leaflet just phospholipids. There are surface exposed proteins embedded in the OM, including porins that function as channels for the acquisition of nutrients and opacity proteins that play a role in bacterial adhesion. Meningococci are surrounded by a polysaccharide capsule. Pili protrude through the membranes and the capsule. Adapted from (Poolman J.T., 1995)(Rosenstein N.E. et al., 2001) and (Steeghs 2001b.)

The class 4 OMP is the reduction-modifiable protein M (RmpM), which is a homologue of *E. coli* OmpA. It is constitutively expressed by all menigococcal strains and is antigenically invariable (Prinz T. et al., 2000). RmpM anchors the meningococcal outer membrane to the periplasmic space and is firmly associated with other OMPs such as porins and iron limitation inducible OMPs (Fig 1.3) (Klugman K.P. et al., 1989).

The opacity proteins Opa and Opc comprise the class 5 OMP family. Opa proteins are predicted to form 8-stranded β barrels in the outer membrane, exposing 4 loops to the surface. Opc spans the membrane 10 times exposing 5 surface loops. These opacity proteins recognise different receptors on host cells and aid bacterial adhesion and invasion. Opa_{CEA} mediates attachment to the carcinoembryonic antigen CD66, which is related to the cell adhesion molecule (CEACAM) family. Opa_{HS} and Opc both bind heparin sulphate proteoglycans and extra cellular matrix proteins fibronectin and vitronectin (de Vries F.P. et al., 1998)(Virji M. et al., 1996) and reviewed in (Hauck C.R. et al., 2003)).

In certain growth conditions *N. meningitidis* can express other OMPs in addition to the 5 major classes of OMP. For example, in iron limiting growth conditions they express receptors for haem, lactorferrin and tansferrin. With the completion of genome sequences for one serogroup A strain and one serogroup B strain of *N. meningitidis* new OMPs with as yet unknown functions are being identified (Parkhill J. et al., 2000)(Tettelin H. et al., 2000).

Lipopolysaccharide (LPS) is present in the outer leaflet of the outer membrane (Fig 1.3). LPS is composed of a hydrophobic lipid A moiety anchoring it in the outer membrane, and a core hydrophilic oligosaccharide exposed on the bacterial surface (Fig 1.4). Meningococcal LPS is often referred to as lipo-oligosaccharide (LOS) as it lacks the O antigen found in the LPS of other *Enterobacteriaceae*. *N. meningitidis* is further divided into 12 immunotypes based on the heterogeneous LPS outer core (Andersen S.R. et al., 1996)(Scholten R.J. et al., 1994). The lipid A of meningococcal LPS is much more conserved than the heterogeneous outer oligosaccharide core. LPS is negatively charged and has a strong affinity for divalent cations, which together form a strong permeability barrier in the outer membrane into which OMPs are integrated.

The biosynthesis pathway of lipid A in *E. coli* has been worked out. In the first step of lipid biosynthesis the enzyme lpxA-acyltransferase catalyses the transfer of the acyl chain from (R)-3-hydroxy-myristol-acyl carrier protein (ACP) to the 3-OH hexose of UDP-N-acetylglucosamine (Coleman J. et al., 1988). Mutations in the early stages of the lipid A biosynthesis pathway in *E. coli* are conditionally lethal (Galloway S.M. et al., 1990). However an early block in lipid A biosynthesis in *N. meningitidis* was found to be non-lethal (Steeghs L. et al., 1998). Lipid A biosynthesis was interrupted by the insertion of an inactivation cassette in the *lpxA* gene of *N. meningitidis*. Although this *lpxA-* mutant was viable, it had a reduced growth rate. It was found to be totally deficient in LPS as shown by SDS-page and ELISA (Steeghs L. et al., 1998). Further studies of the OMPs from the LPS deficient and the wild type *N. meningitidis* showed that expression of the major OMPs was not affected, although the phospholipid composition had changed in the absence of LPS

Oligosaccharide core
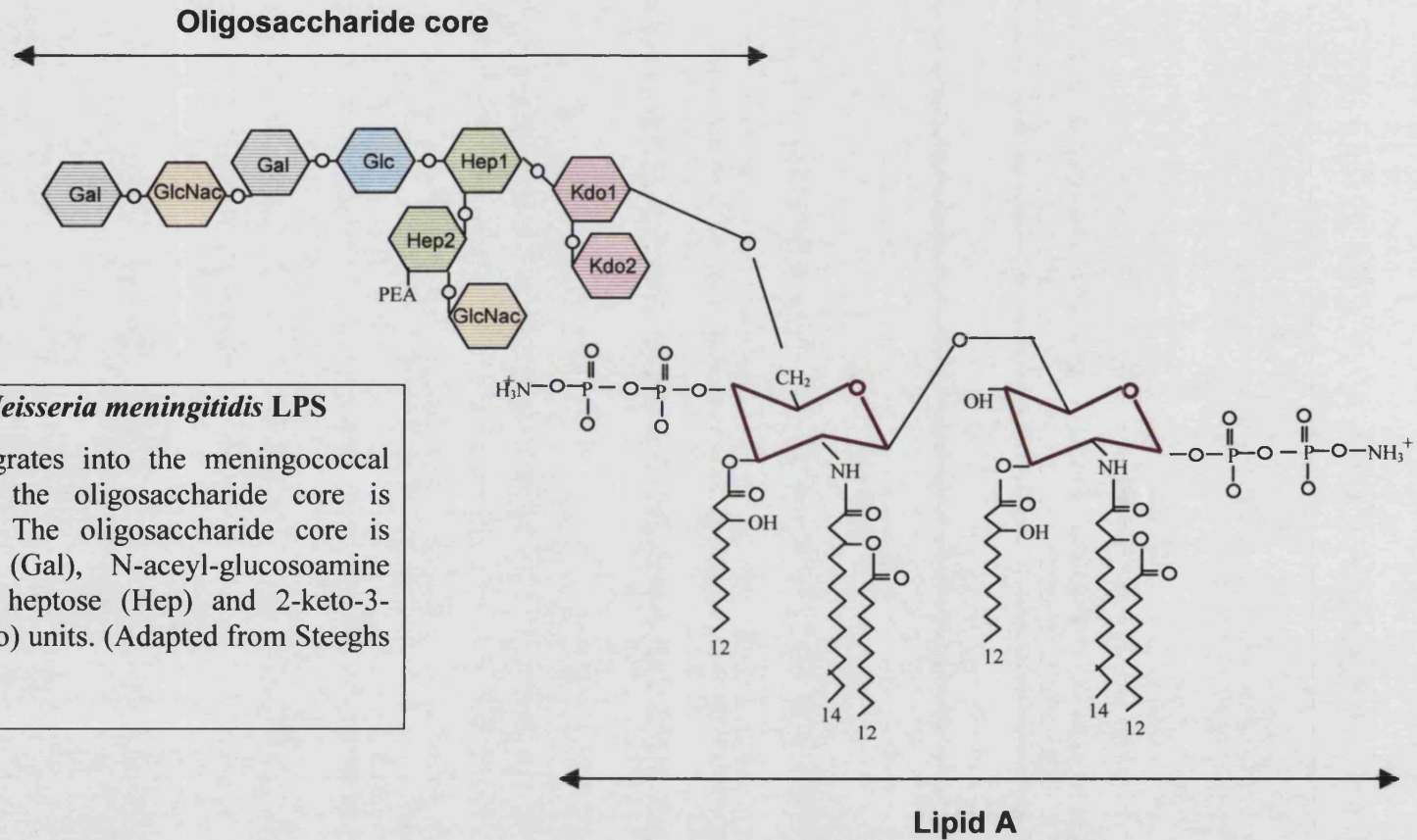
Figure 1.4 Schematic of *Neisseria meningitidis* LPS

The lipid A moiety integrates into the meningococcal outer membrane, whilst the oligosaccharide core is exposed on the surface. The oligosaccharide core is composed of galactose (Gal), N-aceyl-glucosoamine (GlcNac), glucose (Glc), heptose (Hep) and 2-keto-3-deoxyoctulosonic acid (Kdo) units. (Adapted from Steeghs 2001b)

Lipid A

(Steeghs L. et al., 2001a). Expression of the polysaccharide capsule was essential for

viability of the LPS deficient *lpxA- N. meningitidis*.

1.3.3 Colonisation and carriage

The natural environment for *N. meningitidis* is the nasopharynx where the bacteria

reside as commensal organisms. Disease results when meningococci cross the

nasopharyngeal epithelium and enter the blood stream. In Europe, asymptomatic

carriage occurs in ~10% of the population with invasive disease occurring in 0.3-7.1

per 100,000 (Balmer P. et al., 2002). *N. meningitidis* is an occasional pathogen,

invasive disease occurs accidentally during normal bacteria and host interactions.

The factors that result in invasive disease are not fully understood, immune status of

the host and the bacterial strain are both important along with environmental

conditions. Immune defects that compromise the host can promote invasive disease.

These include deficiencies in late complement components, in protein C and in

mannose binding lectin (Vogel U. et al., 1999).

Transmission of N. meningitidis is via aerosol. Colonisation occurs following

adherence of bacteria to epithelial cells of the nasopharynx. N. meningitidis is highly

variable and new variants with different virulence and transmissibility factors are

continuously generated. Meningococci have a natural competency for

transformation, horizontal DNA exchange and recombination, which creates a wide

genetic diversity (Taha M.K. et al., 2002). The ability of meningococci to vary their

expression of different molecules in a process known as phase variation results in

even greater bacterial diversity (de Vries F.P. et al., 1996) .

Different bacterial structures are important for different stages of the bacteria's life cycle. Initial adherence to epithelial cells is mediated by type IV pili, which bind to the widely expressed CD46 (membrane cofactor protein MCP). Attachment of the bacteria is accompanied by elongation of the host cell micovilli and filopodia. Optimal adhesion is through coordinated regulation of different structures on the bacterial surface. After initial attachment pili and capsule expression are down regulated by the transcriptional regulatory protein crgA, allowing intimate association between the bacteria and the host plasma membrane. This is mediated by the surface proteins Opa and Opc, which are both important in attachment and colonisation of non-encapsulated bacteria (Merz A.J. et al., 2000).

The ability of meningococci to vary expression of the polysaccharide capsule is important in many stages of its life cycle. Transmission of meningococci from the nasopharynx is increased as the expression of capsule aids shedding. Capsule expression also protects bacteria in the blood stream from opsinisation and bacteriolysis (see below).

1.3.4 Invasion and the immune response

Invasion occurs via bacterial directed endocytosis by epithelial cells. Internalised bacteria survive and dived within epithelial cells in phagocytic vacuoles. This process is aided by PorB, which translocates the target cell membrane and inhibits maturation of the vacuoles (Rudel T. et al., 1996). Traversal of epithelial membranes into sub-epithelial stromal tissues occurs without obvious disruption to lateral junctional complexes or epithelial barrier properties.

In the blood stream capsule expression and LPS sialyation aid survival by conferring

resistance to complement and phagocytosis, enabling meningococci to grow and

divide. During this growth blebs are released, which are outer membrane vesicles

rich in lipopolysaccharide, OMPs and lipids. This blebbing process contributes to the

high level of endotoxin (LPS) observed in the blood stream of patients with septic

shock (Brandtzaeg P. et al., 2002)(Namork E. et al., 2002). In fatal cases of

meningococcal septicaemia 95% had plasma endotoxin levels higher than 100 EU/ml

(Brandtzaeg P. et al., 2002).

LPS released into the blood stream is a potent stimulator of inflammatory pathways,

inducing the release of both pro- and anti-inflammatory cytokines, chemokines,

soluble receptors and colony stimulating factors (reviewed in (Hackett S.J. et al.,

2001)). The pro inflammatory cytokine TNFα plays an important role in the clinical

manifestations of severe meningococcal disease. TNFα works synergistically with

IL-1 inducing hypotension with a fall in systemic vascular resistance and central

venous pressure along with an increased cardiac output. This results in pulmonary

oedema and haemorrhage (reviewed in (Hackett S.J. et al., 2001)). Levels of other

cytokines, including IL-1, IL-6, IL-8, IL-10 and IL-12 have been found to positively

correlate with TNFα levels. Along with elevated levels of pro-inflammatory

cytokines in patients with severe meningococcal disease, elevated levels of the anti-

inflammatory cytokine IL-10 have also been observed. Severe meningococcal

disease is associated with a disregulation of cytokine levels where the balance

between pro and anti-inflammatory cytokines is no longer maintained.

LPS activates cells via the TLR4/CD14 signalling complex and is the major

stimulator for the release of cytokines and other inflammatory mediators during

meningococcal disease. However other components expressed by *N. meningitidis*

also contribute to the release of cytokines as shown by the release of TNFα, IL-1α

and IL-6 by monocytes stimulated with LPS deficient *N. meningitidis* (Uronen H. et

al., 2000).

1.3.5 Vaccine development

Vaccines based on the polysaccharide capsule for serogroups A, C, Y and W135

were developed 25 years ago but these do not induce protective immunity in children

under the age of 2 and do not offer any long term protective memory (Balmer P. et

al., 2002b)(Pollard A.J. et al., 2000). To improve the immunogenicity,

polysaccharide was conjugated to a protein carrier. These conjugate vaccines induce

bactericidal antibodies and immunological memory in young children (Richmond P.

et al., 2001). In November 1999 a national immunisation programme with

meningococcal serogroup C conjugated vaccines was introduced in the UK. The

introduction of the vaccine has had a significant impact, resulting in an 86.7%

reduction in disease caused by serogroup C meningococci (Balmer P. et al., 2002a).

Contrary to prior concerns, there has been no increase in the overall incidence of

serogroup B disease since the introduction of the serogroup C conjugate vaccine.

Meningococci can switch their capsule and change their serogroup in vitro by

horizontal DNA exchange and transformation (Swartley J.S. et al., 1997). However,

there is no evidence of an increase in capsule switching from serogroup C to

serogroup B following the vaccination programme. A slight increase in the incidence

of disease caused by serogroup W135 isolates has been observed, though this was

associated with pilgrims returning from the Hajj (Balmer P. et al., 2002a).

The development of an effective serogroup B vaccine remains the major challenge in

the prevention of meningococcal disease. Serogroup B isolates account for 73% of

meningococcal disease cases in the UK (903 in 2001) (Balmer P. et al., 2002a). The

serogroup B capsular polysaccharide is poorly immunogenic. It shares homology

with the glycopeptides of the neural cell adhesion molecules (NCAMs), which

results in the immunological tolerance of the serogroup B capsular polysaccharides.

Approaches based on components other than capsular polysaccharides are being

explored to find a suitable serogroup B vaccine. One area under investigation is the

development of vaccines with outer membrane vesicles (OMVs), which contain LPS

and OMPs. Trials have been undertaken in both Cuba and Norway using OMV

vaccines for serogroup B N. meningitidis and efficacies in the range of 50-80% have

been reported (reviewed in (Jodar L. et al., 2002)). However, little protection in

children below the age of 4 was achieved. Also these vaccines only elicit strain

specific protection, though in adults broader responses to heterologous strains were

reported (Jodar L. et al., 2002). The PorA content of the OMV vaccine and to a lesser

extent the Opc protein, elicited the bactericidal antibody response and variations in

PorA (serosuptype) will therefore change the effectiveness of the OMV vaccine.

Approaches using vaccines based on other OMPs, which are conserved between

strains are being investigated. The transferrin protein B (TbpB) and the neisserial

surface protein A (NspA), a membrane protein of unknown function, are both being

explored as potential vaccine candidates (Moe G.R. et al., 2001)(Rokbi B. et al.,

2000)(Rosenstein N.E. et al., 2001)(West D. et al., 2001).

LPS has also been investigated as a potential vaccine candidate, however its

endotoxic properties presents a problem. It is more likely that LPS will be included

as a component of potential vaccines because of its adjuvant properties.

1.4 Dendritic cell interactions with micro-organisms

A wide range of micro organisms have been shown to activate DCs including Gram-

negative bacteria (e.g. *Escherichia coli*), Gram-positive bacteria (e.g. *Staphalococcus*

aureus), protozoa (e.g. *Leishmania major* and *Toxoplasma gondii*), fungi (e.g.

Candida albicans) and viruses (e.g. influenza virus). Purified products derived from

micro-organisms including LPS, peptidoglycan, lipoteichoic acid and outer

membrane proteins have also been shown to induce DC activation *in vitro* (reviewed

in (Sousa C. et al., 1999a)).

The DC response is not the same to each micro-organism. Differences in the

expression of DC surface molecules and the release of cytokines by DCs may affect

the immune response mounted against the micro-organism. For example the fungus

C. albicans exists as unicellular yeasts or as filamentous hyphae and DCs can

discriminate between the two forms of the fungi. DC interactions with the unicellular

yeast results in DC maturation and IL-12 production and a Th1 response, whereas

DCs activated by hyphae did not produce IL-12 and induced a Th2 response. The

hyphae form were also able to escape from the phagosomes (d'Ostiani C.F. et al.,

2000).

DCs respond to viral infection by activating a potent cytotoxic T lymphocyte (CTL)

response and producing type I interferons (IFNα/β). Infection of monocyte derived

DCs by influenza virus results in increased expression of surface MHC class I and

interferon production (Cella M. et al., 1999b). The influenza virus double stranded

RNA was found to be a key component in eliciting this immune response. Though

myeloid DCs have been shown to be activated by viral antigens, plasmacytoid DCs

are the major type I interferon producing cells. They are activated by viruses and

viral components and can induce the stimulation or virus specific CD4$^+$ and CD8$^+$ T

cells (Cella M. et al., 2000)(Fonteneau J.F. et al., 2003).

Some viruses however have harnessed DC biology to aid their pathogenesis. The best

characterised example is Human Immunodeficiency Virus (HIV), which infects DCs

at the site of infection, usually mucosal surfaces. The DCs then traffic to the lymph

nodes where the virus infects CD4$^+$ T cells (reviewed in (Bhardwaj N., 1997)(Klagge

I.M. et al., 1999)). The measles virus also utilizes DC biology. DCs infected with

measles virus are prevented from maturing and are thus unable to prime an anti-

measles T cell response (reviewed in (Bhardwaj N., 1997)(Klagge I.M. et al., 1999)).

1.4.1. Dendritic cells and pattern recognition receptors

The pattern recognition receptors of the innate immune system recognise a wide

variety of micro-organisms and are a subject of great interest. DCs express many of

these receptors that bind to micro-organisms including DEC-205 and the mannose

receptor, these bind and internalise mannosylated antigens and target them to

intracellular processing compartments (Kato M. et al., 2000),(Mahnke K. et al.,

2000). DCs also express scavenger receptors that bind a diverse array of ligands

including lipoteichoic acid and lipopolysaccharides and induce phagocytosis (Dunne

D.W. et al., 1994)(Peiser L. et al., 2000). DCs express other receptors that are

important in the internalisation of opsonised particles such as the Fc family of

receptors that bind immunoglobulin complexed particles (Fanger N.A. et al., 1996);

complement receptors that bind and internalise components of the complement

pathway opsonised to micro-organisms (e.g. C1q and MBL).

These receptors play a fundamental role in the internalisation of various micro-

organisms, but it is unlikely that they can distinguish between many different

pathogens as they bind to ligands common to a wide range of micro-organisms.

Other receptors such as Toll-like receptors (TLRs) are likely to play a role in the

signalling of pathogens to DCs explaining how DCs can mount subtly differing

responses to different pathogens.

The TLRs are a recently described family of receptors expressed by many cells of the

immune system. TLRs are homologous to the Drosophila Toll protein, which is

important in the development of the embryonic dorsal ventral axis. Drosophila Toll

was first thought to have an immune function because its cytoplasmic domain shares

homology with that of the mammalian interleukin-1 receptor (IL-1R) (Gay N.J. et al.,

1991). The Spätzle/Toll/Cactus pathway in Drosophila induces the production of the

anti-fungal peptide drosomycin during fungal infection (Lemaitre B. et al., 1996).

Data base searches initially revealed 5 homologues of Drosophila Toll in the human

genome termed Toll-like receptors (TLRs) 1-5. Now the total number of identified

human TLRs is 10 (Means T.K. et al., 2000)(Rock F.L. et al., 1998). The 10 human

TLRs and Drosophila Toll are type 1 transmembrane proteins with a conserved

intracellular domain also common to IL-1R known as Toll/IL-1R (TIR) domains.

Though mammalian TLRs were predicted to have an immune function, it has not

been easy to identify agonistic ligands. The C3HeJ mouse is hypo-responsive to LPS

due to a single missense mutation within the *tlr4*gene (Watson J. et al., 1978)

(Hoshino K. et al., 1999)(Qureshi S.T. et al., 1999). The importance of TLR4 in LPS

signalling was confirmed when DCs from TLR4 knock out mice were found not to

respond to LPS (Kaisho T. et al., 2001b)(Kaisho T. et al., 2001a). Several other

accessory molecules have been implicated in the binding and recognition of LPS,

including CD14, LPS binding protein (LBP) and MD-2. Together with TLR 4, these

molecules form an LPS binding complex (Fig 1.5). Other ligands for TLR 4 include

heat shock protein 60 (HSP60), one of the first endogenous ligands identified to

signal through TLRs (Ohashi K. et al., 2000).

TLR 2 is involved in the recognition of a broad range of microbial products

including peptidoglycan from Gram-positive bacteria, bacterial lipoproteins,

mycobacterial cell-wall lipoarabinomannan (LAM), yeast cell walls and LPS from *P.*

gingivalis (Hirschfeld M. et al., 1999)(Hirschfeld M. et al., 2001), (Lien E. et al.,

1999)(Means T.K. et al., 1999b)(Means T.K. et al., 1999a)(Schwandner R. et al.,

1999)(Underhill D.M. et al., 1999). This broad range of molecules that signal

through TLR2 can be partly explained by the co-operation of TLR2 with other TLRs,

namely TLR1 and TLR6. TLR2 forms heterodimers with TLR6 in response to

macrophage-activating lipopeptide 2kDa (MALP-2) (Takeuchi O. et al., 2001) and

blocking of either TLR2 or TLR6 inhibits responses to peptidoglycan or yeast zymosan (Ozinsky A. et al., 2000).

Recent studies have shown that TLR3 responds to double stranded RNA (ds RNA), which is a product of most viruses at some point in their life cycle (Alexopoulou L. et al., 2001). Flagellin, the principle structural component of bacterial flagella in both Gram-positive and Gram-negative bacteria signals through TLR5 (Hayashi F. et al., 2001) and TLR7 is activated by imadazoquinolins, which are synthetic anti-viral complexes. However, the natural ligand for TLR7 has yet to be identified, though it is likely to be a viral specific molecule (Hemmi H. et al., 2002). Bacterial DNA signals through TLR9, which unlike mammalian DNA is rich in unmethylated CpG motifs and has immunogenic effects (reviewed in (Krieg A.M., 2000) & (Hemmi H. et al., 2000)). TLR9 knock out mice are unresponsive to CpG DNA.

The signalling pathway common to the IL-1R and the TLR family resulting in the activation of nuclear factor $\varkappa$B (NF$\varkappa$B) has been well studied. NF$\varkappa$B is a transcription factor that has a central role in the control of expression of a wide variety of genes involved in immune responses, including cytokines IL-1, IL-6, IL-8 and TNFα, and co-stimulatory molecules CD80 and CD86. Myeloid differentiation factor 88 (MyD88) is an adapter protein with a TIR domain that associates with TLRs and the IL-1R through homophillic interactions of their TIR domains. MyD88 recruits IL-1 receptor kinase (IRAK), and in turn IRAK recruits another adaptor protein called TNF receptor associated factor 6 (TRAF6). The transcription factor NF$\varkappa$B is activated and released to translocate to the nucleus by the degradation of I$\varkappa$B (Fig 1.5).
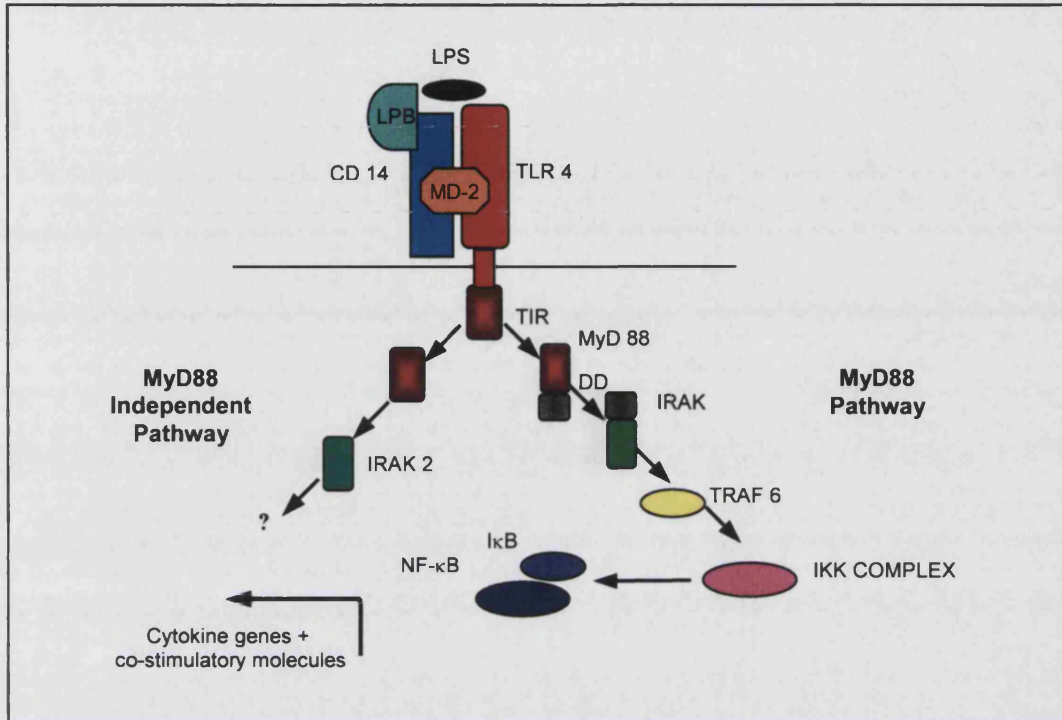
Figure 1.5 The TLR 4 signalling pathway

The LPS signalling complex consists of TLR4, MD-2, LBP and CD14. The TIR domain of TLR4 binds the TIR domain of MyD88, which in turn interacts with IRAK via their death domains (DD). The IKK complex phosphorylates IκB releasing NFκB, which translocates to the nucleus controlling many genes including pro-inflammatory cytokines and co-stimulatory molecules. The MyD88 independent pathway observed in TLR 4 signalling is also shown. Adapted from (Bowie A. et al., 2000)(Underhill D.M. et al., 2002)

The MyD88/IRAK pathway is common to almost all TLRs and shares many elements with IL-1R signalling. There are however other signalling pathways activated by TLRs including the TLR3 MyD88 independent pathway and the TLR4 MyD88 independent pathway (Oshiumi H. et al., 2003)(Yamamoto M. et al., 2002). The TLR4 MyD88 independent pathway results in the increase of co-stimulatory molecules in DCs but not cytokine production.

The TLR family play a crucial role in the innate immune response. Analysis by RNA expression shows that the antigen presenting cells (APCs) DCs and macrophages express the full repertoire of TLRs 1-10 (Muzio M. et al., 2000b)(Muzio M. et al., 2000a). Signalling of TLRs in DCs triggers maturation, including increased expression of surface MHC and co-stimulatory molecules, as well as production of pro-inflammatory cytokines (Kaisho T. et al., 2001b). As the maturation of DCs is essential for the stimulation of naïve T cells, the control of the adaptive immune system by the innate immune system is at least in part regulated through TLRs.

1.4.2 Dendritic Cells and *Neisseria meningitidis*

The Gram-negative human pathogen *N. meningitidis* induces DC activation and maturation. DCs stimulated with an LPS deficient group B *N. meningitidis* produced a different cytokine response to the wild type bacteria (Dixon G.L. et al., 2001). Wild type *N. meningitidis* induced DC IL-12 production, whereas in the absence of LPS not IL-12 production was detected. This study also showed the importance of LPS in the context of the bacteria, as the addition of exogenous LPS did not restore the cytokine response to that of the wild type *N. meningitidis* (Dixon G.L. et al., 2001). The different DC response to the LPS deficient *N. meningitidis* was shown in further

studies, these also showed no difference in the induction of DC cytokine production

between the wild type *N. meningitidis* and a capsule deficient mutant. The un-

encapsulated bacteria bound more readily to DCs and were internalised quicker than

the capsulated wild type organism, but cytokine production remained the same

(Kolb-Maurer A. et al., 2001).(Unkmeir A. et al., 2002).

1.5 Aims

The aim of the following work was to investigate further the phenotype of DCs

following stimulation with *N. meningitidis*, and the importance of LPS as a

component of the bacterial outer membrane. Along with DC activation as measured

by surface marker expression and cytokine production, other aspects of DC biology

were explored. These included the migratory capabilities of DCs following

stimulation with *N. meningitidis*, as well as DC interactions with T cells following

stimulation with *N. meningitidis*. The ability of DCs to migrate to the secondary

lymphoid organs and present antigen to T cells is a crucial part of a DC's function.

Both these aspects of DC biology must be considered when deciphering the critical

components of *N. meningitidis* in terms of potential vaccines.

Chapter 2

Materials and Methods

2.1 Introduction

General methods common to more than one chapter are explained here in detail. More specific methods are covered in each of the results chapters. All reagents, equipment and antibodies used are listed in this section.

2.2 Reagents and equipment

2.2.1 General reagents

Below is a list of all reagents used in this work.

Reagent	Supplier
Accutase	TCS cell works, Buckingham, UK
Agarose	Invitrogen, Paisley, UK
Bovine serum albumin (BSA)	Sigma, Poole, UK
Brefeldin A	Sigma, Poole, UK
Cellfix	BD Bioscience, Oxford, UK
CFSE	Molecular Probes, Leiden, Netherlands
Cholera toxin	Sigma, Poole, UK
Citifluor (glycerol & PBS)	Cititfluor, London, UK
Collagenase type 2	Invitrogen, Paisley, UK
Dimethylsulphoxide (DMSO)	ICN Biomedical, Basingstoke, UK
DNA ladder (50bp)	Invitrogen, Paisley, UK
Deoxynucleotide triphosphates (DNTPs)	Promega, Southampton, UK
Ethenediaminetetraacetic acid, disodium salt (EDTA)	BDH Merck, Poole, UK
Ethanol	BDH Merck, Poole, UK
Foetal calf serum (FCS)	Hyclone, Cramlington, UK
Gelatin 300 bloom	Sigma, Poole, UK
Gel loading solution	Sigma, Poole, UK
Gentamicin	Invitrogen, Paisley, UK
Glycerol	BDH Merck, Poole, UK

Reagent	Supplier
Gonococcal agar	BD bioscience, Oxford, UK
Granulocyte-monocyte colony stimulating factor (GMCSF)	Schering-Plough, Henilworth, NJ, USA
Hank's balanced salt solution, without Phenol Red (HBSS)	Invitrogen, Paisley, UK
Hinf 1 restriction enzyme	Promega, Southampton, UK
Recombinant human interleukin-2 (rIL-2)	Chiron, Emeryville, CA, USA
Recombinant human interleukin-4 (rIL-4)	Schering-Plough, Henilworth, NJ, USA
Ionomycin	Sigma, Poole, UK
N-[2-Hydroxyethyl]piperazine-N'-[2-ethansulfonic acid] (HEPES)	Invitrogen, Paisley, UK
Kanamycin	Sigma, Poole, UK
L-Glutamine	Invitrogen, Paisley, UK
Lipopolysaccharide (E.coli) 0111B:4	Sigma, Poole, UK
Lymphoprep	Nycomed (Amersham), Little Chalfont, UK
MCBD 131 medium	Invitrogen, Paisley, UK
MIP3β	Peprotech EC ltd, London, UK
Monoparin sodium heparin	CP Pharmaceuticals, Wrexham, UK
Muller Hinton broth	DIFCO, BD bioscience, Oxford, UK
Oligonucleotide PCR primers	Sigma-Genosys, Poole, UK
Paraformaldehyde $(CH_2O)_n$	Sigma, Poole, UK
Percoll	Amersham, Little Chalfont, UK
Penicillin/Streptomycin	Invitrogen, Paisley, UK
Permeabilisation solution	Caltag, Burlingame, CA, USA
Pertussis toxin	Sigma, Poole, UK
Phosphate buffered saline tablets (PBS)	Oxoid, Basingstoke, UK
Phytohemagglutinin (PHA)	Sigma, Poole, UK
phorbal 12-myristate 13-acetate (PMA)	Sigma, Poole, UK
Poly (I:C)	Sigma, Poole, UK
Potassium chloride (KCl)	BDH Merck, Poole, UK

Reagent	Supplier
Prostoglandin E2	Sigma, Poole, UK
QIAmp blood kit	Qiagen, Crawley, UK
RPMI 1640 medium with 10mM L-glutamine	Invitrogen, Paisley, UK
RPMI 1640 medium without phenol red	Invitrogen, Paisley, UK
RSA 1 restriction enzyme	Promega, Southampton, UK
Saponin	Sigma, Poole, UK
Sodium azide (NaN$_3$)	BDH Merck, Poole, UK
Staphylococcus enterotoxin B (SEB)	Sigma, Poole, UK
Sodium chloride (NaCl)	BDH Merck, Poole, UK
Sodium hydroxide (NaOH)	BDH Merck, Poole, UK
[methyl ^{3}H]-Thymidine	Amersham, Little Chalfont, UK
Taq polymerase with 10X PCR buffer and MgCl$_2$	Promega, Southampton, UK
TMP solution	Europa Bioproducts, Cambridge, UK
Triton X-100	Sigma, Poole, UK
Trypan blue	Sigma, Poole, UK
Trypsin-EDTA	Invitrogen, Paisley, UK
Recombinant human tumour necrosis factor (rTNFα)	Gift from Dr. K Kotowitz
Tween 20 (Polyoxethylene sorbitan monolaurate)	Sigma, Poole, UK
VITOX	Oxoid, Basingstoke, UK

2.2.2 Antibodies for flow cytometry, tissue culture and confocal microscopy

All the antibodies listed here are monoclonal anti-human antibodies, with the exception of the second and third layers. The conjugated fluorochrome, isotype and species in which the antibodies are derived is given.

Antibody	Isotype	Clone	Supplier
CD1a PE	Mouse IgG2a	NA1/34	Dako, Cambridge, UK
CD3 purified	Mouse IgG2a	S4.1	Caltag, Burlingame, CA, USA
CD3 FITC	Mouse IgG2a	S4.1	Caltag, Burlingame, CA, USA
CD3 PE	Mouse IgG2a	S4.1	Caltag, Burlingame, CA, USA
CD3 Tri-Color®	Mouse IgG2a	S4.1	Caltag, Burlingame, CA, USA
CD4 FITC	Mouse IgG2a	S3.5	Caltag, Burlingame, CA, USA
CD4 PE	Mouse IgG2a	S3.5	Caltag, Burlingame, CA, USA
CD8 Purified	Mouse IgG2a	3B5	Caltag, Burlingame, CA, USA
CD8 FITC	Mouse IgG2a	3B5	Caltag, Burlingame, CA, USA
CD8 PE	Mouse IgG2a	3B5	Caltag, Burlingame, CA, USA
CD11b	Mouse IgG1	2LPM19C	Dako, Cambridge, UK
CD14 purified	Mouse IgG2a	Tük 4	Caltag, Burlingame, CA, USA
CD14 FITC	Mouse IgG2a	Tük 4	Caltag, Burlingame, CA, USA
CD14 PE	Mouse IgG2a	Tük 4	Caltag, Burlingame, CA, USA
CD18	Mouse IgG1	MHM23	Dako, Cambridge, UK
CD19 purified	Mouse IgG1	SJ25-C1	Caltag, Burlingame, CA, USA
CD19 FITC	Mouse IgG1	4G7	BD Bioscience, Oxford, UK
CD25 FITC	Mouse IgG1	CD25-3G10	Caltag, Burlingame, CA, USA
CD25 PE	Mouse IgG1	CD25-3G10	Caltag, Burlingame, CA, USA
CD40 PE	Mouse IgM	14G7	Caltag, Burlingame, CA, USA
CD45RA PE	Mouse IgG2b	MEM 56	Caltag, Burlingame, CA, USA
CD45RO PE	Mouse IgG2a	UCHL1	Caltag, Burlingame, CA, USA
CD 56 purified Ab	Mouse IgG1	MEM-188	Caltag, Burlingame, CA, USA
CD80(B7-1) PE	Mouse IgG1	DAL-1	Caltag, Burlingame, CA, USA
CD83 PE	Mouse IgG2b	HB15	Caltag, Burlingame, CA, USA
CD86 PE	Mouse IgG1	BU63	Caltag, Burlingame, CA, USA
CCR5 PE	Mouse IgG2b	45531	R&D, systems Minneapolis, MN, USA
CCR7 purified Ab	Mouse IgG2a	150503	R&D, systems Minneapolis, MN, USA
DC-SIGN (CD209)	Mouse IgG2b	120507	R&D, systems Minneapolis, MN, USA
HLA-DR (Class II) FITC	Mouse IgG2b	TÜ 36	Caltag, Burlingame, CA, USA
HLA-DR (Class II) PE	Mouse IgG2b	TÜ 36	Caltag, Burlingame, CA, USA
ICAM-1 (CD54)	Mouse IgG1	15.2	Serotec, Oxford, UK
ICAM-2 (CD102)	Mouse IgG1	B-T1	Serotec, Oxford, UK
ICAM-3 (CD50)	Mouse IgG1	KS128	Dako, Cambridge, UK

Antibody	Isotype	Clone	Supplier
LFA-1 (CD11a/CD18)	Mouse IgG2a	38	Serotec, Oxford, UK
L-selectin (CD62L)	Mouse IgG2b	FMC46	Dako, Cambridge, UK
PECAM-1 (CD31)	Mouse IgG1	MBC78.2	Caltag, Burlingame, CA, USA
VLA-4 (CD49d/CD29)	Mouse IgG1	44H6	Serotec, Oxford, UK
IL-1α PE	Mouse IgG1	AS5	BD Bioscience, Oxford, UK
IL-2 PE	Mouse IgG1	5344.111	BD Bioscience, Oxford, UK
IL-4 PE	Mouse IgG1	3010.211	BD Bioscience, Oxford, UK
IL-6 PE	Mouse IgG1	AS12	BD Bioscience, Oxford, UK
IL-8 PE	Mouse IgG1	AS14	BD Bioscience, Oxford, UK
IL-10 PE	Rat IgG1	JES3-9D7	Pharmingen, BD Bioscience, Oxford, UK
IL-12 (p40/p70) PE	Mouse IgG1	C11.5	Pharmingen, BD Bioscience, Oxford, UK
IFNγ FITC	Mouse IgG2b	25723.11	BD Bioscience, Oxford, UK
IFNγ PE	Mouse IgG2b	25723.11	BD Bioscience, Oxford, UK
TNFα PE	Mouse IgG1	6401.1111	BD Bioscience, Oxford, UK
MIP1 α FITC	Mouse IgG2A	14215.41	R&D, systems Minneapolis, MN, USA
RANTES FITC	Mouse IgG1	21445.1-1	R&D, systems Minneapolis, MN, USA
Mouse IgG1 FITC	Mouse IgG1	MOPC-21	Caltag, Burlingame, CA, USA
Mouse IgG1 PE	Mouse IgG1	MOPC-21	Caltag, Burlingame, CA, USA
Mouse IgG2a FITC	Mouse IgG2a	5.205	Caltag, Burlingame, CA, USA
Mouse IgG2a PE	Mouse IgG2a	5.205	Caltag, Burlingame, CA, USA
Mouse IgG2b FITC	Mouse IgG2b	MOPC-195	Caltag, Burlingame, CA, USA
Mouse IgG2b PE	Mouse IgG2b	MOPC-195	Caltag, Burlingame, CA, USA
Mouse IgM PE	Mouse IgM	MOPC-104E	Caltag, Burlingame, CA, USA
Mouse IgG1 PE	Mouse IgG1	X40	BD Bioscience, Oxford, UK
Mouse IgG1 PE	Mouse IgG1	MOPC-21	Pharmingen, BD Bioscience, Oxford, UK
Mouse IgG2a PE	Mouse IgG2a	G155-178	Pharmingen, BD Bioscience, Oxford, UK
Mouse IgG2a	Mouse IgG2a	20102.1	R&D, systems Minneapolis, MN, USA
Mouse IgG2b PE	Mouse IgG2b	20116	R&D, systems Minneapolis, MN, USA

2nd and 3rd Antibody Layers	Supplier
F(ab')$_2$ goat anti mouse IgG FITC	Dako, Cambridge, UK
F(ab')$_2$ goat anti mouse IgG PE	Dako, Cambridge, UK
F(ab')$_2$ goat anti rabbit IgG FITC	Sigma, Poole, UK

2nd and 3rd Antibody Layers	Supplier
$F(ab')_2$ goat anti rabbit IgG PE	Sigma, Poole, UK
$F(ab')_2$ goat anti rabbit IgG Biotin	Dako, Cambridge, UK
Streptavidin FITC	Pharmingen, BD Bioscience, Oxford, UK
Streptavidin CyChrome	Pharmingen, BD Bioscience, Oxford, UK

2.2.3 Antibodies and standards for ELISAs

Antibody pairs and standards (purified recombinant cytokines) for human IL-6, IL-10, IL-12(p40) and TNFα were purchased together as CytoSets™ from BioSource (Camarillo, CA, USA) for ELISA determination.

2.2.4 General consumables

Below is a list of consumables and small pieces of equipment used throughout this work.

Materials	Supplier
Bijou 7 ml	SLS, Wilford, Nottingham, UK
Falcon tubes 5 ml	BD Falcon, BD Bioscience, Oxford, UK
Falcon tubes 15 ml	TPP, Trasadingden, Switzerland
Falcon tubes 50 ml	TPP, Trasadingden, Switzerland
Maxisorb ELISA plates - 96 well	Nalgene Nunc, Rochester, NY, USA
Goat anti-mouse IgG microbeads	Miltenyi Biotech, Bergisch Gladbach, Germany
96 well round bottom plates for FACS staining	Greiner Bio-one, Fricekenhausen, Germany
Tissue culture plates - 6 well	Corning (Costar), Corning, NY, USA
Tissue culture plates - 12 well	Corning (Costar), Corning, NY, USA
Tissue culture plates - 24 well	Corning (Costar), Corning, NY, USA
Tissue culture plates - 96 well	Corning (Costar), Corning, NY, USA
TC flasks 25 cm^3	BD Falcon, BD Bioscience, Oxford, UK

Materials	Supplier
Universal tubes 20 ml	Helena Bioscience
Cryo vials	Nalgene Nunc, Rochester, NY, USA
TC coated flasks (Primira)	BD Falcon, BD Bioscience, Oxford, UK
Filling tube	Universal Hospital Supplies, London, UK
Filter mats (for Harvester)	PerkinElmer (Wallac) Cambridge, UK
Coverslips (for microscopy)	BDH Merck, Poole, Dorset, UK
Macs magnet Stand	Miltenyi Biotech, Bergisch Gladbach, Germany
Mini-Macs Columns	Miltenyi Biotech, Bergisch Gladbach, Germany
Mini-Macs Magnet	Miltenyi Biotech, Bergisch Gladbach, Germany
Slides (for microscopy)	BDH Merck, Poole, Dorset, UK
Wax (for harvester)	PerkinElmer (Wallac) Cambridge, UK
Transwells - 6.5 mm Polycarbonate membrane (5 μm pore)	Corning (Costar), Corning, NY, USA

2.2.5 Equipment

Major Equipment	Supplier
FACScalibur flow cytometer	Becton Dickinson, (BD Bioscience), Oxford, UK
Cell harvester	Dynatech Laboratories, Chantilly, VA, USA
LKB 1218 RackBeta scintillation counter	PerkinElmer (Wallac) Cambridge, UK
MRX microplate reader	Dynatech Laboratories, Chantilly, VA, USA
Confocal microscope	Leica Microsystems UK, Milton Keynes, UK
Phoenix PCR machine	Helena Bioscience, Beaumont, TX, USA
Alpha imager	Alpha Innotech, Cannock, Staffordshire UK
Fluorescent microscope	Leica Microsytsems UK, Milton Keynes, UK

2.2.6 General buffers, solutions and culture media

Milli Q purified water was used to make up the following buffers and solutions where appropriate.

Phosphate buffered saline (PBS)   1 PBS tablet to 100 ml H_2O

10x PBS

67.5 g NaCl
0.625 g Na_2HPO_4
10.5 g KH_2PO_4 pH 4.6
made up to 1000 ml H_2O

FACS washing buffer

1 X PBS
0.02% Sodium Azide
0.5 % BSA

Tris borate EDTA (TBE)

108 g Trizma Base
55 g Boric Acid
7.44 g EDTA
made up to 1000 ml H_2O

ELISA buffer A

8.0 g NaCl
1.42 g $Na_2HPO_4.H_2O$
0.2 g KH_2PO_4 pH 7.4
0.2 g KCl
made up to 1000 ml H_2O

ELISA block

Buffer A
0.5% BSA

ELISA diluent

Buffer A
0.5% BSA
0.1% Tween 20

ELISA wash

9.0g NaCl
1 ml Tween 20
made up to 1000 ml H_2O

MiniMACs buffer

250 ml PBS
1 ml 0.5 M EDTA (pH8)
1.25 g BSA

Cell wash medium	RPMI 1640 5% FCS (heat inactivated [HI])
DC culture medium	RPMI 1640 5% FCS (HI) 2.4 mM L-glutamine 100 U/ml Penicillin/Streptomycin (with 100 ng/ml rGM-CSF and 50 ng/ml rIL-4 when specified)
T cell culture medium	PMI 1640 10% FCS (HI) 2.4 mM L-glutamine 100 U/ml Penicillin/Streptomycin (with 10 U/ml rIL-2 when specified)
Human umbilical cord storage medium	PMI 1640 10 mM L-Glutamine 80 µg/ml Gentamicin 100 U/ml Penicillin/Streptomycin
HUVECS wash medium	RPMI 1640 5% FCS (HI) 10 mM L-Glutamine 80 µg/ml Gentamicin 100 U/ml Penicillin/Streptomycin
HUVECS culture medium	MCBD 131 20% FCS 10 mM L-Glutamine 100 U/ml Penicillin/Streptomycin
Transwell medium	MCBD 131 20% Human Serum (HI) 10 mM L-Glutamine 100 U/ml Penicillin/Streptomycin
Saponin permeabilisation wash	HBSS 0.1% saponin 2mM Hepes buffer 0.05% Sodium Azide

2.3 Cell preparation and culture

2.3.1 Separation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were prepared from venous blood donated by healthy volunteers. Blood was taken by venesection (60-100 ml), carefully transferred to a heparinized flask (10 IU/ml of blood) and diluted with an equal volume of RPMI 1640 medium at 37 °C. The diluted blood was layered onto Ficoll/Hypaque at a density of 1.077 g/ml (lymphoprep) and centrifuged at 400g for 25 minutes at room temperature. The mononuclear cells at the interface were recovered and washed three times in cell wash medium. The PBMC were counted on a haemocytometer with trypan blue to check viability.

2.3.2 Monocyte isolation

Monocytes have a lower nuclear to cytoplasm ratio than peripheral blood lymphocytes (PBL) and they can be separated from PBL by density centrifugation on a discontinuous percoll gradient.

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 34% SIP (1.045 g/ml), 47.5% SIP (1.059 g/ml) and 60% SIP (1.076 g/ml). The PBMC pellet was resuspended in 8 ml of 60% SIP, and 2 ml of this suspension was placed into each of four 15ml tubes. 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 tubes were centrifuged at 1750 g for 45 minutes at room temperature (Fig 2.1) (Hilkens C.M. et al., 1997) (and personal communication with Dr. E deJong).
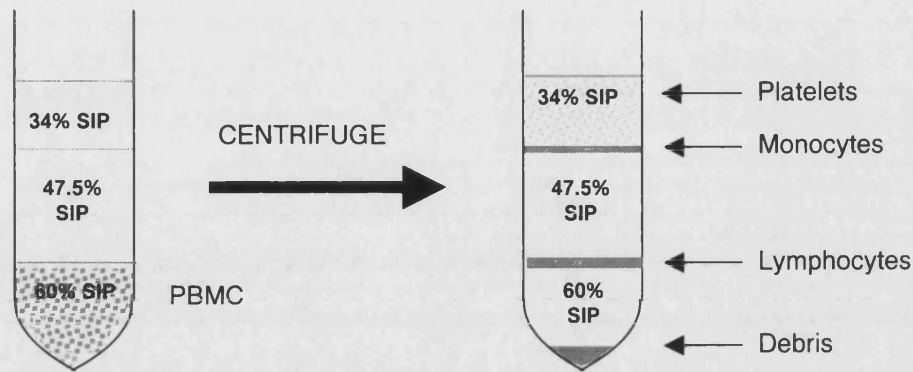
Figure 2.1 Discontinuous percoll gradient separation of monocytes

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. Platelets and aggregated cells formed a suspension in the 34% SIP and lymphocytes formed a layer at the interface between 47.5% and 60% SIP. Any contaminating red blood cells and cell debris pelleted at the bottom of the tube.

Following centrifugation, the upper layer of platelets and aggregated cells was removed and the monocytes harvested from the upper interface. The monocytes were washed three times in cold cell wash medium, counted before the final wash, and then resuspended to a final concentration of 0.5×10^6 cells/ml in DC culture medium. The purity of the monocyte preparation was checked by FACS analysis for contaminating lymphocytes using CD3, CD14 and CD19 antibodies (Fig 2.2). Before purification, PBMC contained between 10-20% CD14$^+$ monocytes. After purification on the discontinuous percoll gradient the upper fraction was found to be contain 95% CD14$^+$ (Fig 2.2). The remaining 5% of contaminating cells in the upper fraction were likely to be T cells (3% CD3$^+$) and B cells (1% CD19$^+$) (Fig 2.2).
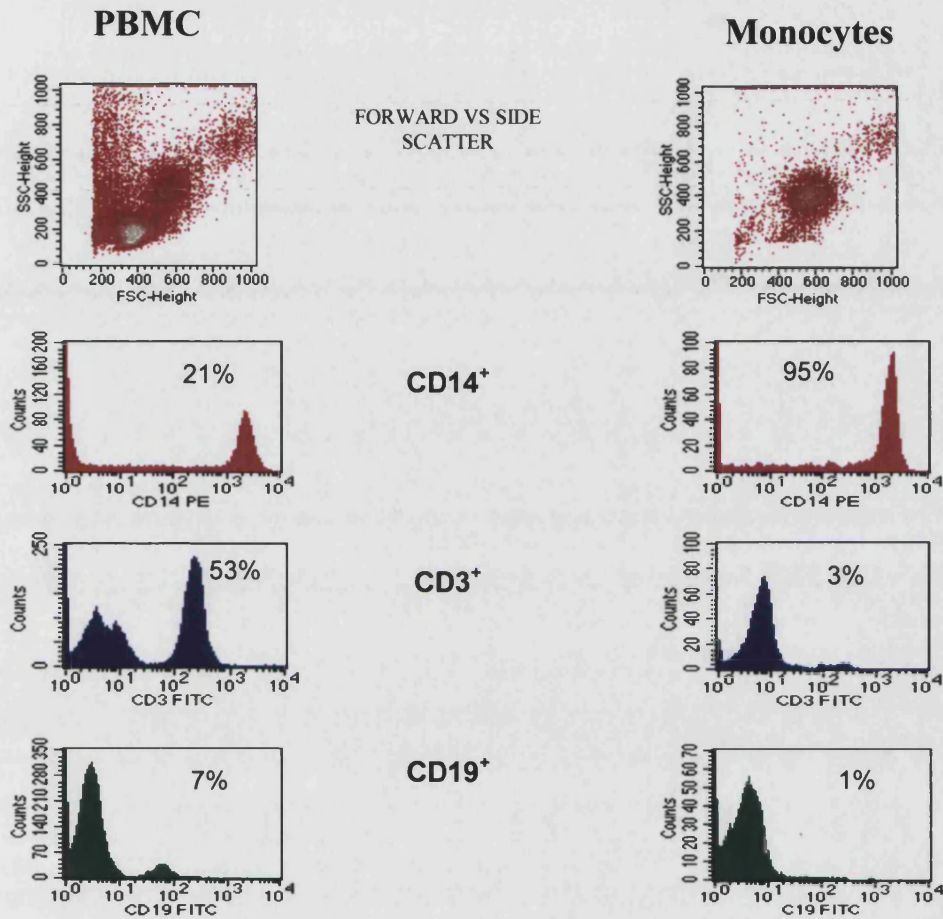
PBMC   **Monocytes**

Figure 2.2 Purity of monocytes following separation by discontinuous percoll density gradient.

The cell components of PBMCs before and after separation on a percoll gradient stained with antibodies to CD14, CD3 and CD19. Cells were not gated.

2.3.3 Freezing and thawing of peripheral blood lymphocytes

Peripheral blood lymphocytes (PBLs) were recovered from the lower lymphocyte rich interface of the percoll gradient, washed twice in cell wash medium and resuspended to a concentration of 10^7 cells/ml in FCS with 10% DMSO. The cell suspension was transferred in 1 ml aliquots to cryo-vials and placed in a -80°C freezer. After 24 hours the cryo-vials were transferred to liquid nitrogen storage.

When T cells were required for an assay, a cryo-vial containing frozen lymphocytes was removed from liquid nitrogen storage and placed into a 37 °C waterbath. The tube was agitated gently until only a small ice clump was left. The cell suspension was then carefully dropped onto 20 ml of RPMI with 10% FCS at 37 °C in a universal tube using a Pasteur pipette. After centrifugation at 300 g for 10 minutes at room temperature, the cell pellet was resuspended in 10 ml of cell wash medium, washed twice and counted on a haemocytometer with trypan blue to check cell viability. T cells were then purified by negative selection.

2.3.4 Purification of CD3$^+$ T cells and CD4$^+$ CD45RA$^+$ T cells by negative selection.

T cells and T cell subsets were purified from PBMC using the MACS magnetic cell sorting system. Cells to be depleted were labelled with lymphocyte subset specific antibodies followed by magnetic anti-immunoglobulin micro beads. After magnetic labelling, the cells were passed through a separation column held in a strong magnet. The column matrix serves to create a high-gradient magnetic field. The magnetically labelled cells are retained in the column while non-labelled cells pass through.

CD3$^+$ lymphocytes were purified from monocyte depleted PBMC. Unwanted cells were labelled with CD14 (10 µg/ml), CD19 (10 µg/ml) and CD56 (10 µg/ml) antibodies for 30 minutes on ice in a final volume of 200 µl. The cells were then washed twice in 10 ml of cold minimacs buffer, resuspended in 80 µl of buffer and 20 µl of goat anti mouse immunoglobulin bead suspension, then incubated for 15 minutes at +4°C with gentle mixing every 5 minutes. The cell and bead mix was then washed in 1.5 ml of cold minimacs buffer, centrifuged at 490 g for 7 minutes and resuspended in 500 µl of buffer. A column was placed in the holding magnet and washed twice with 500 µl of cold minimacs buffer. The cell and bead suspension was added to the top of the column held in the magnet. A sterile 20 ml universal was placed underneath the column to collect the eluted cells. The column was washed three times with 500 µl of cold minimacs buffer. The eluted cells were counted on a haemocytometer and resuspended to 1 x 10^6 cells/ml in T cell culture media.

In experiments where naïve CD4$^+$ CD45RA$^+$ T cells were required, the same negative isolation procedure as above was followed but with the addition of CD8 (10 µg/ml) and CD45RO (10 µg/ml) antibodies in the initial antibody incubation stage. The purity of the isolated cells checked with antibodies to CD4 and CD45RA was found consistently to be 97% CD4$^+$ and 94% CD45RA$^+$ (Fig 2.3)
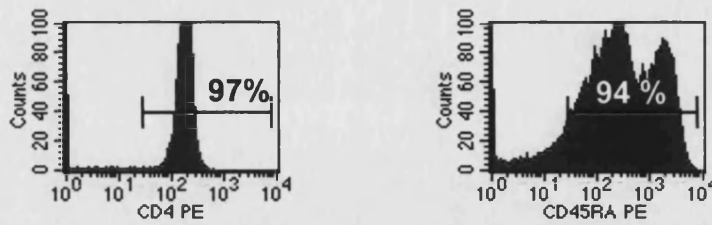
Figure 2.3 Purification of CD4⁺ CD45RA⁺ T cells

After purification with antibodies and beads on a minimacs column, eluted cells were stained with CD4 and CD45RA antibodies and analysed by flow cytometry.

2.3.5 Dendritic cells

The development of *in vitro* culture techniques for generating dendritic cells (DCs) from monocytes has lead to a greater understanding of DC biology and has made it possible for detailed studies on the functions of DCs to be carried out (Sallusto F. et al., 1994)

2.3.5.1 Generating dendritic cells from monocytes

Monocytes were resuspended to 0.5×10^6 cells/ml in DC culture medium supplemented with 100 ng/ml of recombinant human GM-CSF and 50 ng/ml of recombinant human IL-4. The monocytes were added to 12 well tissue culture plates at 1×10^6 cells/well (2 ml/well), or in 24 well plates at 0.5×10^6 cells/well (1 ml/well) and cultured for 6 days at 37 °C in an atmosphere of 5% CO_2 in air. On day 6 the DCs were examined by light microscopy for typical DC morphology of large, irregular shaped veiled cells. The DCs were then gently washed from the wells of the

tissue culture plate in RPMI using sterile Pasteur pipettes and resuspended in fresh

DC culture medium (without GMCSF or IL-4) to a concentration of 10^5-10^6 cells/ml.

The quality of FCS used in culture media proved to be critical, especially in the

development of DCs from monocytes. Batch testing of different FCS with very low

endotoxin content was carried out and the most effective batch of FCS was selected.

FCS was heat inactivated at 56°C for one hour, aliquoted and stored at -20°C.

2.3.5.2 Phenotypic characteristics of dendritic cells

Dendritic cells were identified by flow cytometry using forward and side scatter

characteristics (Fig 2.4 gate R2). DCs were CD1a positive, CD14 low, and negative

for lymphocyte markers CD3 and CD19 (Fig 2.4). DCs without stimulation also

expressed CD40, CD80, CD86 and HLA DR (Fig 3.1).

2.4 Cell phenotyping

In the experiments described in this work, cells were characterised by staining with

monoclonal antibodies for specific surface molecules or for intra cellular cytokine

expression.

2.4.1 Surface staining

Cells were incubated with the appropriate monoclonal antibody (usually 5μg/ml

unless otherwise stated) in 96 well plates on ice in the dark for 30 minutes. For each

monoclonal antibody, a matching isotype control was used to control for non-specific

binding.
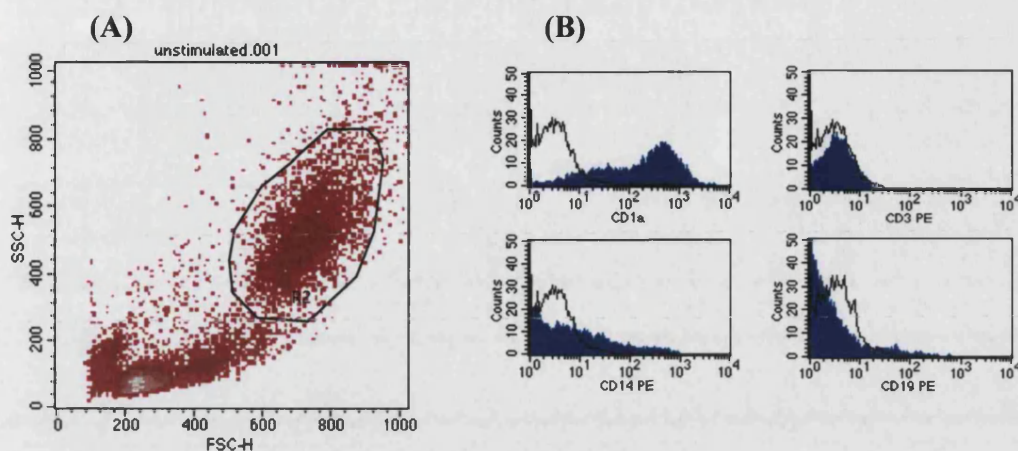
Figure 2.4 Phenotypic characteristics of dendritic cells

DCs were large, granular cells as shown in gate R2 (A). They were CD1a positive, CD14 low and CD19 and CD3 negative. Shaded areas represent cell surface staining with monoclonal antibodies compared to the appropriate isotype control, solid lines (B).

Cells were washed twice in 200 µl of ice cold FACS washing buffer at 200 g for 5 minutes. When directly conjugated monoclonal antibodies were used, the cells were resuspended in 200 µl of Cellfix for analysis by flow cytometry. If a second antibody layer was required, samples were incubated on ice in the dark for 30 minutes with FITC or PE conjugated F(ab')$_2$ goat anti-mouse IgG, then washed and fixed as above.

2.4.2 Intra cellular staining

For the detection of intracellular cytokines, 10 μg/ml of Brefeldin A was added to the cell culture to block the secretion of protein from the Golgi. Cultured cells were harvested, washed in cell wash medium and fixed in 1 ml of 2% paraformaldehyde in PBS for 15 minutes. Cells were then washed with FACS washing buffer, centrifuged at 200 g for 5 minutes, and resuspended in 25 μl of permeabilisation solution. Cytokine specific antibodies and the relevant isotype controls were added for 30 minutes in the dark at room temperature. Cells were washed in 2 ml of FACS washing buffer and fixed in 200 μl Cellfix before analysis by flow cytometry.

2.4.3 Flow cytometry

Fixed cells were analysed on a FACScalibur flow cytometer using CellQuest software. DCs were identified and gated by their distinctive size and granularity characteristics (Fig 2.3). At least 5000 events within the DC gate were collected. For the analysis of T cells a CD3$^+$ gate was used (unless otherwise stated), and at least 20,000 events within this gate were collected.

2.5 Enzyme-linked immunosorbent assay (ELISA)

As well as detecting cytokine production by intracellular staining, secreted cytokines in supernatants were measured by ELISA. Culture supernatants were harvested by centrifugation at 250 g for 10 minutes and then frozen at -80°C until analysed.

The following method is based on the manufacturers instructions. Maxisorb 96-well

ELISA plates were coated with 100 µl/well of coating antibody at 1 µg/ml for 18

hours at 4°C. Plates were washed twice in ELISA wash buffer and blocked for

2 hours with 300 µl/well of blocking solution on a rotator at room temperature. After

4 washes, standards and samples were added (100 µl/well) in duplicates. The

standards were 1:2 serial dilutions of human recombinant protein over the following

ranges; IL-6 (5,000 pg/ml to 2.5 pg/ml); IL-10 (5,000 pg/ml to 2.5 pg/ml);

IL-12(p40) (10,000 pg/ml to 5 pg/ml) and TNFα (10,000 pg/ml to 5 pg/ml) (Fig 2.5).

Samples for IL-6 and IL-10 were used undiluted, samples for IL-12(p40) were tested

at 1:10 dilution and TNFα at 1:100 dilutions. Blank wells were left as negative

controls. The plates were incubated for 18 hours at 4°C and then washed 4 times

followed by the addition of 50 µl/well of biotinylated cytokine specific detection

antibodies. After 2 hours on a rotator at room temperature, the plates were washed 4

times followed by the addition of 100 µl/well of streptavidin-horseradish peroxidase

conjugate. The plates were then incubated for 45 minutes on a rotator in the dark.

After a further 4 washes, 100 µl of the chromogen TMB substrate buffer were added

and the plates left to develop on a rotator in the dark for 20-40 minutes until

sufficient colour appeared. The reaction was stopped by addition of 100 µl/well of

1.8N H_2SO_4. The optical density was measured at 450 nm (reference filter 650 nm)

on a Dynatech MRX microplate reader and analysed using the Revelation software

(Fig 2.5).
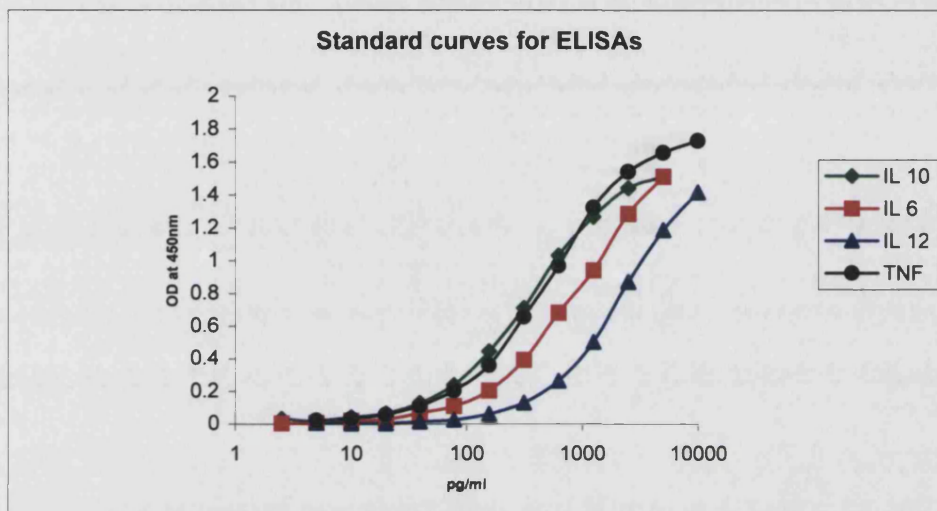
Figure 2.5 ELISA standard curve.

Examples of standard curves for cytokine ELISAs read on the Dynatech MRX microplate reader. The standard curves were formed from serial dilutions of recombinant cytokines starting at 10,000 pg/ml for TNFα and IL-12(p40) and 5,000 pg/ml for IL-10 and IL-6.

2.6 Preparation of *Neisseria meningitidis*

2.6.1 Bacterial strains

The work presented in this thesis is based on a group B clinical isolate of *N. meningitidis* (H44/76) and an isogenic LPS deficient mutant of the same strain (*lpxA-*), both kindly provided by Professor Peter van der Ley, RIVM, Bilthoven, Holland. The serogroup B *N. meningitidis* strain H44/76 (sero (sub)-type (B:15:P1.7,16), ET-5 complex) was isolated from a fatal septicaemia in Norway(Andersen S.R. et al., 1995). The viable LPS deficient isogenic mutant *lpxA-* was constructed by insertional inactivation of the *lpxA* gene with a kanamycin cassette as described previously (Steeghs L. 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 L. et al., 1998). The *lpxA-* isogenic mutant and the parent both showed similar binding patterns to monoclonal antibodies for outer membrane proteins (Steeghs L. et al., 1998)(Steeghs L. et al., 2001a). Purity of the *lpxA-* mutant was maintained by growth on agar plates containing kanamycin (100 μg/ml). A cartoon representation of both the wild type and the LPS deficient isogenic mutant (*lpxA-*) H44/76 are shown in Figure 2.6. When grown in the presence of cmpNANA, wild type H44/76 has sialated LPS.

2.6.2 Growth and preparation of *Neisseria meningitidis*

Growth and preparation of live *N. meningitidis* was carried was carried out in a Class I safety cabinet housed in a category 3 containment facility at negative pressure. Aliquots of *N. meningitidis* strains were stored in Mueller-Hinton broth with 15%

glycerol at -80°C. Bacteria were plated directly from frozen stocks onto fresh gonococcal agar plates supplemented with Vitox, and incubated at 36°C in an atmosphere of 6% CO_2. Organisms were sub-cultured at least once, and harvested in stationary phase at 16 to 18 hours after the previous sub-culture for use. Colonies were streaked from plates with sterile cotton wool swabs into RPMI 1640 (without phenol red). The optical density (OD) of a ten fold dilution of the bacterial suspension was determined using a spectrophotometer at 540 nm. The bacterial suspension was then adjusted to an OD of 1.0. 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 organisms/ml (G. Dixon personal communication).
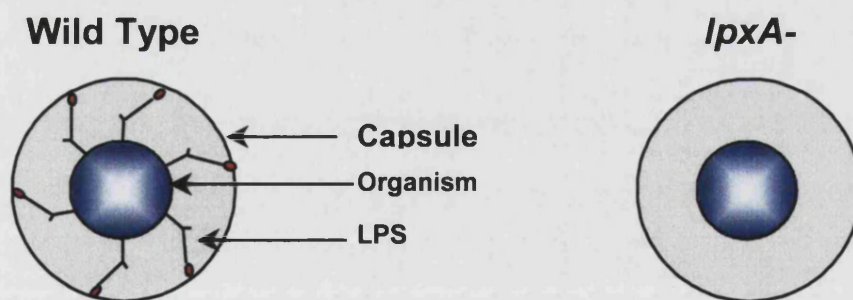
Figure 2.6 Schematic representation of *N. meningitidis* H44/76 wild type and the LPS deficient isogenic mutant *lpxA-*.

The wild type bacterium possesses a capsule and LPS. The *lpxA-* isogenic mutant is totally LPS deficient but possess a capsule. Adapted from (Jack D.L. et al., 1998) and (Dixon 2000).

Bacteria for use in DC activation experiments were fixed in 0.5% paraformaldehyde in PBS for 15 minutes then washed thoroughly in RPMI medium. This treatment rendered bacteria non-viable as judged by viability counts and propidium iodide staining (G. Dixon personal communication). Aliquots of fixed bacteria at a concentration of 10^9 organisms/ml were stored at -80°C until use.

FITC labelled *N. meningitidis* bacteria were used in phagocytosis studies. After fixation, the bacteria were incubated with 0.5 mg/ml of FITC for 20 minutes at 37 °C followed by 4 washes in RPMI 1640 (without phenol red) to remove excess unbound FITC. The OD of the bacterial suspension was adjusted to 1.0 as before. FITC labelling of *N. meningitidis* has no effect on the bacteria's ability to stimulate DCs (H. Uronen personal communication).

Meningococcal LPS from *N. meningitidis* serogroup B strain H44/76 was prepared by Dr. Svein Andersen (Edward Jenner Institute for Vaccine Research, Newbury, UK). Briefly LPS was extracted 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 S.R. et al., 1996).

Chapter 3

Dendritic Cell Activation by *Neisseria meningitidis*

3.1 Introduction

DC maturation is a process that includes the loss of endocytic activity and an increase in surface co-stimulatory and MHC molecules as well as the production of cytokines and chemokines. DC maturation is induced by many different micro-organisms including bacteria, viruses and protozoa as well as by purified microbial products including LPS, peptidoglycans, lipoteichoic acid and CpG DNA and by cytokines TNFα and IL-1β, (reviewed in (Sousa C. et al., 1999)). The Gram-negative bacteria *N. meningitidis* has been shown to induce DC maturation and cytokine production (Dixon G.L. et al., 2001)(Kolb-Maurer A. et al., 2001)(Unkmeir A. et al., 2002)

The work described in this chapter lays the foundations for the rest of this study by establishing conditions for DC activation by *N. meningitidis*, including the role of bacterial concentration.

3.2 Methods

3.2.1 Stimulation of dendritic cells

Monocyte derived DCs were harvested from plates on day 6, washed and resuspended to 5×10^5 cells/ml, then stimulated with wild type *N. meningitidis*, the LPS deficient *lpxA-*, purified meningococcal LPS or other stimuli at varying concentrations for 16-18 hours. Other stimuli used include Prostaglandin E-2 (PGE-2), pertussis toxin (PT), cholera toxin (CT) and poly IC (synthetic viral-like double stranded RNA) as their ability to bias DCs to a Th1 or Th2 response has been reported (de Jong E.C. et al., 2002). Brefeldin A (10 μg/ml) was added to the cultures

for 18 hours when intracellular cytokines were to be measured. Maturation markers

on DCs were measured by flow cytometry, as were intracellular cytokines. Cell

culture supernatants were stored at -80 °C for later analysis by ELISA.

3.3 Results

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

The ability of *N. meningitidis* to induce maturation of DCs was examined by culture

of immature day 6 monocyte-derived DCs with wild type or *lpxA-* strains of *N.*

meningitidis; purified meningococcal LPS or in medium alone for 18 hours.

Stimulation of DCs with the wild type *N. meningitidis* resulted in an increase in

expression of CD40, CD80, CD83, CD86 and HLA-DR, as well as a decrease in

CD14 as compared to DCs cultured in medium alone (Fig 3.1). These changes in

expression of surface markers are consistent with DC maturation. Stimulation with

the LPS deficient *lpxA-* also resulted in DC maturation, though increases in some

markers, especially CD80, CD83 and CD86 were not as great as with the wild type

bacteria. Purified meningococcal LPS also proved to be a good inducer of DC

maturation as measured by an increase in expression of co-stimulatory and activation

markers, although LPS did not induce maturation to the same extent as the wild type

bacteria. Increases in other markers including CD25 and HLA DQ were also seen

(data not shown). DCs cultured in medium alone displayed the characteristics of

immature DCs; i.e. low expression of CD14, low expression of co-stimulatory

molecules CD40, CD80 and CD86 and negative for DC maturation marker CD83.

Figure 3.1 Dendritic cell maturation in response to *N. meningitidis*.

DCs were stimulated for 18 hours in the presence of medium alone, 10 ng/ml of purified meningococcal LPS, 10^7 organisms/ml of wild type or *lpxA- N. meningitidis* or *lpxA-* (10^7 organisms/ml) plus purified LPS (10 ng/ml). The cells were then stained for surface markers. DCs were gated according to size and granularity and analysed for CD14, CD40, CD80, CD83, CD86 and HLA-DR expression as shown (filled histograms) along with appropriate isotype control (solid lines), the MFI (Median Fluorescent Intensity) of each peak is given. The data are a representative of 6 independent experiments.

| | MEDIUM | LPS 10 ng/ml | *lpxA*- 10⁷ CFU/ml | WT 10⁷ CFU/ml | *lpxA*- 10⁷ CFU/ml + LPS 10ng/ml |
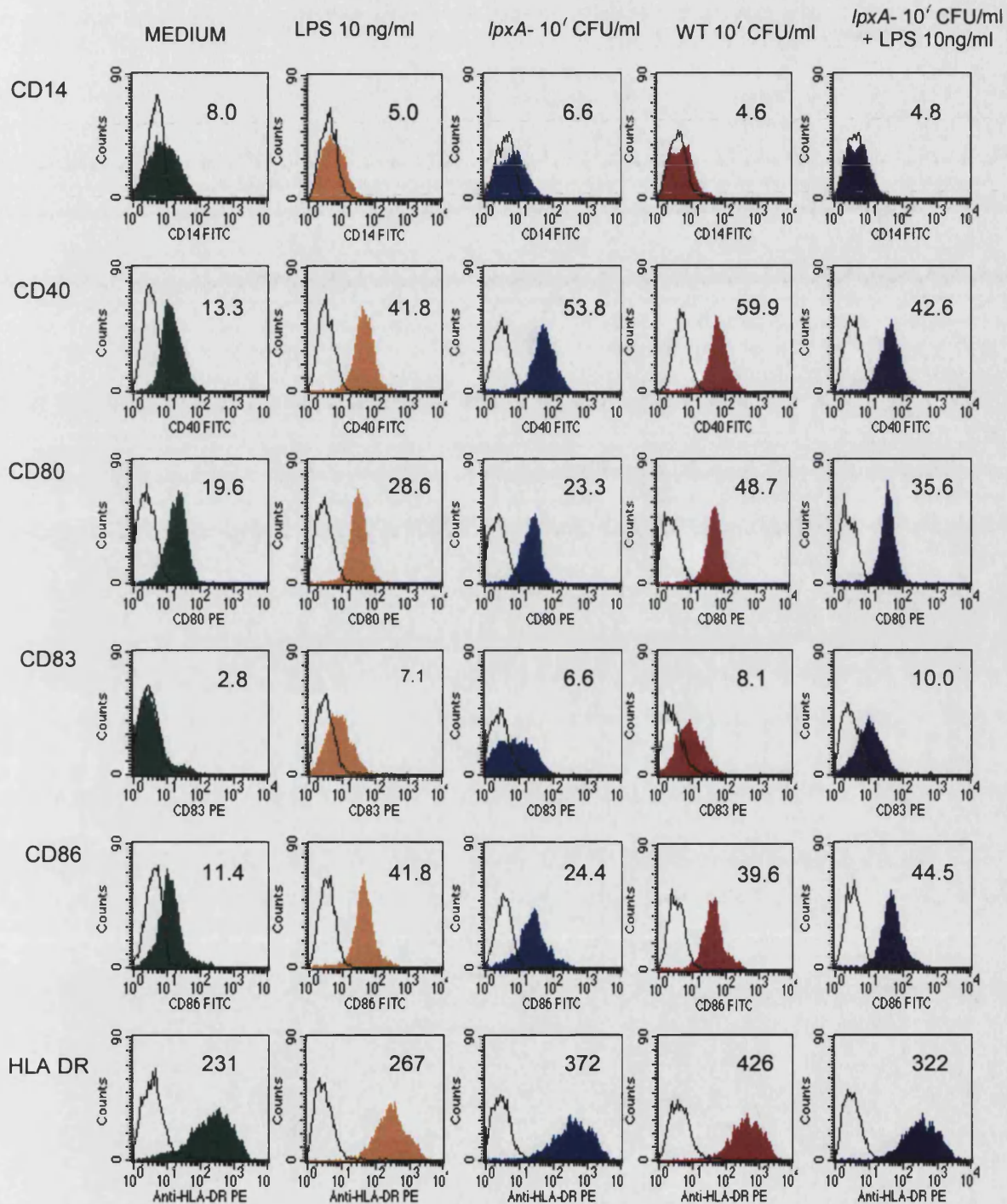
3.3.2 Effect of bacterial concentration on dendritic cell maturation

To determine the dependence of DC maturation on bacterial concentration, DCs were stimulated with wild type and *lpxA-* bacteria at concentrations from 10^5 to 10^7 organisms/ml (Fig 3.2). As before DCs were cultured for 18 hours before antibody staining for surface markers.

DCs responded to both the wild type and *lpxA-* bacteria in a dose dependent manner with an increase in expression of maturation markers corresponding to an increase in bacterial concentration. At all concentrations of bacteria, the wild type organisms induced greater DC maturation than the LPS deficient isogenic mutant *lpxA-* (Fig 3.2). At low concentrations (10^5 organisms/ml), the wild type *N. meningitidis* increased expression of DC CD40, CD80, CD83 and HLA DR; and decreased CD14 typical of DC maturation. However, *lpxA-* only induced an increase in DC surface marker expression at 10^6 organisms/ml and above. At lower concentrations of bacteria there was a greater difference in the ability of the wild type compared to the *lpxA- N. meningitidis* to induce DC maturation, especially in CD80 and CD83 expression.

3.3.3 Dendritic cell cytokine production after stimulation with *N. meningitidis*

To investigate cytokine production by DCs after stimulation with *N. meningitidis*, DCs were cultured for 18 hours with wild type or *lpxA-* H44/76 at 10^7 organisms/ml; purified meningococcal LPS (10 ng/ml); prostaglandin E2 (PGE2) (10^{-6} M) or in medium alone. For analysis by intracellular cytokine staining, brefeldin A was added to the culture medium to block protein secretion from the Golgi.
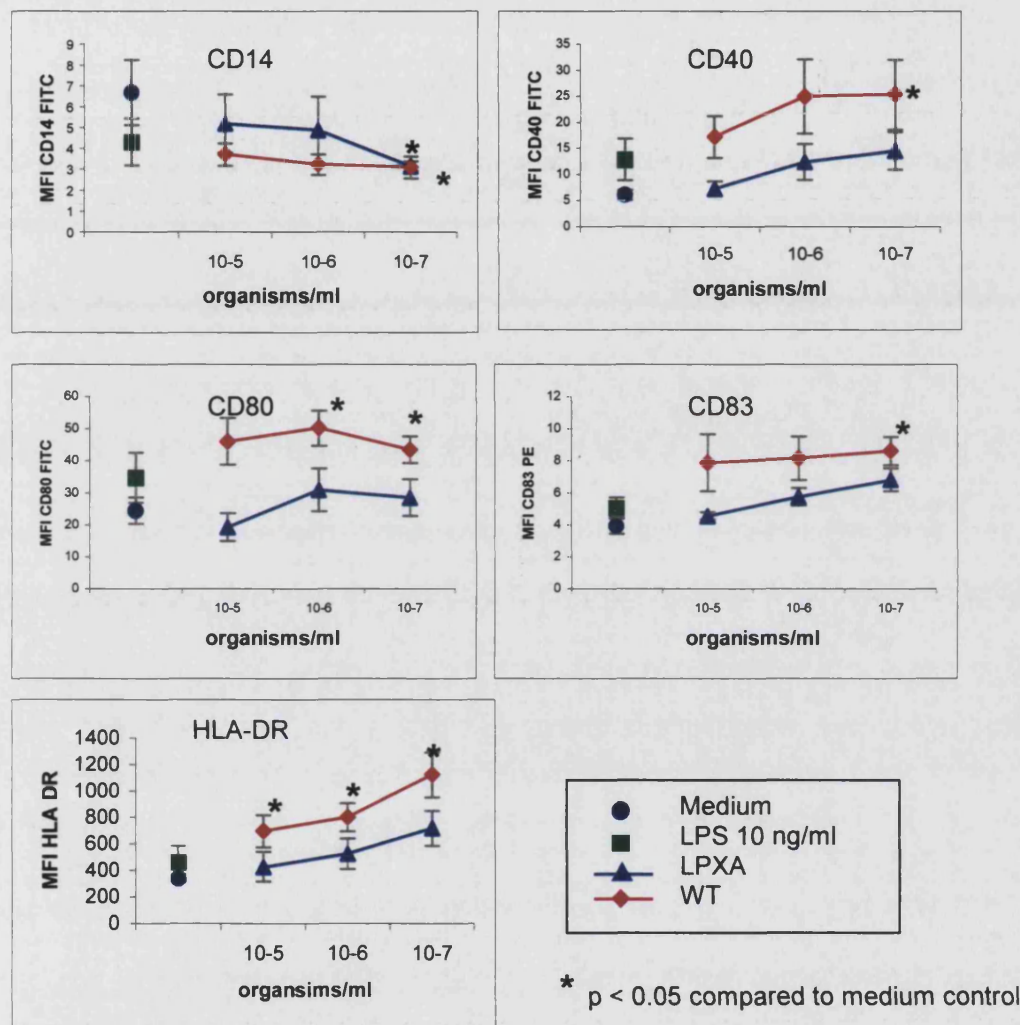
Figure 3.2 Maturation of dendritic cells in response to different concentrations of wild type and *lpxA- N. meningitidis* H44/76

DCs were stimulated for 18 hours in the presence of 10^5, 10^6 or 10^7 organisms/ml of wild type and *lpxA- N. meningitidis* H44/76. DC responses to medium alone and 10 ng/ml of purified meningococcal LPS are also shown. The cells were stained for surface markers CD14, CD40, CD80, CD83 and HLA-DR and the data are expressed as the mean with the SEM of 4 independent experiments. A non-parametric Mann-Whitney U test was used to measure significance of proliferation compared to the medium control.

Intracellular cytokine levels were measured by flow cytometry (Fig 3.3). The wild

type *N. meningitidis* induced DCs to produce IL-1α, IL-6, TNFα and IL-12(p70).

DC stimulation with the *lpxA*- bacteria resulted in IL-1α, IL-6 and TNFα production,

but to a much lesser degree than the wild type, and very little IL-12(p70) production.

Purified meningococcal LPS was found to be a poor inducer of cytokine production

in DCs with only low levels of IL-6 detected. The addition of exogenous purified

LPS to cultures of DCs with the LPS deficient *lpxA*- bacteria did not restore cytokine

production to that observed with the wild type bacteria (Fig 3.3).

Intracellular cytokine staining is a very useful tool for looking at cytokine production

in cells as it allows the identification and gating of specific cell populations.

However, IL-10 was found to be difficult to measure by intracellular staining (data

not shown). ELISAs measure the overall secreted cytokines in cell free culture

supernatants and this enabled DC IL-10 production to be measured. Supernatants

from DC cultures were collected after 18 hours stimulation (without brefeldin A) and

stored at -80°C until used. Sandwich ELISAs for the cytokines IL-6, IL-10,

IL-12(p40) and TNFα were performed on the supernatants (Fig 3.4).

Results obtained by ELISA were consistent with intracellular cytokine assays. DCs

stimulated with the wild type *N. meningitidis* secreted very high levels of IL-10,

IL-12(p40) and TNFα. The *lpxA*-bacteria induced IL-6 but little IL-10, IL-12(p40) or

TNFα. Purified LPS induced very little cytokine production (some IL-6) and PGE2

did not induce any detectable cytokine production (Fig 3.4).
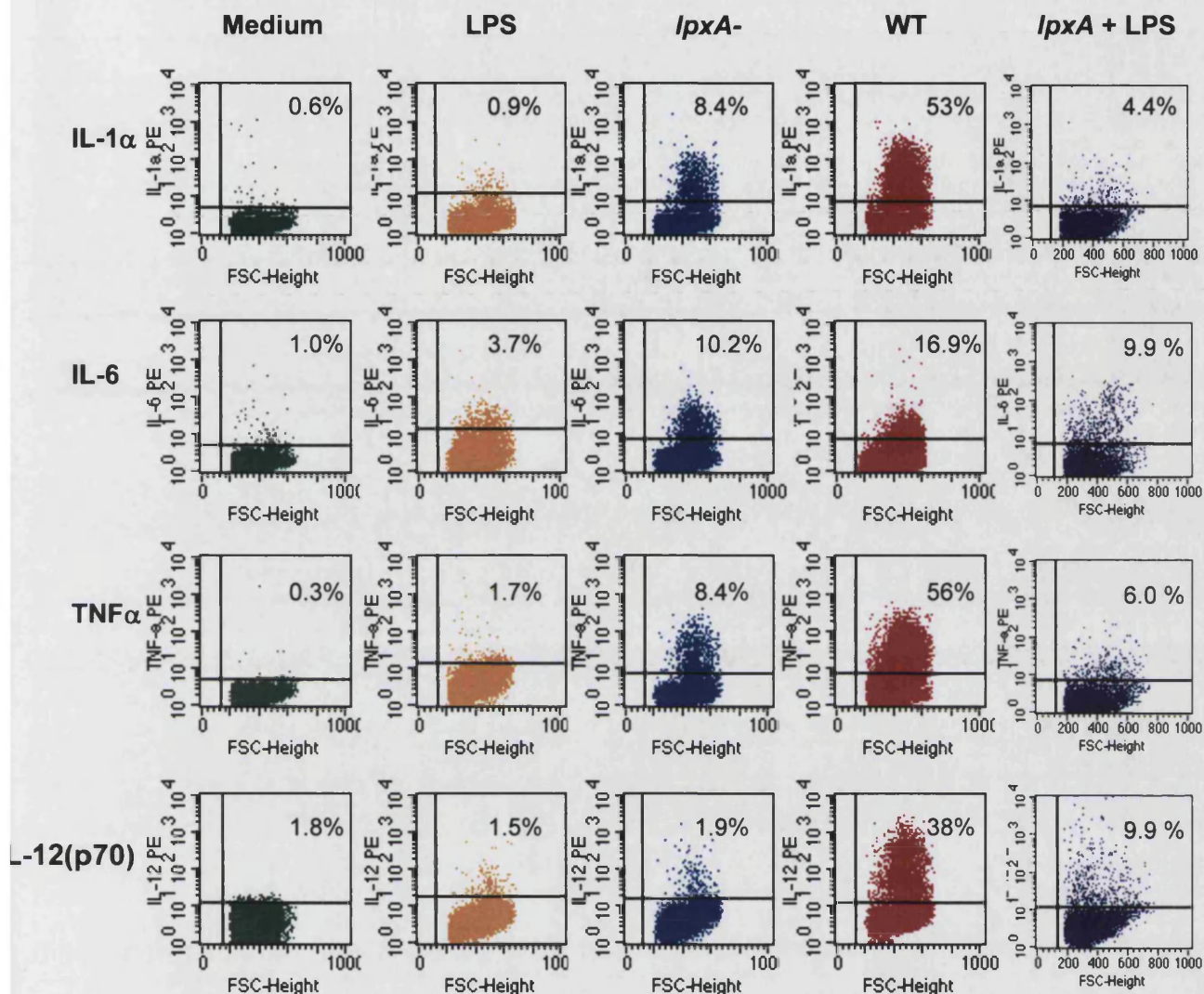
Figure 3.3 Cytokine production by dendritic cells in response to wild type and LPS deficient *lpxA- N. meningitidis*.

DCs were stimulated for 18 hours with 10^7 organisms/ml of wild type or lpxA- *N. meningitidis*, meningococcal LPS (10 ng/ml), *lpxA-* (10^7 organisms/ml) plus purified LPS (10 ng/ml) or medium alone. Cells were permeabilised and stained for intracellular cytokines IL-1α, IL-6, TNFα and IL-12(p70). Data are representative of 5 different experiments. The percentage of positive cells in the upper right quadrant is given.
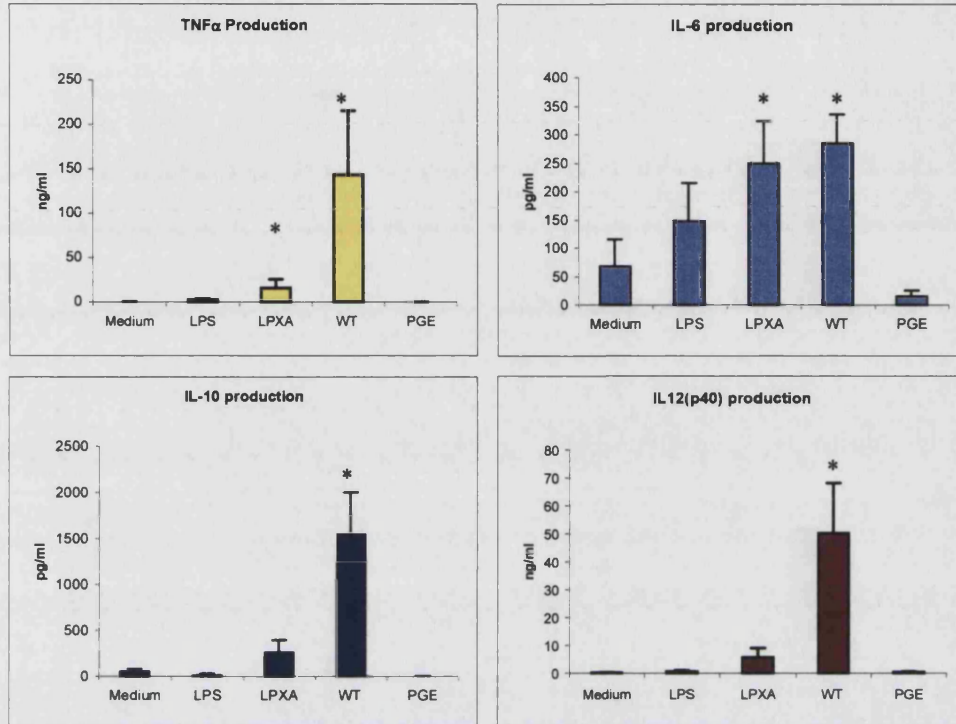
Figure 3.4 Secreted cytokines by dendritic cells measured by ELISA

DCs were stimulated for 18 hours with 10^7 organisms/ml of wild type H44/76 or the LPS deficient *lpxA-*, meningococcal LPS (10 ng/ml), PGE2 (10^{-6} M) or medium alone. Cell free supernatants were collected and cytokines IL-6, IL-10, TNFα and IL-12(p40) were measured by ELISA. Data are expressed as the mean and SEM of 4 separate experiments (* p <0.05 by paired student t test compared to medium control).

3.3.4 Activation of dendritic cells with other stimuli

DCs activated by cholera toxin and prostaglandin E2 (PGE2) have been well characterised and found to induce a Th2 response in naïve T helper cells. Whereas DCs stimulated with poly I:C and pertussis toxin have been shown to induce a Th1 response (de Jong E.C. et al., 2002)(Kalinski P. et al., 1997). For this reason it was important to assess the activation of DCs induced by *N meningitidis* compared to these other characterised stimuli in this system.

DCs were cultured for 18 hours in the presence of cholera toxin (1 µg/ml); PGE2 (10^{-6} M); Poly I:C (20 µg/ml), pertussis toxin (1 µg/ml), wild type *N. meningitidis* (10^7 organisms/ml) or in medium alone. DC maturation was assessed by surface marker expression (Fig 3.5). Cholera toxin, pertussis toxin, Poly I:C and PGE2 induced DC maturation as can be seen by an increase in CD40 and HLA DR and a decrease in CD14 compared to the medium control. Interestingly both cholera toxin and PGE2 caused a decrease in CD83 expression compared to the medium control. The wild type *N. meningitidis* induced the greatest maturation as observed by increases in these surface markers.

DC cytokine production in response to these stimuli was also measured. After 18 hours culture in the presence of cholera toxin (1µg/ml); PGE2 (10^{-6} M); Poly I:C (20 µg/ml) and pertussis toxin (1 µg/ml) cell free supernatants were collected and stored at -80°C. Sandwich ELISAs for the cytokines IL-6, IL-10, IL-12(p40) and TNFα were performed on the supernatants (Fig 3.6).
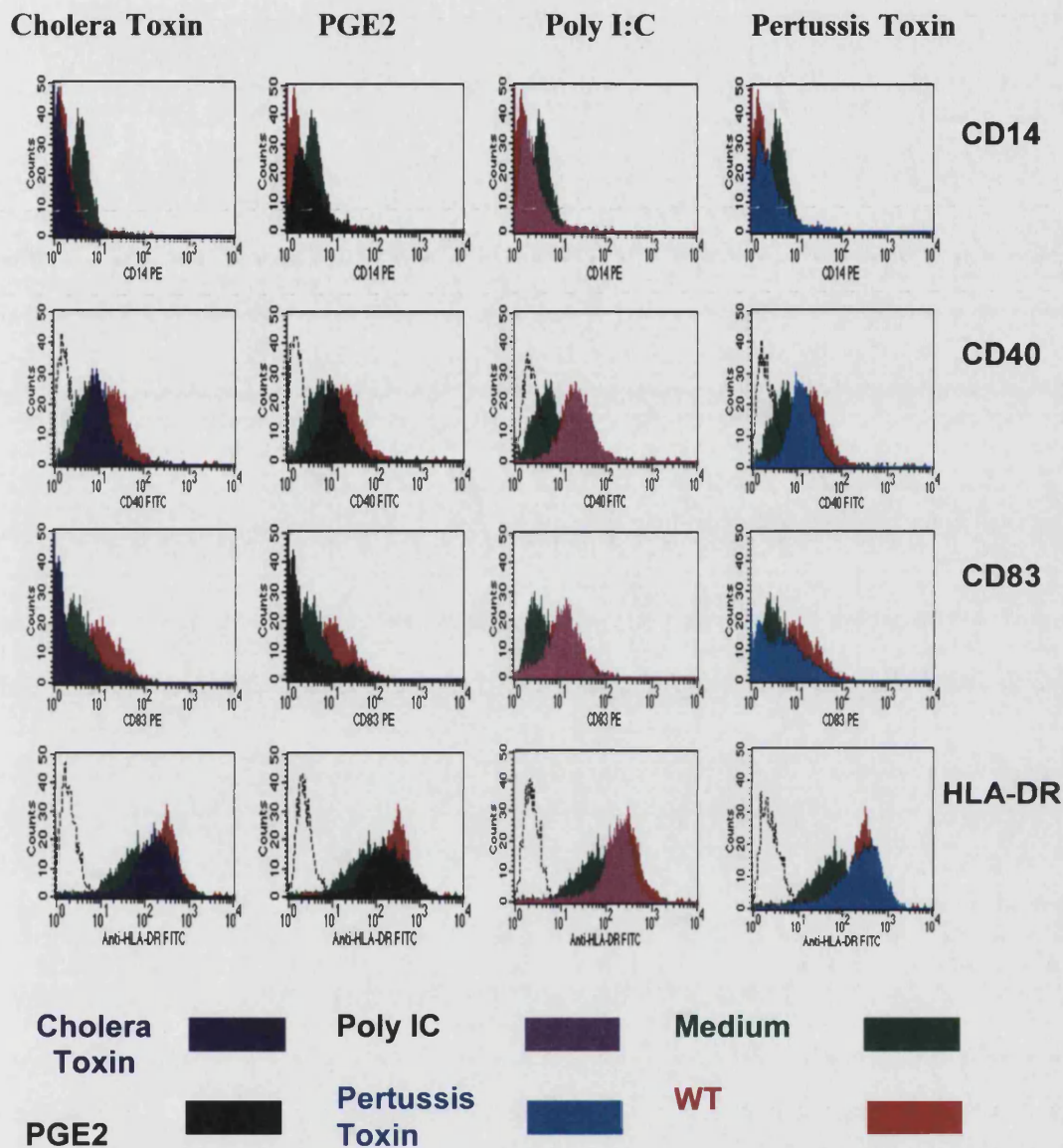
Figure 3.5 Dendritic cell surface marker expression after stimulation with other characterised stimuli

DCs were stimulated for 18 hours in the presence of cholera toxin (1 μg/ml); prostaglandin E2 (PGE2) (10^{-6} M); Poly I:C (20 μg/ml) or pertussis toxin (1 μg/ml). DCs cultured with wild type H44/76 *N. meningitidis* (10^7 organisms/ml) or medium alone are shown for comparison. DCs were then stained for CD14, CD40, CD83 and HLA-DR expression. The data are representative of 3 independent experiments.

Interestingly changes in surface marker expression, with the exception of CD83, indicated that there was little difference in the level of DC stimulation by these microbial products. However when cytokine secretion was analysed by ELISA, Poly I:C and pertussis toxin did induce cytokine production, notably TNFα, IL-6 and IL-12(p40) but little IL-10. Prostaglandin E2 and cholera toxin induced almost no detectable cytokine production. None of the stimuli induced cytokine levels to that of the wild type *N. meningitidis*.

3.4 Discussion

Dendritic cell maturation is associated with several co-ordinated events; including loss of endocytic receptors, increased expression of co-stimulatory molecules (CD40, CD80, CD86), increases in surface MHC molecules, a change in expression of chemokine receptors from CCR5 to CCR7 and cytokine production. These changes that occur during DC maturation facilitate migration, antigen presentation and T cell activation. The morphology of mature DCs is also different to immature DCs. Immature DCs are irregular shaped with a "ruffled" surface. Upon maturation DCs become rounded but with many long and fine dendritic projections (Palucka K. et al., 1999).

LPS is regarded as the major immunogenic component of Gram-negative bacteria, and purified LPS alone can induce DC activation and maturation. The importance of LPS in DC activation was investigated here using the LPS deficient *lpxA*- strain of *N. meningitidis*. Wild type *N. meningitidis* H44/76 induced DC maturation, as shown by
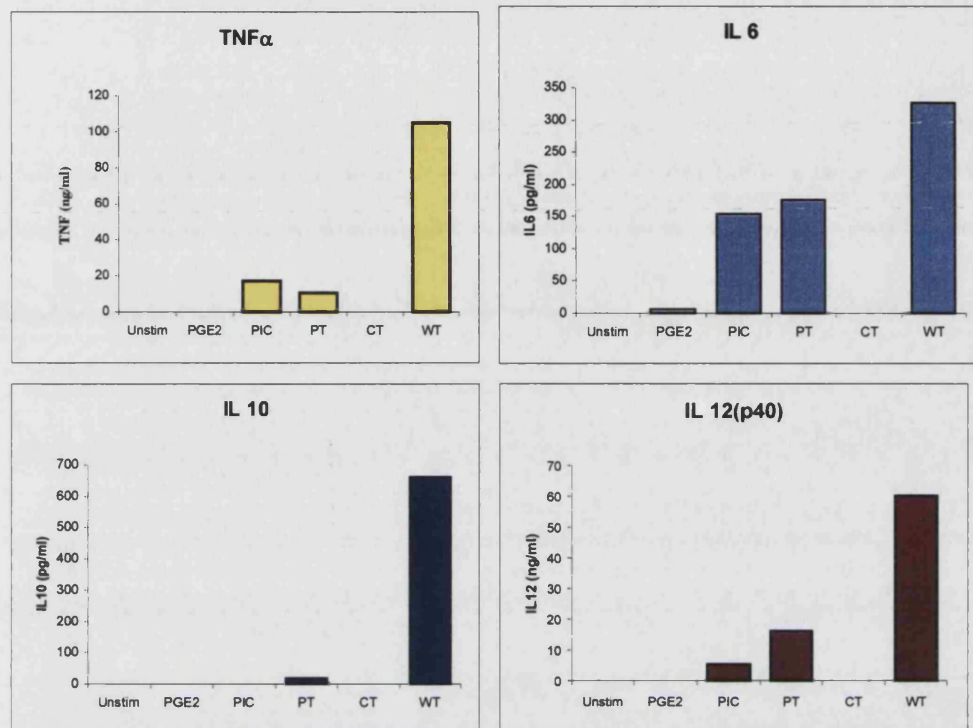
Figure 3.6 Cytokine production by dendritic cells after stimulation with Th1 or Th2 inducing stimuli.

DCs were stimulated for 18 hours in the presence of cholera toxin (CT)(1 μg/ml); prostaglandin E2 (PGE2) (10^{-6} M); Poly I:C (20 μg/ml), pertussis toxin (PT) (1 μg/ml), wild type H44/76 (10^7 organisms/ml) or medium alone. Cell free supernatants were collected and cytokines IL-6, IL-10, TNFα and IL-12(p40) were measured by ELISA. The data are a representative of 3 independent experiments.

an increase in surface co-stimulatory and MHC molecules, and by the production of

cytokines. The *lpxA-* also induced DC maturation, but not to the same extent as the

wild type. Thus bacterial components other than LPS must be responsible for the DC

response to *lpxA-*. Possible candidates are the OMPs PorA and PorB. These are

immunogenic, inducing CD86 expression and proliferation in B cells. They have

been shown to signal via the TLR2 MyD88 dependent pathway (Massari P. et al.,

2002). Bacterial DNA, rich in unmethylated CpG motifs is also immunogenic and

signals via TLR9 and may also account for the DC response induced by *lpxA-*

(Sparwasser T. et al., 1998).

OMP expression by the *lpxA-* bacteria and the wild type is very similar, the only

difference being an increase in cell surface lipoproteins in the outer membranes of

lpxA- organisms. Therefore, the differences observed between DC stimulation with

the wild type compared to the *lpxA-* was most likely due to the absence of LPS. The

lpxA- N. meningitidis were only able to induce DC activation at concentrations of

10^6 organisms/ml or higher, whereas the wild type activated DCs at low

concentrations of bacteria (10^5 organisms/ml), indicating that LPS is an important

component in stimulating DCs at low bacterial concentrations.

The addition of exogenous purified meningococcal LPS with *lpxA-* bacteria did not

reconstitute the DC response to that of the wild type bacteria, even though the LPS

added (10 ng/ml) was equivalent to the LPS content of 10^7 organisms/ml. This is

based on the measurement of the LPS content of *N. meningitidis* by

spectrophotometric analysis of the LPS specific sugar 2-keto-3-deoxyoctonic acid

(KDO), which demonstrated that there are approximately 1.5×10^5 molecules of LPS

per bacterium (Dixon 2000).

The inability of exogenous LPS to restore DC responses to *lpxA-* to that of the wild

type *N. meningitidis* was puzzling and suggested that LPS must be coupled to the

bacteria for optimal DC responses. There are several possible reasons for this. One

explanation could be differences in the structural confirmation of LPS. Purified LPS

molecules aggregate into large supra molecular structures whereas in whole bacteria

LPS molecules are anchored in the outer membrane (Erridge C. et al., 2002). It is

possible that the formation of these aggregates affect the recognition of LPS by its

receptors and hence reduces the endotoxicity of purified LPS preparations compared

to LPS in the membrane of bacteria. A further explanation is that on the surface of

the bacteria LPS would be in close proximity to, and even complexed with outer

membrane proteins. LPS is known to signal through TLR4 (Beutler B., 2000) and the

OMPs on *N. meningitidis* have been shown to signal through TLR2 (Massari P. et al.,

2002). Perhaps simultaneous engagement of both of these receptors, and others, is

required for optimal DC responses. Moreover soluble molecules such as LPS are

taken up by macro-pinocytosis, whereas whole bacteria are internalised by receptor

mediated endocytosis and phagocytosis (reviewed in (Lanzavecchia A., 1996)).

These different methods of uptake deliver purified LPS and *lpxA-* bacteria to separate

compartments of the endocytic pathway and this may affect the way in which DCs

are activated.

In this study, purified meningococcal LPS alone was a poor inducer of DC

activation, especially compared to wild type *N. meningitidis*. Stimulation with

meningococcal LPS did result in an increase of DC surface co-stimulatory and MHC molecules, but very little cytokine production, especially IL-12. This is in contrast to previous studies that have shown DC activation and cytokine production by LPS (Rescigno M. et al., 1998a) (Verhasselt V. et al., 1997). These previous studies used LPS from *Escherichia coli* whereas LPS used here was from *N. meningitidis*. LPS from *N. meningitidis* may not be as potent as the LPS from *E. coli* in the stimulation of DCs. Though this has not been tested directly in DCs, in monocytes *E. coli* LPS is a more potent inducer of IL1β production than LPS from *N. meningitidis* (Erridge C. et al., 2002). Furthermore, following extensive treatment by phenol extraction and ethanol precipitation the *N. meningitidis* LPS used here was highly pure, containing less than 0.3% protein (Andersen S.R. et al., 1996). Commercially available *E. coli* LPS preparations have been found to contain impurities. This situation was highlighted when LPS was found to activate through both TLR2 and TLR4 (Yang R.B. et al., 1999). It was only after purification of LPS preparations that it was found that TLR4 was the LPS signalling receptor and not TLR2 (Tapping R.I. et al., 2000). The impurities in commercially available *E. coli* LPS could explain the previously reported DC maturation and cytokine production.

The tissue factor Prostaglandin E2 and toxin from the extra-cellular bacterium *Vibrio cholerae* are both associated with Th2 responses (de Jong E.C. et al., 2002). In contrast toxin from the intracellular bacteria *Bordetella pertussis* and the synthetic viral RNA molecule poly IC have been shown to induce a Th1 response (Ausiello C.M. et al., 2002)(de Jong E.C. et al., 2002). These stimuli all induced DC maturation in terms of increased surface marker expression. However there were differences in the cytokine production induced by these stimuli. The Th1 inducing

Poly IC and PT both induced DCs to produce cytokines (IL-6, TNFα and IL-12), although the amounts produced were much less than observed with the wild type *N. meningitidis*. In contrast the Th2 inducing CT and PGE2 did not induce any detectable cytokine production by DCs.

These experiments established conditions for DC activation by *N. meningitidis*. Differences were found in DC maturation and cytokine production by the LPS deficient *lpxA-* compared to the wild type *N. meningitidis*. Next DC activation was investigated further by exploring the effects of *N. meningitidis* stimulation on DC migration.

Chapter 4

Dendritic Cell Migration Following Activation

by *Neisseria meningitidis*

4.1 Introduction

Following stimulation by pathogens or inflammatory stimuli, DCs leave the peripheral site of infection and migrate through the tissues to the draining lymph nodes where they interact with naïve T cells. The ability of DCs to migrate is related to changes in expression of adhesion molecules and chemokine receptors.

The role of adhesion molecules in the migration of leukocytes through endothelium has been well studied. Selectins and their mucin ligands are the major molecules involved in tethering and rolling whereas integrins and their immunoglobulin super family ligands mediate firm adhesion. Although not much is known of the adhesion molecules involved in DC migration, it is likely that the same molecules that mediate migration of other leukocytes are involved. Immature and mature DCs express many adhesion molecules including ICAM-1, ICAM-2, ICAM-3 and PECAM-1. ICAM-1 expression increases upon DC stimulation (Starling G.C. et al., 1995) whereas ICAM-2 and ICAM-3 are expressed at a high density on DCs and show little change upon DC maturation (Hart D.N. et al., 1993). The involvement of these molecules in the migration of DCs is not fully understood. The β2 integrin CD18 has been shown to be essential for the migration of DCs to the peripheral tissues, including the lungs (Schneeberger E.E. et al., 2000) and the immunoglobulin-super family adhesion molecule ICAM-1 is important in Langerhans cell migration to lymph nodes (Xu H. et al., 2001).

Chemokines are also very important for DC migration. Immature DCs express chemokine receptors CXCR1, CCR1 and CCR5 and respond to inducible

chemokines produced at inflammatory sites including MCP-1 (CCL2), MIP-1α (CCL3) and RANTES (CCL5) (Sozzani S. et al., 1997). Following activation, DCs increase their expression of CCR7 and decrease expression of CXCR1, CCR1 and CCR5. The increase in CCR7 is thought to be important for DC migration through lymphatics, where the CCR7 ligands MIP-3β (CCL19) and 6Ckine (CCL21) are constitutively expressed (Sallusto F. et al., 1998).

In the previous results section, LPS expressed by the bacteria was shown to be required for optimal DC activation by *N. meningitidis* and cytokine production. In this next section, the requirement for LPS in *N. meningitidis* to induce DC migratory factors such as chemokines, chemokine receptors, adhesion molecules and DC migration were investigated.

4.2 Methods

4.2.1 HUVEC extraction and primary culture

Human umbilical vein endothelial cells (HUVEC) are an easily available and a well characterised source of primary endothelial cells. The method for extraction was originally described by (Gimbrone M.A., Jr. et al., 1974), (Jaffe E.A. et al., 1973) and modified in our laboratory by Dr. Karolena Kotowicz (Kotowicz K. et al., 1996).

Fresh intact cords were collected from the maternity ward of a local hospital with ethical approval and maternal consent. The collected cords were stored in 'human umbilical cord storage medium' and used within 72 hours. Suitable cords were sprayed on their outer surface with 70% IMS and any blood was expressed by gentle

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squeezing into a waste collection pot. Each cord was trimmed by cutting at least 0.5 cm from both ends. One end of the cord was then clamped with artery forceps and the vein at the other end was dilated with blunt forceps, and cannulated with a sterile plastic filling tube, which was secured with small artery forceps. Blood was removed by flushing the vein with warm HUVEC wash medium from a 50 ml syringe. The vein was then filled with 1% collagenase II solution and the cords incubated at 37 °C in 5% CO_2 for 10-20 minutes. After the incubation period, the digest was removed from the cord vein into a sterile Duran bottle and the cord vein was flushed through with an equal volume of HUVEC wash medium. The digest containing HUVECs was transferred to a sterile 50 ml conical tube and centrifuged at 200 g for 7 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in MCBD 131 medium supplemented with 20% FCS, 10 mM L-glutamine and 100 U/ml penicillin/streptomycin. The digest from a large cord (enough to take 20 to 30 ml of collagenase) was resuspended to 10 ml with culture medium and transferred to a 25 cm^3 modified polystyrene tissue culture flask designed for the culture of adherent cells. The cells were incubated at 37 °C and 5% CO_2 for 24 hours and then washed with fresh HUVEC culture medium and inspected by light microscopy. HUVECs have a characteristic cobblestone appearance, and form small clusters. Flasks of HUVECs were subcultured when cells were approaching confluence, usually around 72 hours of culture. To subculture, HUVECs were washed three times in warm non-supplemented RPMI 1640 to remove non-adherent cells and FCS proteins. The cells were then rinsed in 2 ml of accutase and a further 2 ml of accutase added and the flask placed on a shaker at 37 °C in 5% CO_2 for 10 minutes. The cells were inspected by microscopy and when they were rounding and becoming dislodged they were resuspended in fresh culture media

(MCBD 131 with 20% FCS and antibiotics). The cell suspension was divided equally between two 25 cm^3 tissue culture flasks and made up to 10 ml with culture medium. The HUVECs were then cultured at 37 °C in 5% CO_2.

4.2.2. Culture of endothelial cells in transwells

The 6.5 mm transwell inserts have a polycarbonate membrane with 5 μm pores. A solution of 10% 300 bloom gelatine was prepared in dd H_2O and autoclaved. Then 100 μl of gelatine was carefully added to the top of each transwell insert and left to dry overnight at room temperature. The 24 well plate containing the transwells and gelatine was placed in an oven at 60 °C and baked for 1-2 hours. The gelatine was then conditioned by adding 1 ml of MCBD with 20% FCS to each well for at least 2 hours.

Confluent HUVECs were washed in warm RPMI and released from the tissue culture flask with accutase. The cell suspension was transferred to a 50 ml conical tube, washed and resuspended in 2 ml of transwell medium. The cells were counted on a haemocytometer and 100 μl of the HUVEC suspension at 10^6 cells/ml in transwell medium added to the top of each transwell insert (10^5 cells/well) and incubated at 37 °C at 5% CO_2. Every 2 or 3 days the transwell inserts were transferred to new wells with 0.6 ml of fresh transwell media. For each experiment, two transwells were left with only the gelatine coating without HUVECs as controls for measuring resistance (see below) and a control for the migration experiments.

WELL	DAY 8	DAY 10	DAY 12
1	131	166	167
2	138	171	167
3	138	162	164
4	142	173	167
5	130	169	165
6	127	166	168
7	137	171	164
8	133	177	167
9	143	181	179
10	144	179	176
11	136	180	163
12	140	174	177
BLANK	98	107	96

Table 4.1 EVOM readings of HUVEC monolayers grown on transwells

Resistance readings (Ωcm^{-2}) were taken every other day using the EVOM. Duplicate readings in each well were taken from opposite sides of the transwell and the mean given. The monolayers were deemed confluent when the resistance readings no longer increased and were at least $50\Omega^{-2}$ greater than the control of gelatine and media alone (blank).

4.2.3 Integrity of HUVEC monolayers measured by resistance

An electrical voltage ohm meter (EVOM) was used to check the integrity of the endothelial layers in the transwells. The EVOM measures the resistance through the

endothelial layer, which increases as the cells become confluent (Ali M.H. et al.,

1999). The EVOM probe was soaked in 100% ethanol for 20 minutes then rinsed

with fresh RPMI 1640 prior to use. One prong of the probe was placed above the

endothelial monolayer in the upper chamber with the other prong in the lower

chamber. The resistance was measured every second day. Once the resistance had

reached a steady state of around 50 Ωcm^{-2} more than the control transwell   of

gelatine and medium only, and no longer increased, the monolayers were ready.

Typically this occurred after 10-14 days of culture (Table 4.1). Confluence of the

endothelial layers was confirmed by confocal microscopy (not shown).

4.2.4 Dendritic cell migration across endothelial monolayers

DCs were prepared and stimulated with *Neisseria meningitidis* H44/76 wild type, the

LPS deficient isogenic mutant *lpxA-* (both at 10^7 organisms/ml), or in medium alone

for 18 hours as described in section 3.2. If stimulation of the HUVEC monolayers

was required, recombinant TNFα was added to the top of the transwell to give a final

concentration of 1 ng/ml for 18 hours before adding DCs. The stimulated DCs were

washed, resuspended to $5x10^6$ cells/ml in DC culture medium and 100μl of the DC

suspension added to the top of each transwell (5 $x10^5$ per well). Fig 4.1 shows a

schematic representation of the transwell system.

The media containing migrated DCs was recovered from the lower chamber of the

transwell system and centrifuged at 350 g for 5 minutes. The pellet was resuspended

in 100 μl RPMI with 5% FCS and the cells counted on a haemocytometer in trypan

blue. Triplicate counts were made for each condition and time point, and the mean

number of migrated cells was calculated.
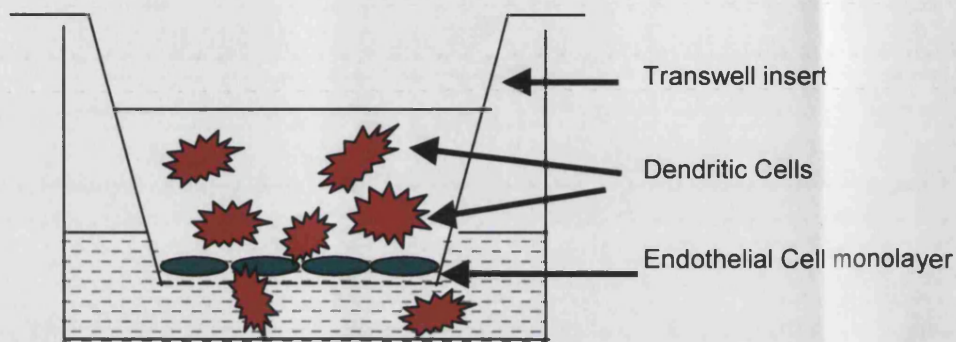
Figure 4.1 Schematic Diagram of the Transwell system

The transwell was placed in the well of a 24 well tissue culture plate. The endothelial monolayer was grown on gelatine on a polycarbonate membrane with 5 μm pores. Dendritic cells migrated across the endothelial membrane, through the polycarbonate membrane and into the chamber below where they were collected and counted.

4.2.5 Dendritic cell migration in the presence of the chemokine Mip3β (CCL19)

To determine DC migration in response to the chemokine Mip3β, the transwell system was used without the endothelial monolayers and gelatine coating. The bottom chamber of the transwell system was filled with 0.6 ml DC culture media supplemented with the chemokine MIP-3β at varying concentrations. Migrated cells were collected from the lower chambers at varying time points and counted as before.

4.3 Results

4.3.1 Expression of adhesion molecules on dendritic cells

DCs were stained for the following adhesion molecules; immunoglobulin super family members ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50) and PECAM-1 (CD31); and integrins VLA-4 (CD49d/CD29), LFA-1 (CD11a/CD18), CD11b and CD18 (Mac-1). DCs were also stained for the DC specific DC-SIGN (dendritic cell specific ICAM3 grabbing non-integrin, CD209) and for L-selectin (CD62L).

Immature DCs were found to express high levels of ICAM-1, ICAM-3, integrins LFA-1 and CD18, and the DC specific DC-SIGN. Immature DCs also expressed low levels of ICAM-2, PECAM-1 and VLA-4, however they did not express any detectable L-selectin (Fig 4.2).

DC migration is closely linked to DC maturation and it is probable that DCs change their expression of adhesion molecules during activation to facilitate migration. DC expression of adhesion molecules following stimulation with *N. meningitidis* was investigated. Immature day 6 monocyte derived DCs were cultured with wild type or *lpxA-* strains of *N. meningitidis*; purified meningococcal LPS or in medium alone for 18 hours and stained with monoclonal antibodies for adhesion molecules and integrins (Fig 4.3). For clarity from this point onwards DCs stimulated with 10^7 organisms/ml of wild type *N. meningitidis* will be referred to as DC_{WT}, those stimulated with 10^7 organisms/ml of *lpxA-* as DC_{LPXA}, DCs stimulated with 10 ng/ml of LPS as DC_{LPS} and DCs cultured in medium alone DC_{medium}.
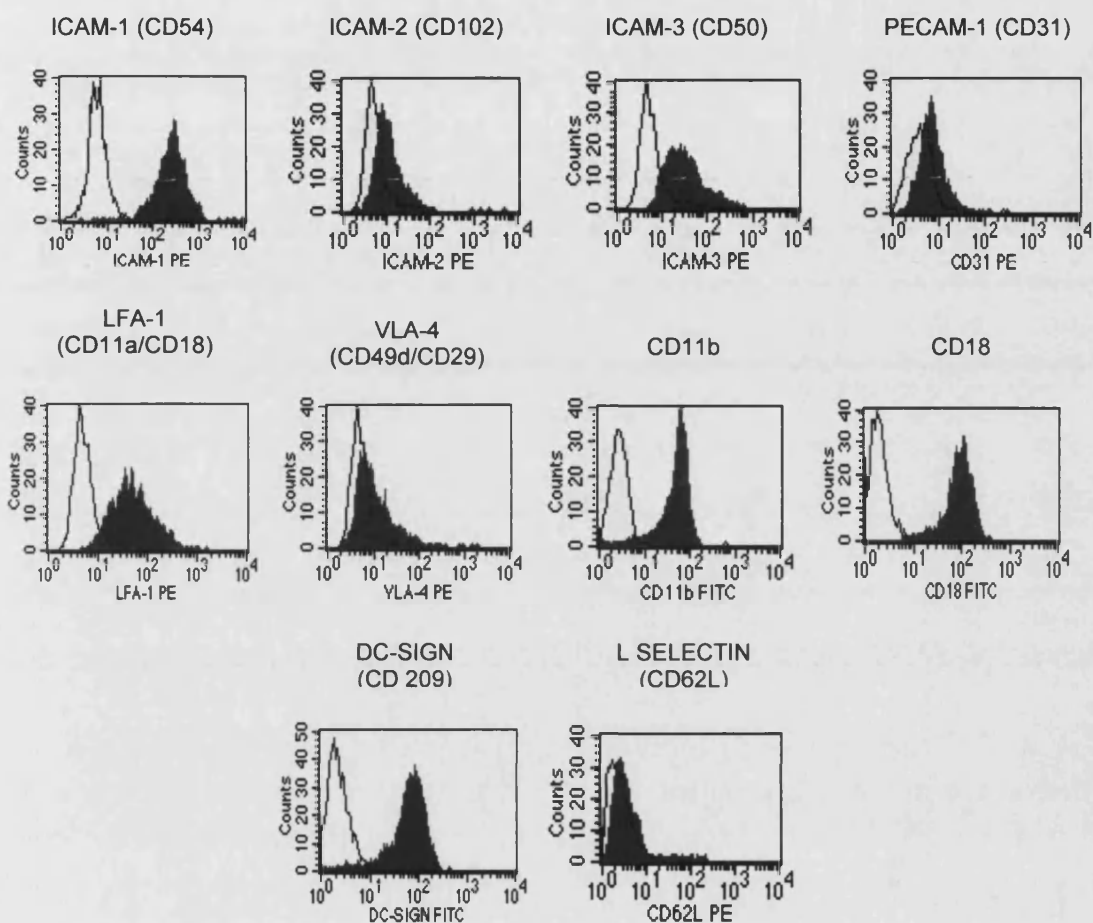
Figure 4.2 Expression of adhesion molecules and integrins on immature dendritic cells

DCs cultured in medium alone were stained for expression of adhesion molecules and integrins. ICAM-1, ICAM-2, ICAM-3, PECAM-1, LFA-1, VLA-4, CD11b, CD18, CD62L and DC-SIGN are shown (filled histograms) along with the appropriate isotype control (solid lines). The data are a representative of 4 different experiments.

DC_{WT} expressed increased PECAM-1 (CD31), VLA-4 and CD62L as shown in Fig

4.3. DC_{LPXA} and DC_{LPS} also expressed increased levels of PECAM-1, VLA-4 and

CD62L, though for PECAM-1 and CD62L this increase was not as great as that seen

on DC_{WT}. Although increases in CD62L were observed on DCs following

stimulation, overall, expression was only detected on about 10% of DCs (Fig 4.3).

CD11b, CD18, ICAM-1, LFA-1 and DC-SIGN were expressed on almost 100% of

DC_{medium}. Following activation there was no change in expression of CD11b, CD18,

LFA-1 or DC-SIGN measured by MFI (data not shown). However DC_{WT}, DC_{LPXA}

and DC_{LPS} all showed increased ICAM-1 expression measured by MFI (Fig 4.3). DC

expression of ICAM-2 and ICAM-3 was found to be highly variable between donors.

There was no overall change in expression of either ICAM-2 or ICAM-3 following

DC stimulation (Fig 4.3).

4.3.2 Dendritic cell expression of chemokine receptors

Chemokines are small chemo-attractive cytokines that play an important role in the

migration of cells. The expression of chemokine receptors by DCs is known to

change during maturation. Immature DCs express CCR5 and mature DCs express

CCR7. In order to investigate the migratory properties of DCs stimulated with *N.*

meningitidis, DC expression of CCR5 and CCR7 was measured following

stimulation with wild type or *lpxA-* strains of *N. meningitidis* H44/76 (Fig 4.4.).

DC_{LPXA} and DC_{WT} both showed a decrease in CCR5 expression compared to

DC_{medium} (Fig 4.4). Interestingly CCR7 expression on DCs increased more than ten

fold following stimulation with wild type *N. meningitidis* but not with *lpxA-* (Fig

4.4).
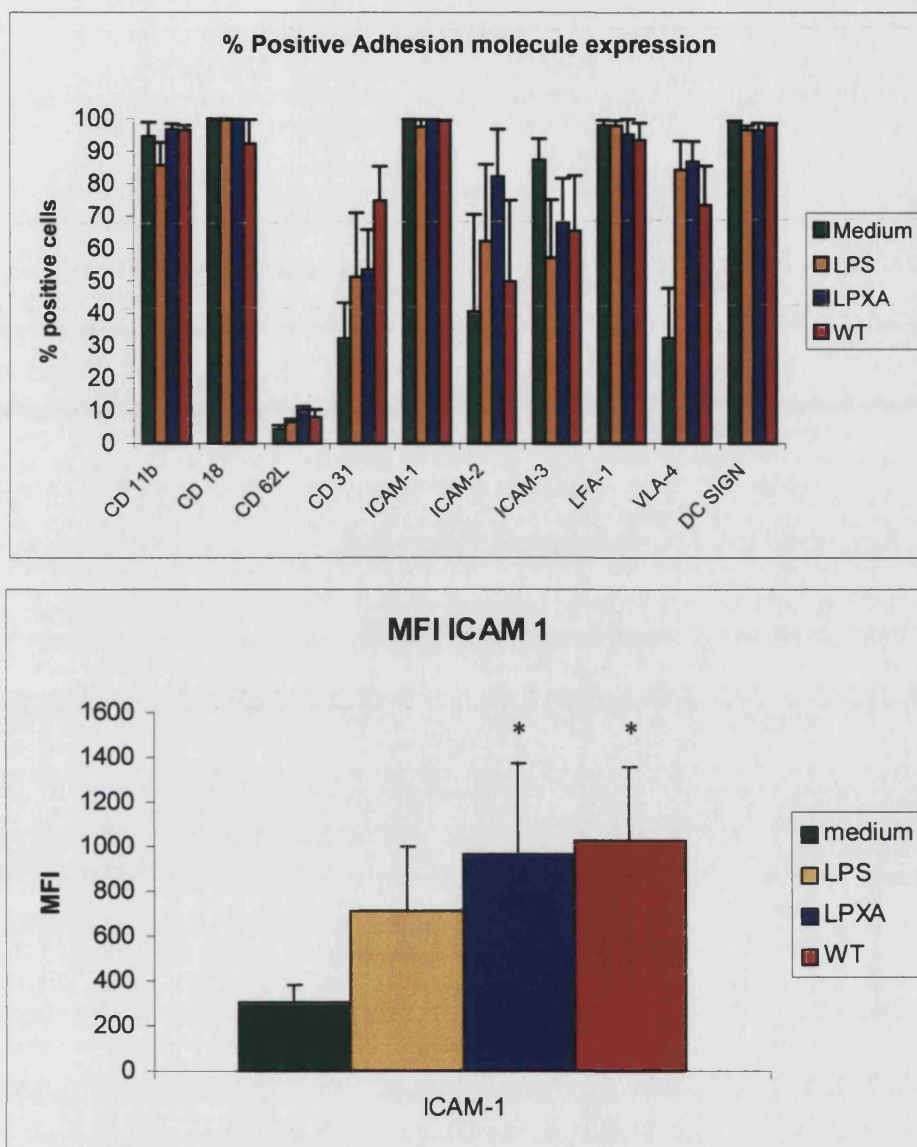
Figure 4.3 Expression of adhesion molecules and integrins on dendritic cells after stimulation with *N. meningitidis*

DCs were stimulated with wild type or *lpxA*- H44/76 *N. meningitidis* (both 10^7 organisms/ml), LPS (10 ng/ml) or medium alone. DCs were stained for ICAM-1, ICAM-2, ICAM-3, PECAM-1, LFA-1, VLA-4, CD11b, CD18, CD62L and DC-SIGN. The percentage of positive cells is shown as well as the MFI for ICAM-1. The data are expressed as the mean and SEM of 4 independent experiments. * indicate p values of <0.05 compared to the medium found by the paired student t-test.
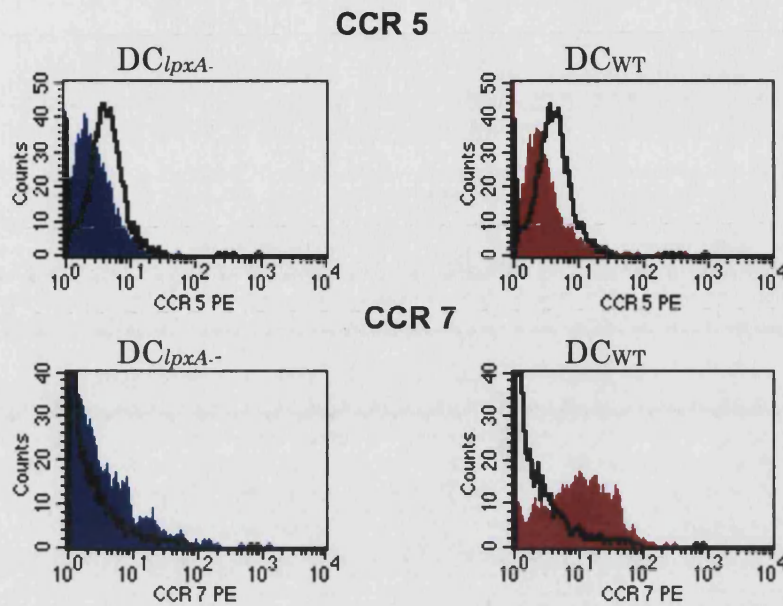
Figure 4.4 Expression of chemokine receptors on dendritic cells after stimulation with *Neisseria meningitidis*

DCs were stimulated for 18 hours with wild type or *lpxA-* H44/76 *N. meningitidis* (both 10^7 organisms/ml) or medium alone and stained for chemokine receptors CCR5 and CCR7. DCs were gated according to size and granularity and fluorescent staining determined by flow cytometry. The black solid lines are cells cultured in medium alone, the red filled histograms are DCs stimulated with wild type *N. meningitidis* and the blue filled histogram are DCs stimulated with the *lpxA-*. The data are a representative of at least 4 independent experiments.

4.3.3 Chemokine production by dendritic cells after stimulation with *N. meningitidis*

Dendritic cells not only express chemokine receptors, they are also capable of producing chemokines RANTES (CCL5), MIP1α (CCL3) and IL-8 (CXCL8) to attract leukocytes. The induction of chemokine production by DCs activated with *N. meningitidis* was investigated. DCs were cultured for 18 hours in the presence of Brefeldin A with wild type or *lpxA- N. meningitidis* (both at 10^7 organisms/ml) or purified meningococcal LPS (10 ng/ml). Intracellular chemokine levels were measured by flow cytometry (Fig 4.5). DC_{WT} produced IL-8 as did DC_{LPXA} though not to the same extent. No IL-8 production was detected by DC_{LPS} (Fig 4.5). There was no detectable production of RANTES or MIP1α by DCs stimulated with *N. meningitidis* or LPS (Fig 4.5).

4.3.4 Dendritic cell migration in the presence of chemokine MIP3β (CCL19)

DC stimulation by wild type *N. meningitidis* results in a decrease of CCR5 and an increase in CCR7 expression, whereas the LPS deficient *lpxA-* does not induce an increase in CCR7 expression. This suggests that wild type but not *lpxA-* might induce migration to MIP3β which is a ligand for CCR7. DC migration in the presence of MIP3β (CCL19) was therefore investigated. The lower chamber of an uncoated transwell was filled with medium containing different concentrations of the chemokine MIP3β. DCs cultured in medium alone, or with wild type or *lpxA- N. meningitidis* were added to the upper chamber of the transwell (5×10^5). Migrated cells were collected from the lower chamber after 6 hours and the cells were counted on a haemocytometer (Fig 4.6).
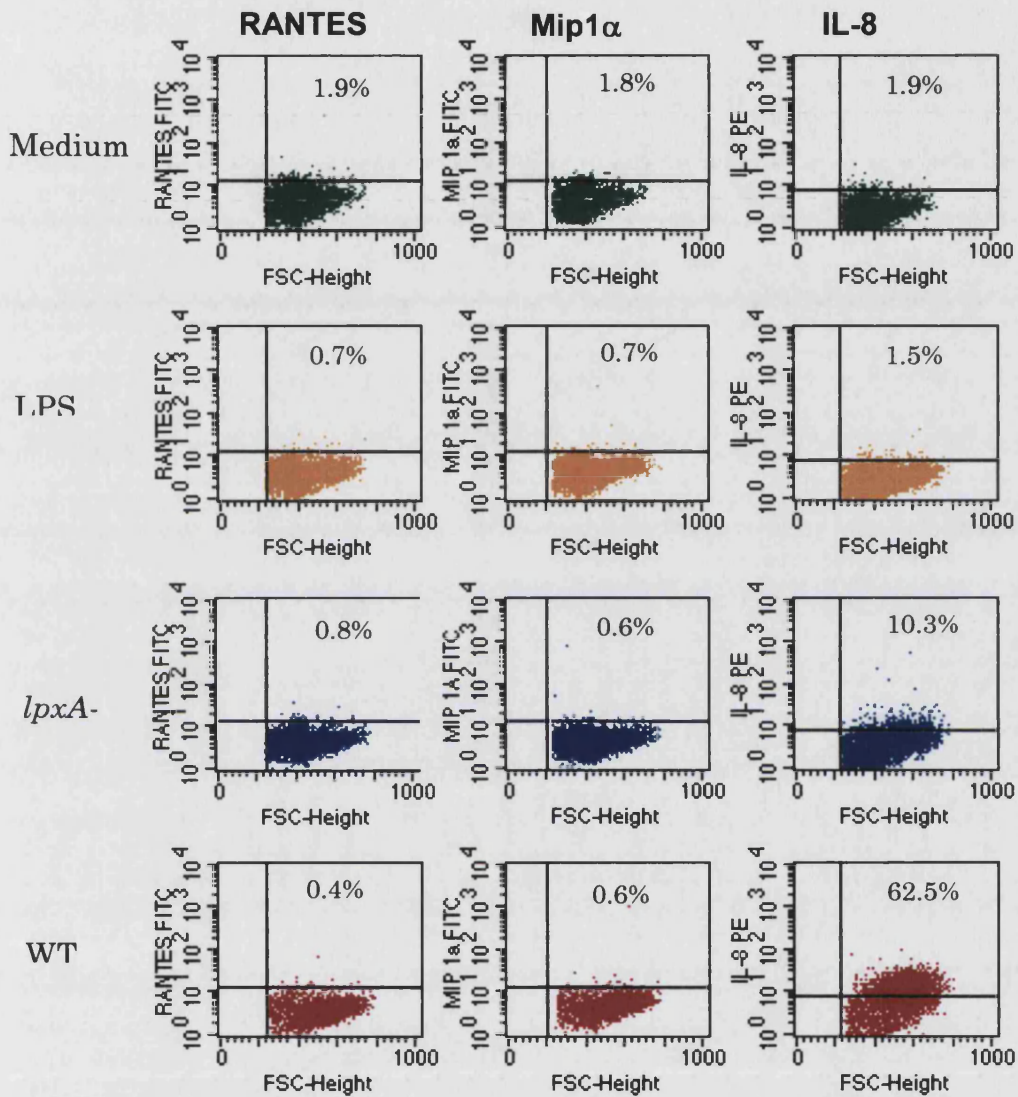
Figure 4.5 Dendritic Cell chemokine production in response to *N. meningitidis*

DCs were stimulated for 18 hours with wild type or LPS deficient *lpxA- N. meningitidis* (both 10^7 organisms/ml), LPS or in medium alone in the presence of Brefeldin A. The cells were permeabilised and stained for intracellular chemokines RANTES, MIP1α and IL-8. Data are a representative of 4 experiments. The percentage of positive cells is given.

Visualisation of the DCs in the upper chamber of the transwell revealed large masses of clumped cells in wells containing DC_{WT}, and some clumping in DC_{LPXA} wells. There was very little or no clumping observed in DC_{medium} wells. In the absence of additional MIP3β, a greater number of DC_{medium} migrated across the transwell than activated DCs, and more DC_{LPXA} migrated than DC_{WT}. The addition of MIP3β to the lower chamber had no effect on the ability of DC_{medium} or DC_{LPXA} to migrate. However the migration of DC_{WT} did increase in the presence of MIP3β, and appeared to respond in a dose dependent manner (Fig 4.6). This pattern of migration is consistent with the expression of CCR7 observed on DCs stimulated by wild type *N. meningitidis*.

4.3.5 Migration of dendritic cells across endothelial cell monolayers

The adhesion and transmigration of leukocytes has been studied in detail (reviewed in (Hogg N. et al., 1995)(Worthylake R.A. et al., 2001)). Expression of adhesion molecules and selectins on endothelial cells is necessary for leukocyte adhesion and diapedisis across endothelial layers. TNFα stimulation is known to increase expression of selectins and adhesion molecules on the surface of endothelial cells. The effect of TNFα stimulation of endothelial cells on the ability of DCs to transmigrate was therefore investigated. HUVEC monolayers were grown to confluence on gelatine coated transwells and stimulated with 1 ng/ml of TNF α, then 5×10^5 monocyte derived DCs were added to the upper transwell chamber. After 24, hours the medium from the lower transwell chamber was collected and the cells were counted on a haemocytometer. As shown in Figure 4.7, DCs migrated more readily

across endothelial cells than gelatine alone. Stimulation of the endothelial layer with

TNFα increased the number of DCs migrating across the endothelial monolayer.
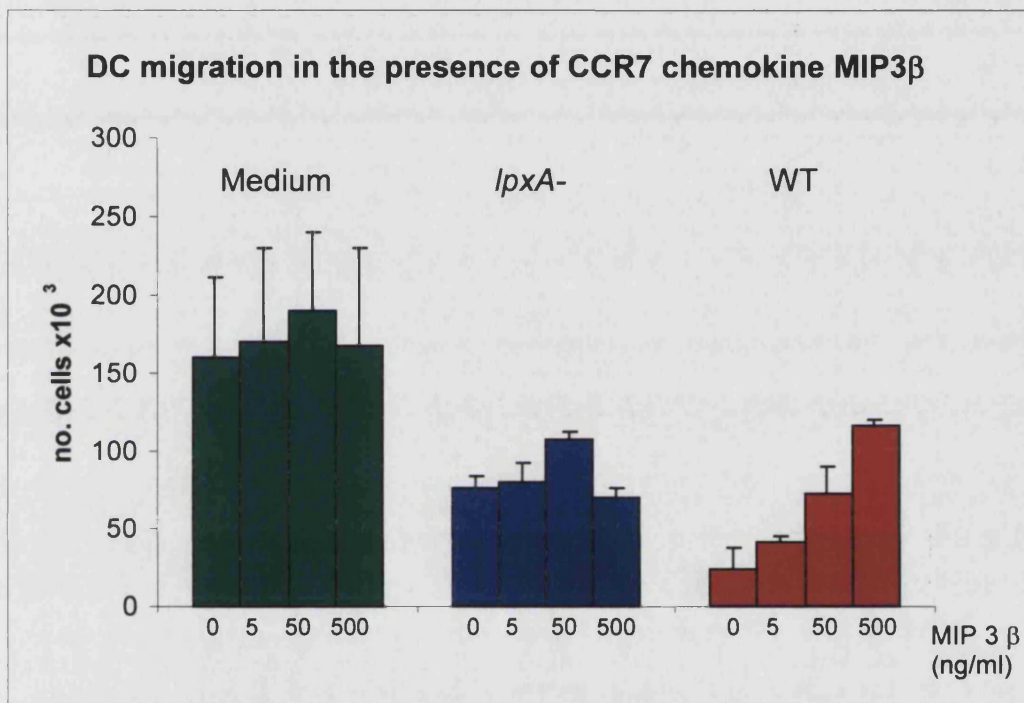
Figure 4.6 Dendritic cell migration in the presence of chemokine MIP3β

DCs stimulated with either wild type or *lpxA- N. meningitidis* (10^7 organisms/ml) or medium alone were added to the upper transwell chamber (5×10^5 cells/transwell). The lower chambers of the transwells contained varying concentrations of the CCR7 specific MIP3β. After 6 hours migrated cells were collected from the lower transwell chamber and counted on a haemocytometer. The data are the mean number of cells recovered in each culture and one SEM of 3 independent experiments.

4.3.6 Effect of dendritic cell activation on migration across endothelial layers

DCs cultured in medium alone or stimulated for 18 hours with 10^7 organisms/ml of wild type *N. meningitidis* were added to the upper chamber of HUVEC coated transwells, which were previously stimulated with 1 ng/ml of TNFα. At various time points the medium was collected from the lower transwell chambers and cells counted on a haemocytometer (Fig 4.8).

These data show that DC migration is a continuous process occurring over 24 hours. More DCs stimulated with wild type *N. meningitidis* migrate across the endothelial monolayer than unstimulated DCs. This increase in migration by stimulated DCs was observed at all time points during the 24 hour time course (Fig 4.8).

4.3.7 Migration of dendritic cells activated with wild type or *lpxA- N. meningitidis*

DCs were stimulated for 18 hours with 10^7 organisms/ml of wild type or *lpxA- N. meningitidis* or medium alone. After washing, 5×10^5 DCs were added to the upper chamber of the HUVEC coated transwell, half of which were previously stimulated with 1 ng/ml TNFα. After 24 hours incubation, cells were collected from the lower chamber of the transwells and counted on a haemocytometer.

A greater number of DC_{WT} migrated across TNFα activated HUVECs than DC_{medium} or DC_{LPXA}(Fig 4.9). DC migration across unstimulated endothelial cells was not effected by *N. meningitidis* activation and was similar in all conditions.
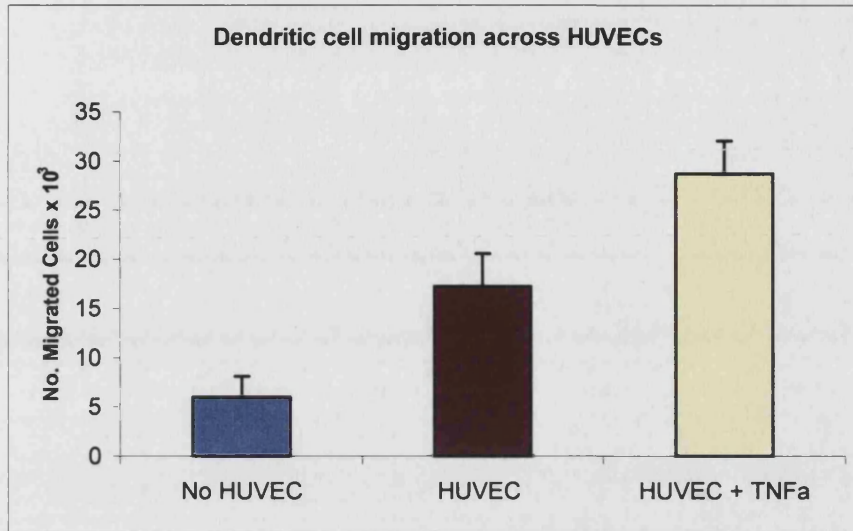
Figure 4.7 Dendritic cell migration across stimulated HUVEC monolayers

HUVEC monolayers were grown on gelatine-coated transwells. When confluence was reached, half of the HUVEC coated transwells were stimulated with 1 ng/ml TNFα. Monocyte derived DCs (5×10^5/well) were added to the upper transwell chamber. After 24 hours, the cells were collected from the lower transwell chambers and counted on a haemocytometer. DC migration across a gelatine layer without HUVECs is also shown. The data are the mean of duplicate wells and are representative of 6 independent experiments.
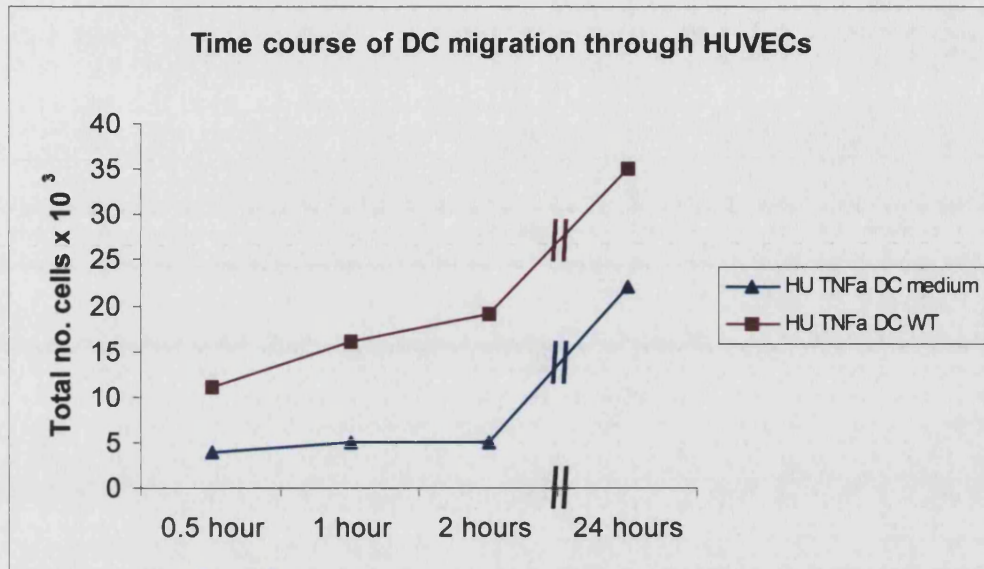
Figure 4.8 Dendritic cell migration across HUVEC monolayers

HUVEC monolayers were grown on gelatine-coated transwells and stimulated with 1 ng/ml TNFα. DCs (5 x 10^5 cells/transwell) stimulated with wild type *N. meningitidis* or grown in medium alone were added to the upper transwell chamber. At 30 minutes, 1 hour, 2 hours and 24 hours, cells were collected from the lower transwell chamber and counted on a haemocytometer. The data are a representative of 6 independent experiments.
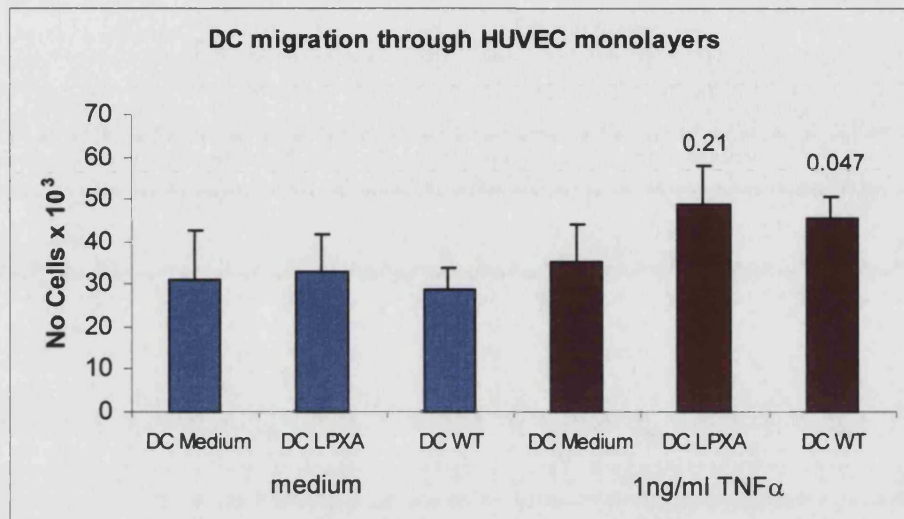
Figure 4.9 Migration of wild type and *lpxA*- stimulated dendritic cells

HUVEC monolayers were grown on gelatine-coated transwells and half were stimulated with 1 ng/ml of TNFα. DCs (5×10^5 cells/transwell) stimulated with wild type or *lpxA- N. meningitidis* (both 10^7 organisms/ml) or grown in medium alone were added to the upper transwell chamber. After 24 hours cells were collected from the lower transwell chamber and counted on a haemocytometer. Data are the mean and SEM of 6 independent experiments. The p values compared to DC_{medium} are shown, as found by the Mann-Whitney U test.

4.4 Discussion

DC migration and maturation are closely linked but the mechanisms controlling DC emigration from the peripheral tissues to the secondary lymphoid organs are not totally understood. Adhesion molecules are important for migration of leukocytes across inflamed endothelium and are likely to play a role in DC migration. Here monocyte derived DCs were found to express a wide variety of adhesion molecules at high densities, including the Ig-superfamily members ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50) and PECAM-1 (CD31). DCs also expressed the selectins LFA-1 (CD11a/CD18), VLA-4 (CD49d/CD29) and MAC-1 (CD11b/CD18) and the DC specific DC-SIGN. Surprisingly DCs were found to be negative or very low for L-selectin (CD62L), which is found on most circulating leukocytes and is important for lymphocyte homing to lymph nodes (Irjala H. et al., 2001).

Of the adhesion molecules studied here, very few changed following DC stimulation with the wild type *N. meningitidis*, the LPS deficient *lpxA-* or purified meningococcal LPS. ICAM-1 increased following DC stimulation with both the wild type and *lpxA- N. meningitidis*, but the increase in ICAM-1 following stimulation with purified meningococcal LPS was not as great. This observation of an increase in DC ICAM-1 expression following stimulation with *N. meningitidis* is consistent with a previous report showing DC ICAM-1 expression increasing following stimulation with IFNγ (Starling G.C. et al., 1995). ICAM-1 has been implicated in the migration of Langerhans cells from the skin to draining lymph nodes. Langerhans cells in ICAM-1 knock out mice were present in the skin epidermis, but migration to lymph nodes following stimulation was severely impaired (Xu H. et al., 2001). Further

experiments revealed that ICAM-1 expression by lymphatic endothelium was more important than ICAM-1 expression by Langerhans cells. The ligands for ICAM-1 include the integrins LFA-1 and Mac-1, which are expressed by lymphocytes and NK cells. Thus ICAM-1 expression by DCs is likely to be important for interactions with these cells. Expression of the integrin VLA-4 ($\alpha4\beta1$ integrin) also increased on DCs following stimulation with *N. meningitidis* wild type, *lpxA-* and purified LPS. There are no reports on the role of VLA-4 in DC migration but VLA-4 binds to fibronectin, MAdCAM-1 and VCAM-1, and could possibly play a role in DC migration. DC expression of PECAM-1 (CD31) did appear to increase following stimulation, especially with the wild type *N. meningitidis*, though this was not statistically significant. The transmigration of leukocytes through vascular endothelium is dependent on PECAM-1 homophillic interactions and thus PECAM-1 maybe important in DC migration (Muller W.A. et al., 1993). Changes in expression of other adhesion molecules were not significant. ICAM-2 and ICAM-3 expression on DCs were found to be especially varied between different donors.

Chemokines play a fundamental role in DC migration and changes in receptor expression on maturing DCs have been observed (Sallusto F. et al., 1998). Immature DCs express CXCR1, CCR1 and CCR5. These receptors are down regulated after DC stimulation, and at the same time expression of CCR7 is increased. In the experiments described here, immature DCs expressed CCR5, which decreased following stimulation with wild type and *lpxA- N. meningitidis*. In contrast CCR7 expression increased following stimulation with wild type *N. meningitidis*, but not with *lpxA-*. The decrease of CCR5 and increase of CCR7 observed on DCs stimulated with wild type *N. meningitidis* is consistent with maturing DCs migrating

from the periphery to the lymph nodes *in vivo*. The absence of CCR7 expression in

DCs stimulated with *lpxA- N. meningitidis* suggests that LPS maybe necessary for *N.*

meningitidis induced DC migration. The necessity of LPS for optimal DC migration

following stimulation with *N. meningitidis* has important implications for vaccine

design. Inefficient migration of DCs following vaccination would inhibit

presentation to T cells in the secondary lymphoid organs, preventing protective

immunity.

DCs are also known to be a source of some chemokines following stimulation. A

previous report (Sallusto F. et al., 1999) showed that monocyte derived DCs are

capable of producing chemokines MIP1α, MIP1β, IL-8 and RANTES following

stimulation with 1 μg/ml salmonella LPS. In the experiments described here DCs did

not produce RANTES or MIP1α following activation with *N. meningitidis*, but they

did produce IL-8. The lack of production of chemokines by DCs stimulated with *N.*

meningitidis could be due to several factors. Firstly, it is possible that *N. meningitidis*

does not induce the production of these chemokines in DCs. However *N.*

meningitidis is a potent stimulator of pro-inflammatory cytokines in DCs (as shown

in Chapter 3) and stimuli that are capable of inducing DC production of cytokines,

including LPS, CD40 ligation and TNFα, have also been shown to induce production

of chemokines (Sallusto F. et al., 1999). Chemokine expression was detected here by

intra-cellular labelling following culture with brefeldin A and it maybe that brefeldin

A interferes with chemokine production or aids the degradation of these chemokines

whilst they are stored in the Golgi. Other studies have used brefeldin A free systems

and measured chemokine production by ELISA in cell culture supernatants or by

mRNA levels. A further possibility for not observing MIP1α or RANTES production

by DCs stimulated with *N. meningitidis* is because the time at which the cells were assayed (18 hours) did not include the period of chemokine production. However, studies with LPS and CD40 ligand stimulation of DCs showed that the optimal time for production of MIP1α was 12 to 18 hours, and the optimal time for RANTES production was 18 to 24 hours (Sallusto F. et al., 1999). Further investigations area needed to explain the lack of chemokine production by DCs stimulated with *N. meningitidis*.

In order to ascertain if the changes in chemokine receptor expression observed following stimulation with *N. meningitidis* are related to the ability of DCs to migrate, experiments were carried out to measure migration in the presence of the CCR7 ligand MIP3β (CCL19). Surprisingly, DCs cultured in medium alone were found to migrate through the 5 μm pores in the transwells most readily. However the rate and total number of migrated DCs cultured in medium alone was not affected by the chemokine MIP3β. Migration of DCs stimulated with the LPS deficient *lpxA-N. meningitidis* were also unaffected by the presence of chemokine. This is probably because DCs in medium alone or stimulated with *lpxA-* do not express the MIP3β receptor CCR7. DCs stimulated with the wild type *N. meningitidis* did respond to the increase in MIP3β in a dose dependent way, which could be attributed to the increase of CCR7 observed on these DCs. The difference seen in migration through the transwell in the absence of chemokine (i.e. DCs cultured in medium alone migrated most readily) may be due to the clustering and clumping of DCs in the upper compartment of the transwell. DCs stimulated with wild type *N. meningitidis* were observed to clump together in tight clusters. DCs stimulated with LPS deficient *lpxA-*

also formed clusters but they were not as large or as frequent as the wild type stimulated DCs. DCs in medium alone did not cluster and were distributed evenly over the transwell. Furthermore it is known that the motility of DCs changes with maturation. Undifferentiated immature DCs are highly motile moving in random directions (Shutt D.C. et al., 2000). This movement of immature DCs may result in their migration though the 5μm pores of the polycarbonate transwell.

The transwell system was also used to investigate the ability of DCs to migrate across a monolayer of endothelial cells (HUVECs). In the absence of endothelial cells, DCs were less unable to migrate through the gelatine layer on the transwells. Stimulation of the endothelial cells with TNFα resulted in an increase in DC transmigration, especially DCs activated with *N. meningitidis*. Activation of endothelial cells is important in the transmigration of leukocytes. TNFα stimulation of endothelium results in an increase in expression of adhesion molecules including CD62E, ICAM-1 and VCAM-1 and an increase in leukocyte binding and transmigration (Bevilacqua M.P. et al., 1987)(Karmann K. et al., 1996). It is probable that the increase in cell adhesion molecules on HUVECs following TNF stimulation is responsible for the increase in DC transmigration. This could be investigated further by studies with blocking antibodies.

The way this transwell system was set up models migration from the luminal side of the endothelium to the tissues. In an *in vivo* situation, maturing DCs would exit the tissues and cross either vascular or lymphatic endothelium in a basal to luminal direction. The ability of DCs to migrate across the basal membrane and through an endothelial monolayer to the luminal surface has been described (D'amico G. et al.,

1998). Moreover HUVECs may not be the best endothelial cells to use in this model. There are similarities between lymphatic and vascular endothelium, but there are also functional differences between the two. Common to other endothelium, lymphatic endothelium expresses CD31, CD34, VE cadherin and von Willibrand factor. The discovery of lymphatic specific markers, podoplannin and the hyaluronan receptor LYVE-1, has made possible the isolation and culture of lymphatic endothelium (Irjala H. et al., 2003)(Kriehuber E. et al., 2001). It would be interesting to investigate DC migration through lymphatic endothelium but difficulties with availability, purification and culture of lymphatic endothelium make this difficult.

In order to further understand the interactions of adhesion molecules during DC migration through endothelial cells following stimulation with *N. meningitidis*, several other lines of investigation need to be followed. Molecules on endothelium responsible for DC adhesion and transmigration could be identified using blocking antibodies. The methods described here explore the migration of DCs through vascular endothelium in a luminal to basal direction. A more accurate model of DC migration from the tissues to the lymphatics could be DC migration through lymphatic endothelium in a basal to luminal direction as shown in a model of reverse transmigration (D'amico G. et al., 1998).

Chapter 5

T Cell Interactions with Dendritic Cells

Activated by *Neisseria meningitidis*

5.1 Introduction

Upon arrival at secondary lymphoid organs, DCs concentrate in T cell areas where they present antigen and deliver other signals to T cells. The combination of MHC-TCR interactions with surface co-stimulatory signals and cytokines is required for activation and polarisation of naive T helper cells to a Th1 or a Th2 phenotype. IL-12 produced by DCs is known to be crucial for Th1 development, but other cytokines including IL-18 and IFNα/β may also play a role. The signals required for Th2 differentiation are less understood. CD80 (B7.1) and CD86 (B7.2) which interact with CD28, OX40 and OX40L, and CD40 and CD40L have been shown to play a role in Th2 development in some instances (de Jong E.C. et al., 2002)(MacDonald A.S. et al., 2002b)(Tanaka H. et al., 2000). IL-4 is crucial in Th2 development, but is not produced by DCs, IL-4 is produced by Th2 cells and helps to prolong the Th2 response (MacDonald A.S. et al., 2002a).

In Chapter 3 it was shown that both the wild type and LPS deficient *N. meningitidis* activated DCs. There were differences in this activation, notably wild type *N. meningitidis* induced IL-12 production in DCs, whereas the *lpxA*- did not. There were also differences in expression of co-stimulatory molecules following DC activation with the wild type compared to the *lpxA*- *N. meningitidis*. These differences in DC activation may be important for T cell differentiation. DC and T cell interactions were therefore investigated following DC activation with *N. meningitidis*.

5.2 Methods

This chapter describes T cell responses to DCs activated by wild type and *lpxA- N. meningitidis*, using highly purified $CD3^+$ T cells and $CD4^+CD45RA^+$ naïve T cells.

5.2.1 T cell culture with activated dendritic cells

DCs were stimulated with wild type *N. meningitidis* (DC_{WT}), the LPS deficient *lpxA-* (DC_{LPXA}), purified meningococcal LPS (DC_{LPS}) or other bacterial products at varying concentrations for 16-18 hours as described in Chapter 3. They were then washed in 10 ml of cell wash medium to remove any excess antigen, counted on a haemocytometer and resuspended to 10^5 cells/ml in T cell culture medium. One hundred micro litres of DC suspension (10^4 DCs) was added to 100 µl of T cells (10^5 T cells) in each well of a 96 well U bottom plate. In some instances DCs were diluted over a range of concentrations starting at 10^5 cells/ml to give DC:T cell ratios of 1:10 to 1:80. The culture plates were placed in a 5% CO_2 incubator at 37 °C for 5 days. As a positive control for T cell proliferation, 10^5 purified T cells were stimulated with 5 ng/ml of PHA, and as a negative control 10^5 purified T cells were cultured without stimulation. All assays were carried out in triplicate.

5.2.2 Measuring T cell proliferation

After 5 days incubation, the DC T cell cultures were pulsed with ^{3}H-thymidine (1 µCi per well) for 16 hours, harvested and ^{3}H-thymidine incorporation determined by liquid scintillation counting. Results are expressed as the mean counts per minute (cpm) of triplicate cultures.

T cell proliferation was also determined by carboxyfluorescein succinimidyl ester (CFSE) staining. The succinimidyl ester of carboxyfluorescein diacetate passively diffuses into cells. It is colourless and non-fluorescent until its acetate groups are cleaved by intracellular esterases to yield highly fluorescent, amine-reactive CFSE, which is inherited by daughter cells upon division. CFSE staining is a useful tool for analysing cell division, as after each division cycle the fluorescence of each cell decreases by a factor of 2, thus identifying the number of divisions cells have undergone. Unlike ^{3}H-thymidine incorporation which only detects cells that divide (undergo DNA synthesis) in the 16 hour period in which thymidine is added to the culture medium, CFSE staining enables the whole population of cells (both dividing and non-dividing) to be analysed and dividing cells can be followed over several cycles. This technique can also be combined with intracellular cytokine staining to show association between proliferation and cytokine production.

T cells were purified by negative selection, washed in RPMI 1640 without supplementary FCS, resuspended to 10^6–10^7 cells/ml in 10 ml of RPMI 1640 and incubated with CFSE at a final concentration of 2.5 μM for 10 minutes at 37 °C. The reaction was stopped by diluting the T cell and CFSE mix with 40 ml of RPMI-1640 with 10% FCS. The cells were washed a further 3 times in cell wash medium, counted on a haemocytometer and resuspended to 10^6 cells/ml in T cell culture medium.

CFSE labelled T cells (5×10^5) were combined in culture with DCs (5×10^4) for 5 days. T cells were then labelled with an anti-CD3 PE antibody (5 μg/ml), or other T cell markers and cell proliferation determined by flow cytometry. In each experiment

20,000 events in the CD3$^+$ gate were collected. In some experiments DC-T cell cultures were visualised by fluorescent microscopy.

5.2.3 Measuring cytokine production in proliferating T cells

After 5 days culture with DCs, CFSE labelled T cells were stimulated with 10 ng/ml of PMA and 1 μg/ml ionomycin in the presence of 10 μg/ml Brefeldin A for 6 hours to induce cytokine production. T cells were first stained with CD3 tri-color® antibody and fixed in 2% paraformaldehyde in PBS at room temperature for 15 minutes. They were then permeabilised (as described in 2.4.2) and stained for intracellular cytokines IL-2, IL-4 and IFNγ.

5.2.4 Dendritic cell priming of CD4$^+$ CD45RA$^+$ naïve T helper cells

Initial work was carried out using CD3$^+$ T cells, which included CD8$^+$ cytotoxic T cells, CD4$^+$ helper T cells, CD45RO$^+$ memory cells and CD45RA$^+$ naïve cells. Naïve T cell responses to DC stimulation were investigated using a method adapted from de Jong E.C. et al., 2002) (and personal communication with Dr de Jong). CD4$^+$CD45RA$^+$ T cells were isolated and cultured with DCs that had been stimulated with *N. meningitidis* wild type or *lpxA-* (10^7 organisms/ml); purified LPS (10 ng/ml); PGE-2 (10^{-6} M) or medium alone. DC (5 x 10^3) were combined with T cells (2 x 10^4) in a 96 well flat bottom plate in the presence of the super antigen SEB (Staphylococcus enterotoxin B) at 100 pg/ml in a total volume of 200 μl. The cells were then cultured for 5 days at 37 °C in 5%CO$_2$ (Fig 5.1). After 5 days, cells from each well of the 96 well plate were transferred to a well in a 24 well plate and diluted to 1 ml with T cell culture medium supplemented with 10 U/ml of IL-2 and cultured for a further 10-12 days at 37 °C in 5%CO$_2$.
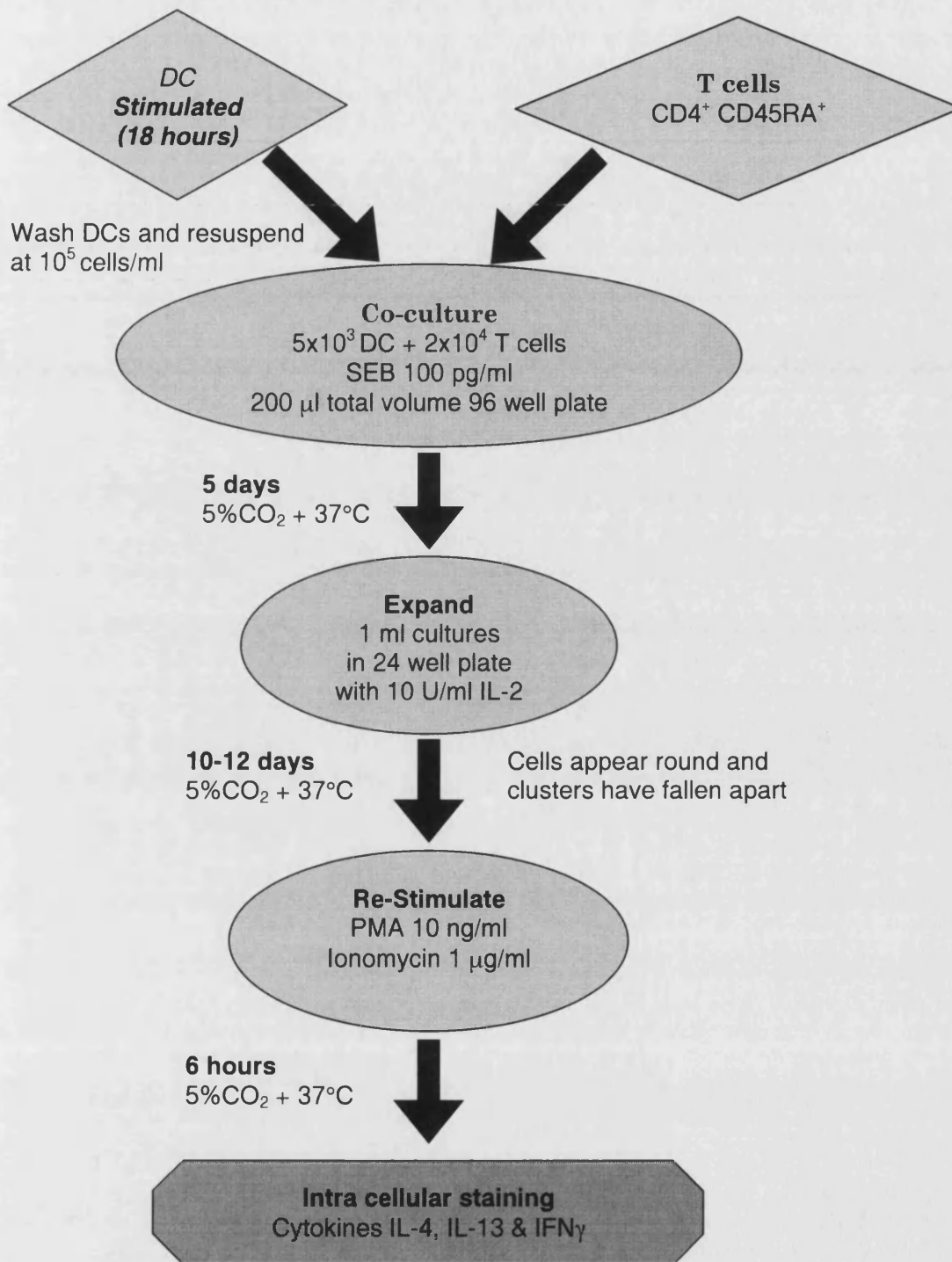
Figure 5.1 Flow chart of dendritic cell and naïve Th cell co-cultures

Culture of DCs and Th cells using super-antigen followed by expansion with IL-2 before restimulation and intra-cellular cytokine detection.

Every second day the wells were subcultured and refreshed with IL-2 supplemented medium (Fig 5.2). After culture for 10-12 days in the presence of IL-2, the cells were observed by light microscopy. Clusters were seen to have fallen apart and T cells had a rounded appearance typical of resting state. The T cells were then restimulated for 6 hours with PMA (10 ng/ml) and ionomycin (1 µg/ml) in the presence of Brefeldin A (10 µg/ml) and then stained for intracellular cytokines IL-4, IL-13 and IFNγ as described in sections 2.4.2 and 5.2.3

5.3 Results

5.3.1 T cell proliferation in response to dendritic cells stimulated with *N. meningitidis* determined by ^{3}H-thymidine incorporation

T cell proliferation in response to DC_{WT}, DC_{LPXA}, DC_{LPS}, and DC_{medium} was determined in allogeneic and autologous combinations by ^{3}H-thymidine incorporation. Immature (non activated) DCs (DC_{medium}) induced T cell proliferation in both autologous and allogeneic MLRs above the background of T cells alone. Stimulated DCs induced similar levels of T cell proliferation at DC:T cell ratios of 1:5 and 1:10. At DC:T cell ratios of 1:20 and 1:40 proliferation was reduced. At a ratio of 1:80 proliferation was almost the same as the background response by T cells on their own (Fig 5.2). DC_{LPS} induced a greater T cell response at lower DC:T cell ratios than DC_{medium}. DC_{WT} and DC_{LPXA} induced greater T cell proliferation at all concentrations than DC_{medium} (Fig 5.2).
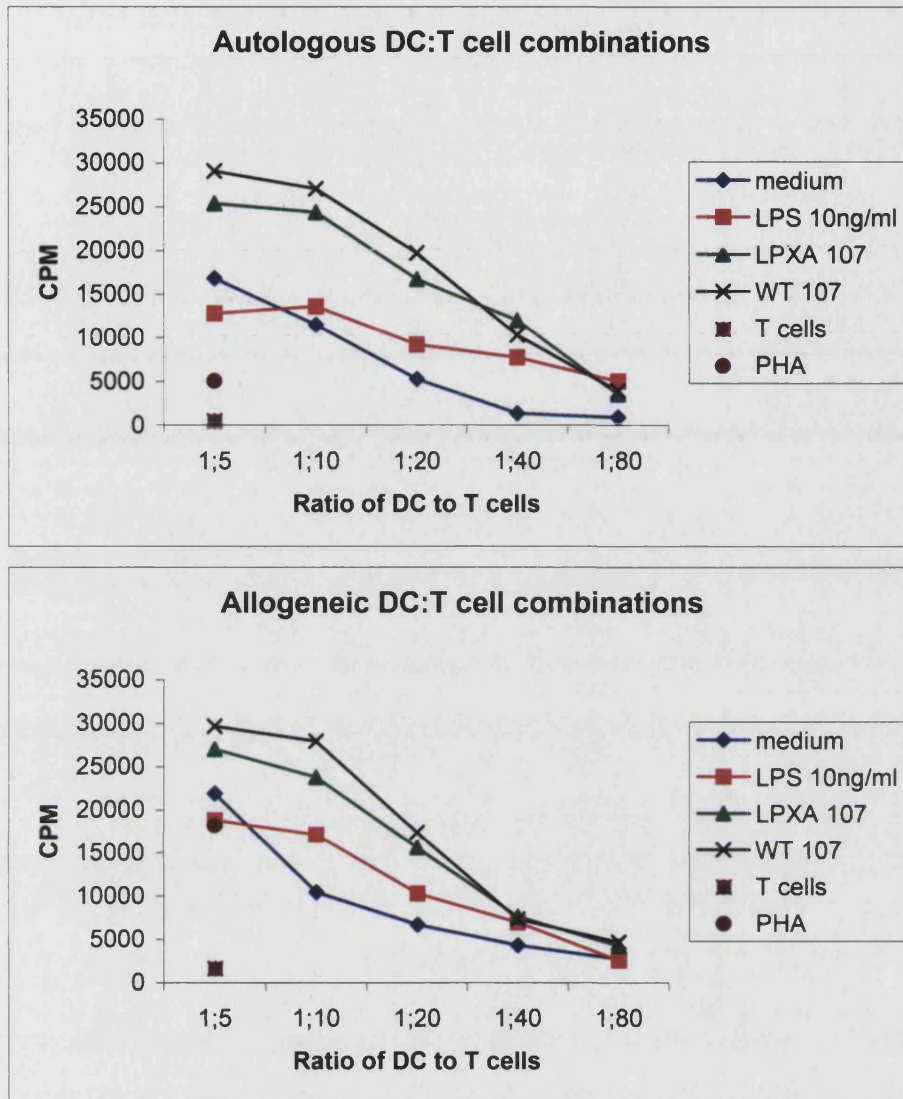
Figure 5.2 T cell proliferation in response to activated dendritic cells

DCs were stimulated for 18 hours with wild type or LPS deficient *lpxA- N. meningitidis* (10^7 organisms/ml), purified LPS (10 ng/ml) or in medium. DC (10^4 cells) were cultured with autologous or allogeneic T cells at ratios of 1:5 to 1:80 (DC:Tcells). T cells cultured alone or with PHA (5 ng/ml) were included as controls. Results are expressed as the mean counts per minute (CPM) of triplicate cultures. Data are representative of one experiment from four.

Wild type H44/76 *N. meningitidis* is a strong inducer of DC maturation and activation even at low concentrations (10^5 organisms/ml), whereas the LPS deficient *lpxA-* only induces DC activation at concentrations of 10^7 organisms/ml or higher (Fig 3.2, Chapter 3). Therefore T cell proliferation in response to DCs activated with different concentrations of wild type and *lpxA- N. meningitidis* were investigated. DCs were cultured with autologous and allogeneic T cells for 5 days at a ratio of 1:10. In these experiments DC_{WT} induced significantly greater T cell proliferation than the medium control at 10^5, 10^6 and 10^7 organisms/ml (Fig 5.3), whereas DC_{LPXA} only induced significant T cell proliferation at 10^7 organisms/ml (Fig 5.3).

In the experiment shown, DC stimulation with varying concentrations of *N. meningitidis* had no effect on allogeneic T cell proliferation. This maybe due to the high proliferative response of T cells cultured with all allogeneic DC including DC_{medium} (Fig 5.3).

The previous experiments showed that optimal T cell proliferation occurred at a ratio of 1:10 (DC:T cells). Also, DCs stimulated with *N. meningitidis* bacteria at 10^7 organisms/ml resulted in the greatest T cell proliferation. In order to investigate further the difference between T cell proliferation induced by DC_{WT}, DC_{LPXA} or DC_{LPS}, DCs activated with wild type or *lpxA- N. meningitidis* (10^7 organisms/ml), purified LPS (10 ng/ml) or medium alone were cultured with autologous or allogeneic T cells at a ratio of 1:10. T cell proliferation was measured by ^{3}H-thymidine incorporation (Fig 5.4). In allogeneic DC:T cell combinations, pre-stimulation of DCs had no effect on T cell proliferation when compared to control DCs cultured in medium only. In autologous combinations, both DC_{WT} and DC_{LPXA}
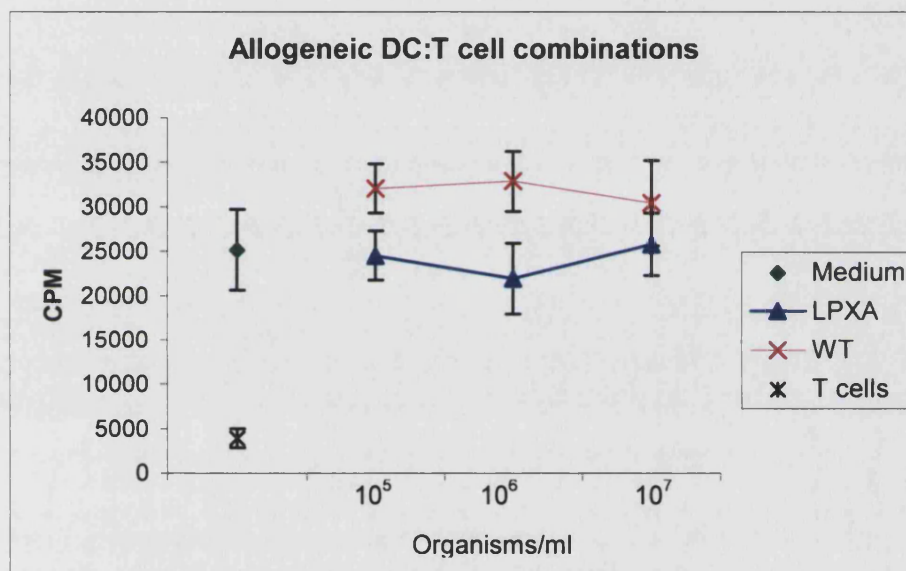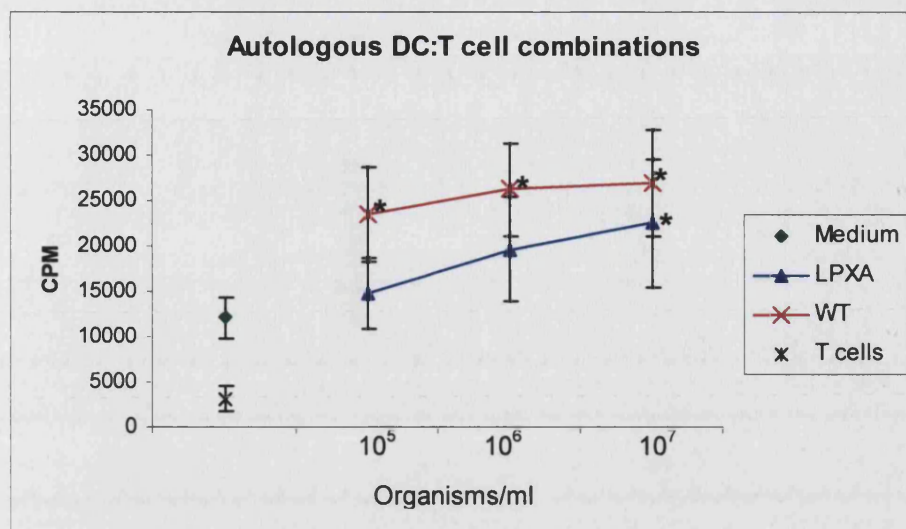
Figure 5.3 T cell proliferation in response to dendritic cells activated with varying concentrations of *N. meningitidis*.

DCs were stimulated with 10^5, 10^6 or 10^7 organisms/ml of wild type or *lpxA- N. meningitidis*. DCs (10^4) were then cultured with T cells (10^5) for 5 days and proliferation was measured by ^{3}H-thymidine incorporation. The data are expressed as the mean and SEM of 8 independent experiments, in which triplicate cultures were performed and the mean CPM taken. A non-parametric Mann-Whitney U test was used to measure significance of proliferation compared to the medium control.
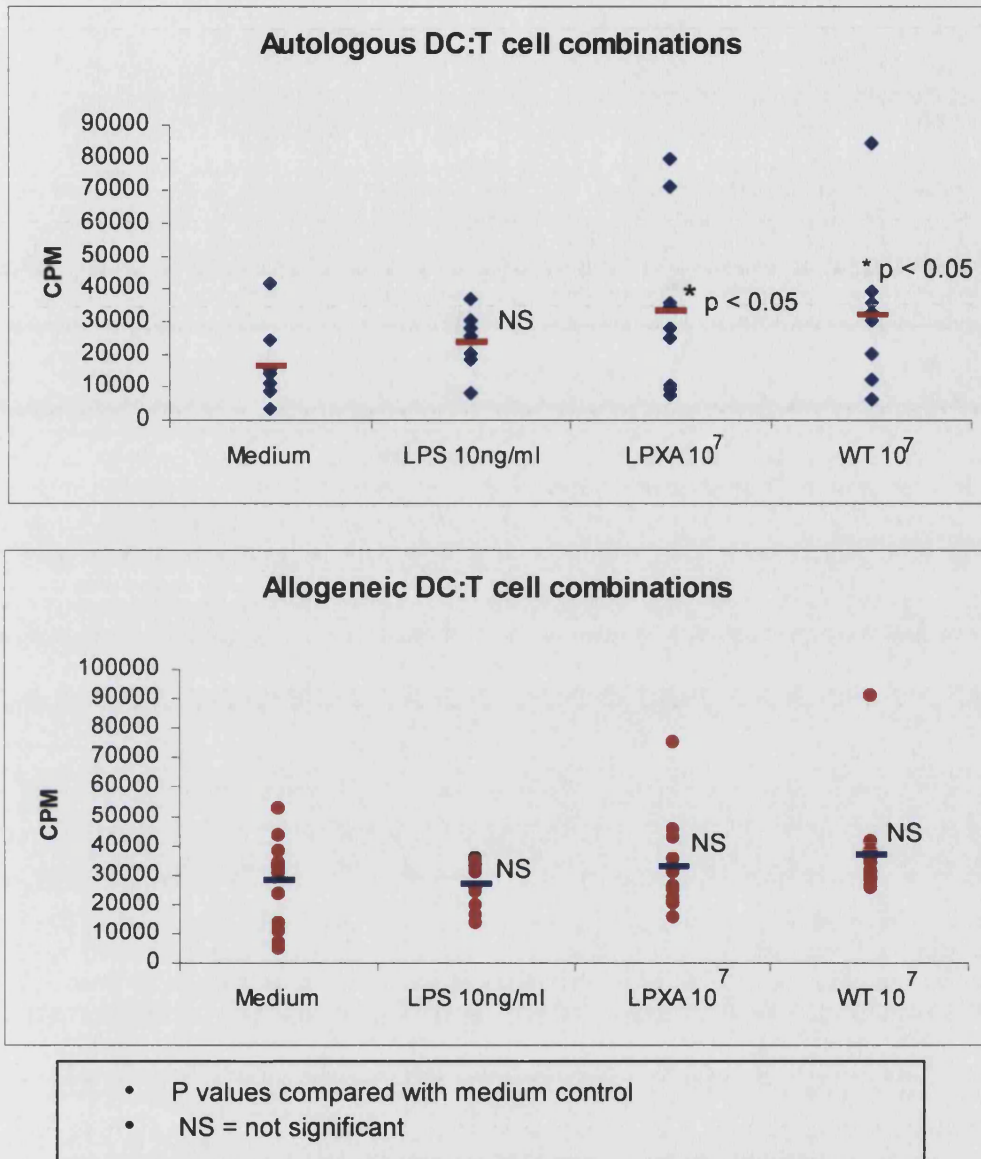
Figure 5.4 T cell proliferation in response to dendritic cells stimulated with *Neisseria meningitidis*.

DCs activated by wild type or *lpxA- N. meningitidis* (10^7 organisms/ml), LPS (10 ng/ml) or medium alone were combined with T cells in autologous or allogeneic cultures at a ratio of 1:10 (10^4 DC and 10^5 T cells). Proliferation was measured by ^{3}H-thymidine uptake after 5 days. Each point represents an individual experiment and is the mean CPM of triplicate cultures. A Mann-Whitney U test was used to measure significance of increased proliferation against the medium control.

induced greater T cell proliferation than DCs cultured in medium. In contrast, LPS stimulation of DCs had no significant effect on their ability to induce T cell proliferation (Fig 5.4).

5.3.2 CFSE staining for measuring cytokine production in proliferating T cells

CFSE staining was used to determine cytokine production in dividing and non-dividing cells. To obtain a baseline picture of T cell proliferation purified $CD3^+$ cells were stained with CFSE and incubated for 5 days with PHA (5 ng/ml) (Fig 5.5).

Proliferation of T cells after activation with PHA gives distinct CFSE peaks corresponding to consecutive generations of daughter cells. The first peak on the right hand side consists of cells which have not divided, and 5-6 cell cycles of decreasing CFSE intensity can be seen from right to left (Fig 5.5.).

Next, CFSE staining of T cells was used to assess T cell proliferation after incubation with DCs that had been activated with wild type or *lpxA- N. meningitidis* (10^7 organisms/ml), purified LPS (10 ng/ml) or medium alone. Fluorescent microscopy was used to visualise the DC and T cell co-cultures at day five. T cells labelled with CFSE can be seen interacting with non-labelled DCs (Fig 5.6). The differences in intensity of green stain on the different T cells is due to the different numbers of proliferation cycles they have undergone.
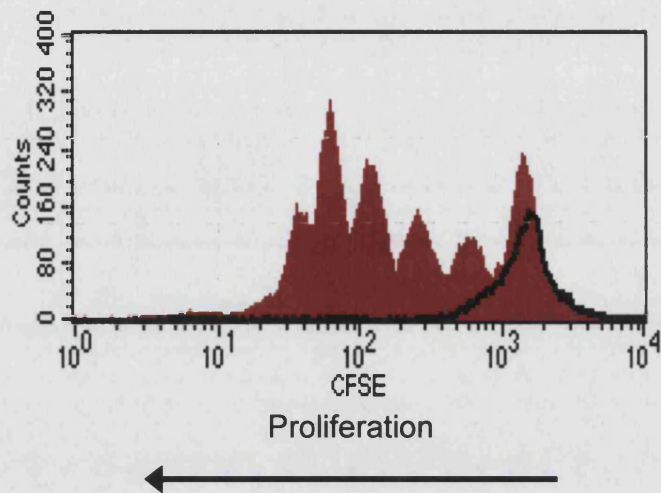
Figure 5.5 PHA induced proliferation of CFSE labelled T cells.

Purified T cells were labelled with CFSE and cultured for 5 days in the presence of 5 ng/ml PHA. Cells were then stained with CD3 PE antibody and analysed for CFSE staining (fluorescence channel 1) in the CD3 gate. Non-dividing CFSE labelled T cells cultured without PHA are shown (black solid line).
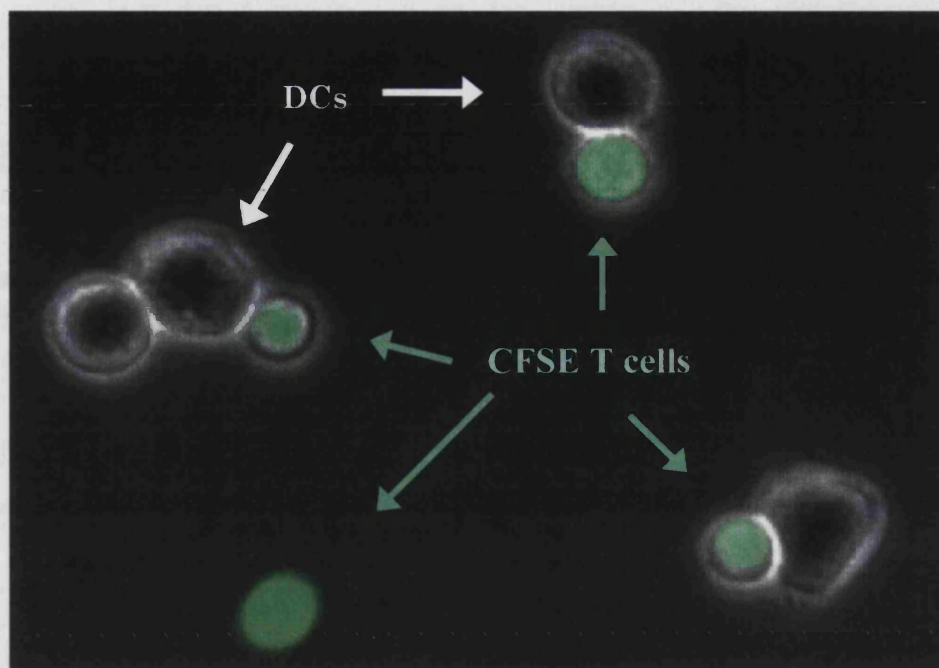
Figure 5.6 CFSE labelled T cells and DCs viewed by fluorescent microscopy.
CFSE labelled T cells and DCs cultured in medium alone after 5 days culture. The image was obtained by fluorescent microscopy and analysed on open lab imaging software.

After 5 days of culture, cells were stained with CD3 PE antibody and analysed for CFSE staining by flow cytometry in the CD3-PE gate. In both the autologous and allogeneic T cell responses, the pattern of proliferation observed was different to that obtained with PHA stimulated T cells. Rather than a clear set of peaks corresponding to T cells at each division cycle, T cells in culture with DCs formed 2 distinct peaks, one of non-dividing T cells (right hand side) and the other a peak of T cells that had all divided 4 or 5 times (Fig 5.7a & b).

No difference in T cell proliferation was observed by CFSE staining in allogeneic combinations with activated and non-activated DCs. In autologous combinations, fewer T cells proliferated than in the allogeneic combinations, although the T cells that entered into proliferation underwent the same number of divisions as in the allogeneic combinations. In the autologous DC:T cell combinations, pre stimulation of DCs with wild type or *lpxA- N. meningitidis* induced significantly greater number of T cells to undergo proliferation than DCs in medium alone (Fig 5.7c). LPS stimulated DCs also induced greater T cell proliferation than medium alone, though this was not statistically significant (Fig 5.7c).

(a) Allogeneic DC:T cell combinations
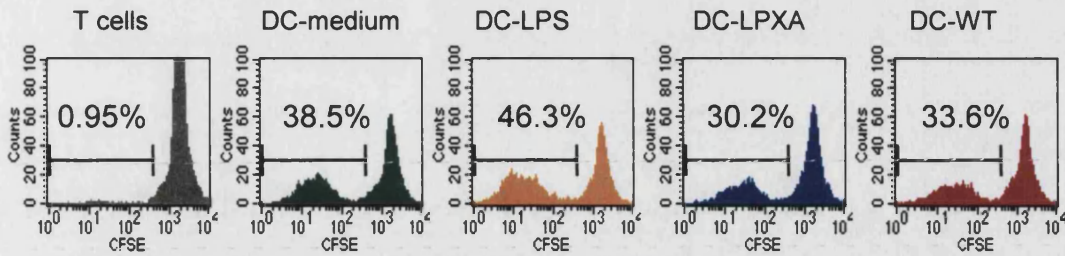
(b) Autologous DC:T cell combinations
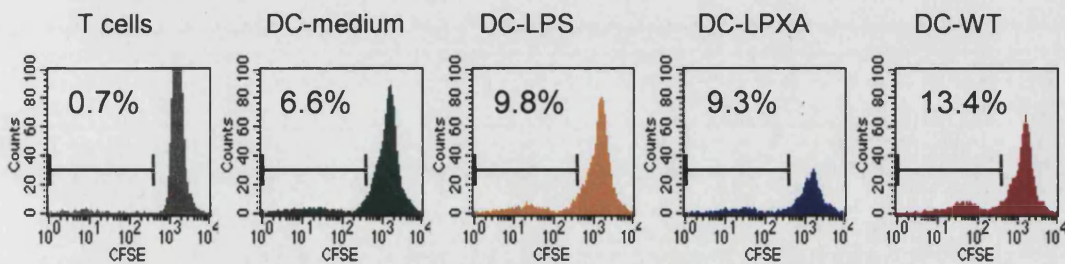
(c) Summary of autologous combinations
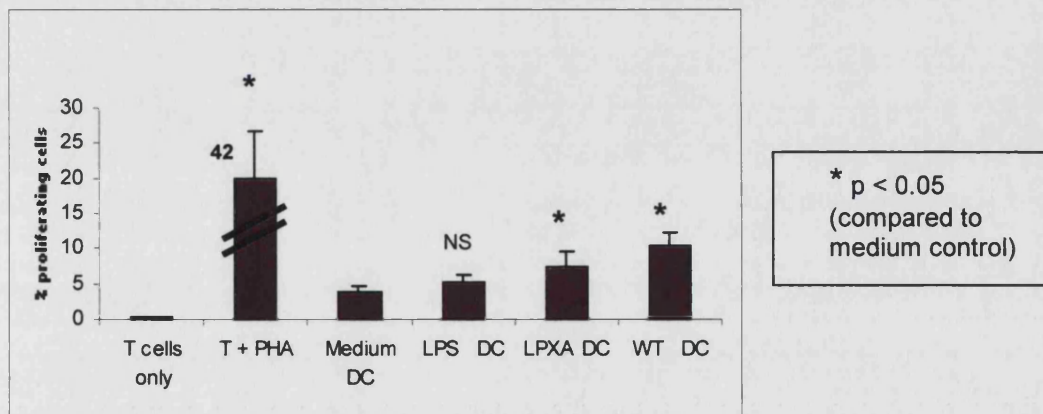
Figure 5.7 T cell proliferation measured by CFSE staining

CFSE labelled T cells were cultured in autologous or allogeneic combination with DCs activated by wild type or *lpxA*- (10^7 organisms/ml), LPS (10 ng/ml) or medium alone for 5 days. Cells were stained with CD3 PE and 20,000 events in this gate were analysed for CFSE staining by flow cytometry. Fig 5.7a shows a representative allogeneic response; Fig 5.7b shows a representative autologous response; and Fig 5.7c is the summary of 10 independent autologous experiments. The mean percent of proliferating cells and 1 SEM is given. A paired student t-test was used to determine significance of increased proliferation compared to the medium control.

It is possible that DCs activated in different ways may preferentially stimulate sub populations of T cells. CFSE labelling allows analysis of the dividing and non-dividing T cells for surface marker expression and cytokine production.

CFSE labelled T cells were cultured for 5 days in autologous combinations with DCs prestimulated with wild type and *lpxA- N. meningitidis* and then stained for surface markers including CD4, CD8, CD45RA, CD45RO and CD25.

Consistent with previous observations, DC_{medium} did not induce many autologous T cells to proliferate and thus it was not possible to phenotype the proliferating cells in this combination. Non-proliferating cells were 77% $CD4^+$, 21% $CD8^+$, 41% $CD45RO^+$, 32% $CD45RA^+$ and less than 1% $CD25^+$ (Fig 5.8). Approximately 13% of the total T cell population cultured with autologous DC_{WT} had divided at least once. In the experiment shown, 55% of the proliferating cells were $CD4^+$ and 29% were $CD8^+$. The majority of proliferating cells were $CD45RO^+$ and $CD25^+$ (94% and 93% respectively). However, there was a small population of proliferating cells that were $CD45RA^+$ (15%). It is possible these cells express both CD45RA and CD45RO (Fig 5.8). DC_{LPXA} did not induce as many T cells to divide as DC_{WT}. The cells that had entered into at least one division represented approximately 2% of the total number of T cells, and 67% expressed CD4, 25% expressed CD8, 71% expressed CD45RO and 67% expressed CD25. Around 50% of the proliferating cells were also $CD45RA^+$. It was not determined if these cells were positive for both CD45RA and CD45RO.

Figure 5.8 Phenotype of proliferating T cells

CFSE labelled T cells (10^5) were cultured with autologous DC_{medium}, DC_{LPXA} or DC_{WT} (10^4). After 5 days cells were stained with CD3 Tri color® and CD4 PE, CD8 PE, CD45RA PE, CD45RO PE or CD25 PE. At least 20,000 events in the $CD3^+$ gate were collected by flow cytometry. Data are a representative of 4 experiments. The percentage of positive cells in the quadrant is given.

T cells + DC_medium

CD4

| 0% | 77% |
| 0% | 2?% |

CD8

| 0% | 21% |
| 0% | 7?% |

CD45RO

| 0% | 41% |
| 0% | 5?% |

CD45RA

| 0% | 32% |
| 0% | 68% |

CD25

| 0% | 1% |
| 0% | 99% |

T cells + DC_LPXA

CD4

| 1% | 69% |
| 0.5% | 29% |

CD8

| 0.5% | 23% |
| 1.5% | 7?% |

CD45RO

| 1.2% | 34% |
| 0.5% | 6?% |

CD45RA

| 1% | 37% |
| 1% | 6?% |

CD25

| 2% | 5% |
| 1% | 92% |

T cells + DC_WT

CD4

| 6% | 53% |
| 5% | 36% |

CD8

| 4% | 19% |
| 10% | 67% |

CD45RO

| 17% | 40% |
| 1% | 42% |

CD45RA

| 2% | 28% |
| 11% | 59% |

CD25

| 13% | 9% |
| 1% | 77% |

← Proliferation

144
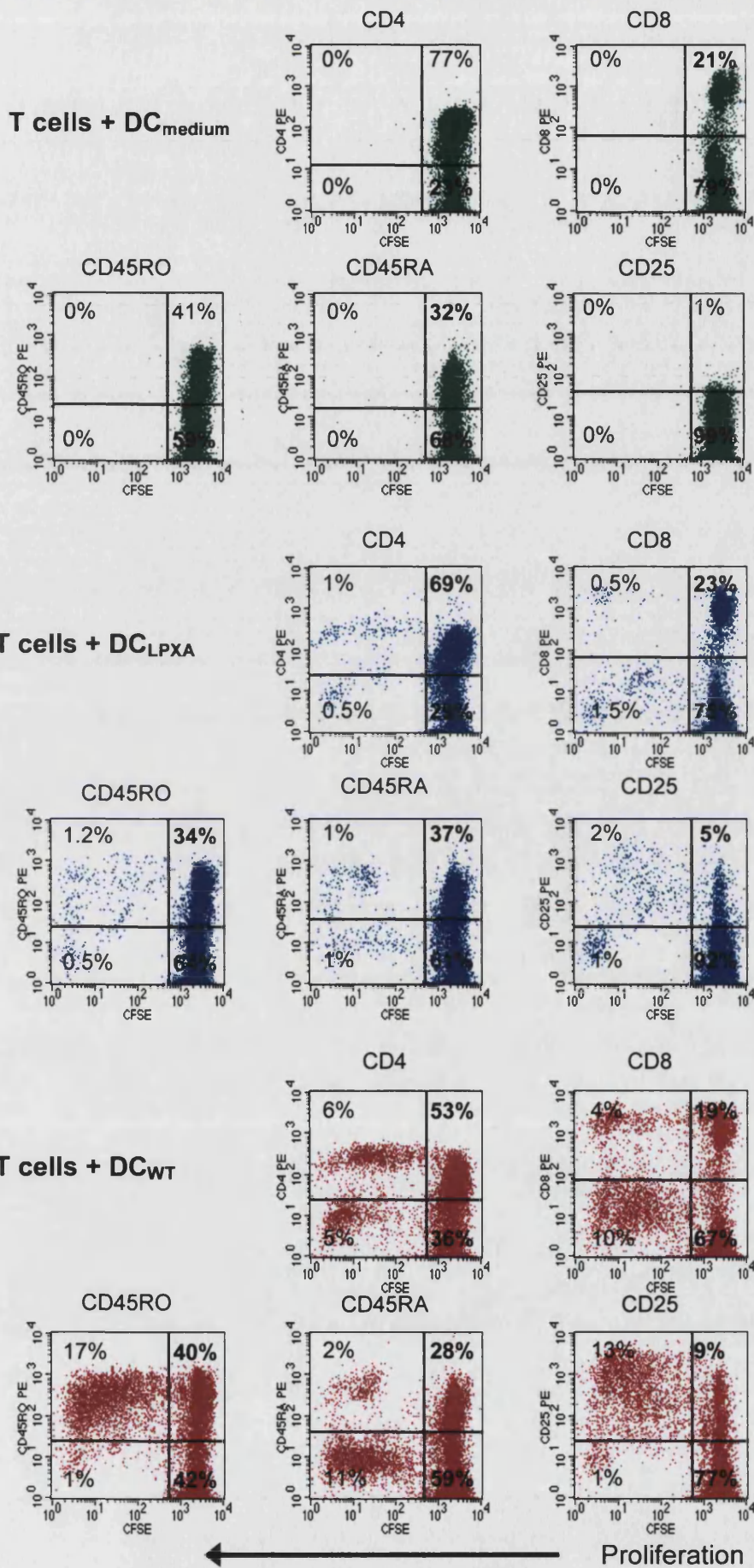
CFSE staining also allows cytokine production by proliferating and non-proliferating T cells to be analysed. CFSE labelled T cells were cultured with autologous DCs for 6 days, and then restimulated with PMA and ionomycin for 6 hours in the presence of brefeldin A. The T cells were stained CD3 and for intracellular cytokines IL-2, IL-4 and IFNγ and analysed by flow cytometry. No IL-4 production was detected (data not shown). IL-2 and IFNγ production are shown (Fig 5.9), where at least 20, 000 events were collected in the CD3$^+$ gate.

As previously observed, only 2-3% of T cells cultured with autologous DC$_{medium}$ divided (Fig 5.9). Of these cells that had undergone at least one division, 50% produced cytokines IFNγ and/or IL-2. DCs stimulated with *N. meningitidis* WT or LPXA, or meningococcal LPS induced a greater number of T cells to proliferate than DC$_{medium}$ (5-9%). In all conditions, approximately 50% of the dividing T cells cultured with stimulated DCs produced IFNγ and/or IL-2. The majority of all the IFNγ and IL-2 producing T cells were non-dividing, though these cytokine producing cells represent only 25% of all the non-dividing cells (Fig 5.9). Twenty six percent of T cells cultured with PHA had divided at least once. However only 20% of the PHA dividing T cells produced cytokines on re-stimulation with PMA and ionomycin compared to 50% of dividing T cells cultured with DCs.
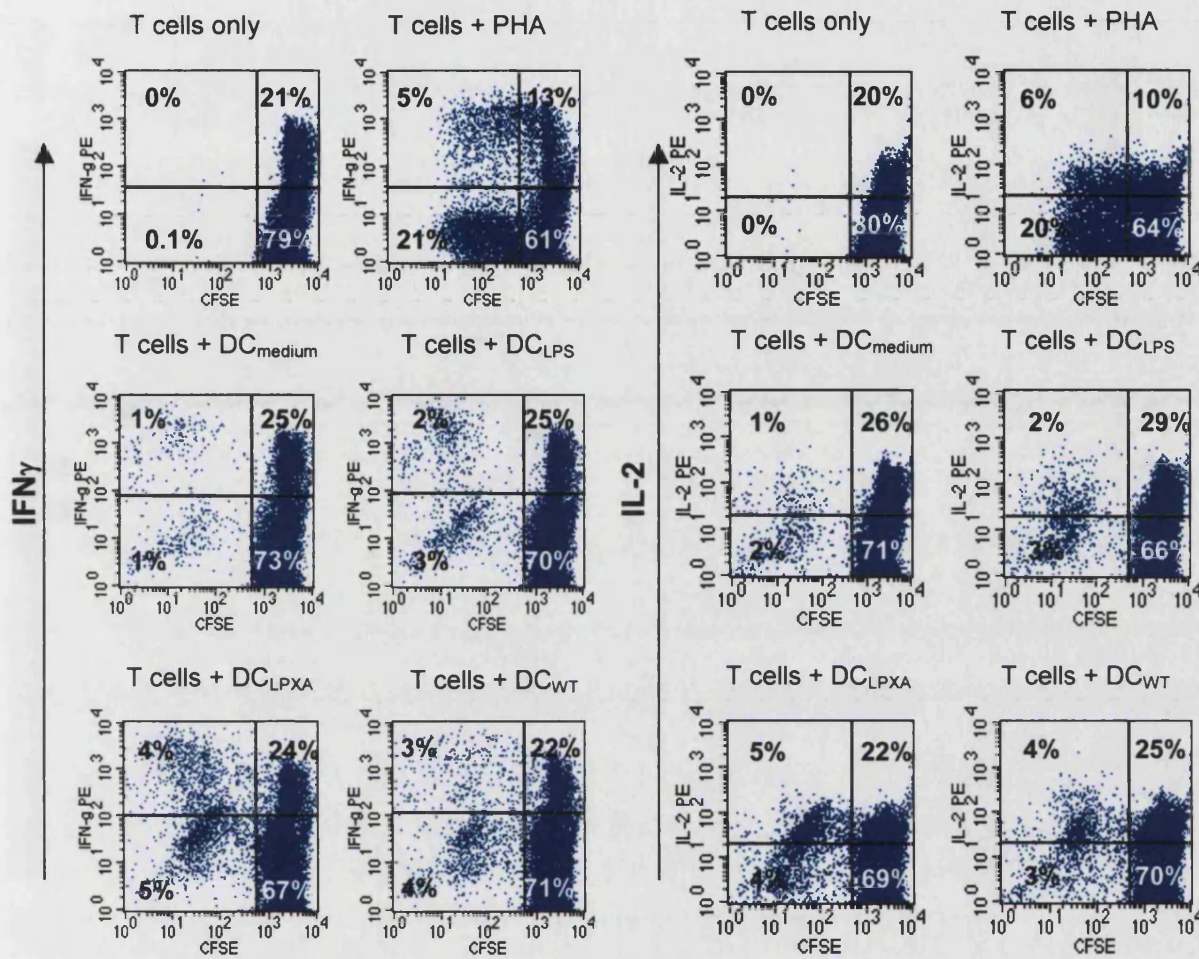
Figure 5.9 Cytokine production by proliferating and non-proliferating T cells
CFSE labelled T cells (10^5) were combined with autologous DCs (10^4) that had been previously stimulated with wild type or *lpxA- N. meningitidis* (10^7 organisms/ml), LPS (10 ng/ml) or medium alone. After 5 days the cells were restimulated for 6 hours with PMA and ionomycin in the presence of brefeldin A, followed by permeabilisation and intracellular staining for IFNγ and IL-2. Cells were stained with CD3 Tri color® and 20,000 events in the CD3$^+$ gate were collected by flow cytometry. Data are representative of 8 experiments. The percentage of positive cells in each quadrant is given.

These experiments showed little difference in cytokine production by T cells cultured with DC_{medium}, DC_{WT} or DC_{LPXA}. However these studies used unfractionated $CD3^+$ cells, which included $CD4^+$, $CD8^+$, $CD45RA^+$, and $CD45RO^+$ T cells, many of which were precomited to specific cytokine production (i.e. $IL-2/IFN\gamma$). The unique function of DCs as professional antigen presenting cells is their ability to prime and activate naïve T cells. The logical next direction was therefore to investigate the ability of DCs to prime and activate naïve T cells after stimulation with *N. meningitidis*.

5.3.3 Dendritic cell activation of naïve helper T cells

DCs that had previously been stimulated with *N. meningitidis* were cultured with purified $CD4^+$ $CD45RA^+$ T cells at ratios from 1:10 to 1:80 DC:T cell. After 5 days proliferation was determined by ^3H –thymidine incorporation and liquid scintillation counting. Prostaglandin E2 (PGE2) was also used to stimulate DCs before culture with naïve T cells as a control for Th2 induction, since PGE2 stimulated DCs are known to promote a Th2 response by naïve T cells (Hilkens C.M. et al., 1995).

DCs induced proliferation of naïve T cells at DC:T cell ratios of 1:10 and 1:20. DC_{WT} and DC_{LPXA} both induced greater T cell proliferation than DCs cultured in medium alone. DCs stimulated with PGE2 did not increase T cell proliferation above that induced by DC_{medium} (Fig 5.10).
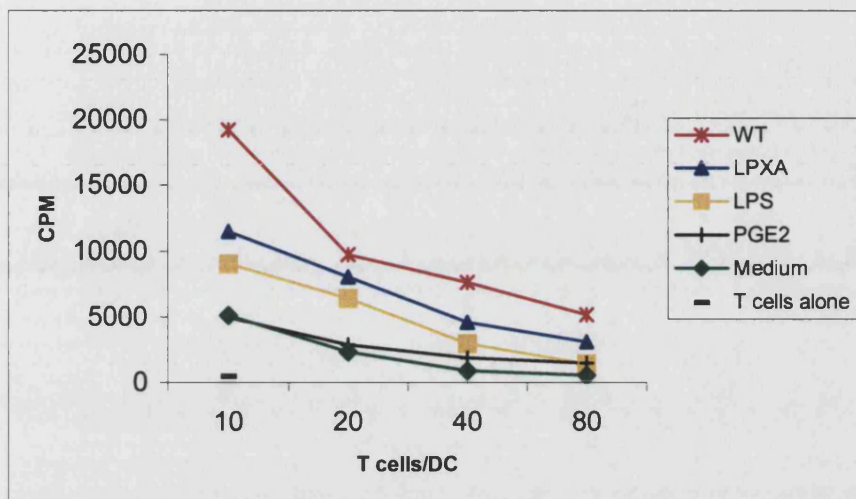
Figure 5.10 Proliferation of naïve T cells after culture with DCs

Purified CD4$^+$CD45RA$^+$ T cells (10^5) were cultured with DCs at DC:T cell ratios of 1:10 to 1:80. DCs were previously stimulated with *N. meningitidis* wild type or *lpxA-* (10^7 organisms/ml), purified meningococcal LPS (10 ng/ml), Prostaglandin E2 (10^{-6} M) or in medium alone. Proliferation was measured by ^{3}H-thymidine uptake after 5 days. Results are expressed as the mean counts per minute (CPM) of triplicate cultures. Data are from one representative experiment.

Naïve T helper cells can develop into a Th1, a Th2 or a Th0 phenotype upon stimulation. Various different methods were tried to determine the effects of DCs activated with *N. meningitidis* on naïve T helper cell development, the most successful of which was the method adapted from (de Jong E.C. et al., 2002). Purified naïve CD45RA$^+$ T cells (2 x 10^4) were cultured with DCs (5 x 10^3) previously stimulated with wild type or *lpxA- N. meningitidis*, purified meningococcal LPS, PGE2 or in medium alone, together with super antigen SEB. The initial culture period with DCs and SEB is to polarise naïve Th cells, followed by an expansion of these cells in IL-2. Cells were restimulated with PMA and ionomycin for 6 hours in the presence of brefeldin A before intra cellular cytokine staining for IL-4, IL-13 and IFNγ (Fig 5.11).

Naïve T cells cultured without DCs produced very little cytokine upon restimulation with PMA and ionomycin (Fig 5.11). T cells that were cultured with DC$_{medium}$ produced cytokines (IL-4, IL-13 & IFNγ) upon restimulation. T cells cultured with DC$_{WT}$ produced more Th1 cytokine (IFNγ) and less Th2 cytokines (IL-4 & IL-13) compared to T cells that were cultured with DC$_{medium}$. In contrast DC$_{LPXA}$ and DC$_{LPS}$ induced a T cell profile similar to that of the DC$_{medium}$. PGE2 is known to induce Th2 polarising effects and was used as a control (Fig 5.11 & 5.12). T cells cultured with DCs stimulated by PGE2 produced more Th2 cytokines and less Th1 cytokines than the medium control. A summary of 10 independent experiments is shown in Figure 5.12.
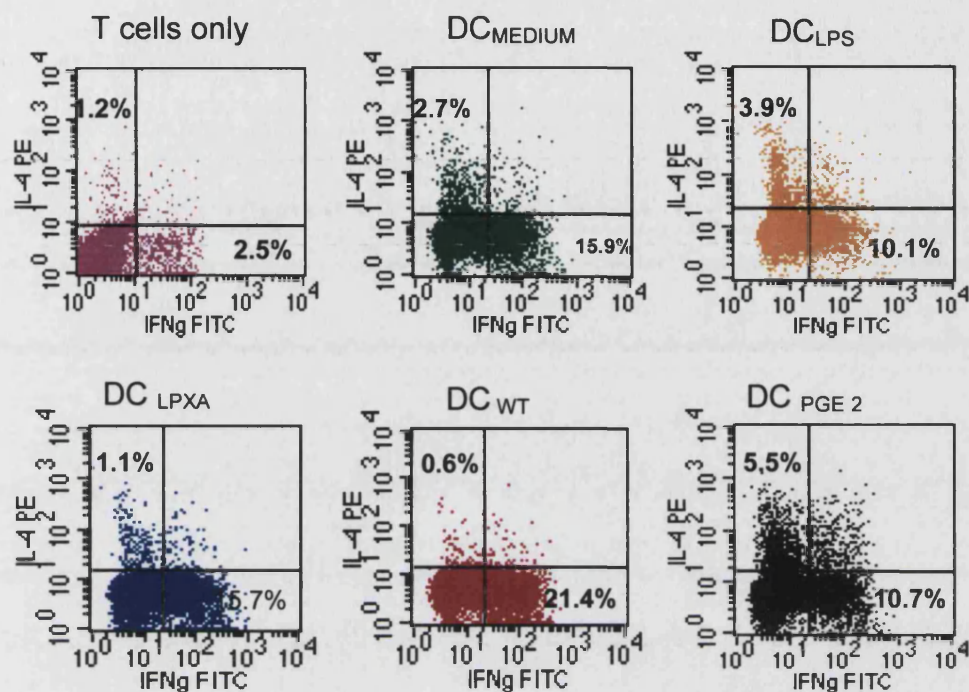
Figure 5.11 IL-4 and IFNγ production by naïve CD45RA⁺ T cells after culture with activated dendritic cells

DCs (5 x 10^3) stimulated with wild type or *lpxA- N. meningtidis*, purified LPS, PGE-2 or medium alone were cultured with purified CD4⁺CD45RA⁺ T cells (2 x 10^4) in the presence of super antigen SEB for five days and then expanded in IL-2 for a further 10 days. The cells were then restimulated for 6 hours with PMA and ionomycin in the presence of brefeldin A followed by permeabilisation and intracellular staining for IL-4 and IFNγ. Data are a representative of 10 experiments; the percentages of positive cells in the quadrants are given.

Figure 5.12 Bar charts representing cytokine production by naïve CD45RA⁺ T cells after culture with dendritic cells.

DCs (5 x 10³) stimulated with wild type or *lpxA- N. meningtidis*, purified LPS, PGE-2 or medium alone were cultured with purified CD4⁺CD45RA⁺ T cells (2 x 10⁴) in the presence of super antigen SEB for five days and then expanded in IL-2 for a further 10 days. The cells were then restimulated for 6 hours with PMA and ionomycin in the presence of brefeldin A followed by permeabilisation and intracellular staining for IL-4, IL-13 and IFNγ. Data are expressed as the mean and SEM of 10 separate experiments. $p < 0.05$ compared to the medium control (*) and wild type *N. meningitidis* (**) are given (paired student t-test).
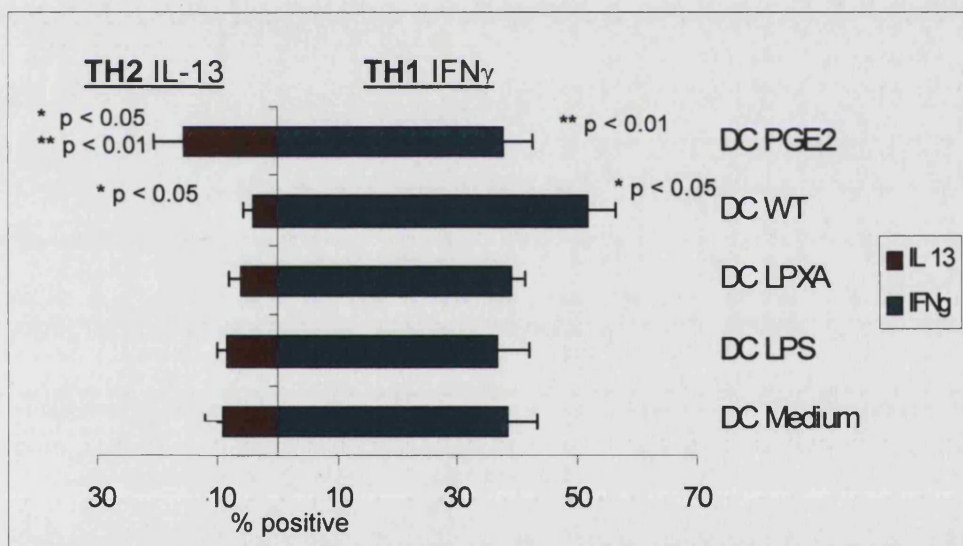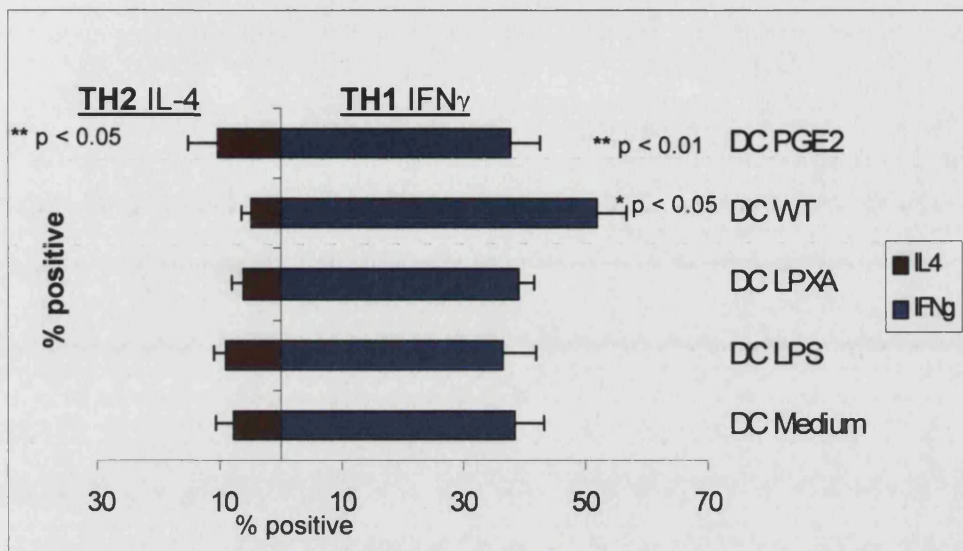
* P values compared with medium control

** P values DC$_{PGE2}$ compared with DC$_{WT}$

5.4 Discussion

Dendritic cells have the capacity to stimulate T cells in both allogeneic and autologous combinations (Crow M.K. et al., 1982)(Scheinecker C. et al., 1998). This stimulatory capacity is much greater than that of other antigen presenting cells such as B cells and macrophages (Crow M.K. et al., 1982). In autologous combinations, DCs stimulated with wild type or *lpxA- N. meningitidis* enhanced T cell proliferation compared to DCs cultured in medium alone. The increase in T cell proliferation in culture with DCs stimulated with bacteria maybe due to the increase of peptide loaded MHC molecules on the surface of DCs interacting with the TCR on T cells and/or an increase in co-stimulatory molecules on the DCs interacting with CD28 on T cells (Bouloc A. et al., 2000)(Howland K.C. et al., 2000)(Scheinecker C. et al., 1998). This is supported by experiments that showed DCs stimulated with wild type or *lpxA- N. meningitidis* had increased surface expression of HLA DR molecules and B7 co-stimulatory molecules CD80 and CD86 (Fig 3.1, chapter 3). The interactions of co-stimulatory molecules on DCs with those on T cells is important for T cell stimulation and activation. (i.e. CD80/CD86 on DCs interacting with CD28 on T cells) (Bouloc A. et al., 2000)(Howland K.C. et al., 2000)(Scheinecker C. et al., 1998)(Wells A.D. et al., 1997). CD40-CD40L interactions have also been shown to play an important role in T cell stimulation. (Howland K.C. et al., 2000), and an increase in CD40 expression by DCs was observed following stimulation with *N. meningtidis*.

Initial experiments to investigate T cell proliferation used ^{3}H-thymidine incorporation. This is a commonly used technique to measure proliferation of *in vitro* cell cultures and gives an overall indication of cell division within the culture during

the period that ^{3}H-thymidine is added. However, this method gives no information on individual cell populations and only cells that divide within the pulse time are detected. One failing of the ^{3}H-thymidine method is that all the cells dividing in the pulse time will incorporate the same amount of ^{3}H-thymidine as half of the cells dividing twice in the same time. It is therefore impossible to know precisely how many cells have divided and at what rate division occurs (Lyons A.B. et al., 1994). An alternative method is labelling with CFSE as it documents all of the divisions that occur during the culture period, and each generation of cells can be identified.

The overall results obtained with CFSE staining were similar to those observed by ^{3}H-thymidine incorporation but this technique provided additional information. The CFSE staining revealed that a small population of T cells cultured with DCs divided many times, as opposed to the whole T cell population dividing just a few times during the culture period. This was seen for T cells in both autologous and allogeneic combinations. In contrast, T cells stimulated with PHA were shown to form many peaks of decreasing CFSE labelling. The reason for the different profiles obtained with MLR and PHA is not clear. Most of the T cells enter into at least one division with PHA, but it is not known if T cells stop after a certain number of divisions or if there is a delay in T cells entering division with PHA stimulation to form these characteristic peaks of division.

DCs stimulated with wild type *N. meningitidis* at 10^5, 10^6 and 10^7 organisms/ml induced T cell proliferation significantly above controls with DC_{medium}. DCs stimulated with *lpxA-* at 10^7 organisms/ml also induced induced significant T cell proliferation compared to controls. Whereas DCs stimulated with 10^5 or

10^6 organisms/ml of *lpxA-* did not induce significant T cell proliferation. This is consistent with DC maturation observed in chapter 3. The wild type *N. meningitidis* induced DC maturation at concentrations as low as 10^5 organisms/ml, where as at low concentrations (less than 10^6 organisms/ml) *lpxA-* was shown not to activate DCs.

Both CD4$^+$ and CD8$^+$ T cells were found to proliferate in combination with DCs. As might be expected, the proliferating T cells expressed the IL-2 receptor (CD25$^+$) and CD45RO, an activation marker found on effector/memory T cells, and were negative for CD45RA. Since purified CD45RA$^+$ T cells were shown to proliferate following stimulation by DCs (Fig 5.10), it is likely that CD45RA$^+$ T cells convert to a CD45RO$^+$ phenotype following stimulation. There was no detectable difference in expression of surface markers on T cells cultured with DCs in autologous or allogeneic combinations or when DCs were stimulated with wild type or *lpxA- N. meningitidis*. However some of the proliferating T cells responding to DC$_{lpxA}$ were CD45RA$^+$. These may have been double positive cells as only 0.5% of proliferating cells were negative for CD45RO.

Naïve CD4$^+$ T cells have the potential to differentiate into IFNγ producing (Th1) or IL-4 producing (Th2) cells. It has been suggested that a third signal is needed for T cell polarisation (Kalinski P. et al., 1999). It was not possible to use antigen specific T cell responses alone to investigate cytokine responses in naïve T cells, as the number of antigen specific naïve T cells is too small. Therefore to investigate cytokine responses in naïve T cells a system was used where engagement of the TCR by allogeneic MHC surrogated the peptide specific response. This signal was further

enhanced by the binding of super antigen SEB. This is signal 1 from the 3 signal

hypothesis (Kalinski P. et al., 1999). Signal two is produced by co-stimulatory

molecules and is dependent on the maturation state of the DCs induced by pathogen

stimulation. The third polarising signal is not fully understood, but is also thought to

be pathogen specific and produced by the DCs. IL-12 is a known Th1 stimulator.

Individuals with a mutation in their IL-12 receptor have impaired Th1 responses and

are prone to infections by intracellular bacteria (de Jong R. et al., 1998). Studies with

neutralising antibodies (Giacomini E. et al., 2001) and knockout mice (MacDonald

A.S. et al., 2002a) have also shown IL-12 is essential for good Th1 development.

IL-18 and type 1 interferons (IFNα/β) are also factors involved in Th1 development

(Giacomini E. et al., 2001). Wild type *N. meningitidis* is a strong inducer of DC

maturation and IL-12 production (as shown in Chapter 3). This could explain the Th1

biased response in naïve T cells activated by DC_{WT}. The LPS deficient *lpxA*- does

induce DC maturation but without IL-12 production (section 3.3.3). The T cell

response to DC_{lpxA} was similar to that induced by DC_{medium} with neither a Th1 or Th2

bias. Purified meningococcal LPS also induced DC maturation without IL-12

production, and the naive T cell response to DC_{LPS} was also neither a Th1 nor a Th2

response, but similar to that of T cells cultured with DC_{medium}. In contrast DC_{PGE2} did

induce a Th2 bias in responding T cells. Interestingly, PGE2 increased DC surface

marker expression, including co-stimulatory molecules and HLA-DR, but there was

no stimulate detectable DC cytokine production (Chapter 3).

The DC signal for Th2 development is not clear, though several co-stimulatory

molecules including CD28-CD80/CD86 and CD40-CD40L are known to play a role

(de Jong E.C. et al., 2002). Th2 responses have been considered to be a default

process that occurs when T cells are stimulated without Th1 inducing cytokines. Consistent with this hypothesis, *Leishmania major* usually induces a Th1 response, but results in a Th2 response in IL-12 knock out mice (Mattner F. et al., 1996). In contrast it has been shown that an extract from a filarial worm will induce a Th2 response *in vitro* in the presence of IL-12 (Whelan M. et al., 2000). Also IL-12 knock out mice infected with non-lethal doses of *Toxoplasma gondii* or *Mycobacterium avium* were found not to develop a Th2 response (Jankovic D. et al., 2002). This suggests that a Th2 response is not the default pathway occurring in the absence of IL-12 and might explain why DCs stimulated with *lpxA*- expressed high surface MHC and co-stimulatory molecules and produced inflammatory cytokines, but not IL-12, did not a induce a strong Th1 nor a Th2 response.

Though significant, the changes in T cell cytokine production between those stimulated with DC_{WT}, DC_{PGE2} and DC_{medium} were not large. There are several potential explanations for this. Firstly variation between individual donors may disguise the overall effect. Responses of some individuals are naturally biased towards Th1 whilst others are Th2 biased (Hammad H. et al., 2001). Also other cytokines, such as IL-18 and IFNα, produced by DCs may be involved in inducing a Th1 response but these were not measured. It is important to note however, that although these differences between DCs stimulated in different ways were not large, they were reproducible. Also published literature using similar techniques have found noteable but not large differences in T cell differentiation (de Jong E.C. et al., 2002).

In summary we found that DCs activated with the wild type *N. meningitidis* stimulated T cells to proliferate and induced a Th1 biased response in naïve T cells, whereas DCs stimulated with *lpxA-* induced T cell proliferation but not a Th1 response. The lack of polarisation seen with *lpxA-* or LPS stimulated DCs is probably a consequence of the low or absent IL-12 production by these DCs.

Chapter 6

Dendritic Cell Expression of Toll-Like Receptors (TLRs)

6.1 Introduction

The recent discovery of the Toll-Like Receptor family (TLRs) has transformed our understanding of the recognition of pathogens by the innate immune system. Since the discovery of this family, much work has been done to characterise ligands that activate TLRs. Many different molecules expressed by pathogens have been found to signal through various TLRs, including LPS, lipoproteins, bacterial DNA and flagellin, though much of this work has been performed on transfected cell lines.

Human myeloid DCs have been shown to express mRNA for TLRs 1, 2, 3, 4, 5, 6, 8 and 10, but not TLRs 7 or 9 (Kadowaki N. et al., 2001)(Visintin A. et al., 2001). Studies using transfected HeLa cells have shown that both TLR4 and TLR2 are important for the recognition and signalling of wild type *N. meningitidis*. However the LPS deficient *lpxA*- organism however was found to signal through TLR2 and not TLR4 (Ingalls R.R. et al., 2001)(Pridmore A.C. et al., 2001). These findings suggest that TLR2 and TLR4 are both likely to be involved in DC recognition of *N. meningitidis*.

Due to the lack of antibodies for many of the TLR family members, much of the work analysing expression in primary cells has been by PCR or Northern blots to detect mRNA. Though mRNA levels can be a good indicator of protein, the correlation is not always clear. Factors such as mRNA stability and post translation modification may effect over all protein levels. Also detection of mRNA in cells does not give any clues as to the eventual location of the protein. The development of polyclonal antibodies specific for TLRs 1-4 by colleagues at GSK meant that expression of TLRs by DCs could be investigated at the protein level.

There is a naturally occurring polymorphism in the human *tlr4* gene, which is known to disrupt TLR4-mediated responses to *E. coli* LPS (Arbour N.C. et al., 2000). This polymorphism was used to further investigate the functional role of TLR4 signalling in response to *N. meningitidis*. The polymorphism consists of two point mutations in the Human TLR4 gene that co-segregate. The first is a substitution of A (adenine) to G (guanine) resulting in amino acid 299 changing from aspartic acid to glycine. The second is a cystine to thymine change, resulting in a change from threonine to isoleucine at amino acid 399. Both mutations are in the fourth exon of TLR4, which codes for part of the extra cellular domain. These polymorphisms were found to occur with an allelic frequency of 6.6% in the studied population (Arbour N.C. et al., 2000). Individuals with this phenotype had an impaired response to inhaled LPS. Moreover THP-1 cells transfected with mutated TLR4 Asp299Gly had impaired NFκB signalling, though the Thr399Ile had no effect (Arbour N.C. et al., 2000). Unlike the mouse TLR4 mutation that is in the intracellular TIR domain, the human TLR4 polymorphisms occur in the extracellular region and their ability to disrupt TLR4 signalling is not yet understood.

This next chapter aims to investigate TLR expression on dendritic cells and the relevance for responding to *N. meningitidis* in individual with the TLR4 polymorphism Asp299Gly.

6.2 Methods

6.2.1 Staining of blood leukocytes and dendritic cells with polyclonal antibodies against Toll-Like Receptors 1-4

There are few commercially available antibodies against the TLR family. Dr. Ginnette

Squires and her team at GSK have produced and affinity purified rabbit polyclonal

antibodies to human TLRs 1, 2, 3 and 4 raised against TLR peptides. The peptides used

for immunisation mapped to the following sequences within the extra cellular domain of

each TLR (table 6.1). The specificity of these antibodies has been proven by ELISA,

fluorescent immunohistochemistry, Western blotting and specific blocking assays as

reported (Fenhalls G. et al., 2002).

	Position	Amino acid Sequence	Stock
TLR1	170-194	VLGETYGEKEDPEGLQDFNTESLHI	50 µg/ml
TLR2	295-321	FRASDNDRVIDPGKVETLTIRRLHIPR	260 µg/ml
TLR3	145-169	KQKNLITLDLSHNGLSSTKLGTQVQ	45 µg/ml
TLR4	223-245	FKEIRLHKLTLRNNFDSLNVMKT	152 µg/ml

Table 6.1 Peptide sequence for human TLR antibody production
Peptide sequences used to produce antibodies against human TLR proteins by rabbit immunisation. Antibodies were affinity purified on peptide columns

Antibody for each of the TLRs or rabbit IgG was added at a final concentration of

10 µg/ml to 200 µl of blood diluted 1:1 in RPMI 1640 or to day 6 monocyte derived

DCs at 5×10^5 cells/ml and incubated on ice for 30 minutes. The cells were then washed

with cold FACS wash and the second layer of FITC or PE conjugated F(ab')$_2$ goat anti

rabbit IgG added at 5 µg/ml for 30 minutes on ice in the dark. The samples were washed

once again with cold FACS wash. Erythrocytes in blood samples were lysed with 1 ml

Lysing Solution for 1-2 minutes until the samples became translucent. The cells were

washed again in cold FACS wash followed by fixation in CellFix solution. Fixed cells

were analysed by flow cytometry on a FACScalibur using CellQuest software. For

whole blood leukocytes, at least 3000 events were collected within the gate

corresponding to the forward and side scatter characteristics of monocytes. For DCs at

least 5000 events within the DC gate were collected.

6.2.2 Intracellular Dendritic Cell TLR staining

Day 6 DCs were fixed in 1 ml of 2% paraformaldehyde in PBS for 15 minutes and then

permeabilised by washing twice in saponin wash solution (HBSS with 0.1% saponin,

2mM Hepes buffer and 0.02% Sodium Azide). To block non specific binding DCs were

incubated with 10% human serum in saponin wash for 15 minutes at room temperature,

then washed once again in saponin wash and resuspended to 5×10^5 cells/ml and

transferred in 200 µl aliquots to 5 ml Falcon tubes. TLR antibody or normal rabbit IgG

was added to each of the tubes at a final concentration of 10 µg/ml and incubated for

30 minutes at room temperature. DCs were washed twice in saponin wash and the

second antibody layer of biotinilated F(ab')$_2$ goat anti-rabbit IgG was added at 5 µg/ml

for 30 minutes, followed by two further washes with saponin wash. The final layer of

streptavidin conjugated PE, FITC or cy5 was added for a further 30minutes in the dark

at room temperature. The DCs were then washed twice in saponin wash, fixed with

200 µl CellFix and analysed by flow cytometry. At least 5000 events within the DC gate

were collected.

Confocal microscopy was also used to analyse intracellular TLR staining. Approximately 10 µl (about 5000 DC) of cell suspension stained intracellularly with TLR antibodies were dropped on to polylysine coated microscope slides and left to air-dry overnight at room temperature in the dark. The cells were mounted with citifluor and sealed with a cover slip. Confocal images were obtained using a Leica SP2 scanning microscope system fitted with appropriate filters sets. Between 10 and 20 optical sections of approximately 0.5 µm spanning the entire DC were taken. Images were projected and superimposed with Leica confocal imaging software.

6.2.3 TLR polymorphisms

Two co-segregating missense mutations in the human TLR 4 gene have been described (Arbour N.C. et al., 2000). Of these polymorphisms, the Asp299Gly interrupted TLR4-mediated LPS signalling. A screen for this mutation was performed on blood samples from 50 healthy volunteers using a simple PCR (Polymerase Chain Reaction) based method and restriction enzyme digest. DNA was extracted using a QIAamp Blood mini kit from 200 µl samples of blood collected by venesection, according to the manufacturer's instructions. Genomic DNA was amplified by PCR using 12.5 µl of aqueous DNA (approximately 10 ng/µl), 0.75 µl of each of the primers, 5 µl of dNTP mix (2 mM each of dATP, dCTP, dGTP, and dTTP) 5 µl of 10X PCR buffer, 3 µl of $MgCl_2$, 0.5 µl AmpliTaq Gold DNA polymerase, and sterile water to make the volume up to 50 µl. PCRs were carried out at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1 minute. These cycles were followed by a final extension step of 72 °C for 10 minutes. Primer pairs designed

specifically to incorporate the 896-nucleotide substitution were ordered from Sigma-

Genosys

Forward   GAAATGAAGGAAACTTGGAAAAGTT

Reverse   CATTTGTCAAACAATTAAATAAGTCAATAGTA

The presence of PCR product was confirmed by running 5 μl of product with 1 μl of

loading dye on a 2% agarose gel containing 0.5 μg/ml ethidium bromide in 1X TBE

running buffer for 1 hour. The gel was visualised under UV light.

Enzyme digests were carried out in 20 μl reactions with 14.6 μl of amplified PCR

product, 0.4 μl BSA, 1 μl of Rsa 1 enzyme and 4 μl of corresponding buffer. The

reaction was incubated at 37 °C for 2 hours. Products were analysed by running 15 μl of

enzyme product with 3 μl of loading dye along side 15 μl of un-cut PCR product on a

2% agarose gel containing 0.5 μg/ml ethidium bromide in 1X TBE running buffer for

1 hour. The gel was visualised under UV light.

6.2.4 Monocyte TNFα and IL-6 production in response to LPS

The function of the TLR4 polymorphism was tested by monocyte responses to LPS

using a whole blood assay (Uronen H. et al., 2000). One ml of blood was taken by

venesection from TLR4 Asp299Gly and healthy human volunteers known not to have

this polymorphism. The blood was transferred to a heparinized 15 ml sterile falcon tube

(10 IU/ml sodium heparin) and diluted in an equal volume of RPMI-1640 medium. The

cells were stimulated with 10^7 organisms/ml of *N. meningitidis* wild type or *lpxA-*, or

with 10 ng/ml purified meningococcal LPS in the presence of brefeldin A (10 μg/ml)

for 4 hours at 37 °C. Intracellular TNFα and IL-6 was detected as in section 2.3.6.

6.3 Results

6.3.1 Expression of TLRs by blood leukocytes

Blood leukocytes were stained with polyclonal rabbit antibodies against human TLRs 1,

2 and 4. Following lysis of erythrocytes, cells were analysed on the FACScalibur and

the different populations gated on forward and right angle scatter (Fig 6.1).

In preliminary experiments gate R1 was shown to contain mainly $CD14^+$ cells

(monocytes), gate R2 to contain $CD3^+$ and $CD19^+$ cells (T and B lymphocytes) and gate

R3 to contain $CD66a^+$ cells (neutrophils). Monocytes and neutrophils (gates R1 and R3)

expressed TLR1, TLR2 and TLR4. Whereas some lymphocytes (gate R2) expressed

very low levels of TLR1 and TLR2, but all were negative for TLR4 (Fig 6.1).

6.3.2 Expression of TLRs on the surface of Dendritic Cells

Day 6 immature Dendritic cells were stained for expression of TLRs 1, 2, 3 and 4 using

the rabbit polyclonal antibodies and a second layer of PE conjugated $F(ab')_2$ goat anti

rabbit IgG. No surface expression of TLRs 1, 2, 3 or 4 could be detected on DCs (Fig

6.2).
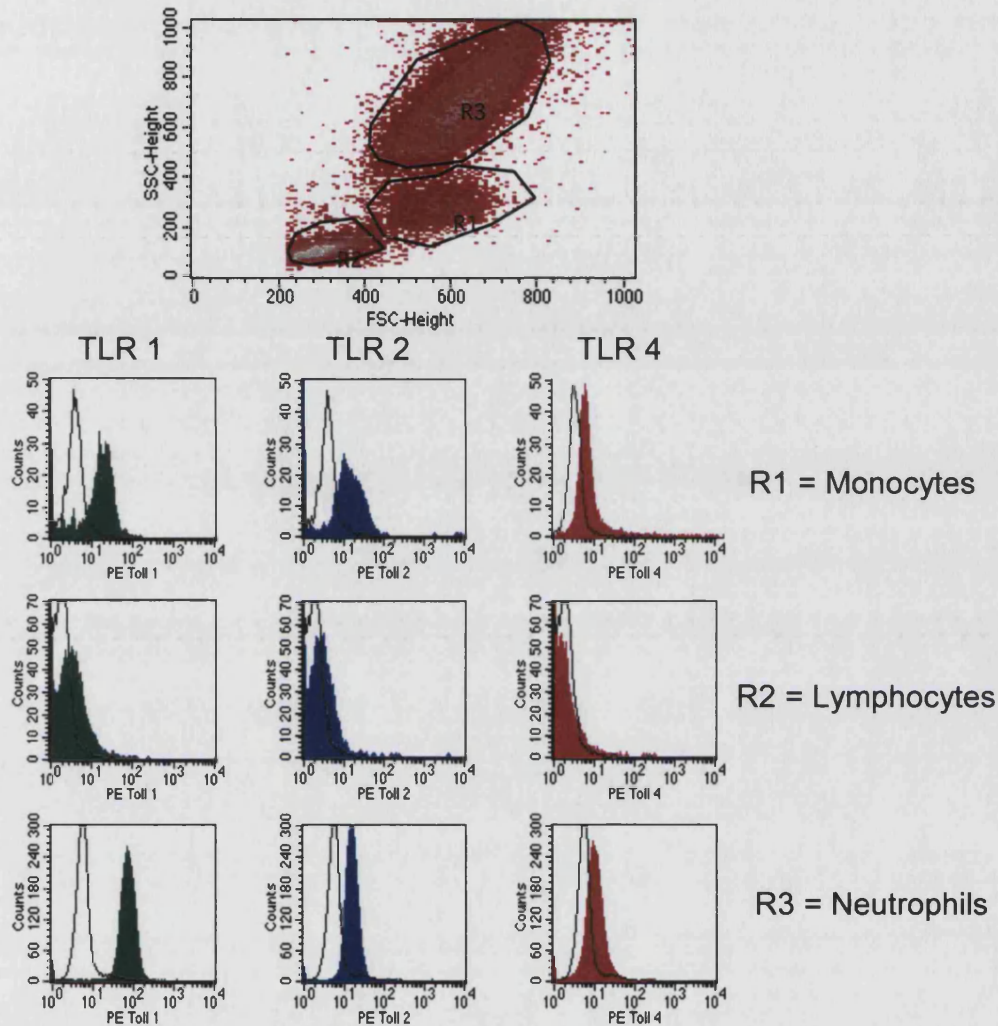
Figure 6.1 Expression of TLR 1, 2 and 4 on cells in whole blood

Whole blood was stained with polyclonal antibodies to TLRs with a PE conjugated F(ab')₂ goat anti rabbit IgG second layer. Three distinct cell populations were identified by their forward and right angle scatter. Expression of TLR 1, 2 and 4 are shown (filled histograms) compared to normal rabbit IgG used as a control (solid lines). Data are a representative of 3 independent experiments.
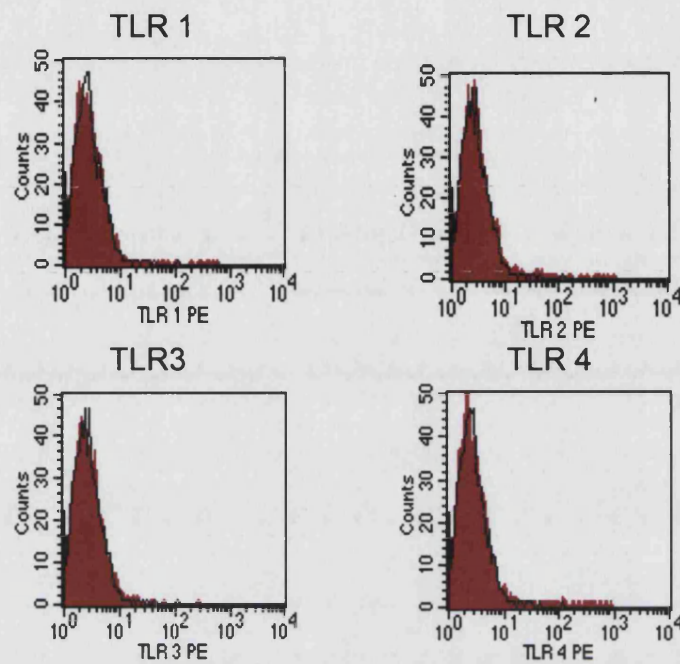
Figure 6.2 Surface staining for Toll Like Receptors on dendritic cells

Day 6 immature DCs were stained with polyclonal antibodies to TLRs 1, 2, 3 and 4 with a PE conjugated second layer. DCs were gated according to size and granularity. Expression of TLRs 1, 2, 3 and 4 are shown (filled histograms) along with normal rabbit IgG used as a control (solid lines). Data are a representative of 6 independent experiments.

6.3.3 Surface expression of TLRs on dendritic cells following stimulation with *N. meningitidis*

There have been some reports that DC TLR expression changes following stimulation (Visintin A. et al., 2001). To determine whether this could explain the absences of TLRs on immature DCs, DCs were stained for expression of TLR2 and TLR4 after activation. There was no surface expression of TLR2 or TLR4 following stimulation with *N. meningitidis* wild type, *lpxA-* or purified meningococcal LPS (Fig 6.3).

6.3.4 Intracellular TLR staining in dendritic cells

No significant TLR expression was detected on the surface of DCs, however mRNA analysis has shown that DCs do express RNA for TLRs 1-5 (Thoma-Uszynski S. et al., 2000)(Visintin A. et al., 2001). Also there have been reports of intracellular TLR expression in epithelial cells and macrophages. This suggests that TLRs may be expressed internally by DCs. To see whether TLR expression could be detected intracellularly, immature monocyte derived DCs were fixed in 2% paraformaldehyde and permeabilised with saponin and stained with the rabbit polyclonal antibodies for TLRs 1-4.

DCs were found to express intra cellular TLRs 1, 2, 3 and 4 by flow cytometry (Fig 6.4). Activation of DCs with *N. meningitidis* had no effect on the intracellular expression of TLRs 1, 2, 3 and 4 as measured by FACS (data not shown).

The location and distribution of the TLRs within DCs was investigated by confocal microscopy. Strong intracellular staining of TLRs 1, 2, 3 and 4 in DCs was observed by confocal scanning microscopy. With all the TLRs the staining appeared to be throughout the cytoplasm with areas of high density found in the para-nuclear region, especially with TLR 4 (Fig 6.5).
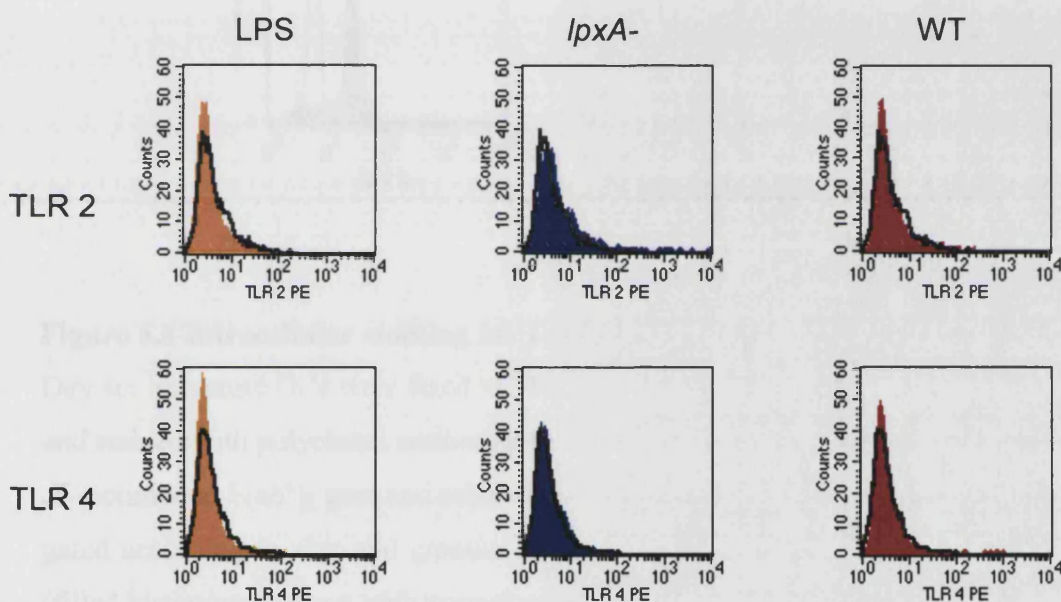
Figure 6.3 TLR expression in dendritic cells following stimulation with *N. meningitidis*

Day 6 immature DCs were cultured for 6 hours in the presence of medium alone, 10 ng/ml of purified meningococcal LPS, or 10^7 organisms/ml of wild type or *lpxA- N. meningitidis*. The cells were then stained with polyclonal antibodies for TLRs 2 and 4 with a PE conjugated second layer. Expression of TLRs following stimulation (filled histograms) is compared to DCs cultured in medium alone (solid lines). Data are representative of 5 independent experiments.
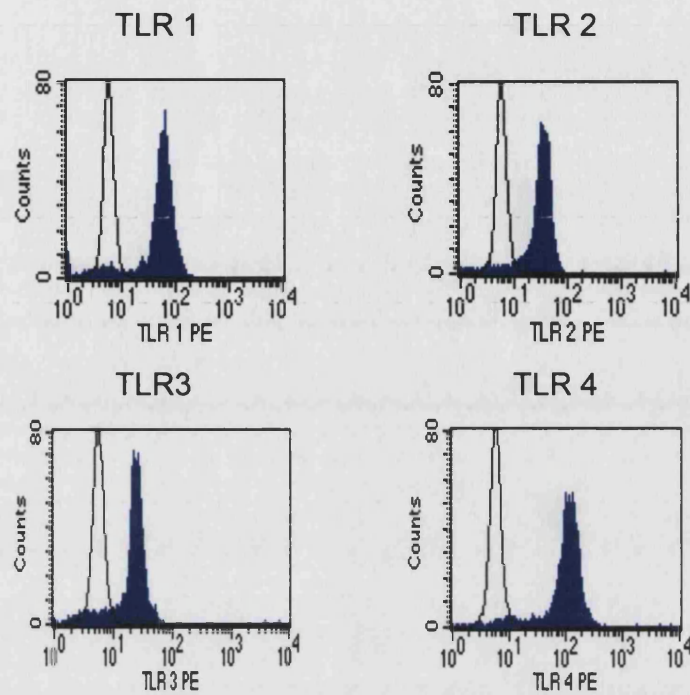
Figure 6.4 Intracellular staining for Toll Like Receptors in DCs

Day six immature DCs were fixed in 2% paraformaldehyde, permeabilised with saponin and stained with polyclonal antibodies to TLRs 1, 2, 3 and 4 followed by a second layer of biotinilated $F(ab')_2$ goat anti rabbit IgG and by streptavidin conjugated PE. DCs were gated according to size and granularity. Expression of TLRs 1, 2, 3 and 4 are shown (filled histograms) along with normal rabbit IgG used as a control (solid lines). Data are a representative of 6 independent experiments.

Figure 6.5 Intracellular dendritic cell expression of TLRs

Day 6 immature DCs were fixed in 2% paraformaldehyde, permeabilised with saponin and stained with polyclonal antibodies to TLRs 1, 2, 3 and 4 followed by a second layer of biotinilated $F(ab')_2$ goat anti rabbit IgG and then streptavidin conjugated FITC. Normal rabbit IgG was used as a control. Staining is throughout the cytoplasm for all TLRs, with areas of high density found in the para-nuclear region (white arrows). Data are a representative of at least 6 independent experiments.

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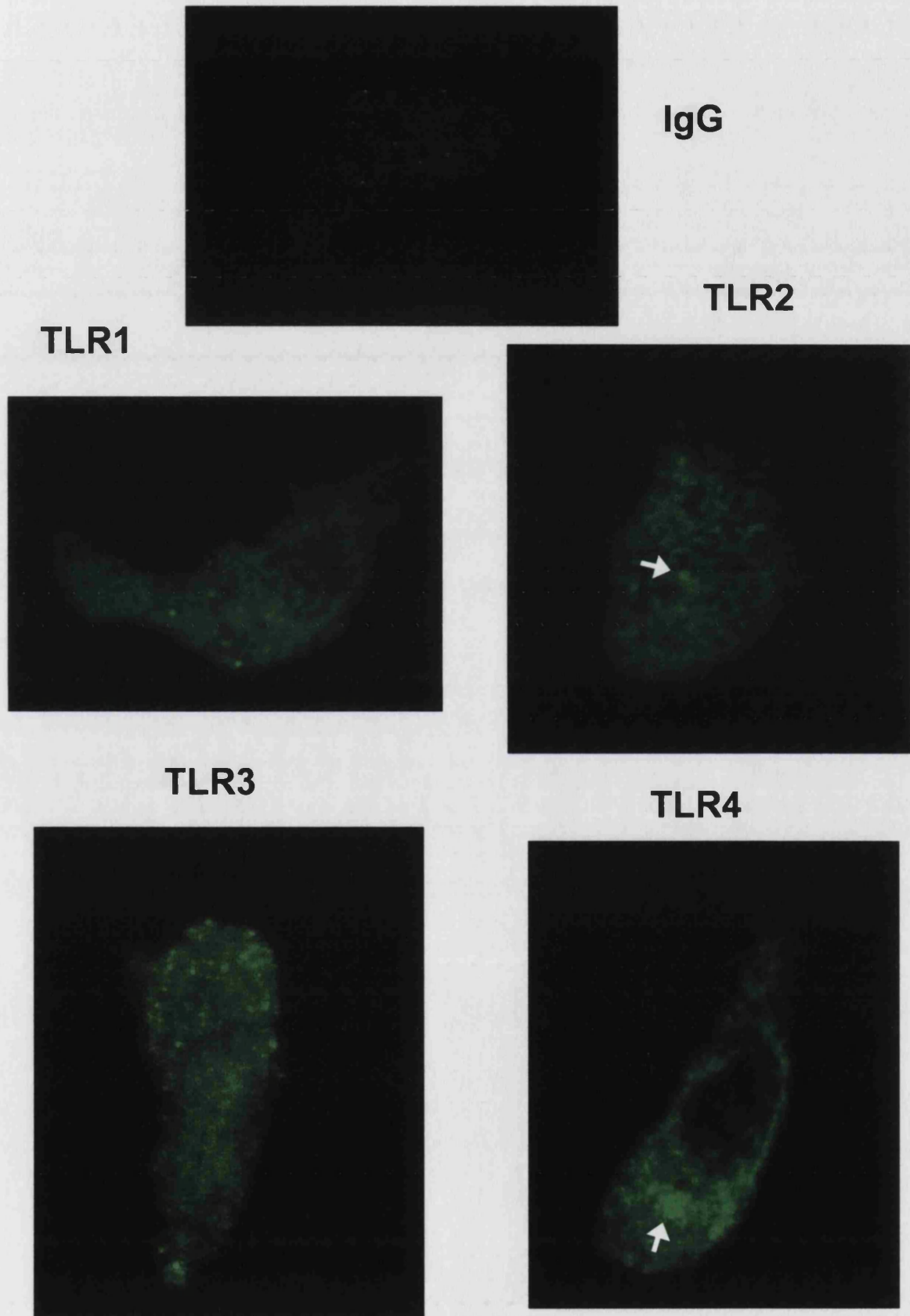
Figure 6.6 TLR expression in dendritic cells after phagocytosis of *N meningitidis*

Day 6 DCs were incubated with 10^8 organisms/ml of FITC labelled wild type *N. meningitidis* (green) for 2 hours. The DCs were fixed in 2% paraformaldehyde permeabilised with saponin and stained with polyclonal antibodies to TLRs 2 & 4 and then a second layer of biotinilated F(ab')$_2$ goat anti rabbit IgG followed by streptavidin conjugated cy5 (red).

TLR 2
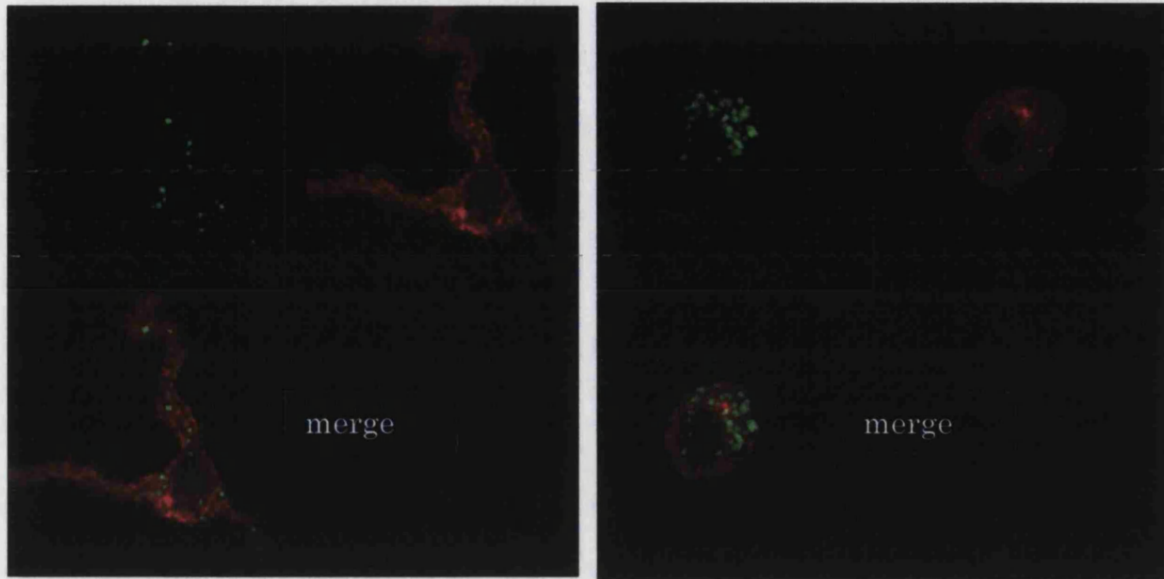
TLR 4
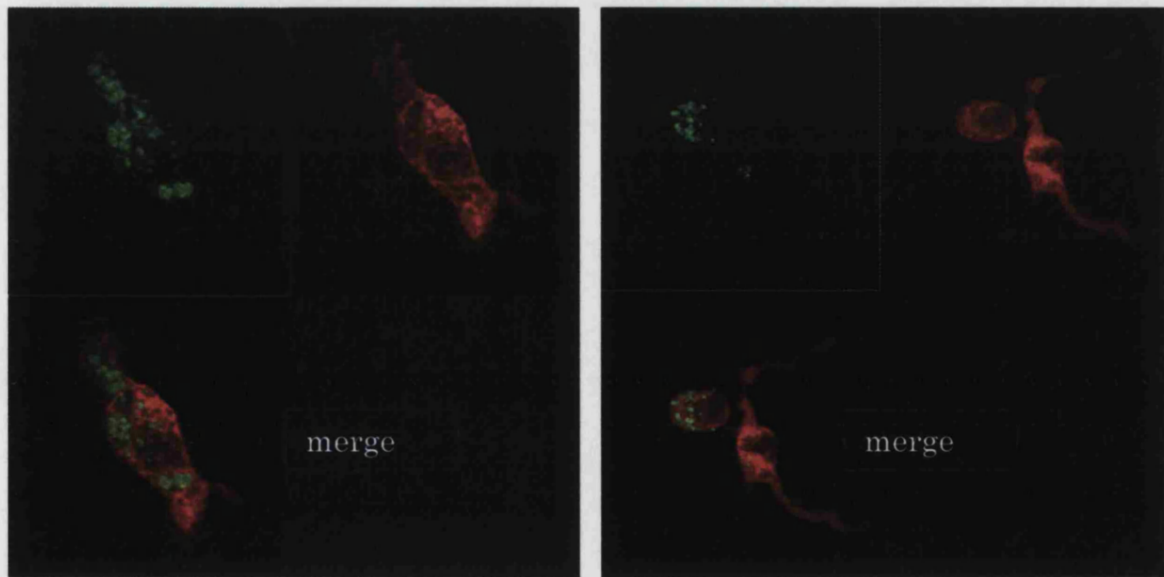
To investigate the intracellular TLR location and distribution in relation to phagocytosed bacteria, DCs were stimulated with 10^8 organisms/ml of FITC labelled wild type *N. meningitidis* and TLR expression examined by confocal microscopy. As shown in Fig 6.6, there was wide a distribution of the FITC labelled bacteria and TLRs 2 and 4 inside the DCs. However there was no evidence of co-localisation as would be indicted by yellow colour in merged images (Fig 6.6).

6.3.5 TLR 4 Polymorphism

In order to investigate the potential role of TLR4 in *N. meningitidis* recognition and signalling the functional human TLR4 polymorphism was investigated.

6.3.5.1 Detection of theTLR4 polymorphism by PCR and enzyme digestion

The Asp399Gly polymorphism in *tlr4* was detected using simple PCR followed by restriction enzyme digestion. The PCR was designed so that the product would incorporate a restriction enzyme site for Rsa 1 only if the original DNA template contained the TLR4 polymorphism. Prior to digestion, the PCR product was 138 base pairs (bp). When the polymorphism was present, digestion with Rsa1 resulted in a product of 118 bp and a 20 bp fragment that was lost from the gel.

Individuals without the polymorphism were identified by the presence of a single band at 138 bp (lanes 1-5, 7-11 & 13 Fig 6.7). Heterozygous individuals had two bands seen at 138 bp and 118 bp (lanes 6, 12&14 Fig 6.7). Homozygous individuals would have one band at 118 bp assuming complete digestion but none were found in the population studied.
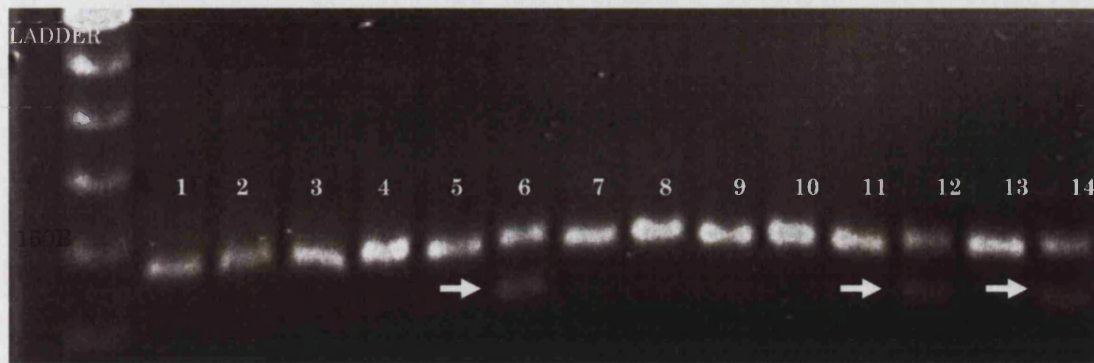
Figure 6.7 Detection of the *tlr*4 polymorphism by restriction enzyme digestion

The region containing the TLR4 polymorphism was amplified by PCR, followed by restriction enzyme digestion by Rsa1. The PCR product was 138 bp and the digested product, when the polymorphism was present, was 118bp (white arrows). The digested fragment of 20bp was not seen. Odd numbered lanes contain PCR product before digestion, even numbered lanes contain the enzyme digest product.

Of the 50 healthy adult human volunteers analysed for this TLR4 polymorphism, 4 heterozygous individuals for polymorphism Asp399Gly were identified. This corresponds to an allelic frequency of 4%, which compares with the allelic frequency of 3.3-7.9% described in the literature (Arbour N.C. et al., 2000).

6.3.5.2 Effect of TLR4 polymorphism on monocyte responses to LPS and *N. meningitidis*

The functionality of the TLR4 polymorphism was tested by monocyte cytokine production in response to LPS or *N. meningitidis* bacteria. Whole blood collected from Asp299Gly individuals and from healthy volunteers known not to have the polymorphism was cultured with 10^7 organisms/ml wild type or *lpxA- N. meningitidis*, or with 10 ng/ml purified meningococcal LPS for 4 hours. Intracellular TNFα and IL-6 production was measured in $CD14^+$ monocytes (Fig 6.8)

There was no detectable difference in cytokine production by monocytes from individuals with the TLR polymorphism compared to wild type controls in response to purified meningococcal LPS or the wild type *N. meningitidis* (fig 6.8). However the response to *lpxA-* was more varied between the controls, with 2 out of 3 individuals having a response lower than the polymorphic individuals (Fig 6.8).
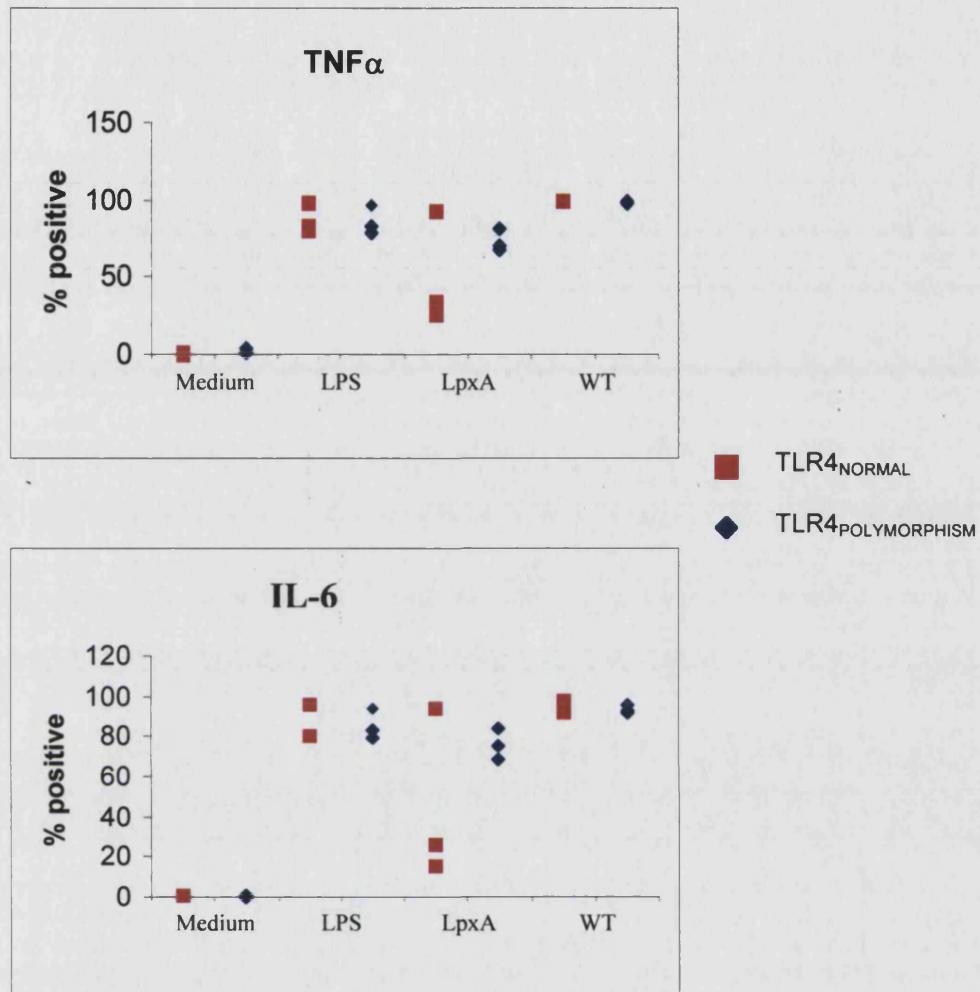
Figure 6.8 Effect of TLR4 Asp399Gly polymorphism on TNFα and IL-6 production in monocytes

Whole blood from individuals with and with out the TLR4 polymorphisms was cultured with 10^7 organisms/ml of wild type or *lpxA- N. meningitidis*, purified meningococcal LPS (10 ng/ml) or in medium alone for 4 hours. Following erythrocyte lysis, cells were stained for intracellular TNF and IL-6 production. At least 3000 events were collected in the CD14$^+$ gate. Data are representative of 3 individual experiments.

6.4 Discussion

The first part of this chapter describes TLR expression in DCs and other cells. Monocytes were found to express surface TLR1 TLR2 and TLR4, agreeing with other studies although expression can be highly variable between different donors, ranging from undetectable levels to a few thousand TLR4 molecules per cell (Visintin A. et al., 2001). Neutrophils were found to express surface TLRs 1, 2 and 4, whilst lymphocytes expressed very low levels of these molecules. These findings are consistent with mRNA analysis of TLR expression which found PMNs and monocytes expressed TLRs 1, 2, 3, 4 & 5, and T cells, B cells and NK cells only expressed low levels of TLR1 mRNA (Muzio M. et al., 2000a)(Muzio M. et al., 2000b).

TLR expression has been studied during the differentiation of DCs from monocytes in culture with GMCSF and IL-4. During differentiation, mRNA levels for TLRs 1, 2, 4 & 5 decreased, whilst mRNA levels of TLR3 and MD-2 increased (Thoma-Uszynski S. et al., 2000)(Visintin A. et al., 2001). RNA levels of TLRs in immature DCs have been reported to increase transiently following activation in the first 3 hours after which mRNA levels decreased (Visintin A. et al., 2001). In the absence of appropriate antibodies, surface expression of DC TLRs has not been generally described. Using polyclonal TLR antibodies raised against peptides, no surface expression for any of the TLRs tested could be detected on DCs (Figs 6. and 6.). Furthermore no surface TLR2 or TLR4 expression was detected on DCs following stimulation with wild type or *lpxA- N. meningitidis* or purified meningococcal LPS.

The lack of detectable surface TLRs on immature and activated DCs described here together with the many reports of TLR mRNA expression in DCs as well as reports of

intracellular TLR detection in other systems (Hornef M.W. et al., 2002)(Underhill D.M. et al., 1999) led to the investigation of intracellular TLRs in DCs. Intracellular TLR 1, 2, 3 and 4 were all readily detected in DCs by FACS analysis. By comparison lymphocytes, which were also negative for surface TLRs, did not express any intracellular TLRs (data not shown). Confocal imaging revealed that TLR staining was present throughout the cytoplasm in a vesicular pattern. There were concentrated areas of staining in a para-nuclear location, particularly with TLR4. Intracellular TLR4 concentrated around the Golgi has been detected in epithelial cells, monocytes and transfected Hek293 cells (Hornef M.W. et al., 2002)(Latz E. et al., 2002). TLR4 complexed with MD-2 has also been observed to traffic from the Golgi to the plasma membrane (Latz E. et al., 2002)(Nagai Y. et al., 2002).

The intracellular localisation of TLR molecules may be fundamental in DC biology. DCs rapidly phagocytose and endocytose pathogens. Studies with latex beads have shown that phagocytosis is not dependent on a danger signal from the pathogen. Phagocytosis of bacteria has also proven to be fundamental in DC cytokine production, especially IL-12 (Uronen-Hanson et al 2003). It seems possible that DCs phagocytose potential pathogens and then sample the phagosome with TLRs. The DC can then mount the appropriate response to the phagocytosed pathogen. This hypothesis was tested by confocal imaging of internalised *N. meningitidis* and TLRs 2 & 4 in DCs. Internalised bacteria were found in close proximity to TLRs 2 & 4 but there was no evidence for colocalisation which would show up as yellow staining in the merged images. This apparent lack of co-localisation between TLRs and the phagocytosed bacteria may occur if the contact between TLRs and the bacteria is transient and not detectable after 2 hours. Alternatively only a few TLR molecules maybe needed to

interact with the bacteria in order to signal appropriately. There is also the possibility that contact between TLRs and phagocytosed bacteria does not happen. The methods used here are not sensitive enough to detect interactions of individual TLRs. Further investigations and more sensitive time courses need to be undertaken on order to verify these speculations.

Both TLR2 and TLR4 have been shown to play a role in the detection and signalling of *N. meningitidis* (Ingalls R.R. et al., 2000)(Pridmore A.C. et al., 2001). Although TLR4 signalling has been implicated in the response to *N. meningitidis* by TLR4 transfected cell lines, the role of TLR4 in monocyte or DC responses to *N. meningitidis* has not been investigated. This was approached here by examining responses of monocytes with a naturally occurring mutation that has been reported to decrease signalling to LPS. The allelic frequency of this polymorphism was found in this study to be 4%, which compares well with other studies where allelic frequency ranges between 3.3% and 7.9% have been reported (Arbour N.C. et al., 2000)(Read R.C. et al., 2001). Of the 50 healthy volunteers screened, 4 individuals were found to be heterozygous for the Asp299Gly substitution.

No difference in the monocyte response to *N. meningitidis* wild type and purified meningococcal LPS was found between those with the Asp299Gly substitution and wild type individuals. There are several possible explanations why this TLR4 polymorphism does not affect *N. meningitidis* signalling. The individuals identified with the Asp299Gly substitution were all heterozygotes, and may be one copy of the functional TLR4 gene is enough in heterozygotes. Though decreased responses to inhaled LPS have been observed in heterozygotes (Arbour N.C. et al., 2000). However the decreased

responses reported were to *E. coli* LPS, whereas *N. meningitidis* LPS was used here. It is possible that the functional polymorphism affects the binding of *E. coli* LPS to TLR4 but has no effect on the binding of LPS from *N. meningitidis*. The structure of the lipid A is thought to determine the interaction of LPS with TLR4, and the structure of *N. meningitidis* lipid A is different to that of *E. coli* Lipid A (Netea M.G. et al., 2002). A study of patients with meningococcal disease found no correlation with the Asp299Gly polymorphism and the likelihood or severity of *N. meningitidis* infections (Read R.C. et al., 2001). Extensive genetic studies however, have revealed that not one particular TLR4 mutation can be linked to susceptibility or severity of meningococcal disease, but collectively significantly more rare TLR4 coding variants were found in patients with meningococcal disease than a control population (Smirnova I. et al., 2003). Interestingly decreased responses to the LPS deficient *lpxA-* was seen in two out of three individuals without the Asp299Gly polymorphism. The *lpxA-* bacteria have been shown to signal through TLR2. The data here could be explained by other genetic differences between individuals, perhaps related to TLR2 or TLR2 adaptor molecules. Though genetic studies found no TLR2 variants were over represented in the meningococcal patients studied (Emonts M. et al., 2003)(Smirnova I. et al., 2003).(Emonts M. et al., 2003)

A further explanation for why the TLR4 polymorphism does not affect responses to *N. meningitidis* is that TLR4 is not the major receptor for the detection and signalling to *N. meningitidis*. This explanation could also account for why there was no co-localisation observed between TLR4 and phagocytosed *N. meningitidis* and why the TLR4 polymorphism has no effect on the outcome of meningococcal disease (Read R.C. et al., 2001). The studies that have implicated TLR4 in the detection of *N. meningitidis* have

been with transfected cell lines, which may not express the receptor, or receptors that

signal for *N. meningitidis*. In this case, TLR-4 maybe a secondary receptor that is not

required for *N. meningitidis* signalling in the presence of other receptors.

Chapter 7

General Discussion

7.1 Current vaccine strategies

Polysaccharide based conjugate vaccines have offer successful protection against group A and C *N. meningitidis*, the structural homology shared between the group B polysaccharide capsule and the glycopeptides of human NCAM have prevented this approach for group B organisms. Therefore current vaccine strategies are focussed on bacterial proteins rather than the polysaccharide capsule.

Considerable attention has been directed at outer membrane vesicle (OMV) based vaccines. OMVs are prepared easily by detergent extraction from meningococcal bacterial cells. They contain outer membrane proteins, mainly porins, and can be prepared with reduced levels of LPS. The high endotoxicity of LPS means that its presence is undesirable in a vaccine because of the potential host response. This OMV based approach has been largely unsuccessful as these vaccines only elicit strain specific protection and do not offer protection in infants (reviewed (Jodar L. et al., 2002)). Vaccine candidates based on conserved antigens are under investigation, including the transferrin protein B (TbpB) and the neisserial surface protein A (NspA) (Rokbi B. et al., 2000)(West D. et al., 2001).

Vaccine development is a lengthy and costly process. Components of the bacteria must be identified and purified before their ability to induce immunity is tested. Even if a potential vaccine is identified, its efficacy and safety must be thoroughly investigated before a clinical trial is considered. Testing the immunogenicity of potential vaccines for group B *N. meningitidis* is more complicated as it is a human specific pathogen with no suitable animal model for disease. Even when a vaccine shows potential immunogenicity in an animal model, does not necessarily translate to

disease protection in humans. Transgenic mice expressing human CD46 have recently been described. *N. meningitidis* binds to human CD46 expressed on epithelial cells, leading to bacterial invasion. Thus these transgenic mice are susceptible to meningococcal disease and present a potentially useful model for investigating the protectiveness of vaccines against disease (Johansson L. et al., 2003).

7.2 Dendritic cells as an *in vitro* model system highlights the importance of LPS

As an alternative model, activation of human monocyte derived DCs were used to investigate the immunogenicity of *N. meningitidis* and the importance of LPS. Although this approach has its limitations, DCs have an advantage in that they are involved in all stages of generating an immune response. All of these stages can be measured *in vitro* including inflammatory cytokine production, antigen uptake and processing, migration, T cell activation and subsequent B cell antibody production. This thesis examined these different stages of DC responses to *N. meningitidis*. There were two main objectives for this work. Firstly to examine aspects of basic DC biology, including the role of LPS in DC activation. Secondly to gather useful information on which properties of the bacteria might be important for a vaccine.

Monocytes can differentiate into DCs following transendothelial migration into the tissues and/or phagocytosis (Randolph G.J. et al., 1998)(Randolph G.J. et al., 1999). Following the initial recognition and uptake of bacteria DCs release pro-inflammatory cytokines, including IL-1, IL-6 and TNFα. DC activation is further

enhanced by the local release of pro-inflammatory cytokines by tissues at the site of

infection. LPS is thought to be the major component of Gram-negative bacteria in

inducing this pro-inflammatory response. It was found here that wild type *N.*

meningitidis induced IL-1α, IL-6 and TNFα production in DCs. The LPS deficient

lpxA- was found to induce some cytokine production, but to a lesser extent than the

wild type. Thus demonstrating that LPS is important, but is not the only contributing

factor in this response and other bacterial components are involved.

Following activation and maturation by a pathogen DCs must migrate from the

peripheral tissues to the draining lymph nodes in order to present antigen to naïve T

cells. This migration is facilitated by changes in DC chemokine receptor expression.

In vitro experiments here found that a change in chemokine receptor expression from

CCR5 to CCR7 occurred following stimulation with wild type *N. meningitidis*, which

was proven to be functional as these DCs responded to MIP3β. Stimulation with the

LPS deficient *lpxA-* only induced a decrease in CCR5 expression without an increase

in CCR7 and there was no response to MIP3β. This would imply that LPS expression

is required for optimal migration of DCs from the peripheral tissues to the secondary

lymphoid organs *in vivo* following *N. meningitidis* stimulation. The emigration of

DCs from the tissues would involve their crossing of endothelial layers. This was

modelled here by using an *in vitro* HUVEC based system. Optimal DC migration

required activation of both DCs and endothelium, which is a likely situation during

N. meningitidis infection.

Following migration to the T cell areas of the secondary lymphoid organs, DCs

present antigen to T cells. The ability of DCs to activate T cells is fundamental in the

initiation of an adaptive immune response. The T cell stimulatory capabilities of DCs

activated by *N. meningitidis* were assessed here. DCs stimulated with either the wild

type or *lpxA- N. meningitidis* both induced T cell proliferation. However differences

were found in T cell polarisation. DCs stimulated with wild type *N. meningitidis*

induced a strong Th1 response, demonstrated by IFNγ production but with very little

IL-4. DCs stimulated with *lpxA-* induced neither a strong Th1 response nor a strong

Th2 response.

T helper cells control the antibody isotype switching in B cells by CD40 ligation and

cytokine production. Protection against *N. meningitidis* is ultimately reliant on the

induction of the correct antibody response. For group A and C organisms IgG2

antibodies against capsular polysaccharides elicit protection, however IgG1 and IgG3

antibody sub types are protective against group B infections (Pollard A.J. et al.,

1999)(Vermont C.L. et al., 2002). How Th cells activated by DCs in this study will

influence antibody isotype switching has not yet been addressed.

The requirement of LPS in the bacteria to activate DCs, induce CCR7 expression and

migration, and to stimulate Th cell polarisation suggest that LPS maybe an important

component in vaccines to generate effective immune responses against *N.*

meningitidis. The findings of this study are consistent with a previous study which

showed that *lpxA-* was a poor inducer of bactericidal antibodies compared to the wild

type *N. meningitidis* in mice. Interestingly this study found that the response to outer

membrane complexes (OMCs) derived from the *lpxA-* organism could be restored to

wild type OMC responses with the addition of exogenous LPS (Steeghs L. et al.,

1999). However the addition of LPS with heat killed *lpxA-* whole organisms did not

enhance the response. One suggested reason for this difference is that the negatively

charged LPS molecules could associate with the available membrane complexes of

the OMCs, but could not associate with the membranes of the heat killed

encapsulated bacteria.

The use of an *in vitro* system as a model for an immune response to a pathogen does

have limitations. For example the effects of other cells that are involved in the onset

of meningococcal disease, including neutrophils, macrophages and endothelial cells,

are not considered in this situation (Dixon G.L. et al., 1999)(Heyderman R.S. et al.,

1999). Also factors present in serum such as complement, immunoglobulin or

cytokines are not taken into account (reviewed (Hackett S.J. et al., 2001))(Vogel U.

et al., 1999). These additional aspects may affect DC interactions with *N.*

meningitidis and should be considered in future studies.

7.3 The future for group B *N. meningitidis* vaccine design

The toxicity of LPS has meant that most vaccine strategies using OMVs or purified

components of group B bacteria have avoided its inclusion. One approach to this

problem is to engineer bacteria to express modified LPS that does not have the

toxicity of wild type LPS. An isogenic mutant of *N. meningitidis* has been created

which has the same adjuvant capabilities as the wild type organism but with reduced

toxicity (van Der L.P. et al., 2001). This mutant *lpxL1* was created by the insertional

inactivation of the *lpxL* gene, which interrupts lipid A acyloxyacylation resulting in

LPS with a penta- instead of hexa- acylated lipid A. Experiments in mice have shown

that *lpxL1* induced equivalent antibody titres to the wild type *N. meningitidis* but

reduced TNFα production (van Der L.P. et al., 2001). This mutant should be

examined in the DC assays described here, and if it activates DCs similarly to the

wild type bacteria it could potentially be considered as a basis for a vaccine.

Further *N. meningitidis* LPS mutants have been created by replacing the *lpxA* gene

with the equivalent gene from *E. coli* or *P. aeruginosa*. In both strains a 10-fold

reduction in their ability to induce TNFα production was observed, but their adjuvant

activity compared to the wild type was not affected (Steeghs L. et al., 2002). These

findings enable the toxicity of LPS to be separated from its adjuvant capabilities. The

interaction of these organisms with human DCs also needs to be investigated.

The work described here represents a good model for assessing immune function and

potential vaccine design, although there are other aspects still to be addressed. Most

notable is the resulting antibody response. DC and T cell interactions with B cells

following *N. meningitidis* stimulation needs to be explored as B cell stimulation and

antibody production represent a fundamental aspect in any long-term protective

immune response, which is the ultimate goal for a successful vaccine. Interactions of

the wild type *N. meningitidis* induced Th1 cells and B cells could be assessed for

antibody isotype switching. The Ig class and subclass of antibody produced by B

cells following culture with DC activated T cells could be measured along with the

bactericidal properties of the antibodies.

The H44/76 strain of *N. meningitidis* was used exclusively in the work presented

here. This bacterium was isolated from a fatal case of septicaemia, and displays

many characteristics of meningococci that have entered and survived in the blood

stream. Including expression of a polysaccharide capsule and LPS with sialyated

terminal sugars (de Vries F.P. et al., 1996), both of which aid survival in the blood stream by conferring resistance to complement and phagocytosis. It is likely, however, that newly invaded bacteria encountering DCs in the sub-epithelial stromal tissues will not express capsule but may express Opa and Opc, which are crucial in inducing bacterial invasion across the nasopharynx endothelium. Future studies using this system of assessing DC interactions with *N. meningitidis* should take into account this phase variable proteins and assess their relevance in DC activation.

A successful vaccine for group B meningococcal infections has still not been achieved. The recent publication of the complete genome for a group B organism has altered the approach to vaccine design (Tettelin H. et al., 2000). The use of PCR and cloning techniques allowed for the identification of 29 surface exposed proteins, against which antibodies were raised. By assessing these proteins in a variety of *N. meningitidis* strains, epitopes conserved across multiple strains were identified (reviewed Adu-Bobie 2003). This novel approach to identifying potential vaccine candidates offers promise in the continuing search for a group B vaccine, and the ability to assess these candidates promptly and easily in an *in vitro* DC system may well result in a successful vaccine in the near future.

Reference List

Abdi K. IL-12: the role of p40 versus p75. *Scand.J.Immunol.* 2002. **56.** 1-11.

Adu-Bobie J., Capecchi B., Serruto D., Rappuoli R. and Pizza M. Two years into reverse vaccinology. *Vaccine.* 2003. **21.** 605-610.

Aicher A., Hayden-Ledbetter M., Brady W.A., Pezzutto A., Richter G., Magaletti D., Buckwalter S., Ledbetter J.A. and Clark E.A. Characterization of human inducible costimulator ligand expression and function. *J Immunol.* 2000. **164.** 4689-4696.

Akira S. The role of IL-18 in innate immunity. *Curr.Opin.Immunol.* 2000. **12.** 59-63.

Albert M.L., Sauter B. and Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature.* 1998. **392.** 86-89.

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

Alexopoulou L., Holt A.C., Medzhitov R. and Flavell R.A. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature.* 2001. **413.** 732-738.

Ali M.H., Schlidt S.A., Chandel N.S., Hynes K.L., Schumacker P.T. and Gewertz B.L. Endothelial permeability and IL-6 production during hypoxia: role of ROS in signal transduction. *Am.J.Physiol.* 1999. **277.** L1057-L1065.

Andersen S.R., Bjune G., Lyngby J., Bryn K. and Jantzen E. Short-chain lipopolysaccharide mutants of serogroup B Neisseria meningitidis of potential value for production of outer membrane vesicle vaccines. *Microb.Pathog.* 1995. **19.** 159-168.

Andersen S.R., Bryn K., Thorseng K. and Jantzen E. Heterogeneity of lipopolysaccharides in *Neisseria meningitidis* revealed by thin-layer chromatography combined with monoclonal antibodies. *J Microbiol.Methods.* 1996. **25.** 187-194.

Arbour N.C., Lorenz E., Schutte B.C., Zabner J., Kline J.N., Jones M., Frees K., Watt J.L. and Schwartz D.A. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat.Genet.* 2000. **25.** 187-191.

Ausiello C.M., Fedele G., Urbani F., Lande R., Di Carlo B. and Cassone A. Native and genetically inactivated pertussis toxins induce human dendritic cell maturation and synergize with lipopolysaccharide in promoting T helper type 1 responses. *J Infect.Dis.* 2002. **186.** 351-360.

Balmer P., Borrow R. and Miller E. Impact of meningococcal C conjugate vaccine in the UK. *J.Med.Microbiol.* 2002. **51.** 717-722.

Balmer P. and Miller E. Meningococcal disease: how to prevent and how to manage. *Curr.Opin.Infect.Dis.* 2002. **15.** 275-281.

Banchereau J., Schuler-Thurner B., Palucka A.K. and Schuler G. Dendritic cells as vectors for therapy. *Cell.* 2001. **106.** 271-274.

Beutler B. Tlr4: central component of the sole mammalian LPS sensor. *Curr.Opin.Immunol.* 2000. **12.** 20-26.

Bevilacqua M.P., Pober J.S., Mendrick D.L., Cotran R.S. and Gimbrone M.A., Jr. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc.Natl.Acad.Sci.U.S.A.* 1987. **84.** 9238-9242.

Bhardwaj N. Interactions of viruses with dendritic cells: a double-edged sword. *J.Exp.Med.* 1997. **186.** 795-799.

Bleijs D.A., Geijtenbeek T.B., Figdor C.G. and van Kooyk Y. DC-SIGN and LFA-1: a battle for ligand. *Trends Immunol.* 2001. **22.** 457-463.

Bouloc A., Bagot M., Delaire S., Bensussan A. and Boumsell L. Triggering CD101 molecule on human cutaneous dendritic cells inhibits T cell proliferation via IL-10 production. *Eur.J Immunol.* 2000. **30.** 3132-3139.

Bowie A. and O'Neill L.A. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *J Leukoc Biol.* 2000. **67.** 508-514.

Brandtzaeg P. and van Deuren M. Current concepts in the role of the host response in Neisseria meningitidis septic shock. *Curr.Opin.Infect.Dis.* 2002. **15.** 247-252.

Brasel K., De Smedt T., Smith J.L. and Maliszewski C.R. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood.* 2000. **96.** 3029-3039.

Brown K.A., Bedford P., Macey M., McCarthy D.A., Leroy F., Vora A.J., Stagg A.J., Dumonde D.C. and Knight S.C. Human blood dendritic cells: binding to vascular endothelium and expression of adhesion molecules. *Clin.Exp.Immunol.* 1997. **107.** 601-607.

Castellano F., Chavrier P. and Caron E. Actin dynamics during phagocytosis. *Semin.Immunol.* 2001. **13.** 347-355.

Caux C., Vanbervliet B., Massacrier C., Azuma M., Okumura K., Lanier L.L. and Banchereau J. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J Exp.Med.* 1994. **180.** 1841-1847.

Cella M., Salio M., Sakakibara Y., Langen H., Julkunen I. and Lanzavecchia A. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp.Med.* 1999. **189.** 821-829.

Cella M., Jarrossay D., Facchetti F., Alebardi O., Nakajima H., Lanzavecchia A. and Colonna M. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat.Med.* 1999. **5.** 919-923.

Cella M., Facchetti F., Lanzavecchia A. and Colonna M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat.Immunol.* 2000. **1.** 305-310.

Chambers C.A. The expanding world of co-stimulation: the two-signal model revisited. *Trends Immunol.* 2001. **22.** 217-223.

Chapoval A.I., Ni J., Lau J.S., Wilcox R.A., Flies D.B., Liu D., Dong H., Sica G.L., Zhu G., Tamada K. and Chen L. B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. *Nat.Immunol.* 2001. **2.** 269-274.

Chen A.I., McAdam A.J., Buhlmann J.E., Scott S., Lupher-ML J., Greenfield E.A., Baum P.R., Fanslow W.C., Calderhead D.M., Freeman G.J. and Sharpe A.H. Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. *Immunity.* 1999. **11.** 689-698.

Chow A., Toomre D., Garrett W. and Mellman I. Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. *Nature.* 2002. **418.** 988-994.

Coleman J. and Raetz C.R. First committed step of lipid A biosynthesis in Escherichia coli: sequence of the lpxA gene. *J.Bacteriol.* 1988. **170.** 1268-1274.

Crow M.K. and Kunkel H.G. Human dendritic cells: major stimulators of the autologous and allogeneic mixed leucocyte reactions. *Clin.Exp.Immunol.* 1982. **49.** 338-346.

D'amico G., Bianchi G., Bernasconi S., Bersani L., Piemonti L., Sozzani S., Mantovani A. and Allavena P. Adhesion, transendothelial migration, and reverse transmigration of in vitro cultured dendritic cells. *Blood.* 1998. **92.** 207-214.

d'Ostiani C.F., Del Sero G., Bacci A., Montagnoli C., Spreca A., Mencacci A., Ricciardi-Castagnoli P. and Romani L. Dendritic cells discriminate between yeasts

and hyphae of the fungus Candida albicans. Implications for initiation of T helper cell immunity in vitro and in vivo. *J Exp.Med.* 2000. **191**. 1661-1674.

Davis D.M. Assembly of the immunological synapse for T cells and NK cells. *Trends Immunol.* 2002. **23**. 356-363.

de Baey A. and Lanzavecchia A. The role of aquaporins in dendritic cell macropinocytosis. *J.Exp.Med.* 2000. **191**. 743-748.

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

de Jong R., Altare F., Haagen I.A., Elferink D.G., Boer T., Breda Vriesman P.J., Kabel P.J., Draaisma J.M., van Dissel J.T., Kroon F.P., Casanova J.L. and Ottenhoff T.H. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. *Science.* 1998. **280**. 1435-1438.

de Vries F.P., Cole R., Dankert J., Frosch M. and van Putten J.P. Neisseria meningitidis producing the Opc adhesin binds epithelial cell proteoglycan receptors. *Mol.Microbiol.* 1998. **27**. 1203-1212.

Dhodapkar M.V., Steinman R.M., Krasovsky J., Munz C. and Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp.Med.* 2001. **193**. 233-238.

Dixon G.L., Heyderman R.S., Kotovicz K., Jack D.L., Andersen S.R., Vogel U., Frosch M. and Klein N. Endothelial adhesion molecule expression and its inhibition by recombinant Bactericidal/Permeability-increasing protein are influenced by the capsulation and lipooligosaccharide structure of neisseria meningitidis [In Process Citation]. *Infect.Immun.* 1999. **67**. 5626-5633.

Dixon G.L. The influence of bacterial structure on the host inflammatory response to *Neisseria meningitidis*. *PhD thesis.* 2000.

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

Dunne D.W., Resnick D., Greenberg J., Krieger M. and Joiner K.A. The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. *Proc.Natl.Acad.Sci.U.S.A.* 1994. **91.** 1863-1867.

Dustin M.L. and Cooper J.A. The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. *Nat.Immunol.* 2000. **1.** 23-29.

Emonts M., Hazelzet J.A., de Groot R. and Hermans P.W. Host genetic determinants of Neisseria meningitidis infections. *Lancet Infect.Dis.* 2003. **3.** 565-577.

Engering A., Geijtenbeek T.B., van Vliet S.J., Wijers M., van Liempt E., Demaurex N., Lanzavecchia A., Fransen J., Figdor C.G., Piguet V. and van Kooyk Y. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol.* 2002. **168.** 2118-2126.

Erridge C., Bennett-Guerrero E. and Poxton I.R. Structure and function of lipopolysaccharides. *Microbes.Infect.* 2002. **4.** 837-851.

Fanger N.A., Wardwell K., Shen L., Tedder T.F. and Guyre P.M. Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells. *J.Immunol.* 1996. **157.** 541-548.

Fenhalls G., Squires G.R., Stevens-Muller L., Bezuidenhout J., Amphlett G., Duncan K. and Lukey P.T. Associations between Toll-like Receptors and IL-4 in the Lungs of Patients with Tuberculosis. *Am.J.Respir.Cell Mol.Biol.* 2002.

Finne J., Bitter-Suermann D., Goridis C. and Finne U. An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues. *J.Immunol.* 1987. **138.** 4402-4407.

Fonteneau J.F., Gilliet M., Larsson M., Dasilva I., Munz C., Liu Y.J. and Bhardwaj N. Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity. *Blood.* 2003. **101.** 3520-3526.

Fossum S. Dendritic leukocytes: features of their in vivo physiology. *Res.Immunol.* 1989. **140.** 883-891.

Galloway S.M. and Raetz C.R. A mutant of Escherichia coli defective in the first step of endotoxin biosynthesis. *J.Biol.Chem.* 1990. **265.** 6394-6402.

Gatti E., Velleca M.A., Biedermann B.C., Ma W., Unternaehrer J., Ebersold M.W., Medzhitov R., Pober J.S. and Mellman I. Large-scale culture and selective maturation of human Langerhans cells from granulocyte colony-stimulating factor-mobilized CD34+ progenitors. *J Immunol.* 2000. **164.** 3600-3607.

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

Geijtenbeek T.B., Krooshoop D.J., Bleijs D.A., van Vliet S.J., van Duijnhoven G.C., Grabovsky V., Alon R., Figdor C.G. and van Kooyk Y. DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. *Nat.Immunol.* 2000. **1.** 353-357.

Geissmann F., Dieu-Nosjean M.C., Dezutter C., Valladeau J., Kayal S., Leborgne M., Brousse N., Saeland S. and Davoust J. Accumulation of immature langerhans cells in human lymph nodes draining chronically inflamed skin. *J Exp.Med.* 2002. **19;196.** 417-430.

Giacomini E., Iona E., Ferroni L., Miettinen M., Fattorini L., Orefici G., Julkunen I. and Coccia E.M. Infection of Human Macrophages and Dendritic Cells with

Mycobacterium tuberculosis Induces a Differential Cytokine Gene Expression That Modulates T Cell Response. *J Immunol.* 2001. **166.** 7033-7041.

Gimbrone M.A., Jr., Cotran R.S. and Folkman J. Human vascular endothelial cells in culture. Growth and DNA synthesis. *J.Cell Biol.* 1974. **60.** 673-684.

Girolomoni G., Caux C., Dezutter-Dambuyant C., Lebecque S. and Ricciardi-Castagnoli P. Langerhans cells: still a fundamental paradigm for studying the immunobiology of dendritic cells. *Trends Immunol.* 2002. **23.** 6-8.

Grakoui A., Bromley S.K., Sumen C., Davis M.M., Shaw A.S., Allen P.M. and Dustin M.L. The immunological synapse: a molecular machine controlling T cell activation. *Science.* 1999. **285.** 221-227.

Greenwald R.J., McAdam A.J., Van der W.D., Satoskar A.R. and Sharpe A.H. Cutting edge: inducible costimulator protein regulates both Th1 and Th2 responses to cutaneous leishmaniasis. *J Immunol.* 2002. **168.** 991-995.

Grouard G., Rissoan M.C., Filgueira L., Durand I., Banchereau J. and Liu Y.J. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J.Exp.Med.* 1997. **185.** 1101-1111.

Hackett S.J., Thomson A.P. and Hart C.A. Cytokines, chemokines and other effector molecules involved in meningococcal disease. *J.Med.Microbiol.* 2001. **50.** 847-859.

Hammad H., Charbonnier A.S., Duez C., Jacquet A., Stewart G.A., Tonnel A.B. and Pestel J. Th2 polarization by Der p 1--pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. *Blood.* 2001. **98.** 1135-1141.

Hart D.N. and Prickett T.C. Intercellular adhesion molecule-2 (ICAM-2) expression on human dendritic cells. *Cell Immunol.* 1993. **148.** 447-454.

Hauck C.R. and Meyer T.F. 'Small' talk: Opa proteins as mediators of Neisseria-host-cell communication. *Curr.Opin.Microbiol.* 2003. **6.** 43-49.

Hayashi F., Smith K.D., Ozinsky A., Hawn T.R., Yi E.C., Goodlett D.R., Eng J.K., Akira S., Underhill D.M. and Aderem A. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature.* 2001. **410.** 1099-1103.

Hemmi H., Takeuchi O., Kawai T., Kaisho T., Sato S., Sanjo H., Matsumoto M., Hoshino K., Wagner H., Takeda K. and Akira S. A Toll-like receptor recognizes bacterial DNA. *Nature.* 2000. **408.** 740-745.

Hemmi H., Kaisho T., Takeuchi O., Sato S., Sanjo H., Hoshino K., Horiuchi T., Tomizawa H., Takeda K. and Akira S. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat.Immunol.* 2002. **3.** 196-200.

Heufler C., Koch F., Stanzl U., Topar G., Wysocka M., Trinchieri G., Enk A., Steinman R.M., Romani N. and Schuler G. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur.J Immunol.* 1996. **26.** 659-668.

Heyderman R.S., Ison C.A., Peakman M., Levin M. and Klein N.J. Neutrophil response to Neisseria meningitidis: inhibition of adhesion molecule expression and phagocytosis by recombinant bactericidal/permeability-increasing protein (rBPI21). *J.Infect.Dis.* 1999. **179.** 1288-1292.

Hilkens C.M., Vermeulen H., van Neerven R.J., Snijdewint F.G., Wierenga E.A. and Kapsenberg M.L. Differential modulation of T helper type 1 (Th1) and T helper type 2 (Th2) cytokine secretion by prostaglandin E2 critically depends on interleukin-2. *Eur.J.Immunol.* 1995. **25.** 59-63.

Hilkens C.M., Kalinski P., de B.M. and Kapsenberg M.L. Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. *Blood.* 1997. **90.** 1920-1926.

Hirschfeld M., Kirschning C.J., Schwandner R., Wesche H., Weis J.H., Wooten R.M. and Weis J.J. Cutting edge: inflammatory signaling by Borrelia burgdorferi lipoproteins is mediated by toll-like receptor 2. *J.Immunol.* 1999. **163.** 2382-2386.

Hirschfeld M., Weis J.J., Toshchakov V., Salkowski C.A., Cody M.J., Ward D.C., Qureshi N., Michalek S.M. and Vogel S.N. Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect.Immun.* 2001. **69.** 1477-1482.

Hogg N. and Berlin C. Structure and function of adhesion receptors in leukocyte trafficking. *Immunol.Today.* 1995. **16.** 327-330.

Hornef M.W., Frisan T., Vandewalle A., Normark S. and Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp.Med.* 2002. **195.** 559-570.

Hoshino K., Takeuchi O., Kawai T., Sanjo H., Ogawa T., Takeda Y., Takeda K. and Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J.Immunol.* 1999. **162.** 3749-3752.

Howland K.C., Ausubel L.J., London C.A. and Abbas A.K. The roles of CD28 and CD40 ligand in T cell activation and tolerance. *J Immunol.* 2000. **164.** 4465-4470.

Iezzi G., Scotet E., Scheidegger D. and Lanzavecchia A. The interplay between the duration of TCR and cytokine signaling determines T cell polarization. *Eur.J Immunol.* 1999. **29.** 4092-4101.

Inaba K., Witmer-Pack M., Inaba M., Hathcock K.S., Sakuta H., Azuma M., Yagita H., Okumura K., Linsley P.S., Ikehara S. and . The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. *J.Exp.Med.* 1994. **180.** 1849-1860.

Inaba K., Turley S., Iyoda T., Yamaide F., Shimoyama S., Reis e Sousa, Germain R.N., Mellman I. and Steinman R.M. The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. *J.Exp.Med.* 2000. **191.** 927-936.

Ingalls R.R., Lien E. and Golenbock D.T. Differential roles of TLR2 and TLR4 in the host response to Gram-negative bacteria: lessons from a lipopolysaccharide-deficient mutant of Neisseria meningitidis. *J.Endotoxin.Res.* 2000. **6.** 411-415.

Ingalls R.R., Lien E. and Golenbock D.T. Membrane-associated proteins of a lipopolysaccharide-deficient mutant of Neisseria meningitidis activate the inflammatory response through toll-like receptor 2. *Infect.Immun.* 2001. **69.** 2230-2236.

Irjala H., Johansson E.L., Grenman R., Alanen K., Salmi M. and Jalkanen S. Mannose receptor is a novel ligand for l-selectin and mediates lymphocyte binding to lymphatic endothelium. *J Exp.Med.* 2001. **194.** 1033-1042.

Irjala H., Elima K., Johansson E.L., Merinen M., Kontula K., Alanen K., Grenman R., Salmi M. and Jalkanen S. The same endothelial receptor controls lymphocyte traffic both in vascular and lymphatic vessels. *Eur.J.Immunol.* 2003. **33.** 815-824.

Jack D.L., Dodds A.W., Anwar N., Ison C.A., Law A., Frosch M., Turner M.W. and Klein N.J. Activation of complement by mannose-binding lectin on isogenic mutants of Neisseria meningitidis serogroup B. *J.Immunol.* 1998. **160.** 1346-1353.

Jaffe E.A., Nachman R.L., Becker C.G. and Minick C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J.Clin.Invest.* 1973. **52.** 2745-2756.

Jakob T. and Udey M.C. Regulation of E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells by inflammatory mediators that mobilize Langerhans cells in vivo. *J.Immunol.* 1998. **160.** 4067-4073.

Janeway-CA J. Presidential Address to The American Association of Immunologists. The road less traveled by: the role of innate immunity in the adaptive immune response. *J.Immunol.* 1998. **161.** 539-544.

Jankovic D., Liu Z. and Gause W.C. Th1- and Th2-cell commitment during infectious disease: asymmetry in divergent pathways. *Trends Immunol.* 2001. **22.** 450-457.

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

Jodar L., Feavers I.M., Salisbury D. and Granoff D.M. Development of vaccines against meningococcal disease. *Lancet.* 2002. **359.** 1499-1508.

Johansson L., Rytkonen A., Bergman P., Albiger B., Kallstrom H., Hokfelt T., Agerberth B., Cattaneo R. and Jonsson A.B. CD46 in meningococcal disease. *Science.* 2003. **301.** 373-375.

Jonuleit H., Schmitt E., Schuler G., Knop J. and Enk A.H. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp.Med.* 2000. **192.** 1213-1222.

Kadowaki N., Antonenko S., Lau J.Y. and Liu Y.J. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. *J Exp.Med.* 2000. **192.** 219-226.

Kadowaki N., Ho S., Antonenko S., Malefyt R.W., Kastelein R.A., Bazan F. and Liu Y.J. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp.Med.* 2001. **194.** 863-869.

Kaech S.M., Wherry E.J. and Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. *Nature Rev.Immunol.* 2002. **2.** 251-262.

Kaisho T. and Akira S. Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. *Trends Immunol.* 2001. **22.** 78-83.

Kaisho T., Takeuchi O., Kawai T., Hoshino K. and Akira S. Endotoxin-induced maturation of myd88-deficient dendritic cells. *J Immunol.* 2001. **166.** 5688-5694.

Kalinski P., Hilkens C.M., Snijders A., Snijdewint F.G. and Kapsenberg M.L. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol.* 1997. **159.** 28-35.

Kalinski P., Hilkens C.M., Wierenga E.A. and Kapsenberg M.L. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today.* 1999. **20.** 561-567.

Karmann K., Min W., Fanslow W.C. and Pober J.S. Activation and homologous desensitization of human endothelial cells by CD40 ligand, tumor necrosis factor, and interleukin 1. *J.Exp.Med.* 1996. **184.** 173-182.

Kato M., Neil T.K., Fearnley D.B., McLellan A.D., Vuckovic S. and Hart D.N. Expression of multilectin receptors and comparative FITC-dextran uptake by human dendritic cells. *Int.Immunol.* 2000. **12.** 1511-1519.

Kellermann S.A., Hudak S., Oldham E.R., Liu Y.J. and McEvoy L.M. The CC chemokine receptor-7 ligands 6Ckine and macrophage inflammatory protein-3 beta are potent chemoattractants for in vitro- and in vivo-derived dendritic cells. *J Immunol.* 1999. **162.** 3859-3864.

Klagge I.M. and Schneider-Schaulies S. Virus interactions with dendritic cells. *J.Gen.Virol.* 1999. **80 (Pt 4).** 823-833.

Klugman K.P., Gotschlich E.C. and Blake M.S. Sequence of the structural gene (rmpM) for the class 4 outer membrane protein of Neisseria meningitidis, homology of the protein to gonococcal protein III and Escherichia coli OmpA, and construction

of meningococcal strains that lack class 4 protein. *Infect.Immun.* 1989. **57.** 2066-2071.

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

Kotowicz K., Callard R.E., Friedrich K., Matthews D.J. and Klein N. Biological activity of IL-4 and IL-13 on human endothelial cells: functional evidence that both cytokines act through the same receptor. *Int.Immunol.* 1996. **8.** 1915-1925.

Krieg A.M. The role of CpG motifs in innate immunity. *Curr.Opin.Immunol.* 2000. **12.** 35-43.

Kriehuber E., Breiteneder-Geleff S., Groeger M., Soleiman A., Schoppmann S.F., Stingl G., Kerjaschki D. and Maurer D. Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp.Med.* 2001. **194.** 797-808.

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

Lanzavecchia A. Mechanisms of antigen uptake for presentation. *Curr.Opin.Immunol.* 1996. **8.** 348-354.

Latz E., Visintin A., Lien E., Fitzgerald K.A., Monks B.G., Kurt-Jones E.A., Golenbock D.T. and Espevik T. Lipopolysaccharide Rapidly Traffics to and from the Golgi Apparatus with the Toll-like Receptor 4-MD-2-CD14 Complex in a Process That Is Distinct from the Initiation of Signal Transduction. *J Biol Chem.* 2002. **277.** 47834-47843.

Le Naour F., Hohenkirk L., Grolleau A., Misek D.E., Lescure P., Geiger J.D., Hanash S. and Beretta L. Profiling changes in gene expression during differentiation

and maturation of monocyte-derived dendritic cells using both oligonucleotide microarrays and proteomics. *J Biol Chem.* 2001. **276.** 17920-17931.

Lemaitre B., Nicolas E., Michaut L., Reichhart J.M. and Hoffmann J.A. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell.* 1996. **20;86.** 973-983.

Lenschow D.J., Walunas T.L. and Bluestone J.A. CD28/B7 system of T cell costimulation. *Annu.Rev.Immunol.* 1996. **14.** 233-258.

Lien E., Sellati T.J., Yoshimura A., Flo T.H., Rawadi G., Finberg R.W., Carroll J.D., Espevik T., Ingalls R.R., Radolf J.D. and Golenbock D.T. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J.Biol.Chem.* 1999. **274.** 33419-33425.

Liu Y.J. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell.* 2001. **106.** 259-262.

Luft T., Jefford M., Luetjens P., Toy T., Hochrein H., Masterman K.A., Maliszewski C., Shortman K., Cebon J. and Maraskovsky E. Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. *Blood.* 2002. **100.** 1362-1372.

Luster A.D. The role of chemokines in linking innate and adaptive immunity. *Curr.Opin.Immunol.* 2002. **14.** 129-135.

Lutz M.B., Rovere P., Kleijmeer M.J., Rescigno M., Assmann C.U., Oorschot V.M., Geuze H.J., Trucy J., Demandolx D., Davoust J. and Ricciardi-Castagnoli P. Intracellular routes and selective retention of antigens in mildly acidic cathepsin D/lysosome-associated membrane protein-1/MHC class II-positive vesicles in immature dendritic cells. *J.Immunol.* 1997. **159.** 3707-3716.

Lyons A.B. and Parish C.R. Determination of lymphocyte division by flow cytometry. *J Immunol Methods.* 1994. **171.** 131-137.

MacDonald A.S. and Pearce E.J. Cutting edge: polarized Th cell response induction by transferred antigen-pulsed dendritic cells is dependent on IL-4 or IL-12 production by recipient cells. *J Immunol.* 2002. **168.** 3127-3130.

MacDonald A.S., Straw A.D., Dalton N.M. and Pearce E.J. Cutting edge: Th2 response induction by dendritic cells: a role for CD40. *J Immunol.* 2002. **168.** 537-540.

Mahnke K., Guo M., Lee S., Sepulveda H., Swain S.L., Nussenzweig M. and Steinman R.M. The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J Cell Biol.* 2000. **151.** 673-684.

Maldonado-Lopez R. and Moser M. Dendritic cell subsets and the regulation of Th1/Th2 responses. *Semin.Immunol.* 2001. **13.** 275-282.

Massari P., Henneke P., Ho Y., Latz E., Golenbock D.T. and Wetzler L.M. Cutting edge: Immune stimulation by neisserial porins is toll-like receptor 2 and MyD88 dependent. *J Immunol.* 2002. **168.** 1533-1537.

Massari P., Ram S., Macleod H. and Wetzler L.M. The role of porins in neisserial pathogenesis and immunity. *Trends Microbiol.* 2003. **11.** 87-93.

Massari P., King C.A., Ho A.Y. and Wetzler L.M. Neisserial PorB is translocated to the mitochondria of HeLa cells infected with Neisseria meningitidis and protects cells from apoptosis. *Cell Microbiol.* 2003. **5.** 99-109.

Mattner F., Magram J., Ferrante J., Launois P., Di Padova K., Behin R., Gately M.K., Louis J.A. and Alber G. Genetically resistant mice lacking interleukin-12 are susceptible to infection with Leishmania major and mount a polarized Th2 cell response. *Eur.J.Immunol.* 1996. **26.** 1553-1559.

Means T.K., Lien E., Yoshimura A., Wang S., Golenbock D.T. and Fenton M.J. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J.Immunol.* 1999. **163**. 6748-6755.

Means T.K., Wang S., Lien E., Yoshimura A., Golenbock D.T. and Fenton M.J. Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. *J.Immunol.* 1999. **163**. 3920-3927.

Means T.K., Golenbock D.T. and Fenton M.J. The biology of Toll-like receptors. *Cytokine Growth Factor Rev.* 2000. **11**. 219-232.

Medzhitov R. and Janeway C.A., Jr. Innate immunity: impact on the adaptive immune response. *Curr.Opin.Immunol.* 1997. **9**. 4-9.

Moe G.R., Zuno-Mitchell P., Lee S.S., Lucas A.H. and Granoff D.M. Functional activity of anti-Neisserial surface protein A monoclonal antibodies against strains of Neisseria meningitidis serogroup B. *Infect.Immun.* 2001. **69**. 3762-3771.

Montoya M.C., Sancho D., Bonello G., Collette Y., Langlet C., He H.T., Aparicio P., Alcover A., Olive D. and Sanchez-Madrid F. Role of ICAM-3 in the initial interaction of T lymphocytes and APCs. *Nat.Immunol.* 2002. **3**. 159-168.

Morelli A.E., Zahorchak A.F., Larregina A.T., Colvin B.L., Logar A.J., Takayama T., Falo L.D. and Thomson A.W. Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood.* 2001. **98**. 1512-1523.

Muller W.A., Weigl S.A., Deng X. and Phillips D.M. PECAM-1 is required for transendothelial migration of leukocytes. *J Exp.Med.* 1993. **178**. 449-460.

Muzio M., Bosisio D., Polentarutti N., D'amico G., Stoppacciaro A., Mancinelli R., van't Veer C., Penton-Rol G., Ruco L.P., Allavena P. and Mantovani A. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol.* 2000. **164**. 5998-6004.

Muzio M., Polentarutti N., Bosisio D., Prahladan M.K. and Mantovani A. Toll-like receptors: a growing family of immune receptors that are differentially expressed and regulated by different leukocytes. *J Leukoc Biol.* 2000. **67.** 450-456.

Nagai Y., Akashi S., Nagafuku M., Ogata M., Iwakura Y., Akira S., Kitamura T., Kosugi A., Kimoto M. and Miyake K. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat.Immunol.* 2002. **3.** 667-672.

Namork E. and Brandtzaeg P. Fatal meningococcal septicaemia with "blebbing" meningococcus. *Lancet.* 2002. **360.** 1741-.

Nassif X., Beretti J.L., Lowy J., Stenberg P., O'Gaora P., Pfeifer J., Normark S. and So M. Roles of pilin and PilC in adhesion of Neisseria meningitidis to human epithelial and endothelial cells. *Proc.Natl.Acad.Sci.U.S.A.* 1994. **91.** 3769-3773.

Netea M.G., van Deuren M., Kullberg B.J., Cavaillon J.M. and van der Meer J.W. Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? *Trends Immunol.* 2002. **23.** 135-139.

Ohashi K., Burkart V., Flohe S. and Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J.Immunol.* 2000. **164.** 558-561.

Oppmann B., Lesley R., Blom B., Timans J.C., Xu Y., Hunte B., Vega F., Yu N., Wang J., Singh K., Zonin F., Vaisberg E., Churakova T., Liu M., Gorman D., Wagner J., Zurawski S., Liu Y., Abrams J.S., Moore K.W., Rennick D., Waal-Malefyt R., Hannum C., Bazan J.F. and Kastelein R.A. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity.* 2000. **13.** 715-725.

Oshiumi H., Matsumoto M., Funami K., Akazawa T. and Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat.Immunol.* 2003. **4.** 161-167.

Ozinsky A., Underhill D.M., Fontenot J.D., Hajjar A.M., Smith K.D., Wilson C.B., Schroeder L. and Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc.Natl.Acad.Sci.U.S.A.* 2000. **97.** 13766-13771.

Palucka K. and Banchereau J. Dendritic cells: a link between innate and adaptive immunity. *J.Clin.Immunol.* 1999. **19.** 12-25.

Parkhill J., Achtman M., James K.D., Bentley S.D., Churcher C., Klee S.R., Morelli G., Basham D., Brown D., Chillingworth T., Davies R.M., Davis P., Devlin K., Feltwell T., Hamlin N., Holroyd S., Jagels K., Leather S., Moule S., Mungall K., Quail M.A., Rajandream M.A., Rutherford K.M., Simmonds M., Skelton J., Whitehead S., Spratt B.G. and Barrell B.G. Complete DNA sequence of a serogroup A strain of Neisseria meningitidis Z2491. *Nature.* 2000. **404.** 502-506.

Peiser L., Gough P.J., Kodama T. and Gordon S. Macrophage class A scavenger receptor-mediated phagocytosis of Escherichia coli: role of cell heterogeneity, microbial strain, and culture conditions in vitro. *Infect.Immun.* 2000. **68.** 1953-1963.

Pollard A.J., Galassini R., van der Voort E.M., Booy R., Langford P., Nadel S., Ison C., Kroll J.S., Poolman J. and Levin M. Humoral immune responses to Neisseria meningitidis in children. *Infect.Immun.* 1999. **67.** 2441-2451.

Pollard A.J. and Levin M. Vaccines for prevention of meningococcal disease. *Pediatr.Infect.Dis.J.* 2000. **19.** 333-344.

Pond L. and Watts C. Functional early endosomes are required for maturation of major histocompatibility complex class II molecules in human B lymphoblastoid cells. *J Biol Chem.* 1999. **274.** 18049-18054.

Poolman J.T. Development of a meningococcal vaccine. *Infect.Agents Dis.* 1995. **4.** 13-28.

Pridmore A.C., Wyllie D.H., Abdillahi F., Steeghs L., van Der L.P., Dower S.K. and Read R.C. A Lipopolysaccharide-Deficient Mutant of Neisseria meningitidis Elicits Attenuated Cytokine Release by Human Macrophages and Signals via Toll-like Receptor (TLR) 2 but Not via TLR4/MD2. *J Infect.Dis.* 2001. **183.** 89-96.

Prigozy T.I., Naidenko O., Qasba P., Elewaut D., Brossay L., Khurana A., Natori T., Koezuka Y., Kulkarni A. and Kronenberg M. Glycolipid antigen processing for presentation by CD1d molecules. *Science.* 2001. **291.** 664-667.

Prinz T. and Tommassen J. Association of iron-regulated outer membrane proteins of Neisseria meningitidis with the RmpM (class 4) protein. *FEMS Microbiol.Lett.* 2000. **183.** 49-53.

Pulendran B., Banchereau J., Maraskovsky E. and Maliszewski C. Modulating the immune response with dendritic cells and their growth factors. *Trends Immunol.* 2001. **22.** 41-47.

Qureshi S.T., Lariviere L., Leveque G., Clermont S., Moore K.J., Gros P. and Malo D. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J.Exp.Med.* 1999. **189.** 615-625.

Ramachandra L., Song R. and Harding C.V. Phagosomes are fully competent antigen-processing organelles that mediate the formation of peptide:class II MHC complexes. *J.Immunol.* 1999. **162.** 3263-3272.

Ramachandra L. and Harding C.V. Phagosomes acquire nascent and recycling class II MHC molecules but primarily use nascent molecules in phagocytic antigen processing. *J.Immunol.* 2000. **164.** 5103-5112.

Randolph G.J., Beaulieu S., Lebecque S., Steinman R.M. and Muller W.A. Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science.* 1998. **282.** 480-483.

Randolph G.J., Beaulieu S., Pope M., Sugawara I., Hoffman L., Steinman R.M. and Muller W.A. A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. *Proc.Natl.Acad.Sci.U.S.A.* 1998. **95.** 6924-6929.

Randolph G.J., Inaba K., Robbiani D.F., Steinman R.M. and Muller W.A. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity.* 1999. **11.** 753-761.

Randolph G.J. Is maturation required for langerhans cell migration? *J Exp.Med.* 2002. **19;196.** 413-416.

Read R.C., Pullin J., Gregory S., Borrow R., Kaczmarski E.B., di Giovine F.S., Dower S.K., Cannings C. and Wilson A.G. A Functional Polymorphism of Toll-like Receptor 4 Is Not Associated with Likelihood or Severity of Meningococcal Disease. *J Infect.Dis.* 2001. **184.** 640-642.

Res P.C., Couwenberg F., Vyth-Dreese F.A. and Spits H. Expression of pTalpha mRNA in a committed dendritic cell precursor in the human thymus. *Blood.* 1999. **94.** 2647-2657.

Rescigno M., Martino M., Sutherland C.L., Gold M.R. and Ricciardi-Castagnoli P. Dendritic cell survival and maturation are regulated by different signaling pathways. *J.Exp.Med.* 1998a. **188.** 2175-2180.

Rescigno M., Citterio S., Thery C., Rittig M., Medaglini D., Pozzi G., Amigorena S. and Ricciardi-Castagnoli P. Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. *Proc.Natl.Acad.Sci.U.S.A.* 1998b. **95.** 5229-5234.

Richmond P., Borrow R., Goldblatt D., Findlow J., Martin S., Morris R., Cartwright K. and Miller E. Ability of 3 different meningococcal C conjugate vaccines to induce immunologic memory after a single dose in UK toddlers. *J.Infect.Dis.* 2001. **183.** 160-163.

Rissoan M.C., Soumelis V., Kadowaki N., Grouard G., Briere F., de Waal M.R. and Liu Y.J. Reciprocal control of T helper cell and dendritic cell differentiation [see comments]. *Science.* 1999. **283.** 1183-1186.

Roake J.A., Rao A.S., Morris P.J., Larsen C.P., Hankins D.F. and Austyn J.M. Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J Exp.Med.* 1995. **181.** 2237-2247.

Robbiani D.F., Finch R.A., Jager D., Muller W.A., Sartorelli A.C. and Randolph G.J. The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes. *Cell.* 2000. **103.** 757-768.

Rock F.L., Hardiman G., Timans J.C., Kastelein R.A. and Bazan J.F. A family of human receptors structurally related to Drosophila Toll. *Proc.Natl.Acad.Sci.U.S.A.* 1998. **95.** 588-593.

Rokbi B., Renauld-Mongenie G., Mignon M., Danve B., Poncet D., Chabanel C., Caugant D.A. and Quentin-Millet M.J. Allelic diversity of the two transferrin binding protein B gene isotypes among a collection of Neisseria meningitidis strains representative of serogroup B disease: implication for the composition of a recombinant TbpB-based vaccine. *Infect.Immun.* 2000. **68.** 4938-4947.

Rosenstein N.E., Perkins B.A., Stephens D.S., Lefkowitz L., Cartter M.L., Danila R., Cieslak P., Shutt K.A., Popovic T., Schuchat A., Harrison L.H. and Reingold A.L. The changing epidemiology of meningococcal disease in the United States, 1992-1996. *J.Infect.Dis.* 1999. **180.** 1894-1901.

Rosenstein N.E., Perkins B.A., Stephens D.S., Popovic T. and Hughes J.M. Meningococcal disease. *N.Engl.J.Med.* 2001. **344.** 1378-1388.

Rudel T., Schmid A., Benz R., Kolb H.A., Lang F. and Meyer T.F. Modulation of Neisseria porin (PorB) by cytosolic ATP/GTP of target cells: parallels between

pathogen accommodation and mitochondrial endosymbiosis. *Cell.* 1996. **85.** 391-402.

Saint-Vis B., Fugier-Vivier I., Massacrier C., Gaillard C., Vanbervliet B., Ait-Yahia S., Banchereau J., Liu Y.J., Lebecque S. and Caux C. The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J.Immunol.* 1998. **160.** 1666-1676.

Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell.* 2000. **101.** 455-458.

Sallusto F. and Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp.Med.* 1994. **179.** 1109-1118.

Sallusto F., Cella M., Danieli C. and Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp.Med.* 1995. **182.** 389-400.

Sallusto F., Schaerli P., Loetscher P., Schaniel C., Lenig D., Mackay C.R., Qin S. and Lanzavecchia A. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur.J Immunol.* 1998. **28.** 2760-2769.

Sallusto F., Palermo B., Lenig D., Miettinen M., Matikainen S., Julkunen I., Forster R., Burgstahler R., Lipp M. and Lanzavecchia A. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur.J Immunol.* 1999. **29.** 1617-1625.

Scandella E., Men Y., Gillessen S., Forster R. and Groettrup M. Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood.* 2002. **100.** 1354-1361.

Scheinecker C., Machold K.P., Majdic O., Hocker P., Knapp W. and Smolen J.S. Initiation of the autologous mixed lymphocyte reaction requires the expression of costimulatory molecules B7-1 and B7-2 on human peripheral blood dendritic cells. *J Immunol.* 1998. **161.** 3966-3973.

Schneeberger E.E., Vu Q., LeBlanc B.W. and Doerschuk C.M. The accumulation of dendritic cells in the lung is impaired in CD18-/- but not in ICAM-1-/- mutant mice. *J.Immunol.* 2000. **164.** 2472-2478.

Scholten R.J., Kuipers B., Valkenburg H.A., Dankert J., Zollinger W.D. and Poolman J.T. Lipo-oligosaccharide immunotyping of Neisseria meningitidis by a whole-cell ELISA with monoclonal antibodies. *J.Med.Microbiol.* 1994. **41.** 236-243.

Schulz O., Edwards D.A., Schito M., Aliberti J., Manickasingham S., Sher A. and Reis e Sousa. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. *Immunity.* 2000. **13.** 453-462.

Schwandner R., Dziarski R., Wesche H., Rothe M. and Kirschning C.J. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J.Biol.Chem.* 1999. **274.** 17406-17409.

Schwartz B., Moore P.S. and Broome C.V. Global epidemiology of meningococcal disease. *Clin.Microbiol.Rev.* 1989. **2 Suppl.** S118-S124.

Shutt D.C., Daniels K.J., Carolan E.J., Hill A.C. and Soll D.R. Changes in the motility, morphology, and F-actin architecture of human dendritic cells in an in vitro model of dendritic cell development. *Cell Motil.Cytoskeleton.* 2000. **46.** 200-221.

Sigal L.J., Crotty S., Andino R. and Rock K.L. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature.* 1999. **398.** 77-80.

Smirnova I., Mann N., Dols A., Derkx H.H., Hibberd M.L., Levin M. and Beutler B. Assay of locus-specific genetic load implicates rare Toll-like receptor 4 mutations in meningococcal susceptibility. *Proc.Natl.Acad.Sci.U.S.A.* 2003. **100.** 6075-6080.

Snijders A., Kalinski P., Hilkens C.M. and Kapsenberg M.L. High-level IL-12 production by human dendritic cells requires two signals. *Int.Immunol.* 1998. **10.** 1593-1598.

Sousa C., Yap G., Schulz O., Rogers N., Schito M., Aliberti J., Hieny S. and Sher A. Paralysis of dendritic cell IL-12 production by microbial products prevents infection-induced immunopathology. *Immunity.* 1999. **11.** 637-647.

Sousa C., Sher A. and Kaye P. The role of dendritic cells in the induction and regulation of immunity to microbial infection. *Curr.Opin.Immunol.* 1999. **11.** 392-399.

Sousa C.R., Hieny S., Scharton-Kersten T., Jankovic D., Charest H., Germain R.N. and Sher A. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *J Exp.Med.* 1997. **186.** 1819-1829.

Sozzani S., Luini W., Borsatti A., Polentarutti N., Zhou D., Piemonti L., D'amico G., Power C.A., Wells T.N., Gobbi M., Allavena P. and Mantovani A. Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. *J Immunol.* 1997. **159.** 1993-2000.

Sozzani S., Allavena P., D'amico G., Luini W., Bianchi G., Kataura M., Imai T., Yoshie O., Bonecchi R. and Mantovani A. Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J Immunol.* 1998. **161.** 1083-1086.

Sozzani S., Allavena P., Vecchi A. and Mantovani A. The role of chemokines in the regulation of dendritic cell trafficking. *J Leukoc.Biol.* 1999. **66.** 1-9.

Sparwasser T., Koch E.S., Vabulas R.M., Heeg K., Lipford G.B., Ellwart J.W. and Wagner H. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur.J Immunol.* 1998. **28.** 2045-2054.

Stahl P.D. The mannose receptor and other macrophage lectins. *Curr.Opin.Immunol.* 1992. **4.** 49-52.

Starling G.C., McLellan A.D., Egner W., Sorg R.V., Fawcett J., Simmons D.L. and Hart D.N. Intercellular adhesion molecule-3 is the predominant co-stimulatory ligand for leukocyte function antigen-1 on human blood dendritic cells. *Eur.J.Immunol.* 1995. **25.** 2528-2532.

Steeghs L., den Hartog R., den Boer A., Zomer B., Roholl P. and van Der L.P. Meningitis bacterium is viable without endotoxin. *Nature.* 1998. **392.** 449-450.

Steeghs L., Kuipers B., Hamstra H.J., Kersten G., van A.L. and van-der L.P. Immunogenicity of outer membrane proteins in a lipopolysaccharide-deficient mutant of Neisseria meningitidis: influence of adjuvants on the immune response. *Infect.Immun.* 1999. **67.** 4988-4993.

Steeghs L., de Cock H., Evers E., Zomer B., Tommassen J. and van Der L.P. Outer membrane composition of a lipopolysaccharide-deficient Neisseria meningitidis mutant. *EMBO J.* 2001a. **20.** 6937-6945.

Steeghs L. Lipid A engineering in vaccine development against *Neisseria meningitidis. PhD thesis.* 2001b.

Steeghs L., Berns M., ten Hove J., de Jong A., Roholl P., van Alphen L., Tommassen J. and van Der L.P. Expression of foreign LpxA acyltransferases in Neisseria meningitidis results in modified lipid A with reduced toxicity and retained adjuvant activity. *Cell Microbiol.* 2002. **4.** 599-611.

Steinman R.M. and Swanson J. The endocytic activity of dendritic cells. *J.Exp.Med.* 1995. **182.** 283-288.

Stoll S., Delon J., Brotz T.M. and Germain R.N. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science.* 2002. **296.** 1873-1876.

Summers K.L., Hock B.D., McKenzie J.L. and Hart D.N. Phenotypic characterization of five dendritic cell subsets in human tonsils. *Am.J Pathol.* 2001. **159.** 285-295.

Swartley J.S., Marfin A.A., Edupuganti S., Liu L.J., Cieslak P., Perkins B., Wenger J.D. and Stephens D.S. Capsule switching of Neisseria meningitidis. *Proc.Natl.Acad.Sci.U.S.A.* 1997. **94.** 271-276.

Takeuchi O., Kawai T., Muhlradt P.F., Morr M., Radolf J.D., Zychlinsky A., Takeda K. and Akira S. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int.Immunol.* 2001. **13.** 933-940.

Tanaka H., Demeure C.E., Rubio M., Delespesse G. and Sarfati M. Human monocyte-derived dendritic cells induce naive T cell differentiation into T helper cell type 2 (Th2) or Th1/Th2 effectors. Role of stimulator/responder ratio. *J Exp Med.* 2000. **192.** 405-412.

Tang A., Amagai M., Granger L.G., Stanley J.R. and Udey M.C. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature.* 1993. **361.** 82-85.

Tapping R.I., Akashi S., Miyake K., Godowski P.J. and Tobias P.S. Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for escherichia and salmonella lipopolysaccharides. *J Immunol.* 2000. **165.** 5780-5787.

Tettelin H., Saunders N.J., Heidelberg J., Jeffries A.C., Nelson K.E., Eisen J.A., Ketchum K.A., Hood D.W., Peden J.F., Dodson R.J., Nelson W.C., Gwinn M.L., DeBoy R., Peterson J.D., Hickey E.K., Haft D.H., Salzberg S.L., White O.,

Fleischmann R.D., Dougherty B.A., Mason T., Ciecko A., Parksey D.S., Blair E., Cittone H., Clark E.B., Cotton M.D., Utterback T.R., Khouri H., Qin H., Vamathevan J., Gill J., Scarlato V., Masignani V., Pizza M., Grandi G., Sun L., Smith H.O., Fraser C.M., Moxon E.R., Rappuoli R. and Venter J.C. Complete genome sequence of Neisseria meningitidis serogroup B strain MC58. *Science*. 2000. **287**. 1809-1815.

Thoma-Uszynski S., Kiertscher S.M., Ochoa M.T., Bouis D.A., Norgard M.V., Miyake K., Godowski P.J., Roth M.D. and Modlin R.L. Activation of toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. *J Immunol*. 2000. **165**. 3804-3810.

Thornton A.M. and Shevach E.M. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J.Immunol*. 2000. **164**. 183-190.

Tommassen J., Vermeij P., Struyve M., Benz R. and Poolman J.T. Isolation of Neisseria meningitidis mutants deficient in class 1 (porA) and class 3 (porB) outer membrane proteins. *Infect.Immun*. 1990. **58**. 1355-1359.

Trombetta E.S., Ebersold M., Garrett W., Pypaert M. and Mellman I. Activation of lysosomal function during dendritic cell maturation. *Science*. 2003. **299**. 1400-1403.

Tsai C.M., Frasch C.E. and Mocca L.F. Five structural classes of major outer membrane proteins in Neisseria meningitidis. *J.Bacteriol*. 1981. **146**. 69-78.

Tseng S.Y., Otsuji M., Gorski K., Huang X., Slansky J.E., Pai S.I., Shalabi A., Shin T., Pardoll D.M. and Tsuchiya H. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp.Med*. 2001. **193**. 839-846.

Turley S.J., Inaba K., Garrett W.S., Ebersold M., Unternaehrer J., Steinman R.M. and Mellman I. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science*. 2000. **288**. 522-527.

Underhill D.M., Ozinsky A., Hajjar A.M., Stevens A., Wilson C.B., Bassetti M. and Aderem A. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens [see comments]. *Nature.* 1999. **401.** 811-815.

Underhill D.M. and Ozinsky A. Toll-like receptors: key mediators of microbe detection. *Curr.Opin.Immunol.* 2002. **14.** 103-110.

Unkmeir A., Kammerer U., Stade A., Hubner C., Haller S., Kolb-Maurer A., Frosch M. and Dietrich G. Lipooligosaccharide and polysaccharide capsule: virulence factors of Neisseria meningitidis that determine meningococcal interaction with human dendritic cells. *Infect.Immun.* 2002. **70.** 2454-2462.

Uronen H., Williams A.J., Dixon G., Andersen S.R., van Der L.P., van Deuren M., Callard R.E. and Klein N. Gram-negative bacteria induce proinflammatory cytokine production by monocytes in the absence of lipopolysaccharide (LPS). *Clin.Exp.Immunol.* 2000. **122.** 312-315.

Uronen-Hansson H, Allen J.S, Dixon G., Osman M., vand Der L.P., Steeghs L., Wong S., Callard R.E., Klein N. Human dendritic cell activation by *Neisseria meningitides*: phagocytosis depends on expression of lipopolysaccharide (LPS) by the bacteria and is required for optimal cytokine production. Submitted for publication. 2003.

van Der L.P., Steeghs L., Hamstra H.J., ten Hove J., Zomer B. and van Alphen L. Modification of lipid A biosynthesis in Neisseria meningitidis lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. *Infect.Immun.* 2001. **69.** 5981-5990.

van der Merwe P.A. Formation and function of the immunological synapse. *Curr.Opin.Immunol.* 2002. **14.** 293-298.

Verhasselt V., Buelens C., Willems F., De Groote D., Haeffner-Cavaillon N. and Goldman M. Bacterial lipopolysaccharide stimulates the production of cytokines and

the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway. *J Immunol.* 1997. **158.** 2919-2925.

Vermont C.L., van Dijken H.H., van Limpt C.J., de Groot R., van Alphen L. and van Den Dobbelsteen G.P. Antibody avidity and immunoglobulin G isotype distribution following immunization with a monovalent meningococcal B outer membrane vesicle vaccine. *Infect.Immun.* 2002. **70.** 584-590.

Virji M., Makepeace K., Ferguson D.J. and Watt S.M. Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. *Mol.Microbiol.* 1996. **22.** 941-950.

Visintin A., Mazzoni A., Spitzer J.H., Wyllie D.H., Dower S.K. and Segal D.M. Regulation of toll-like receptors in human monocytes and dendritic cells. *J Immunol.* 2001. **166.** 249-255.

Vogel U. and Frosch M. Mechanisms of neisserial serum resistance. *Mol.Microbiol.* 1999. **32.** 1133-1139.

Watson J., Kelly K., Largen M. and Taylor B.A. The genetic mapping of a defective LPS response gene in C3H/HeJ mice. *J.Immunol.* 1978. **120.** 422-424.

Watts C. and Amigorena S. Antigen traffic pathways in dendritic cells. *Traffic.* 2000. **1.** 312-317.

Watts C. Antigen processing in the endocytic compartment. *Curr.Opin.Immunol.* 2001. **13.** 26-31.

Watts C. Immunology. Antigen presentation--losing its shine in the absence of GILT. *Science.* 2001. **294.** 1294-1295.

Wells A.D., Gudmundsdottir H. and Turka L.A. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and

CD28 differentially regulate the induction and duration of a proliferative response. *J Clin.Invest.* 1997. **100.** 3173-3183.

West D., Reddin K., Matheson M., Heath R., Funnell S., Hudson M., Robinson A. and Gorringe A. Recombinant Neisseria meningitidis transferrin binding protein A protects against experimental meningococcal infection. *Infect.Immun.* 2001. **69.** 1561-1567.

Whelan M., Harnett M.M., Houston K.M., Patel V., Harnett W. and Rigley K.P. A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. *J Immunol.* 2000. **164.** 6453-6460.

Whitmire J.K. and Ahmed R. Costimulation in antiviral immunity: differential requirements for CD4(+) and CD8(+) T cell responses. *Curr.Opin.Immunol.* 2000. **12.** 448-455.

Worthylake R.A. and Burridge K. Leukocyte transendothelial migration: orchestrating the underlying molecular machinery. *Curr.Opin.Cell Biol.* 2001. **13.** 569-577.

Xu H., Guan H., Zu G., Bullard D., Hanson J., Slater M. and Elmets C.A. The role of ICAM-1 molecule in the migration of Langerhans cells in the skin and regional lymph node. *Eur.J Immunol.* 2001. **31.** 3085-3093.

Yamamoto M., Sato S., Mori K., Hoshino K., Takeuchi O., Takeda K. and Akira S. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J.Immunol.* 2002. **169.** 6668-6672.

Yang R.B., Mark M.R., Gurney A.L. and Godowski P.J. Signaling events induced by lipopolysaccharide-activated toll-like receptor 2. *J.Immunol.* 1999. **163.** 639-643.

Extra References

Balmer P. and Miller E. Meningococcal disease: how to prevent and how to manage. *Curr.Opin.Infect.Dis.* 2002. **15.** 275-281.

Brandtzaeg P. and van Deuren M. Current concepts in the role of the host response in Neisseria meningitidis septic shock. *Curr.Opin.Infect.Dis.* 2002. **15.** 247-252.

de Vries F.P., van Der E.A., van Putten J.P. and Dankert J. Invasion of primary nasopharyngeal epithelial cells by Neisseria meningitidis is controlled by phase variation of multiple surface antigens. *Infect.Immun.* 1996. **64.** 2998-3006.

Hackett S.J., Thomson A.P. and Hart C.A. Cytokines, chemokines and other effector molecules involved in meningococcal disease. *J.Med.Microbiol.* 2001. **50.** 847-859.

Merz A.J. and So M. Interactions of pathogenic neisseriae with epithelial cell membranes. *Annu.Rev.Cell Dev.Biol.* 2000. **16.** 423-457.

Namork E. and Brandtzaeg P. Fatal meningococcal septicaemia with "blebbing" meningococcus. *Lancet.* 2002. **360.** 1741-.

Rudel T., Schmid A., Benz R., Kolb H.A., Lang F. and Meyer T.F. Modulation of Neisseria porin (PorB) by cytosolic ATP/GTP of target cells: parallels between pathogen accommodation and mitochondrial endosymbiosis. *Cell.* 1996. **85.** 391-402.

Taha M.K., Deghmane A.E., Antignac A., Zarantonelli M.L., Larribe M. and Alonso J.M. The duality of virulence and transmissibility in Neisseria meningitidis. *Trends Microbiol.* 2002. **10.** 376-382.

Uronen H., Williams A.J., Dixon G., Andersen S.R., van Der L.P., van Deuren M., Callard R.E. and Klein N. Gram-negative bacteria induce proinflammatory cytokine production by monocytes in the absence of lipopolysaccharide (LPS). *Clin.Exp.Immunol.* 2000. **122.** 312-315.

Vogel U. and Frosch M. Mechanisms of neisserial serum resistance. *Mol.Microbiol.* 1999. **32.** 1133-1139.