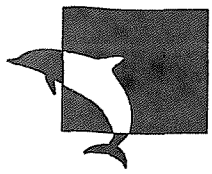

UNIVERSITY OF SOUTHAMPTON

Effects of Bacterial Adjuvants on Human Antigen Presenting Cells

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ABSTRACT

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Doctor of Philosophy

EFFECTS OF BACTERIAL ADJUVANTS ON HUMAN ANTIGEN PRESENTING CELLS

By Tamara Al-Bader

Dendritic cells (DC) are key participants in the development of immune responses. DCs have the ability to recognize various components of pathogens through highly conserved pattern recognition receptors. Maturation of DCs by whole bacteria or their components results in their ability to stimulate naïve as well as primed T-cells. Antigen-specific activation of $CD4^+$ T-helper cells by DCs is influenced by a number of factors, which include strength and duration of co-stimulation and production of inflammatory mediators. This, together with the nature of the pathogen mediates polarization of distinct Th subsets necessary for development of appropriate immune responses. In this thesis, the hypothesis addressed is that different bacterial components may behave as adjuvants by modulating DC function through changes in co-stimulatory molecule expression and production of soluble mediators in a way that would differentially stimulate T-cell proliferation. The dendritic cells used in this study are monocyte-derived dendritic cells (mo-DC). Synthetic oligodeoxynucleotiedes (ODN) containing CpG motifs and outer membranes of *Neisseria meningitidis* were investigated as bacterial-derived adjuvants.

Immunomodulatory effects of bacterial DNA are attributed to the presence of CpG-motifs. Similarly, ODN containing CpG-motifs are reported to possess stimulatory activity on a variety of immune cells. One aim of this study was to examine the effects of CpG-ODN on CD80, CD86, CD40 and HLA-DR expression and cytokine production by monocytes and mo-DCs. The results revealed striking differences between the effects of CpG ODN on monocytes and mo-DCs. Monocytes treated with CpG markedly up-regulated CD86 expression as well as induced production of IL-1β, IL-6, IL-10 and IL-12p40. Similar changes in phenotype were observed in CpG-treated monocytes obtained from non-atopic and atopic individuals. By contrast, mo-DCs treated with CpG ODN failed to show any changes in expression of co-stimulatory molecules and HLA-DR, production of cytokines and did not affect mo-DC driven allogeneic T-cell proliferation. These findings indicate that the *in vitro* differentiation of monocytes to mo-DC alters their responsiveness to CpG DNA and correlates with the loss of Toll-like receptor (TLR) 9 during this differentiation. Expression of TLR9 was observed on human epidermal Langerhans' cells (LC) and not on $CD34^+$-derived DCs suggesting that LC are CpG responsive cells. Differential expression of TLR2, TLR4 and TLR9 on monocytes and distinct DC subsets suggests that activation by bacterial components is facilitated following recognition through specific receptors.

Serogroup B *Neisseria meningitidis* is a major cause of life-threatening meningitis to which no effective vaccine is available. Initiation of innate and acquired immune responses to *N. meningitidis* is likely to be dependent on cellular responses of dendritic cells to antigens present in the outer membrane (OM) of the meningococcus. In this study, the responses of mo-DC to wild type meningococci, a mutant deficient in lipooligosaccharide (LOS), and their corresponding isolated OM, were investigated. The ability of purified recombinant meningococcal class I porin to modulate mo-DC function was also examined. Wild type OM and purified class I porin selectively up-regulated Toll-like receptor (TLR) 4 mRNA expression and induced mo-DC maturation, reflected by increased production of chemokines, pro-inflammatory cytokines and CD83, CD80, CD86, CD40 and MHC class II molecules. In contrast, LOS-deficient OM selectively up-regulated TLR2 mRNA expression, and induced relatively moderate increases in both cytokine production and expression of CD86 and MHC class II molecules. Mo-DCs exposed to OM, from wild type or LOS-deficient mutant exhibited enhanced capacity to drive and polarize allogeneic naïve T-cells. Moreover, mo-DCs exposed to purified class I porin augmented their capacity to stimulate autologous tetanus-toxoid specific T-cell proliferation. Study of actions of meningococcal OM proteins on DC may facilitate novel approaches in manipulating desired immune responses with appropriately designed vaccines.

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ABBREVIATIONS

APC	Antigen presenting cell
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CpG ODN	Cytosine-phosphate-Guanine Oligodeoxynucleotide
cSMAC	Central supramolecular activating complex
CTL	Cytotoxic T-lymphocyte
CXCR	CXC-chemokine receptor
DC	Dendritic cell
DC1 or DC2	Dendritic cell type 1 or type 2
dsRNA	Double-stranded RNA
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Ig	Immunoglobulin
IL-	Interleukin-
iNOS	Inducible nitric oxide synthase
LC	Langerhans' cells
LOS	Lipo-oligosaccharide
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC class I/II	Major histocompatibility complex class I/II
MLR	Mixed leukocyte reaction
Mo-DC	Monocyte-derived dendritic cell
Mo-LC	Monocyte-derived Langerhans' cell
NK	Natural killer cell
NO	Nitric oxide
OM	Outer membranes (*Neisseria meningitidis*)
PAMP	pathogen-associated molecular pattern
PCR	Polymerase chain reaction
Poly (I:C)	Polyinosinic-polycytidylic acid
Por	Porin (*Neisseria meningitidis*)
PRR	Pattern recognition receptor
RT	Reverse transcription
SCF	Stem cell factor
STAT 4/6	Signal transducer and activator of transcription 4/6
Th1/2	T-helper cell type 1 / 2
TIR	Toll/IL-1R homology
TLR	Toll-like receptor
TNF	Tumour necrosis factor

CHAPTER 1

General Introduction

1

1.1 ANTIGEN PRESENTATION

1.1.1 Antigen presenting cells (APCs)

APCs are important regulators of the immune system (Steinman, 1991). In order to initiate a primary immune response, presentation of antigen to T-cells is dependent on APCs. Various cells posses this function, including dendritic cells (DC) and macrophages. Cells of the monocyte/macrophages lineage are derived from myelomonocytic stem cells in the bone marrow (Grage-Griebenow *et al.*, 2001; Ziegler-Heitbrock, 2000). These cells give rise to monoblasts that then develop into monocytes. Monocytes circulate in the blood and have a half-life of 1-3 days. Following this, they migrate into various tissues where they develop into specialized macrophages and serve in primary defence against foreign invaders. DC precursors originate from $CD34^+$ stem cells in the bone marrow and give rise to distinct DC lineages that reside in a variety of tissues (Caux *et al.*, 1996). DCs are described as 'professional antigen presenting cells' due to their inherent ability to take up and process antigen present in the environment, migrate from the periphery to the lymphoid organs and present this antigen to T-cells in order to mount an immune response (Steinman, 1991).

1.1.2 T-cells

In humans, the T lymphocyte pool consists of three populations; naïve, memory and effector T-cells. Naïve and memory T-cell pool generally maintain constant size throughout their life cycles, whereas the effector T-cell pool are able to expand upon activation, followed by apoptosis or further development into memory T-cells (Dutton *et al.*, 1998; Visintin *et al.*, 2001; Swain, 1994; Croft, 1994).

Upon encountering antigen (presented by professional APC) for the first time, naïve $CD4^+$ and $CD8^+$ T-cells differentiate into effector cells, a process normally completed within 4-5 days following antigen exposure (Dutton *et al.*, 1998). Additional activation of these effector T-cells leads to mass proliferation, a process referred to as

clonal expansion. The $CD4^+$ effector cells develop into helper T-cells (Th) whose role is to aid in directing the activities and responses of various cell types; they therefore participate in both cell-mediated and humoral immunity. Precursor Th0 cells are capable of differentiating into three subsets, Th1, Th2 or Th3 that may be distinguished by their production of distinct cytokines (Mosmann and Sad, 1996; Fukaura *et al.*, 1996). $CD8^+$ effector cells develop into cytotoxic T-cells (Tc) with functions enabling specific destruction of target cells and, therefore, are important participants in cell-mediated immunity. Precursor Tc cells are capable of differentiating into to Tc1 or Tc2 subsets (Vukmanovic-Stejic *et al.*, 2000).

Secondary immune responses are brought about following repeated exposure to antigen. The higher frequency of antigen-specific effector cells resulting from clonal expansion during the primary immune response contributes to the rapid development of initiating the secondary response. Compared with the activation of naïve precursor T-cells, effector T-cell activation may be brought about at lower antigen concentrations and co-stimulation may not be essential (Swain, 1994; Swain, 1999). Memory T-cells maintain the polarized cytokine production pattern established following effector cell differentiation during the primary immune response (Dutton *et al.*, 1998). This further contributes to quick onset of the memory T-cell derived effector immune response. In the presence of polarizing cytokines, such as IL-12 within the environment, memory effector T-cells may be influence to express distinct cytokine profiles suggesting that these memory cells are not stable (Vieira *et al.*, 2000; Smits *et al.*, 2001).

1.1.3 APC-T cell interaction

Initial interaction of T-cells with APCs leads to the formation of 'immunological synapses' at the sites of contact between these two cells. Synapse formation precedes T-cell activation and is associated with rapid clustering of TCR/CD3 complexes binding to peptide/MHC complexes on APC. This initial triggering signal aided by CD4/CD8 is known as 'signal 1' and ensures the specificity of the response. Interaction of T cells and APCs requires the rearrangement of membrane proteins in order to coordinate subsequent kinase and phosphatase signalling events. This may be brought about following the downregulation or exclusion of surface molecules such as CD43 and CD45 (Ostberg *et al.*, 1998; Thomas, 1999), respectively, from the

membrane microdomains, enabling antigen-receptor engagement. Moreover, it has been proposed that the ligation of adhesion molecule CD2 stabilizes antigen-receptor engagement as a result of segregating larger molecular weight proteins such as CD45, CD43 and LFA-1 from the APC - T-cell contact area (Dustin *et al.*, 1997; Grakoui *et al.*, 1999).

Optimal Th-cell activation is brought about by the generation of a range of distinct signalling events produced during the recognition of antigen on the surface of professional APCs. 'Signal 2' is provided by the binding of co-stimulatory molecules on T-cells with their co-receptors on APCs. Some co-stimulatory/adhesion molecules may provide essential second signals for T-cell activation, but others may act largely by enhancing TCR triggering (by stabilizing synapse formation and/or recruiting intracellular signalling molecules). In addition, some co-stimulatory/adhesion molecules (eg-CD28) are important for inducing cytokine (eg- IL-2) synthesis by T-cells, whereas others (eg-CD40L) maintain or induce activation of APCs. A third activation signal ('Signal 3') is generated via cytokines and soluble mediators, produced by both the T-cells and APCs, that subsequently promotes the expansion and differentiation of antigen specific Th-cells. These events are suggested to take place in the supramolecular structure of the "immune synapse".

1.1.3.1 Immunological synapse

The cognate interactions of naïve T-cells with APCs require physical cell-cell contact to enable signal induction and subsequent T-cell activation (Bromley *et al.*, 2001). Early studies demonstrated the ability of APCs, such as DCs, and T-cells to form clusters in an antigen-independent manner (Steinman and Cohn, 1974). The formation of an immunological synapse between APC and T-cell enables efficient contact between cells brought about by the ligation of several receptors (Grakoui *et al.*, 1999; Wulfing and Davis, 1998; Monks *et al.*, 1998).

At the beginning of synapse formation, low avidity interactions between adhesion molecules take place to stabilize contact between cells (Grakoui *et al.*, 1999). LFA-1 expressed on resting T-cells binds to ICAM-1 on the APC. This contact occurs at the centre of the synapse area and is referred to as junction formation. At this point, TCR

engagement with MHC-peptide complexes on the surface of the APC is facilitated. During TCR engagement, the LFA-1/ICAM-1 complexes move to the periphery leaving the TCR/MHC-peptide complexes at the centre of the now 'mature' immunological synapse. The transport of the TCR-MHC to the centre, and LFA-1 to the periphery has been shown to be actin-mediated and depends on several factors. These include the nature of the peptide, strength of LFA-1/ICAM-1 interactions and ligation of other co-stimulatory molecules such as CD28 to CD80/CD86. TCR signalling also leads to clustering of CD2 on T-cells and enhanced LFA-3 binding which reinforces the contact between APC and T-cells (Dustin *et al.*, 1998). The final stage of the synapse formation involves stabilizing the TCR/MHC in the central cluster (central supramolecular activation clusters or cSMAC) and the adhesion and co-stimulatory molecules in the circumference of cSMAC, referred to as peripheral SMAC (pSMAC) (Wulfing and Davis, 1998; Monks *et al.*, 1998). It has been put forward that a role of adhesion and co-stimulatory molecules is to amplify the signal transmitted by the TCR by arranging the TCR-MHC cluster formation in the cSMAC (Grakoui *et al.*, 1999).

A newly described receptor implicated in establishing first contact between DCs and resting T-cells is DC-Specific ICAM-3 Grabbing Non-integrin (DC-SIGN) (Geijtenbeek *et al.*, 2000). DC-SIGN is exclusively expressed by DCs and has been demonstrated to mediate high affinity adhesion between DCs and ICAM-3 on resting T-cells. The authors proposed that the initial contact brought about by DC-SIGN/ICAM-3 interactions transiently stabilizes the DC-T-cell contact allowing the DC to interact with various resting T-cells to ensure optimal TCR engagement and subsequent induction of primary immune responses (Geijtenbeek *et al.*, 2000).

1.1.3.2 'Signal 1' – T-cell receptor / CD3 complex

Cell surface TCR is a heterodimer consisting of an αβ chain or sometimes as a γδ dimer and is non-covalently associated with the invariant CD3-γ,-δ,-ε chains and TCR–ζ chains. The CD3 and TCR chains are members of the immunoreceptor signal transduction family that contain immunoreceptor tyrosine-based activation motifs (ITAMs). Interaction between the TCR and antigen-MHC complex together with co-receptors CD4 or CD8, leads to activation of protein tyrosine kinase (PTK) and phosphorylation of ITAMs within the cytoplasmic tail of the CD3 chain. Activation

of PTKs results in recruitment and tyrosine phosphorylation of enzymes such as phopholipase Cγ1 (PLCγ1), and adaptor proteins such as LAT (linker for activation of T-cells). In addition, PTKs activated by antigen receptors couple to phosphatidylinositol-3-kinase (PI-3 kinase) and control the activity of Ras- and Rho-family GTPases. Phosphorylation of downstream substrates subsequently initiates signal-transduction cascades that contribute to the reorganization of the cytoskeleton and activation of genes necessary for adequate T-cell proliferation and differentiation (van Leeuwen and Samelson, 1999; Acuto and Cantrell, 2000).

1.1.3.3 'Signal 2' – Co-stimulation by accessory molecules

T-cell expansion is regulated by a number of factors. Following TCR interaction with peptide/MHC complexes, co-stimulatory signals are generated following the interaction between surface molecules on an APC and their respective ligands on the T-cell, leading to T-cell activation. A number of molecules have been suggested to possess co-stimulatory function, with some of these accessory molecules participating at different stages of T-cell activation. Lack of co-stimulation fails to induce expression of CD40L (CD154) on T-cells leading to ineffective production of IL-2 and subsequently to inadequate T-cell proliferation (Shu *et al.*, 1995). Therefore, the production of multiple co-stimulatory signals possibly acting in collaboration with each other would ensure the generation of effective and prolonged responses.

Understanding the mechanisms and functions of co-stimulatory molecules may potentially be beneficial as targets for adjuvant action to ensure prolonged T-cell responses and possibly promote the production of memory responses. There are multiple co-stimulatory molecules on APCs that upon binding to their respective counterparts, influence T-cell activation. Some of these include, B7-molecules that bind CD28/CTLA-4 on T-cells, CD40 that binds to CD40L on T-cells, OX40 (CD134) that binds to OX40L on T-cells, LFA-1, 2 that bind CD2 on T-cells, Inducible co-stimulator (ICOS) ligand (B7h, B7RP-1, LICOS or B7-H2) that binds ICOS on T-cells, 4-1BB ligand that binds 4-1BB (CD137) on T-cells and Signalling Lymphocyte Activation Molecule (SLAM) ligand that binds SLAM on T-cells (Hathcock *et al.*, 1994; Watts and DeBenedette, 1999; Chambers and Allison, 1997;

Gramaglia *et al.*, 1998; Hutloff *et al.*, 1999; Shuford *et al.*, 1997; Salomon and Bluestone, 1998).

1.1.3.3.1 B7-molecules

CD80 (B7-1) and CD86 (B7-2) are members of the immunoglobulin superfamily and share limited sequence homology in their extracellular domains (June *et al.*, 1994). CD86 is constitutively expressed on APCs such as dendritic cells and monocytes/macrophages and is up-regulated further following microbial encounter or stimulation with inflammatory mediators (McLellan *et al.*, 1995; Creery *et al.*, 1996; Hathcock *et al.*, 1994). Low levels of CD80 are expressed on resting human monocytes, but expression is up-regulated following exposure to lipopolysaccharide (LPS) of gram negative bacteria (Schmittel *et al.*, 1995; Hathcock *et al.*, 1994). In contrast to monocytes, higher levels of CD80 are expressed on human dendritic cells (Hathcock *et al.*, 1994). The surface density of co-stimulatory molecules, in particular CD80 and CD86, has been shown to be a critical factor in determining the efficiency of T-cell activation by human APCs (van Gool *et al.*, 1996). High levels of surface CD80/CD86 expression on APCs are more efficient at activating T-cells than those expressing low levels of CD80/CD86. The density of other adhesion and MHC class II molecules are reported to have minimal effects in antigen presentation compared to CD80/CD86 (Kiertscher and Roth, 1996).

CD28 is the putative ligand for members of the B7 family and is constitutively expressed on naïve and activated T-cells (Freeman *et al.*, 1992). Following engagement between CD28 and CD80/CD86, CD28 expression is reduced. CD80 engagement with CD28 has been shown to be more effective at CD28 down regulation than CD86 (Krummel and Allison, 1995). The ligation of CD80/CD86 to CD28 molecules on T-cells together with signalling through the TCR results in a number of effects. The production of high levels of cytokines such as IL-2, as a result of this ligation provides important T-cell survival signals, therefore, preventing the induction of T-cell anergy or tolerance. Other activating signals include the increased expression of CD25 (IL-2R) and entry into the cell cycle as well as expression of anti-apoptotic proteins of the Bcl-XL family (Thompson, 1995) and up-regulation of chemokine receptors. Interaction between CD80/CD86-CD28 also stimulate the expression of other co-stimulators such as CD40L (De Boer *et al.*, 1993).

If antigen presentation is not accompanied by co-stimulation, anergy, a state of functional unresponsiveness may result. Another member of the CD28 family, cytotoxic T lymphocyte antigen 4 (CTLA-4), expressed on T-cells is capable of binding to CD80/CD86 on APCs. Interaction of CD28 with its ligands CD80/CD86 is weaker than the interaction with CTLA-4. It has been suggested by some studies that T cell anergy may be the result of CD80/CD86-CTLA-4 interactions. CTLA-4 is induced on T-cells after activation and, upon binding to its ligand, blocking signals are transduced that inhibit the transcription of IL-2 and, therefore, play a role in limiting the antigen-induced proliferative response of activated T-cells (Krummel and Allison, 1995).

Although certain antigens are capable of providing enough stimulus to activate T-cells through strong TCR signals, co-stimulation via CD28 plays a role in augmenting and sustaining T-cell responses. In turn, this promotes Th-cell expansion and differentiation. There are conflicting reports suggesting CD80 and CD86 to differentially mediate the development of Th1 or Th2 cells (Lenschow *et al.*, 1994; Freeman *et al.*, 1995). Some evidence suggests CD80 plays a role in directing Th1 differentiation whilst CD86 is involved in directing Th2 differentiation. T-cells from myelin basic protein-specific transgenic mice cultured in the presence of a blocking mAb against CD86, produced predominantly IFN-γ, whereas addition of blocking mAb against CD80 resulted in IL-4 production by T-cells (Kuchroo *et al.*, 1995). These findings are in accordance with a study using an *in vivo* murine model of asthma, where it was found that administration of blocking anti-CD86 mAb into OVA sensitised A/J mice resulted in abolished allergen-induced airway hyper-responsiveness, pulmonary eosinophilia and reduced serum IgG1 and IgE levels. In contrast, this was not observed following the administration of blocking anti-CD80 mAb (Keane-Myers *et al.*, 1998a). However using a model of graft rejection, commonly believed to be Th1-mediated, rejection was inhibited using blocking anti-CD86 mAb while blocking anti-CD80 mAb failed to inhibit rejection (Lenschow *et al.*, 1995).

ICOS-Ligand has been identified as a B7-like molecule and is constitutively expressed on monocytes and DCs and on peripheral B-cells of some donors. Engagement of ICOS can augment induction of both Th1 and Th2 cytokines, but

circumstances effectively costimulate Th2 responses (Tesciuba *et al.*, 2001; Ozkaynak *et al.*, 2001). ICOS is expressed similarly on both Th1 and Th2 lines after primary stimulation but remains high on Th2 lines after repeated activation steps. Ligands for program death-1 (PD-1) molecules, expressed on activated T-cells, are also B7 family members, PD-L1 (B7-H1) and PD-L2 (B7-DC) (Tseng *et al.*, 2001). Interaction of PD-1 with either PD-L1 or PD-L2 results in inhibition of T and B responses (Freeman *et al.*, 2000). Expression of PD-L1 and PD-L2 is not expressed on resting B cells, monocytes or DCs. In human monocytes, IFN-γ treatment (but not LPS) results in induction of PD-L1 and PD-L2. Whereas, on DCs LPS plus IFN-γ treatment induces PD-L1 and PD-L2 mRNA expression (Latchman *et al.*, 2001).

1.1.3.3.2 CD40

CD40 belongs to the tumour necrosis factor receptor family and is expressed on a broad range of cell types including APCs. On freshly isolated human monocytes expression of CD40 is low, but may be induced in the presence of cytokines such as GM-CSF, IL-3 and IFN-γ (Stout and Suttles, 1996) and following exposure to pathogens (Caux *et al.*, 1994; van Kooten and Banchereau, 1997; Sousa *et al.*, 1997). On macrophages, peripheral blood DCs and mo-DCs, CD40 is expressed in high levels and upon activation with inflammatory mediators, such as TNF-α and LPS, expression is enhanced (Sallusto and Lanzavecchia, 1994; Ulevitch and Tobias, 1999).

CD40 binds to its putative receptor CD40-ligand (CD154, CD40L), expressed mainly on activated Th-cells, resulting in the secretion of cytokines such as IL-1α/β, IL-6, IL-8, IL-10, IL-12 and TNF-α (Caux *et al.*, 1994; Cella *et al.*, 1996). Following antigen stimulation of naïve T-cells, CD40L expression is observed after approximately 3-5hrs and decreases by 15-20hrs (Jaiswal *et al.*, 1996; Croft, 1994). Additional consequences of CD40-CD40L interactions include enhanced survival of APCs as well as increased nitric oxide production and tumoricidal activity by the APCs. CD40 ligation can influence the phenotype of APCs by up-regulating the expression of MHC class I and II molecules as well as various co-stimulatory molecules such as ICAM-1, CD80, CD86 and LFA-3 (Koch *et al.*, 1996; Noelle, 1996). On human mo-DCs it is reported that CD40 associates with membrane rafts (sphingolipid- and

cholesterol-rich plasma membrane microdomains) following receptor engagement, to initiate successive DC-mediated signalling events for the induction of IL-1α/β mRNA and IL-12 mRNA via MEK/ERK and p38 MAPK pathways, respectively (Vidalain *et al.*, 2000).

In vitro studies investigating the consequences of co-culturing APCs with T-cells have revealed that blocking the interaction between CD40 and CD40L results in reduced $CD4^+$ Th-cell proliferation (Grewal *et al.*, 1996). The authors suggested this to be the consequence of a lack in CD40 signalling through the APC as well as altered signalling to the Th-cells via CD40L, which in turn may modify additional co-stimulation and cytokine production required for adequate Th-cell proliferation. Moreover, synchronized interactions by co-stimulators CD80/CD86 and CD40 with their respective ligands is reported to play a role in efficient Th-cell activation (Yang and Wilson, 1996). Together, these studies highlight an important function of CD40-CD40-ligand interactions for the development of effector Th-cells. Furthermore, *in vivo*, it has been shown that CD40 triggering by Th-cells enables DCs to activate cytotoxic T-cells (Bennett *et al.*, 1998; Ridge *et al.*, 1998).

A variety of cytokines may be produced following CD40 ligation. There is inconclusive evidence as to whether this action preferentially influences the development of either Th1 or Th2-cell subsets. However, production of IL-12 by the APC after CD40 ligation is regarded to play an important role in skewing T-helper cells towards a Th1 type (Heufler *et al.*, 1996).

1.1.3.4 T-cell polarization – role of 'Signal 3'

T effector cells are categorized based on their patterns of cytokine production. There are various factors implicated in the regulation of naïve $CD4^+$ T-cell differentiation into Th1 or Th2 effectors cells. These distinct T-cell subsets were first demonstrated in mice and are distinguished by their cytokine profiles (Mosmann and Coffman, 1989). Murine Th1 cells are defined by the production of IL-2, IFN-γ, TNF-β, GM-CSF and protect against intracellular pathogens (Mosmann and Coffman, 1989). These Th1 cytokines in turn influence the activation of cytotoxic T-cells, therefore, play a role in the induction of cell-mediated immunity (Yang and Wilson, 1996).

Murine Th2 cells preferentially produce IL-3, IL-4, IL-5, IL-10 and IL-13 in response to extracellular microorganisms and parasites. These cytokines play a role in humoral immunity involving antibody-producing B-cells. A third Th subset, Th3 cells, shows many phenotypic similarities to the Th2 subset, with the exception of producing high levels of TGF-β (Groux *et al.*, 1997; Fukaura *et al.*, 1996).

Human Th-cell subsets are not as clearly defined as in the murine system due to cross-production of cytokines between the subsets in response to variable stimuli, such as pathogens. The human Th1 and Th2 signature cytokines are similar to those from mice. IL-10 however has been shown to be produced by both Th1 and Th2 subsets (Sornasse *et al.*, 1996). In addition, IL-10 has been shown to suppress antigen presentation, thereby inducing tolerance (Saint-Vis *et al.*, 1998; Steinbrink *et al.*, 1997).

The factors implicated in directing the differentiation of Th-cells into either Th0, Th1, Th2 or Th3 subsets include 1) the nature and dose of the antigen (Hosken *et al.*, 1995) 2) the intensity of the TCR activation signals (Constant *et al.*, 1995), 3) the strength and nature of co-stimulatory signals provided to the Th-cell by the APC (June *et al.*, 1994; Gause *et al.*, 1997), 4) the cytokines produce by the APC together with the cytokines present in the surrounding environment (O'Garra and Murphy, 1996) and 5) the genetic background of the naïve Th-cell (Hsieh *et al.*, 1995).

As mentioned above, 'signal 3' is initiated by soluble mediators either present in the surrounding environment or produced by APCs and these play a role in influencing Th-cell differentiation. Production of IL-12 by activated APCs including macrophages and dendritic cells induces the development of Th1 differentiation (Szabo *et al.*, 1997; Trinchieri, 1995). IL-12 production by APCs may in turn act on NK cells to produce IFN-γ that also aids the development of Th1 cells. In viral infections, release of large amounts of type I IFN by APC may suppress IL-12 production (McRae *et al.*, 2000). Nonetheless, type I IFN can substitute for IL-12 in these cases and promote IFN-γ (and IL-10) synthesis by T-cells via a STAT2/STAT4-dependent pathway. IL-4 is the most efficient promoter of Th2 differentiation (Nelms *et al.*, 1999). IL-4 has been shown to be produced by $CD4^+$ $NK1.1^+$ T-cells and γδT-cells (Yoshimoto and Paul, 1994). Naïve T-cells stimulated with low antigen

concentrations or with low-affinity TCR binding peptides produce IL-4 when CD28-mediated co-stimulation is provided, whereas higher concentrations of antigen even in conjunction with CD28 co-signals do not (Tao *et al.*, 1997). APC-derived IL-6 has also been shown to promote Th2 development *in vitro* by enhancing IL-4 production during priming (Rincon *et al.*, 1997). There is now also increasing evidence that naïve $CD4^+$ T-cells provide a source of IL-4 necessary for their development into Th2 effector cells via an autocrine pathway (Croft and Swain, 1995; Yang *et al.*, 1995). Recently, IL-4 was reported to provide a negative feedback to DCs causing them to secrete bioactive IL-12 (Hochrein *et al.*, 2000).

Once polarization has occurred, the differentiation state is reinforced by cytokines from one T-cell subset that inhibits the development of the opposite T-cell subset. Thus, IL-4 and IL-10 have been shown to inhibit the production of IFN-γ and IL-12 and conversely, IFN-γ inhibits the differentiation of the Th2 subset (Gajewski and Fitch, 1988).

1.1.3.5 Role of cytokines

Cytokines play an important role in directing Th1 and Th2 immune responses. APCs such as DCs and macrophages express mRNA transcripts and secrete a range of cytokines in response to various inflammatory stimuli (Saint-Vis *et al.*, 1998; Henderson *et al.*, 1996). Lipopolysaccharide (LPS) from Gram-negative bacteria is a potent inducer of monocyte/macrophages and DCs for the production of IL-12, TNF-α and IL-6 (Verhasselt *et al.*, 1997; Reis e Sousa *et al.*, 1999). Influenza virus-stimulated mo-DC also produce TNF-α and IL-6, but differ from LPS stimulated mo-DCs in that they produce small amounts of IL-15 and do not produce IL-12 (Cella *et al.*, 1999a; Verhasselt *et al.*, 1997).

1.1.3.5.1 IL1α and IL1β

IL-1α and IL-1β are two pro-inflammatory cytokines that have been shown to promote Th1 differentiation by partially inhibiting IL-4 production (Sandborg *et al.*, 1995). Human $CD4^+$ clones specific for purified protein derivative (PPD) of Mycobacterium tuberculosis, that are high IFN-γ and low IL-4 producers, induced higher levels of IL-1β production upon contact with APC (Chizzolini *et al.*, 1997). In

contrast, tetanus toxoid specific $CD4^+$ clones displaying Th2 cytokine profiles produced lower levels of IL-1β but higher levels of IL-1 receptor antagonist (IL-1Ra) (Chizzolini *et al.*, 1997). Production of IL-1β by APCs is also suggested to play a role in augmenting IFN-γ production by NK cells by acting in conjunction with IL-12 (Hunter *et al.*, 1995). The interaction between CD40 and CD40 ligand is reported to be critical for the activation of IL-1 synthesis by monocytes (Wagner *et al.*, 1994).

IL-1 receptor belongs to the IL-1R/toll-like receptor superfamily (O'Neill and Dinarello, 2000). The IL-1 receptor possesses three extracellular immunoglobulin (Ig) domains and a conserved Toll/IL-1R (TIR) domain. During binding of IL-1 to the IL-1 type I receptor complex formation with IL-1 receptor accessory protein (IL-1RAcP) is necessary in order to initiate signal transduction (O'Neill and Dinarello, 2000).

1.1.3.5.2 IL-6

Inflammatory stimuli, such as LPS, result in the production of IL-6 by human DCs and monocyte/macrophages (Saint-Vis *et al.*, 1998; Verhasselt *et al.*, 1997). In mice, IL-6 is reported to be able to polarize naïve $CD4^+$ T-cells into effector T-cells displaying Th2 cytokine profiles by stimulating the initial production of IL-4 by $CD4^+$ T-cells (Rincon *et al.*, 1997). In humans IL-6, unlike IL-12, does not inhibit IL-4 production by phytohaemagglutinin (PHA) stimulated PBMCs or PHA stimulated-purified T-cells (Sandborg *et al.*, 1995).

1.1.3.5.3 IL-10

IL-10 is known to have potent immune-modulating effects on DC and macrophages and acts to preferentially inhibit Th1 priming (Aste-Amezaga *et al.*, 1998). IL-10 suppresses T-cell induced as well as anti-CD40 mAb triggered IL-12 production by DC (Koch *et al.*, 1996). Also, IL-10 markedly inhibits DC-driven IFN-γ production by purified murine $CD4^+$ and $CD8^+$ T-cells (Macatonia *et al.*, 1993). Immature DCs exposed to IL-10 show a decreased ability to stimulate $CD4^+$ T-cells in a mixed lymphocyte reaction (MLR), *in vitro.* IL-10-mediated effects on immature human DC are also reported to render them tolerogenic (Steinbrink *et al.*, 1997). Moreover, IL-10 pre-treated DCs exhibited baseline levels of CD83, CD56 and CD86 expression

indicating the inability of IL-10 to develop fully mature DC (Steinbrink *et al.*, 1997). Exposure of mature DCs to IL-10 results in suppressed IL-12 production and such cells have been shown to suppress IFN-γ production by T-cells (De Smedt *et al.*, 1997; Macatonia *et al.*, 1993). A diminished expression of surface CD80 is observed on IL-10 treated murine DCs and Langerhans' cells (Ozawa *et al.*, 1996; De Smedt *et al.*, 1997). On monocytes/macrophages, IL-10 is shown to affect overall antigen presenting function, and in particular, to inhibit surface expression of MHC class II molecules (Koppelman *et al.*, 1997). It may be plausible that the production of IL-10 by APCs in response to stimuli or similarly the presence of IL-10 in the local environment, contributes to the low expression of MHC class II molecules on APCs. In turn, this may serve to deliver low antigen signals to T-cells which may preferentially promote Th2-dominated responses (Constant *et al.*, 1995).

1.1.3.5.4 IL-12

IL-12 is a heterodimeric cytokine, made up of two covalently linked subunits, designated p35 and p40 (Szabo *et al.*, 1997; Trinchieri, 1995). Most cell types express low levels of p35 constitutively whereas p40 synthesis is inducible and restricted to a subset of hematopoietic cells including B cells, macrophages, granulocytes and DCs (Trinchieri, 1995). The p35 subunit of IL-12 is not active on its own but, upon interaction with the p40 chain, biological activity is displayed. The secretion of the isolated p35 protein has not been detected. Cells that produce the biologically active 70kd IL-12 heterodimer secrete the isolated p40 chain, usually at higher levels than the heterodimer (Trinchieri, 1995). IL-12p70 binds to the IL-12R expressed on T-cells which in turn induces the production of IFN-γ (Gately *et al.*, 1998; Szabo *et al.*, 1997). The IL-12R is made up of two subunits IL-12Rβ1 and IL-12Rβ2 (Gately *et al.*, 1998). Expression of both these subunits at the cell surface is necessary for high-affinity IL-12 binding. IL-12Rβ2 is believed to be the signal transducing subunit and contains conserved tyrosine residues in the cytoplasmic region (Gately *et al.*, 1998; Rogge *et al.*, 1997). Studies using human Th2 cells have revealed the lack of surface IL-12Rβ2 expression due to downregulation by IL-4, IL-10 and TGF-β2, which may explain the unresponsiveness of Th2 cells to IL-12 (Szabo *et al.*, 1997; Rogge *et al.*, 1997; Wu *et al.*, 1997).

A newly identified protein, p19, is reported to complex with the p40 subunit of IL-12 to form the biologically active cytokine IL-23 (Oppmann *et al.*, 2000). Human and mouse DCs produce detectable levels of secreted IL-23 which displays functions similar to IL-12. Engagement of the p19p40 complex to the IL-12Rβ1 activates Stat4 and stimulates IFN-γ production and proliferation in human PHA blast T-cells and CD45RO T-cells (Oppmann *et al.*, 2000).

IL-12 plays a central role in mediating Th1 development (Heufler *et al.*, 1996; Hilkens *et al.*, 1997). IL-12 also stimulates NK cells and fosters CTL development. In response to certain microbial stimuli, such as LPS and *Staphylococcus aureus* Cowan strain I, human monocytes/macrophages and dendritic cells produce IL-12p40 and IL-12p70 (Trinchieri, 1995; Hunter *et al.*, 1995). DCs also produce IL-12 p70 as a result of CD40 ligation and without additional bacterial stimuli (Langenkamp *et al.*, 2000). Although mature DCs produce IL-12, bi-directional signalling between T-cells and APC may be required for secretion of large amounts of IL-12. The presence of IFN-γ augments IL-12p70 production (Lamont and Adorini, 1996). Naïve Th cells do not produce high levels of IL-12, unless exogenous IFN-γ is present, whereas activated memory Th cells are efficient IL-12 inducers even in the absence of exogenous IFN-γ (Heufler *et al.*, 1996; Hilkens *et al.*, 1997).

1.2 DENDRITIC CELLS

DCs have been described as 'professional' APCs on the basis of their ability to present antigen and induce primary immune responses, therefore permitting establishment of immunological memory (Steinman, 1991; Mackay, 1999). Immature DC are specialized to sample and take up antigen in the periphery and process it for presentation to T-cells in the secondary lymphoid tissue (Nijman *et al.*, 1995). DCs also play a role in the clearance of infectious agents (Brightbill *et al.*, 1999; Janeway, 1992). DC maturation, brought about by external factors within the environment such as whole bacteria or components of bacteria, generally involves the redistribution of MHC molecules from the intracellular endocytic compartments to the surface, down-regulation of antigen uptake, changes in co-stimulatory and adhesion molecules as well as chemokine receptors, cytoskeleton re-orgainisation and the secretion of soluble mediators. DCs ultimately acquire the capacity to migrate to secondary lymphoid organs where adequate presentation of processed antigen to T-cells takes place (Steinman, 1991).

1.2.1 Dendritic cell lineages

DCs are bone marrow-derived and are widely distributed as immature cells throughout the body (lymphoid and non-lymphoid tissues), particularly in tissues that interface with the environment (Figure 1.1). Human and murine DC precursors arise from $CD34^+$ pluripotent haematopoetic stem cells (Galy *et al.*, 1995; Inaba *et al.*, 1992). Following extensive *in vitro* studies, two DC lineages have been identified and classified as lymphoid- and myeloid-derived (Hart, 1997; Wu *et al.*, 1996; Olweus *et al.*, 1997; Dzionek *et al.*, 2000). Different environmental factors influence the differentiation of $CD34^+$ stem cells into the respective DC lineages, ultimately leading to DCs residing in distinct locations and possessing different phenotypes and function (Stumbles *et al.*, 1998; Caux *et al.*, 1996; Rescigno *et al.*, 2001; Trobonjaca *et al.*, 2001; Austyn *et al.*, 1994).

Myeloid DCs found in the skin (dermal dendritic cells and Langerhans' cells) as well as those found in mucosal surfaces, such as the lung and gut, function principally to capture antigens (Cumberbatch *et al.*, 2000; Rescigno *et al.*, 2001; Julia *et al.*, 2002; McWilliam *et al.*, 1996). In the lymphoid tissues DCs are found in the spleen, lymph

nodes and thymus. The DCs found within the T-cell areas of the secondary lymphoid tissues, referred to as interdigitating DCs, are mature and express high levels of costimulatory molecules necessary for efficient T-cell activation. DCs found in the germinal centres are reported to be involved in T-cell dependent memory B-cell responses (Wykes *et al.*, 1998; Dubois *et al.*, 1998). Blood DCs are found in low frequency in the circulation. Detection of fresh uncultured blood DCs is based phenotypically by the absence of leukocyte lineage (lin)-specific antigens, such as CD3, CD14, CD19 and CD56, and the presence of HLA-DR, CD4 or CD33 (Olweus *et al.*, 1997; Robinson *et al.*, 1999; Kohrgruber *et al.*, 1999; Romani *et al.*, 1994; O'Doherty *et al.*, 1993).

1.2.1.1 The myeloid dendritic cell lineage

Myeloid DCs are migratory cells that are recruited to the lymph nodes as veiled cells via the afferent lymphatics under inflammatory conditions. These cells are also believed to be stimulatory, as they are involved in the induction of immune responses following encounter with antigen in the periphery and subsequent presentation of antigen to naïve T-cells in the lymphoid organs (Hart, 1997; Sallusto and Lanzavecchia, 1999).

In vitro, $CD34^+$ stem cells differentiate into DCs in the presence of GM-CSF and TNFα, to express CD1a, CD13, CD33, CD11c, CD4, CD40, CD80, CD86, CD54, CD58 and MHC class II molecules (Caux *et al.*, 1996). Cutaneous lymphocyte-associated antigen (CLA) expressing $CD34^+$ stem cells develop into $CD1a^+$ $CD14^-$ intermediate cells in the presence of GM-CSF and TNF-α (Strunk *et al.*, 1997). This DC subset has been shown to differentiate into the $CD1a^+$, Birbeck $granule^+$, Lag^+ LC *in vitro* and typically express high levels of HLA-DR and HLA-DQ (Caux *et al.*, 1996). $CD34^+$ stem cells also give rise to LCs *in vitro* in the presence of GM-CSF, TNF-α and TGF-β (Reid, 1998). In contrast CLA^- $CD34^+$ stem cells develop into $CD1a^-CD14^+$ DCs in the presence of GM-CSF and TNF-α that give rise to cells resembling dermal DCs. Dermal dendritic cells lack Birbeck granules, express CD32, possess intracytoplasmic factor XIIIa and may sub-divided into further populations: $CD1a^-CD14^-$, $CD1a^+CD14^-$ or $CD1a^-CD14^+$ (Reid, 1998). The $CD1a^-CD14^+$

intermediates may also differentiate into macrophage-like cells in the presence of macrophage-colony-stimulating factor (M-CSF) (Caux *et al.*, 1996). Dermal DCs demonstrate a high efficiency of antigen capture, whereas LCs do not. Moreover, LC lack functional mannose receptors and are poor stimulators of antigen-specific CD4+ T-cell clones when compared to monocyte-derived DCs (Mommaas *et al.*, 1999).

Monocyte-derived DCs (mo-DCs) belong to the myeloid lineage. Monocytes cultured *in vitro* in the presence of GM-CSF and IL-4 or IL-13 lose CD14 expression and develop an immature DC phenotype (Sallusto and Lanzavecchia, 1994; Piemonti *et al.*, 1995). In contrast to mo-DCs cultured in human serum/plasma, culturing in foetal calf serum results in marked up-regulation of CD1a (Pietschmann *et al.*, 2000). Following a maturation step with TNF-α or IL1-β, mo-DCs up-regulate the co-stimulatory molecules CD80, CD86 and CD40 as well as CD83, a DC maturation marker (Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996). In the presence of GM-CSF, IL-4 and TGF-β monocytes cultured *in vitro* for 6 days develop LC-like characteristics (Geissmann *et al.*, 1998).

In vitro, it has been reported that monocytes are capable of differentiating into DCs following migration across an endothelial monolayer in the abluminal to luminal direction; this process has been referred to as reverse transmigration. This situation would occur when cells move from tissues into the afferent lymph. Therefore, veiled DCs in the lymph may originate from monocytes that interact with the endothelium that influences the development of immature DCs (Randolph *et al.*, 1998). *In vivo*, monocytes that phagocytosed sub-cutaneously injected fluorescent microspheres migrate to the lymph nodes where they differentiate into DCs (Randolph *et al.*, 1999). Therefore, these studies suggest monocytes to be a DC precursor *in vivo*. The cytokine environment and presence of differentiation/co-stimulatory signals may be the deciding factors whether monocytes acquire DC or macrophage characteristics and function (Palucka *et al.*, 1998; Vieira *et al.*, 2000).

1.2.1.2 The lymphoid dendritic cell lineage

Lymphoid DCs were initially described in the mouse. Murine thymic and splenic DCs are characterized by $CD8\alpha^+$, $DEC\text{-}205^+$, $CD11b^-$. IL-2 and IL-5 have been shown to drive NK-cell associated ($IL\text{-}2R^+$) DCs from $CD34^+$ progenitors

(Bykovskaja *et al.*, 1998; Bykovskaia *et al.*, 1999). Resident lymphoid DCs are non-migratory cells found in the thymus and spleen and may be responsible for the induction of tolerance in the absence of inflammation (Sallusto and Lanzavecchia, 1999). In humans, there is evidence that $CD4^+$, $CD11c^-$, $CD3^-$ plasmacytoid T cells isolated from tonsils are DC precursors. Plasmacytoid DCs also express $CD45RA^+$ and high levels of the IL-3Rα chain ($CD123^{high}$), and therefore, depend on IL-3 for their survival. Culturing plasmacytoid cells with IL-3 and CD40L results in their obtaining a DC morphology and phenotype that lack myeloid-associated markers (Grouard *et al.*, 1997). Early progenitors of the lymphoid related DC pathway also express CD34, CD10 and CD38 whilst intermediate precursors have been described as $IL\text{-}2R^+$, $CD86^+$, $CD4^+$ and MHC class I and II positive (Galy *et al.*, 1995; Marquez *et al.*, 1998). Recently, surface antigens BDCA-2 and BDCA-4 have been shown to be expressed on non-cultured human $CD123^{high}$ $CD11c^-$ plasmacytoid blood DCs (Dzionek *et al.*, 2000). Human plasmacytoid DCs do not produce IL-12 and are a major source of IFN–α (Siegal *et al.*, 1999; Cella *et al.*, 1999a). Due to their capacity to induce apoptosis and play a role in eliminating potentially self reactive T-cells, it is suggested that lymphoid DCs primarily mediate regulatory rather than stimulatory immune effector functions. Nevertheless, human lymphoid DCs derived from $CD4^+$, $CD11c^-$, $CD3^-$ plasmacytoid T cells have been shown to promote Th2 differentiation (Rissoan *et al.*, 1999).

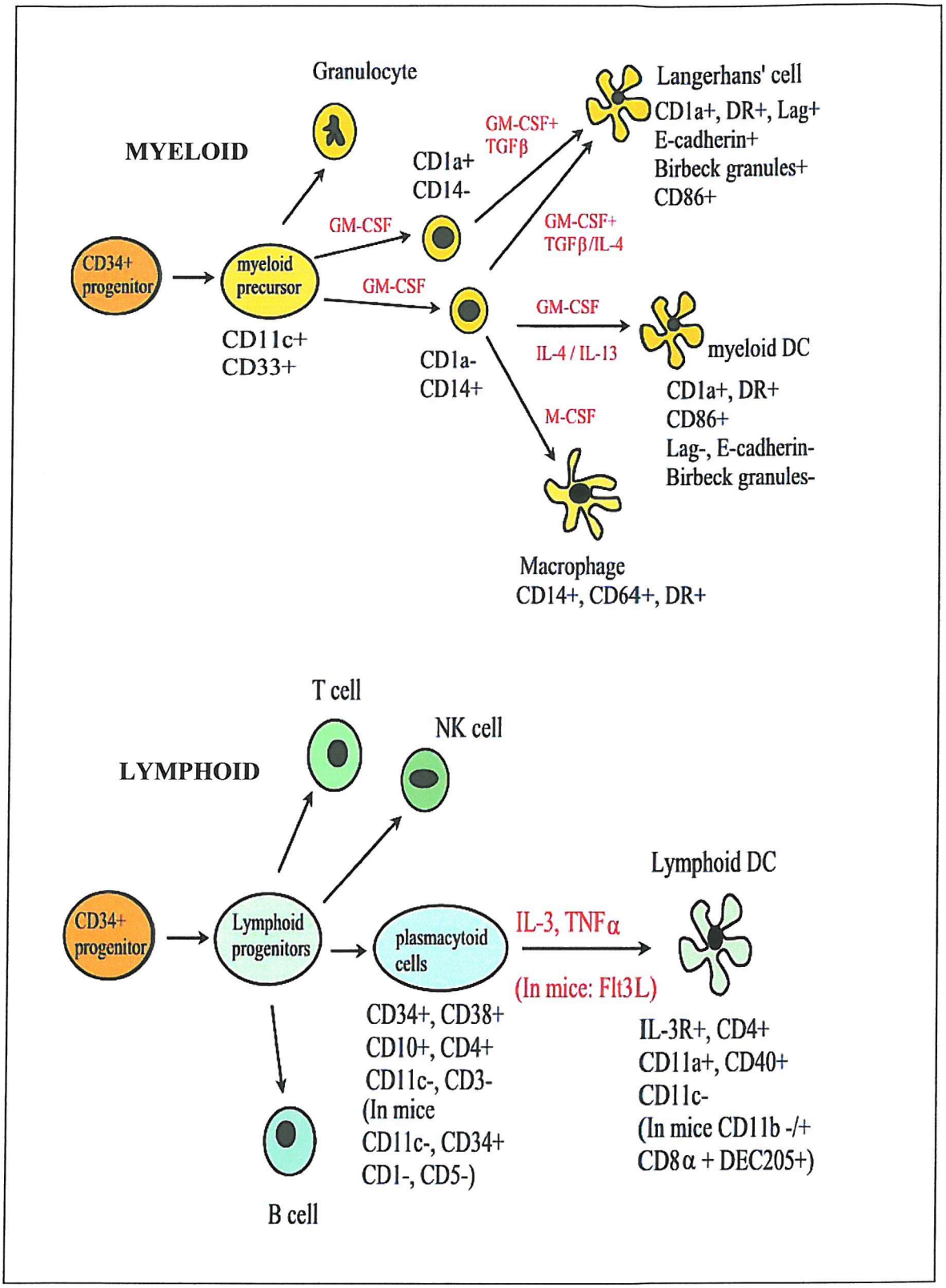


Figure 1.1: Myeloid and lymphoid dendritic cell lineages.

1.2.2 Dendritic cell function

The ability of DCs to present antigen and subsequently induce a potent immune response is brought about by efficient recognition and uptake of antigen, processing and presenting in the context of MHC-peptide complexes for the interaction with TCR on T-cells (Cella *et al.*, 1997a). As described in earlier sections, co-stimulation is necessary to sustain presentation of antigen to T-cells and also influences T-helper cell differentiation (van Gool *et al.*, 1996). In addition, inflammatory signals from the surrounding environment directed at DCs result in altered production and secretion of soluble mediators that aid in influencing T-cell differentiation therefore determining the type of immune response elicited (Kalinski *et al.*, 1999a).

1.2.2.1 Recognition and uptake of antigen

Inducible responses of the innate immune system are triggered following the recognition of pathogens by a set of pattern recognition receptors (PRRs). These receptors can recognize conserved molecular patterns, referred to as pathogen-associated molecular patterns (PAMPs), shared by various organisms (Medzhitov and Janeway Jr, 1998). The recognition of invading organisms in this manner allows the immune system to detect the presence of an infectious microbe, but additionally determines the type of infecting pathogen. In turn, PRRs activate conserved host defense signalling pathways that control the expression of numerous genes to induce an effective immune response for elimination of invading pathogens (Medzhitov and Janeway Jr, 1998).

Unlike macrophages that principally function as scavenger cells, pathogen-induced stimulation of DCs results in early production of mediators that recruit other macrophages, neutrophils, NK cells as well as immature DCs to the inflammatory site. These inflammatory mediators have also been suggested to prolong DC survival (Muzio *et al.*, 2000a; Rescigno *et al.*, 1998a).

PRR may generally be classed as those that mediate phagocytosis and those that lead to the activation of proinflammatory pathways. Immature DCs are very efficient at

taking up antigen and they do this very quickly by a number of mechanisms. Antigen may be captured non-specifically by macropinocytosis, where active membrane ruffling facilitates the in-take of extracellular fluid (Sallusto *et al.*, 1995). DC macropinocytosis is constitutive allowing continuous internalisation of large volume of fluid. In other macropinocytic APCs such as macrophages, growth factors regulate the uptake of antigen non-specifically (Sallusto *et al.*, 1995).

DCs capture large particles by phagocytosis, a process suggested to be mediated via complement receptors (CR) including CR3 (CD11b/CD18, Mac-1) and CR4 (CD11c/CD18, LFA-3), and type II and III Fcγ receptors that function to internalize bacteria (Ingalls and Golenbock, 1995; Fanger *et al.*, 1996). During DC activation, expression of Fcγ receptors is not modulated and signalling via the γ-chain results in immunocomplex antigen presentation (Lanzavecchia, 1990). This mode of antigen uptake may result in efficient antigen presentation on MHC class I molecules (Rescigno *et al.*, 1998b). The DC line, D1, was shown to be capable of presenting ovalbumin (OVA) in association with MHC class I 10^6 fold more efficiently following phagocytosis of OVA-expressing bacteria as compared to the uptake of soluble OVA (Rescigno *et al.*, 1998b). Following the phagocytosis of bacteria, D1 cells develop a mature phenotype with increased levels of MHC I and MHC II, CD80, CD86 and CD40. Similarly, D1 cells that phagocytose apoptotic cells also develop mature phenotypes but do not induce inflammatory responses (Rovere *et al.*, 1998). DCs also express surface CD36 and αvβ5 that are reported to bind and mediate phagocytosis of apoptotic cells (Albert *et al.*, 1998).

DCs can also take up antigen through specialized regions of the plasma membrane, called coated pits, by a process termed receptor-mediated endocytosis. Immature DCs express abundant C-type lectin family members, DEC-205 (in humans known as gp200MR6) and mannose receptors (Engering *et al.*, 1997; Jiang *et al.*, 1995). The mannose receptor, a 180 kDa transmembrane receptor expressed on macrophages and DCs, is specialized in the internalization of carbohydrate antigens (Engering *et al.*, 1997). Pathogens and their products regulate mannose receptor expression on APC (Engering *et al.*, 1997; Sallusto *et al.*, 1995). The endocytic and phagocytic activities of mannose receptors are independently regulated, and combinations of cytokines may act in concert to alter the different activities of the receptor (Raveh *et al.*, 1998).

Mannose receptor-mediated phagocytosis, but not endocytosis, of chitin particles, initiates IL-12 production by APCs (Shibata *et al.*, 1997). Fc receptors, such as FcγRII (CD32), FcγRI (CD64) and CD23, which mediate the internalization of immune complexes are also present (Engering *et al.*, 1997; Sallusto *et al.*, 1995; Fanger *et al.*, 1996). DCs express receptors for heat shock proteins (hsp), gp96 and hsp70, that mediates internalization of hsp-peptide complexes (Todryk *et al.*, 1999).

Epidermal Langerhans' cells contain the lectin Langerin (CD207) which induces the formation of Birbeck granules in the endocytic compartment. Langerin is suggested to play a role in antigen uptake (Valladeau *et al.*, 2000). Murine BDCA2, a type II C-type lectin, expressed in plasmacytoid DCs are also reported to internalise antigen (Dzionek *et al.*, 2001).

1.2.2.2 Toll-like receptors

Members of the Toll-like receptor (TLR) family play a fundamental role in pathogen recognition and activation of innate immunity (Kopp and Medzhitov, 1999). In *Drosophila*, Toll family proteins specify the innate immune responses to microbial infections; Toll is responsible for the response against fungal infection, whereas, 18-wheeler responds to bacterial infection. In addition, *Drosophila* Toll controls dorsal/ventral embryonic development (Hashimoto *et al.*, 1988). TLRs are highly conserved receptors for microbial determinants (Medzhitov and Janeway Jr, 1998). In mammals, the capacity of TLRs to mount an appropriate immune response to invading pathogens resides in its ability to distinguish various pathogens-associated molecular patterns (PAMPs) (Medzhitov and Janeway, Jr., 2002). In humans, 10 Toll-related proteins have been characterized to date; however, TLR2 and TLR4 are the best described PRRs that recognize PAMPs, (Figure 1.2).

Using murine models, LPS is reported to bind to TLR4 (Poltorak *et al.*, 2000). C3H/HeJ and C57BL/10ScCr mice contain mutations in the *Tlr4* gene and are hyporesponsive to LPS (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). Macrophages obtained from C3H/HeJ mice and exposed to LPS are unable to secrete inflammatory cytokines, fail to phagocytose opsonized particles and produce reactive oxygen species or nitric oxide (Poltorak *et al.*, 1998). During the phagocytosis of pathogens,

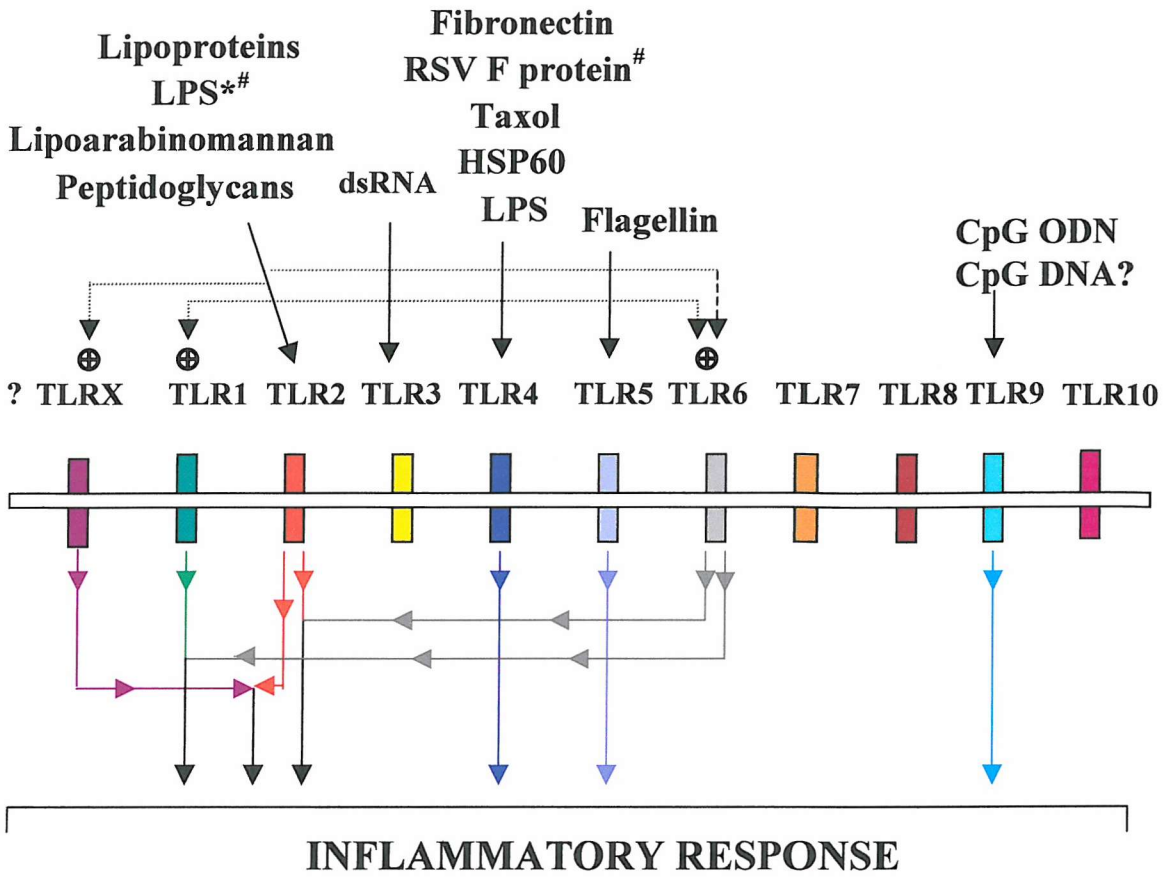
TLRs have also been shown to be recruited to the phagosomes. Here, TLRs may sample antigen, determine the nature of the pathogen and consequently formulate an appropriate immune defence for its elimination (Underhill *et al.*, 1999). In humans, TLR2 is also implicated in conferring responsiveness to LPS by the activation of NFκB transcription factor with induces transcription of inflammatory cytokines. However, this evidence comes from transfection studies in which TLR4 was absent (Yang *et al.*, 1998; Kirschning *et al.*, 1998). This may suggest TLR2 plays a role in recognizing LPS when other LPS binding receptors are absent. Recently, structural variants of LPS (*Leptospira interrogans* and *Porphyromonas gingivalis*) that differ from LPS of gram-negative bacteria have been reported to signal through TLR2 in conjunction with CD14 (Werts *et al.*, 2001; Hirschfeld *et al.*, 2001). In humans, constitutively active TLR4 also induced expression of B7 family members on APC, which are required for efficient T-cell activation (Medzhitov *et al.*, 1997). Therefore, TLR4 is described as an important link between pathogen detection and induction of adaptive immune responses (Medzhitov *et al.*, 1997). TLR4 is also reported to induce inflammatory signalling pathways to respiratory syncytial virus fusion (F) protein, suggesting that TLR4 is not solely be selective for LPS (Kurt-Jones *et al.*, 2000). Moreover, although TLRs are typically described as receptors that recognize microbial structures, non-microbial products such as heat shock protein (hsp) 60 and the extracellular domain A of fibronectin are reported to signal through TLR4 (Ohashi *et al.*, 2000; Kawasaki *et al.*, 2000; Okamura *et al.*, 2001).

TLR2 preferentially mediates inflammatory signalling in response to components of Gram-positive bacteria, yeast and lipoproteins that are found in gram-positive and gram-negative bacteria (Anderson, 2000; Ozinsky *et al.*, 2000; Hertz *et al.*, 2001). There are increasing observations, from studies using transfected cells lines overexpressing the TLRs of interest, that different TLRs could combine together to recognize different foreign components, thereby enhancing the specificity of the determinants recognized as well as the intensity of the signals generated. TLR2 is reported to co-operate with TLR1 and TLR6 for the induction of responses to bacterial peptidoglycans (Ozinsky *et al.*, 2000; Hajjar *et al.*, 2001). Ozinsky and colleagues described TLR1/TLR2/TLR6 complexes to be recruited into macrophage phagosomes where they are able to sample it's contents (Ozinsky *et al.*, 2000). TLR3 has been shown to activate NF-κB in response to double-stranded RNA (Alexopoulou

et al., 2001). TLR5 recognizes flagellin, which is the principal component of bacterial flagella (Hayashi *et al.*, 2001). Recently, a study using TLR9 knock-out mice demonstrated the requirement of this receptor to mediate immune responses induced by short bacterial DNA sequences (Hemmi *et al.*, 2000). The specificity of other TLRs to different foreign agents or pathogens have yet to be elucidated.

Mammalian TLRs are transmembrane molecules comprising multiple extracellular leucine-rich repeats, a single transmembrane domain and an intracellular signalling domain. This signalling domain is closely related to those found in the IL-1R family and has been termed Toll and IL-1 related (TIR) (O'Neill and Dinarello, 2000). The secreted protein, MD-2, is believed to act as an accessory protein to TLR4 and increases responsiveness to LPS (Dziarski *et al.*, 2001; Yang *et al.*, 2000; Shimazu *et al.*, 1999). TLR activation results in recruitment of MyD88, which interacts with TIR domain of the receptor through its C-terminal Toll homology domain. The N-terminal domain of MyD88 interacts with a death domain of serine/threonine protein kinase (IRAK). Phosphorylated IRAK subsequently complexes with TRAF6 adaptor protein inducing a cascade of signalling events leading to the activation of mitogen-activated-protein-kinases (MAPK) and translocation of NF-κB to the nucleus (Takeuchi *et al.*, 2000; Schnare *et al.*, 2000; Hacker *et al.*, 2000; Bowie and O'Neill, 2000).

Human monocytes express mRNA transcripts for TLR-1, 2, 4, 5, 6, and 8. NK cells and B-cells express mRNA for TLR-1, 2, 4, 6 and 9 in B-cells (Muzio *et al.*, 2000b; Hornung *et al.*, 2002). Expression of surface TLR1 on human monocytes is also reported (Wyllie *et al.*, 2000). Human plasmacytoid DCs ($CD11c^{-}CD123^{+}$) express mRNA for TLR-1, 6, 7 and 9 (Hornung *et al.*, 2002). TLR3 transcripts, detected by *in situ* hybridization, were selectively expressed on murine $CD1a^{+}$ DCs present in the T-cell areas of lymph nodes and on human *in vitro* differentiated mo-DC (Muzio *et al.*, 2000b). TLR3 mRNA expression was also shown to be inhibited on mo-DC upon exposure to LPS, IL-1β and TNF-α (Cella *et al.*, 1997b).



⊕ Cooperation between TLRs

LPS	Gram-negative bacteria
LPS*#	*Leptospira interrogans*, *Porphyromonas gingivalis*
?	Unknown
#	Association with CD14
TLRX	As yet unidentified TLR
RSV	Respiratory syncytial virus

Figure 1.2: TLR recognition of microbial components and co-operation between receptors to initiate signalling.

1.2.2.3 Antigen processing and presentation by MHC molecules

DCs resident in peripheral tissues have the capacity to accumulate antigens with high efficiency and broad specificity (Cella *et al.*, 1997b). These antigens are processed and loaded onto MHC class I or class II molecules enabling efficient activation of naïve and primed $CD8^+$ and $CD4^+$, respectively, in the lymphoid regions. Murine bone-marrow derived DCs and human mo-DCs synthesize large numbers of MHC class II molecules that are associated with antigenic-peptides and expressed on the cell surface (Cella *et al.*, 1997b; Pierre *et al.*, 1997). Moreover, synthesis of class II molecules in immature DCs occurs at a high rate, which is further increased by maturation stimuli such as LPS and TNF–α (Watts, 1997; Cella *et al.*, 1997b). In humans, newly synthesized MHC class II molecules present on the cell surface are complexed with an invariant chain (Ii) (Saudrais *et al.*, 1998). Captured antigens are degraded in endosomes and the polypeptides are transported into MHC class II-rich compartments during the maturation process. Peptide loading is brought about following the internalization of MHC-Ii complexes and Ii degradation by cathepsin S (in immature DCs this is inhibited by cystatin C). Subsequently, MHC-peptide complexes are recycled back to the cell surface. Surface expression of antigenic peptides complexed onto MHC class II molecules is short lived, being endocytosed back into the cell and degraded by proteases (Cella *et al.*, 1997b). However, maturation results in an increase in the half-life of the MHC class II-peptide complex on the surface of human DCs due to reduced endocytosis (Cella *et al.*, 1997b). Prolonged expression of these peptide-MHC complexes allows the migrating DC to present the antigenic peptide upon reaching the T-cell areas of secondary lymphoid organs. Human immature DC are also reported to express abundant empty class II MHC molecules at the cell surface, which disappear upon maturation, and which are suggested to act as extracellular receptors that may pick up peptide antigens that are not taken up by other mechanisms (Santambrogio *et al.*, 1999).

The recognition of peptide-MHC complexes by the T-cell antigen receptor provides the first signal that is required for optimal T-cell growth and differentiation. Moreover, a single MHC class II-peptide complex on DCs is able to trigger stimulation of several TCRs (Valitutti *et al.*, 1995).

1.2.2.4 Dendritic cell maturation and migration to lymphoid regions

Immature DCs are induced to mature following exposure to a number of factors. These include 1) mediators produced during tissue inflammation, such as GM-CSF, TNF-α, IL-1β, IL-6, TGF-β and prostaglandins, 2) pathogen-related products, such as LPS, bacterial DNA and double-stranded RNA, and 3) T-cell derived signals (Rescigno *et al.*, 1997; Rescigno *et al.*, 1999; Hartmann *et al.*, 1999; Cella *et al.*, 1999b; Kalinski *et al.*, 1999a; Vieira *et al.*, 2000). Following bacterial encounter, DC survival and maturation is brought about by the induction of MAP-kinase ERK and NF-kB signal pathways (Ammon *et al.*, 2000; Vidalain *et al.*, 2000). Upon maturation, the ability of DCs to capture antigen is lost. This is paralleled by their increased migratory capacity (Cella *et al.*, 1997a; Rescigno *et al.*, 1999). The rate at which DCs migrate from blood to tissues and similarly from tissues to lymph nodes is increased according to various inflammatory stimuli.

Immature DCs expressing chemokine receptors CCR1, CCR2, CCR5 and CXCR1 are attracted by inflammatory chemokines across the endothelium towards sites of inflammation (Sozzani *et al.*, 1998). Migration results in response to monocyte chemotactic protein 1 (MCP), macrophage inflammatory protein 1α and 3α (MIP-1α, MIP-3α) and RANTES (Sallusto and Lanzavecchia, 1999). Blood-borne DCs expressing P-selectin glycoprotein ligand (PSGL)-1 have been shown to exit blood at inflammatory sites by tethering and rolling on P- and E-selectins upregulated by inflammatory stimuli on endothelial cells (Sallusto and Lanzavecchia, 1999). Human β-defensins (HBD) 1 and 2, released in mucosal surfaces and skin in response to microbial invasion, are chemotactic for immature dendritic cells through interaction with CCR6 (Yang *et al.*, 1999).

Maturing DCs are characterized by upregulation of MHC class II, CD80, CD86, CD40, CD54 and CD58 (Sallusto *et al.*, 1995; Rescigno *et al.*, 1997). Chemokines are also produced by DCs following exposure to maturation stimuli. This serves to enhance recruitment of additional immature DCs to the site of inflammation and down-regulate the expression of cognate receptors on maturing DC permitting their migration from the inflamed tissues into the lymphatics (Sallusto and Lanzavecchia, 1999). Maturing DCs up-regulate receptors for constitutive chemokine such as CXCR4, CCR4 and CCR7 resulting in enhanced responsiveness to their appropriate

ligands. The chemokine SDF-1 binds to CXCR4, TARC and MDC binds to CCR4, and MIP-3β and SLC binds CCR7 (Dieu *et al.*, 1998; Sozzani *et al.*, 1998). SDF-1 is constitutively expressed in a broad range of tissues (Bleul *et al.*, 1996), whereas MIP-3β is restricted to lymph nodes, thymus and appendix (Rossi *et al.*, 1997). This suggests an important role for MIP-3β and CCR7 in directing migration of maturing DCs from the periphery to the T-cell regions of the lymph nodes where antigen presentation can occur.

Upon reaching the lymph node, the mature DC lacks the ability to internalize antigen and instead expresses high levels of surface molecules necessary for optimal antigen presentation (Kitajima *et al.*, 1996). Final DC maturation is brought about following the binding with CD40L during T-cell interaction. Resultant effects of CD40-CD40L ligation is the production of IL-12, IL-18, TNF-α and MIP-1α and further upregulation of surface molecules including MHC class II, CD11a, CD54, CD80, CD86 and CD58 (Peng *et al.*, 1998; Thomas and Lipsky, 1994; Cella *et al.*, 1996). In addition, CD40 ligation results in the up-regulation of two survival genes in the DC, bcl-2 and bcl-XL, thereby prolonging the interaction between DC and the T-cell (Wong *et al.*, 1997).

1.2.3 Modulation of dendritic cell activation by pathogens

The nature of the maturation signals received by immature DC from microbial products at the peripheral sites has been referred to 'Signal 0' (Kalinski *et al.*, 1999a; de Jong *et al.*, 2002). Gram-negative and positive bacteria are capable of activating DCs to induce varying levels of maturation (Ulevitch and Tobias, 1999; Henderson *et al.*, 1997). A number of bacterial constituents are also capable of stimulating an immune response, including peptidoglycans, lipoteichoic acids, lipoarabinomannan (LAM), lipopeptides and bacterial DNA (de Jong *et al.*, 2002; Gewirtz *et al.*, 2001; Hertz *et al.*, 2001; Lien *et al.*, 1999; Bauer *et al.*, 2001a).

'Signal 0' together with the type of locally released inflammatory mediators and the quality and quantity of co-stimulation is suggested to be critical for influencing the polarization of an immune response. Distinct APC lineages possess functional differences in their ability to influence the polarization of Th-cell responses during the priming of naïve T-cells, following the encounter with pathogens. Antigen processing

and other immune response mechanisms may be activated or inhibited by microbial components to the benefit of either the host or the pathogen (Brodsky *et al.*, 1999). Current literature suggests that DCs may direct the appropriate immune response towards a cell mediated (Th1) or humoral (Th2) type by responding to pathogen-derived, or induced IL-12 promoting or IL-12 inhibiting factors. In humans, myeloid DCs (also termed DC1) reportedly induce Th1 effector cells whilst plasmacytoid DCs (DC2) induce Th2 differentiation (Rissoan *et al.*, 1999). The reverse scenario has been observed in rodents, both *in vivo* and *in vitro* (Pulendran *et al.*, 1999; Maldonado-Lopez *et al.*, 1999). Myeloid DCs are able to produce high levels of the Th1-inducing cytokine IL-12 (Vieira *et al.*, 2000; Langenkamp *et al.*, 2000). However, it has been observed that mo-DCs can give rise into either DC1 or DC2 depending on the nature of the maturation stimulus influencing IL-12 production (Kalinski *et al.*, 1997; Lambrecht *et al.*, 2000). *In vitro*, the ratio between mature mo-DCs and naïve T-cells in co-cultures is shown to influence polarization of an immune response. At a low DC/T-cell ratio (1:300), where DCs were matured after stimulation with LPS, *Staphylococcus aureus* Cowan 1 or CD40L, naïve T-cells were differentiated into Th2 effector cells. In contrast, a high DC/T cell ratio (1:4) favoured a mixed Th1/Th2 response (Tanaka *et al.*, 2000). Additionally, the length of DC activation with maturation stimuli, such as LPS, poly (I)•poly (C) and TNF-α/IL-β, may influence the polarization of distinct Th-subsets. DCs matured for longer were observed to be in an exhausted state, in terms of IL-12 production, therefore influencing the development of Th2 effector cells (Langenkamp *et al.*, 2000).

Collectively, the ability of immature DC to 1) distinguish the nature of the pathogenic-stimulus (signal 0) through PRRs or pathogen-induced tissue responses (signal 3), 2) display appropriate levels of co-stimulatory molecules and 3) modulate IL-12 production as well as other soluble mediators in response to infection, together with the kinetics of DC activation plays a critical role in determining the type of $CD4^+$ Th immune response induced.

1.2.3.1 'DC1' – myeloid dendritic cells

A known potent activator of macrophages and DCs is LPS, the integral component of the outer membrane of gram-negative bacteria. The lipid portion (Lipid A) is the

toxic portion of LPS and its effects are exerted when gram-negative bacteria lyse as a result of attack by the complement membrane attack complex or by ingestion and killing by phagocytes (Ulevitch and Tobias, 1999). Mo-DCs cultured *in vitro*, in the presence of IL-4 and GM-CSF, do not express CD14, yet are susceptible to activation by LPS through recognition via TLRs, principally TLR4 (Verhasselt *et al.*, 1997; Pulendran *et al.*, 2001; Anderson, 2000). This activation results in the up-regulation of surface expression of MHC class II molecules, CD80, CD86, CD40 and ICAM-1. Moreover, LPS-mediated activation induces production IL-12, IL-1β and TNF-α (Verhasselt *et al.*, 1997; de Jong *et al.*, 2002).

Similarly, ligation of CD40 on DCs with CD40L induces high levels of IL-12p40 and IL-12p70 which influence the priming of $CD4^+$ Th1 cells (Macatonia *et al.*, 1995; Langenkamp *et al.*, 2000). The presence of IFN-γ in conjunction with LPS- or maturation factors IL-1β / TNF-α act synergistically resulting in maturation of mo-DCs with enhanced IL-12 production following CD40 ligation (Caux *et al.*, 1994; Cella *et al.*, 1996). Influenza virus and double-stranded RNA (dsRNA) also activate human DCs (Cella *et al.*, 1999b). This is characterized by the upregulation of MHC molecules, CD83, CD80 and CD86 and production of modest levels of IL-12p70. Also, type I interferon is produced following dsRNA stimulation. The ability of DCs to respond to dsRNA may be attributed the recognition of specific molecular patterns on dsRNA (Cella *et al.*, 1999b).

1.2.3.2 'DC2' – lymphoid dendritic cells

The ability of DCs to drive the development of Th2 responses are less understood. Some evidence suggests that Th2 development may be a default pathway reflected by the lack of IL-12 produced by APCs. Certain mediators or pathogens are capable of affecting the levels of IL-12 produced by DCs. *In vitro* cultured mo-DCs, pre-treated with prostaglandin E2 (PGE_2), have reduced ability to produce IL-12 (Kalinski *et al.*, 1997; Vieira *et al.*, 2000). Subsequent co-culture of these mo-DC with naïve T-cells directs the development of Th2 cells producing high levels of IL-4 and IL-5 and low levels of IFN-γ. The addition of PGE_2 with maturation factors IL-1β and TNF-α to mo-DCs was also shown to drive Th2 differentiation (Kalinski *et al.*, 1997). From this study, it was demonstrated that PGE_2 is able to synergise with IL-1β and TNF-α

to induce mo-DC maturation via cyclic AMP (cAMP) pathways (Kalinski *et al.*, 1997). Prostanoids produced during infection by certain helminths are suggested to possess Th2 skewing properties by affecting cAMP levels (Kalinski *et al.*, 1997).

Schistosoma mansoni eggs have been shown to result in IL-10 production by mo-DCs (Kullberg *et al.*, 1992; Kalinski *et al.*, 1997). IL-10 has been suggested to influence APCs to drive Th2 cell differentiation (Berg *et al.*, 2001; De Smedt *et al.*, 1997). Mo-DCs cultured with IL-10 in combination with IL-1β and TNF-α show reduced IL-12 p70 production (Kalinski *et al.*, 1998). In mice, DCs exposed to IL-10 are capable of inducing Th2 development (De Smedt *et al.*, 1997). Type I interferon's, produced in response to some viral infections, such as measles virus, have recently been shown to inhibit secretion of IL-12p40 by mo-DC leading to reduced T-cell IFN-γ production, therefore, influencing the development of Th2 differentiation (McRae *et al.*, 2000; Servet-Delprat *et al.*, 2000). Other agents reported to inhibit IL-12 production by DC include cholera toxin (CT) and Rauscher Leukemia virus (RLV). CT-treated mo-DCs up-regulated expression of MHC class II, CD80 and CD86, however, *in vitro* priming of naïve $CD4^+CD45RA^+$ T-cell drove the polarization towards a Th2 phenotype (Gagliardi *et al.*, 2000). Mo-DCs produce intracellular IL-4, a Th2 skewing cytokine, in response to RLV with a reduction in IL-12 production (Kelleher *et al.*, 1999) as well as in response to ES-62 antigen from a filarial nematode (Whelan *et al.*, 2000).

Th2 responses may also be preferentially generated at the end of the immune response through contact with exhausted DCs (Langenkamp *et al.*, 2000). Thus, in addition to the origin of the DC (whether myeloid or lymphoid), the nature of the activating stimuli received by the DCs plays and important role in determining the development of Th1 or Th2 immune responses.

1.3 ADJUVANTS

Adjuvants have been widely used in the development of vaccines to increase, prolong or enhance specific immune responses to non-immunogenic vaccine antigens. The potency of an adjuvant is usually judged in terms of levels, duration and localization of antibody responses. New strategies in vaccine development are being employed with use of adjuvants that are capable of inducing antigen-specific cellular immune responses as well as the desired antibody response (Singh and O'Hagan, 1999; Vogel, 2000).

1.3.1 Mechanisms of adjuvant action

Adjuvants have diverse mechanisms of action, therefore, the selection of which adjuvant to use in vaccine development is made on the basis of the route of administration and the type of immune response required for a particular antigen, whether antibody or cell-mediated or for the induction of mucosal immunity (Vogel, 2000; Audibert and Lise, 1993; Czerkinsky *et al.*, 1999).

Adjuvants can strengthen the immune response to an antigen by a number of mechanisms 1) they can increase the immunogenicity of weak antigens such as purified or recombinant antigens, 2) they can enhance the speed and duration of the immune response, 3) they can modulate antibody avidity, specificity, isotype, or subclass distribution, 4) improve antigen delivery to APCs, as well as processing and presentation by the APCs, 5) induce the production of immunomodulatory cytokines.

1.3.2 Types of immunological adjuvants

Adjuvants are classified by their nature, mechanisms of action and physical or chemical properties. However, they may be broadly categorized as immunostimulatory or particulate adjuvants, based on their principal mode of action (Vogel, 2000). The most widely used adjuvant in human vaccines are the aluminium-based salts, also referred to as alum (Gupta, 1998). Toxicity of alum is low and functions to aid the transport of conjugated antigen to the draining lymph node where immune responses are generated. However, the potency of alum as an adjuvant either to induce antibodies against protein subunits or to induce cell-mediated immunity is

low (Gupta, 1998). Furthermore, alum adjuvants can induce IgE antibody responses and have been associated with the development of allergic reactions to the aluminium-based salts themselves, in humans (Relyveld *et al.*, 1998).

1.3.2.1 Immunostimulatory adjuvants

These adjuvants are thought to exert their effects predominantly at the cytokine level through activation of co-stimulatory signals or through related intracellular signalling pathways. This group may be divided further into microbial and non-microbial adjuvants.

1.3.2.1.1 Microbial adjuvants

Freund's complete adjuvant (FCA) is a well-known adjuvant capable of generating potent immune responses (Freund *et al.*, 1937). FCA consists of a water-in-mineral-oil emulsion containing killed mycobacteria. However, due to high toxicity, its use in human vaccines is unfeasible. Muramyl dipeptide, the active component of the mycobacteria in FCA, is being evaluated for use as an adjuvant (Cohen *et al.*, 1996). A derivative of LPS, Monophosphoryl Lipid A (MPL), from gram-negative bacteria such as *Salmonella Minnesota* has been extensively evaluated for use as an adjuvant. The mechanism of action remains to be elucidated, although it is suggested to act similarly to LPS (Gustafson and Rhodes, 1992). MPL has been shown to efficiently induce $CD4^+$ T-cell mediated immunity with increased production of IFN-γ, which promotes the generation of Th1 responses (Sasaki *et al.*, 1997; Thoelen *et al.*, 1998; Ismaili *et al.*, 2002).

1.3.2.1.2 Non-microbial adjuvants

Saponins derived from the Chilean soapbark tree, *Quillaja saponaria* belong to the group of immunostimulatory adjuvants. Through the interaction with cholesterol, saponins are capable of intercalating into cell membranes resulting in pore formation. Isolated pure fractions of Quil A saponin, termed QS21, have been used in clinical trials as adjuvants in vaccines against cancer and infectious diseases (Sjolander *et al.*, 1997). At low doses, QS21 is mildly toxic with a strong ability to induce CTL responses as well as to promote Th1-type responses with the production of IL-2 and

IFN-γ. QS21 has also been employed for use as an adjuvant for DNA vaccines (Sasaki *et al.*, 1998).

The use of cytokines IL-1, IL-2, IFN-γ, IL-12 and GM-CSF as adjuvants to modify or re-direct an immune response has also been explored, but shown little promise for use in vaccines due to 1) dose-related toxicity, 2) their proteinaceous nature resulting in reduced stability and a short *in vivo* half-life (Vogel, 2000; Gupta, 1998). Addition of IL-12 to an alum-adsorbed HIV-1 gp120 vaccine elicited Th1 cytokines and IgG2 and IgG3 production in mice. Exclusion of IL-12 in the same vaccine induced Th2 responses and IgG1 production in mice (Jankovic *et al.*, 1997).

1.3.2.2 Particulate adjuvants

Particulate adjuvants have dimensions comparable to pathogens and facilitate the induction of immune responses by targeting antigen directly into APCs (Gupta, 1998). These groups of adjuvants include emulsions, immunostimulatory complexes (ISCOMs), liposomes and microparticles.

MF59, a squalene oil-in-water emulsion, is a potent adjuvant with low toxicity in humans. MF59 enhances the immunogenicity of influenza vaccine and has been shown to be more effective than alum as an adjuvant for the hepatitis B vaccine. Studies with labelled MF59 have demonstrated the ability of adjuvant to target macrophages and dendritic cells at the site of injection as well as in the lymph nodes (Dupuis *et al.*, 1998). MF59 is a potent inducer of antibody responses. MF59 have also been explored for use as a delivery system for immunostimulatory adjuvants such as MPL and QS21, to increase uptake of immunostimulatory adjuvants by APCs (Gupta, 1998).

Liposomes have been evaluated both as adjuvants and as delivery systems for antigens (Gregoriadis, 1990). The development of polymerized liposomes has been shown to remain in stable form in the gut and therefore, is being investigated for use in mucosal vaccines. ISCOMs are composed of Quil A adjuvant incorporated into lipid particles comprising cholesterol, phospholipids and cell membranes of antigen. The adjuvant effect of ISCOMs is mediated by directly targeting APCs resulting in the production of IL-12 (Smith *et al.*, 1999). In preclinical models, liposomes and

ISCOMS have been shown to greatly improve the induction of CTL responses over that induced by antigen alone or in combination with standard alum adjuvants (Takahashi *et al.*, 1990).

Biodegradable and biocompatible polyesters, poly-lactide-*co*-glycolides (PLGs), have been used for the development of adjuvants through the encapsulation of antigens into PLGs. These adjuvants act as controlled-release delivery system, controlling the rate of release of entrapped antigens (O'Hagan *et al.*, 1991). PLGs mediate their effects following their uptake into DCs and macrophages and the local lymph nodes after intramuscular injection. Compared to alum adjuvants, PLG microparticles are effective for the induction of CTL responses in rodents (Nixon *et al.*, 1996).

1.4 IMMUNOSTIMULATORY DEOXYNUCLEOTIDES

Immunostimulatory sequences (ISS) within bacterial DNA are known to play a role in eliciting potent immune responses. Initial findings of the immunostimulatory properties of bacterial DNA by Tokunaga and colleagues appeared in the 1980s (Mashiba *et al.*, 1988). They reported that mycobacterial DNA activation of NK cells was responsible for the potent antitumor activity of an extract of *Mycobacterium bovis* Bacilles Calmette-Guérin (BCG). Additionally, short synthetic oligodeoxynucleotide (ODN) sequences taken from bacterial genomes exhibit stimulatory activity. The finding that palindromic hexamers containing CpG dinucleotides (Cytosine-phosphate-Guanine) were the active units in the synthetic ODNs led to suggestion that the immunostimulatory capacity of bacterial DNA was attributed in part to the presence of CpG dinucleotides or 'motifs'. Reversal of the central CpG-motif to a GpC motif abrogated its stimulatory effects (Sonehara *et al.*, 1996). Synthetic single stranded ODN containing 5' –purine –purine –C –G –pyrimidine -pyrimidine –3' sequences possess optimal stimulatory properties on murine and certain human immune cells, *in vitro,* in a manner similar to bacterial DNA (Krieg *et al.*, 1995; Klinman *et al.*, 1997).

The frequency of CpG dinucleotides in bacterial genomes is approximately 1 in 16 bases. In vertebrate genomes however, CpG dinucleotides are present at about a quarter of the predicted random frequency. The fewer CpG dinucleotides found in vertebrate DNA is referred to as 'CpG suppression'. An important difference between vertebrate and bacterial DNA is the methylation of cytosine residues at position C-5 in vertebrate DNA, whilst in bacteria DNA this is unmethylated (Bird, 1986). Manipulation of vertebrate DNA to express unmethylated backbones did not present stimulatory activity when added into cultures containing CpG-responsive cells suggesting that the CpG dinucleotides in vertebrate genomes are flanked by bases that constitute immune-neutralizing motifs that block the effects of the immunostimulatory motifs (Krieg *et al.*, 1998a). Poly-G ODN sequences efficiently inhibit the ability of stimulatory CpG motifs to activate NF-KB (Lenert *et al.*, 2001; Chen *et al.*, 2001a). CpG suppression and methylation are highly conserved among all vertebrates (Yi *et al.*, 1996).

1.4.1 Immunostimulatory sequences

The immunostimulatory capacity of synthetic antisense ODN is widely believed to depend on the presence of CpG sequence(s), preferably at the centre of the sequence (Krieg *et al.*, 1995). However, the initial consensus that stimulatory properties of ODN depends on the presence of palindromic hexamer sequences, containing CpG motifs flanked by two 5'-purines and two 3'-pyrimidines, is now controversial. A number of sequences have been reported, that do not conform to this rule but are capable of immunostimulatory activity in both murine and human cells (Van Uden and Raz, 1999). Additionally, some sequences without CpG motifs may be stimulatory to human B-cells (Liang *et al.*, 1996). The presence of 'TpG' motifs in synthetic ODN sequences are also reported to possess mild stimulatory effects (Chu *et al.*, 1997; Yi *et al.*, 1996), whereas, repeated G- and/or C-sequences may specifically neutralize immunostimulatory activity (Krieg *et al.*, 1998a). CpG motifs that appear to be optimal for stimulating murine cells may differ from those that stimulate human cells. Mouse cells respond optimally to GACGTT motif, whilst there is accumulating evidence suggesting that human cells respond to the GTCGTT motif when present in a nuclease-resistant phosphorothioate backbone (Krieg and Wagner, 2000; Krieg *et al.*, 1995). Nuclease-resistant phosphorothioate ODN may be as much as 200-fold more potent than natural phosphodiester ODN for the activation of human B-cells and murine bone marrow and spleen cells (Krieg *et al.*, 1995) (Liang *et al.*, 1996). Phosphorothioate modification of the ODN replaces 1 of the 2 non-bridging oxygens of the internucleotide phosphate with a sulphur atom thereby preventing nuclease activity and extending its half life (Van Uden and Raz, 1999). However, the use of high concentrations of phosphorothioate ODN is reported to non-specifically activate B-cells and murine DCs (Krieg and Wagner, 2000). The number of bases required for maximal potency within the immunostimulatory sequence varies between a 15 - 22-mer ODN. Use of a 6-mer ODN in a cationic lipid transfection demonstrated stimulatory effects (Sonehara *et al.*, 1996).

1.4.2 Cellular activation by CpG ODN

The effects of CpG ODN on immune cells were initially characterized in murine studies. More recently, the immunostimulatory properties of CpG ODN on human cells, *in vitro*, are becoming appreciated. A summary of the effects of CpG ODN on

immune cells is shown in Figure 1.3. *In vitro*, CpG ODN does not directly activate purified murine and human NK cells. CpG-mediated human NK cell activation appears to be indirect and requires the presence of IL-12, TNF-α and IFN-α/β produced by CpG-activated APCs (Van Uden and Raz, 1999). Additionally, CpG ODN does not directly activate purified T-cells. However, when T-cells are treated simultaneously with CpG ODN and anti-CD3-activating antibody, increased production of IL-2, expression of IL-2 receptors and induction of proliferation and differentiation into CTLs are seen (Bendigs *et al.*, 1999).

1.4.2.1 B cell activation

CpG ODN can directly stimulate B-cells to proliferate and produce high levels of IL-6, IL-12 and IL-10 (Yi *et al.*, 1996). The mitogenic effect of CpG ODN on B-cells is independent of IL-6 production as determined from experiments using blocking antibodies and knock out mice (Krieg *et al.*, 1995). In addition, purified B-cells activated with CpG ODN produce polyclonal IgM antibodies that are independent of T-helper cells or exposure to antigen but dependent on IL-6 produced by CpG-activated B-cells. Neutralization of IL-6 produced by B-cells in response to CpG ODN markedly reduces IgM production (Yi *et al.*, 1996). Also, exogenous IFN-γ (such as that produced by NK cells) together with CpG ODN augments IL-6 secretion by splenic B-cells (Yi *et al.*, 1996). CpG-activation also results in the induction of reactive oxygen-dependent intracellular signal pathways and inhibition of B-cells from anti-Ig-induced apoptosis (Yi *et al.*, 1998). Activation of murine B-cells by CpG ODN, both *in vivo* and *in vitro*, results in marked changes in cell surface molecules. These include increased expressions of MHC class II molecules, CD80, CD86, CD40, CD16/32, ICAM-1, IFN-γ receptor and IL-2 receptor. The low affinity IgE receptor, CD23, however is down regulated following CpG ODN activation of B-cells (Yi *et al.*, 1998).

1.4.2.2 Monocyte/Macrophage activation by ODN

CpG ODN-stimulated murine primary macrophages and macrophage cell lines become cytotoxic and produce pro-inflammatory cytokines TNF-α, IL-6, IL-12p40, IL-18 and IFN-α/β (Lipford *et al.*, 1997; Van Uden and Raz, 1999) These cytokines are capable of stimulating the production of IFN-γ by NK cells that in turn, can

activate macrophages further, forming a positive feedback loop. In addition, immunoglobulin isotype switching to IgG2a is induced thereby biasing the development of Th1 response in murine models. *In vivo*, as well as in mixed cell cultures, CpG ODN activation of macrophages is suggested to be indirect, through the production of IFN-γ by other cells. Murine bone marrow-derived macrophages that have been primed with IFN-γ show increased expression of inducible nitric oxide synthase (iNOS) and its product nitric oxide (NO) following treatment with CpG ODN (Stacey *et al.*, 2000). CpG DNA and LPS are reported to synergize to induce macrophage NO production and monocyte cytokine production (Gao *et al.*, 1999; Hartmann and Krieg, 1999). RAW 264 macrophage cell line and spleen cells express increased levels of cyclooxygenase-2 and secrete PGE2 following treatment with CpG ODN (Chen *et al.*, 2001b). Moreover, treatment with CpG ODN also results in a rapid increase of mRNA for a variety of anti-apoptotic genes and cellular proto-oncogenes (Stacey *et al.*, 2000). The anti-apoptotic effects of CpG ODN on bone marrow-derived macrophages are associated with down-regulation of the growth factor CSF-1 (Stacey *et al.*, 2000). In addition to cytokine production following CpG ODN activation, murine bone marrow-derived macrophages are capable of up-regulating cell surface molecules that are involved in antigen processing and presentation; MHC class I molecule, CD40, CD86, ICAM-1 and CD16/32 (IgG receptor for internalization of bound antigen and antibody-dependent cellular cytotoxicity). Human monocyte/macrophage primary cells and cell lines activated with CpG ODN produce IL-6, TNF-α and IL-12p40 (Hartmann *et al.*, 1999; Roman *et al.*, 1997).

1.4.2.3 DC activation by ODN

CpG ODN and bacterial DNA are potent stimuli for murine bone marrow-derived DCs, cultured *in vitro* in the presence of GM-CSF, inducing them to produce high levels of the Th1-type cytokines IL-12, IL-18 and moderate levels of IL-6 and TNF-α (Sparwasser *et al.*, 1998). Cytokine production is paralleled by the up-regulation of MHC class II molecules and co-stimulatory molecules CD86 and CD40 but not CD80 (Sparwasser *et al.*, 1998). Moreover, maturation of BMDCs with CpG ODN is observed in a CD40-CD40 ligand independent manner(Sparwasser *et al.*, 1998).

CpG-activated DCs are capable of eliciting mixed lymphocyte reactions *in vitro* and *in vivo*. Subcutaneous injection of CpG ODN or plasmid DNA leads to the activation of DCs from the draining lymph nodes. Activation of Langerhans' cell (LC)-like murine foetal-skin derived DCs with CpG ODN was observed *in vitro* (Jakob *et al.*, 1998). *In vivo* inoculation of CpG ODN into murine skin resulted in enhanced expression of MHC II and CD86 and accumulation of intracellular IL-12 by LCs (Jakob *et al.*, 1998).

Human myeloid $CD11c^+$ DCs are not activated by CpG ODN/DNA whereas these cells are strongly responsive to LPS (Hartmann *et al.*, 1999). However, the reverse activation pattern is observed for human plasmacytoid ($CD123^+$) DCs. CpG-activation of plasmacytoid DCs results in up-regulation of CD40, CD80, CD86, ICAM-1 (CD54) and MHC class II molecules (Krug *et al.*, 2001a), promotes cell survival and induces production of soluble mediators (IL-8, IP-10, GM-CSF, IL-6, TNFα) (Krug *et al.*, 2001a; Bauer *et al.*, 2001b). Synergistic production of IL-12p70 by CpG-treated plasmacytoid DCs is observed following further stimulation with CD40Ligand (Krug *et al.*, 2001a). Phosphorothioate CpG ODN sequences are more potent than phosphodiester ODNs at stimulating IFN-α production by plasmacytoid DCs (Kadowaki *et al.*, 2001a; Krug *et al.*, 2001b).

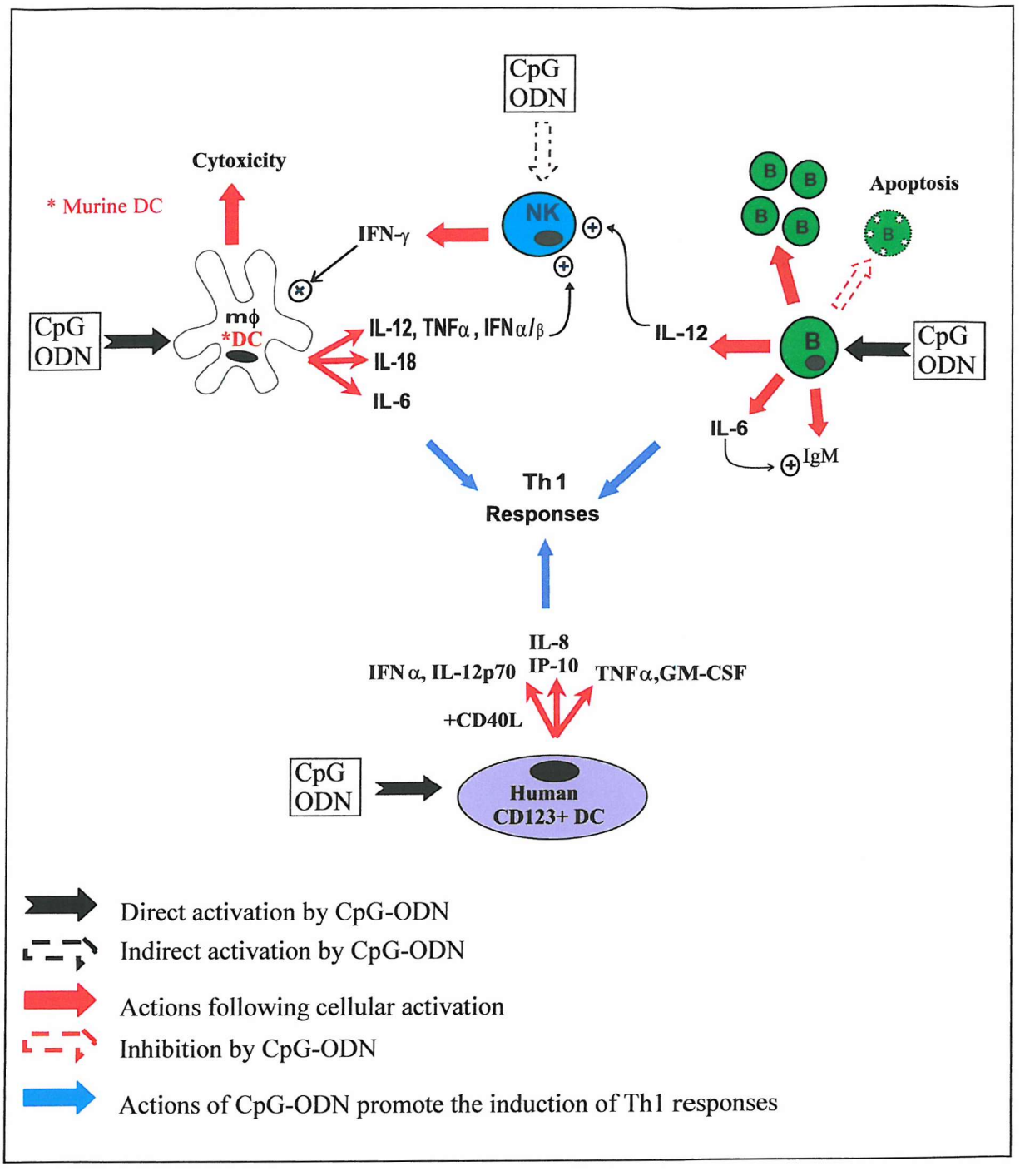


Figure 1.3: Actions of CpG ODN on immune cells.

1.4.3 Mechanisms of immune stimulation by ODN

CpG ODN triggers the induction of potent responses. Therefore, they may potentially act as 'danger signals' or 'Signal 0' and function as a ligand for pattern recognition receptors thereby, activating the innate immune system as occurs with invading pathogens (Wagner, 1999; Kalinski *et al.*, 1999a). A number of reports suggest the presence of surface proteins on immune cells that are capable of recognizing and subsequently binding DNA and synthetic ODN. Similarly, there is a large body of evidence indicating the need for ODN to be internalized in order to result in cellular activation (Figure 1.4).

1.4.3.1 Binding of CpG ODN to surface proteins for cellular activation

Collective evidence suggests that a variety of receptors for DNA and ODN may be present on human cells. Bacterial DNA and ODNs are polyanionic, therefore, they cross cell membranes inefficiently (Loke *et al.*, 1989). Activation of human B-cells by phosphorothioate ODN coupled to sepharose beads is receptor-mediated (Liang *et al.*, 1996; Liang *et al.*, 2000). Binding of ODN to receptors on human B-cells is reported to be fast, specific and initially temperature independent and is necessary for subsequent cellular activation (Liang *et al.*, 2000). However, these findings are contradicted by separate studies showing that sepharose beads used to immobilize ODN could in fact be taken up and induce polyclonal B-cell activation (Manzel and Macfarlane, 1999). Using other methods of ODN immobilization to avoid cellular uptake resulted in non-stimulation (Krieg *et al.*, 1995; Manzel and Macfarlane, 1999). ODN can also bind serum IgM, IgG and IgA suggesting that immunoglobulins on human B-cells might function as a receptor for ODN (Rykova *et al.*, 1994). However, this method of recognition seems unlikely to be involved in the responses of macrophages or DCs.

A 30-kDa protein that binds nucleic acid has been detected by chromatography and on immunoblots of cell membrane preparations of human peripheral blood cells (Bennett *et al.*, 1985). The β2-integrin Mac-1 (CD11b/CD18) is suggested to also function as an ODN binding protein on human macrophages (Benimetskaya *et al.*, 1997). An increase in Mac-1 expression was accompanied by an increase in the internalization of

ODNs. Nucleic acid-binding receptors may also contribute to the activity of ODN. A series of guanine repeats can bind to the type I scavenger receptor by forming base-quartet-stabilized four-stranded helices, thereby promoting immunostimulatory activity of ODN on macrophages and enhancing NK cell lytic activity and IFN-γ production (Pearson *et al.*, 1993). Double stranded DNA (dsDNA) and ODN have also been shown to bind to HLA class II molecules (Filaci *et al.*, 1998). Moreover, preincubation of anti-CD3 stimulated human PBMCs with dsDNA inhibits antigen-specific T-cell proliferation, suggesting that the binding of dsDNA to class II molecules may inhibit antigen presentation (Filaci *et al.*, 1998).

Binding of phosphorothioate CpG ODN to human intestinal epithelial Caco-2 cells is reported to be receptor-mediated involving charge interactions (Beck *et al.*, 1996). Interaction between phosphodiester CpG ODN and thrombin is also reported to involve charge interactions (He *et al.*, 1998). Neutralizing the negative charge of phosphodiester CpG ODN decreased the thrombin-inhibitory activities. Therefore, charge-charge interactions may play a role in binding CpG ODN to their receptors. Additionally, it has been speculated that the reduced binding and stimulatory activities of phosphodiester ODN on B-cells compared to phosphorothioate ODN is due to the distribution of charge in the backbone of phosphorothioate ODN compared with phosphodiester ODN (Liang *et al.*, 2000).

Recently, cellular responses to CpG ODN have been documented to be mediated through TLR9 (Hemmi *et al.*, 2000). Mice deficient for TLR9 exhibited impaired responses to CpG ODN, in terms of splenocyte proliferation and production of IL-6, TNF-α and IL-12p40 by macrophages and dendritic cells, compared to wild type mice (Hemmi *et al.*, 2000). Furthermore, LPS-induced up-regulation MHC class II and co-stimulatory molecules CD40 and CD86 were unaffected in TLR9-deficient mice. These findings suggested the effects of CpG ODN, and not LPS, to be mediated through TLR9. Transfection of TLR9 into normally unresponsive human embryonic kidney cells, causes them to become responsive following stimulation with phosphodiester CpG ODN (Bauer *et al.*, 2001a). Moreover, 293 cells transfected with human TLR9 are shown to become optimally responsive to human-specific motifs and not murine motifs (GTCGTT not GACGTT) suggesting that this receptor is able to distinguish between different species (Bauer *et al.*, 2001a). It is proposed that

TLR9 is present in endosomes where it could interact with CpG ODN (Hemmi *et al.*, 2000), and there is circumstantial evidence suggesting that TLR9 takes up CpG but not non-CpG DNA (Takeshita *et al.*, 2001).

Collectively, these findings suggest the involvement of multiple pathways or receptors that recognize and bind to CpG ODN. Engagement of one or more of these receptors may result in initial cellular activation and subsequent internalization of CpG ODN into the cell. Whether these receptors are selective for certain plasmid DNA as well as single CpG ODN sequences remains to be elucidated.

1.4.3.2 Internalization of CpG ODN for activation

Contrary to the reports suggesting CpG-stimulation is mediated through an extracellular receptor, there is a body of evidence suggesting that CpG ODN exerts its effects following internalization via fluid phase endocytosis. FITC-labelled CpG ODN is taken up by macrophages and localize in the endosomal-lysosomal compartment (Hacker *et al.*, 1998). This uptake is reported not to be CpG-specific as non-CpG may be endocytosed in a comparable manner (Hacker *et al.*, 1998). Also, non-CpG ODN may block the uptake of CpG ODN suggesting that ODN uptake is CpG-motif independent. Blockade of CpG ODN uptake in macrophages by non-CpG ODN competition inhibited TNF-α, IL-6 and IL-12 production (Hacker *et al.*, 1998). Studies using the murine pre-B cell line, WEHI 231 and murine splenocytes demonstrated the need for cellular uptake of CpG ODN in order for cell activation to be achieved (Krieg *et al.*, 1995; Macfarlane and Manzel, 1998). DNA and ODN are internalized via acidified vesicles and may persist in the cells for many hours in an intact form. Lipofection of CpG ODN into spleen cells enhances their immunostimulatory effects, suggesting the presence of an intracellular receptor specific for CpG ODN (Yamamoto *et al.*, 1994). Additionally, blocking the endosomal acidification and processing of CpG ODN to MHC molecules with drugs, such as chloroquine and quinacrine, specifically inhibits the anti-apoptotic effects of CpG ODN on WEHI 231 murine B-cells as well as inhibiting CpG ODN induced secretion of IL-6 by human PBMCs. In contrast, the effects of LPS were not inhibited by chloroquine (Macfarlane and Manzel, 1998). These drugs did not block the putative ODN-cell surface receptor and subsequent uptake of ODN, but they blocked

CpG ODN mediated cytokine release (Hacker *et al.*, 1998). Therefore, an endosomal maturation step appears necessary for the induction of CpG-induced signal transduction pathways (Hacker *et al.*, 1998).

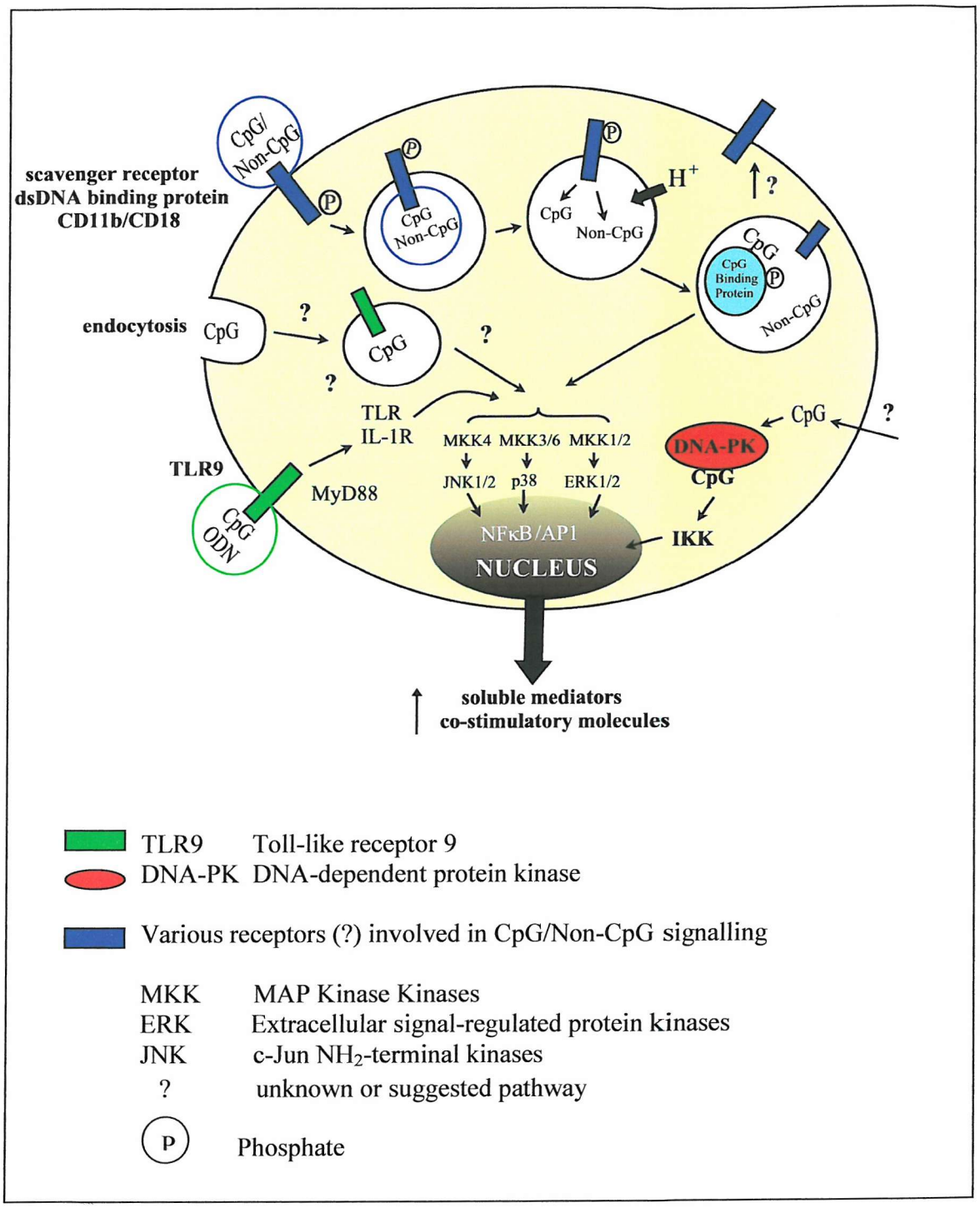


Figure 1.4: Signalling pathways through which CpG ODN might activate immune cells.

1.4.3.3 Signal transduction pathways

Like exposure to LPS, exposure of murine B-cells, macrophages and DC to CpG ODN results in increased levels of reactive oxygen species and NFκB activation (Sester *et al.*, 1999; Stacey *et al.*, 1996; Weinstein *et al.*, 1991). CpG ODN induces ERK activity in murine primary macrophages and the macrophage cell line RAW264.7, with peak activity observed 30 minutes following treatment. Inversion of CpG motifs, GpC, did not induce ERK activity demonstrating that activation depended on CpG-motifs (Hacker *et al.*, 1999). Similarly, LPS induces ERK activation in these models. Upstream activating kinases of ERK are the MAPK/ERK kinases (MEK) 1/2, which are activated by phosphorylation at Ser217/221. Treatment of RAW264.7 cells with CpG ODN and LPS resulted in the induction of phosphorylated forms of MEK 1/2 within 25-45 minutes. These results indicate activation of macrophages by CpG ODN induces ERK activity via a MEK-dependent pathway (Hacker *et al.*, 1999). Secretion of TNF-α from RAW264.7 cells upon CpG ODN activation requires p38 activity (Hacker *et al.*, 1998). Blocking MEK pathways with specific inhibitors subsequently inhibits MEK-dependent ERK activation. In RAW264.7 cells, inhibition of ERK activation significantly suppresses TNF-α secretion but was found to increase IL-12p40 secretion (Hacker *et al.*, 1999).

Bone marrow-derived DCs activated by CpG ODN results in the release of IL-12 and TNF-α at levels similar to those seen following activation by LPS. Methylation or inversion of the CpG-motif in the ODN totally abolished this capacity. CpG ODN also activates stress kinases JNK and p38 in primary $CD11c^+$ bone marrow-derived DCs, with peak activity observed after 30 minutes. In contrast to macrophages, ERK activation was not detected in CpG ODN or LPS-stimulated bone marrow-derived DCs (Hacker *et al.*, 1999). However, a separate study demonstrated LPS-induced ERK activation in splenic DCs (Rescigno *et al.*, 1998a). IL-12 production by bone marrow-derived DCs stimulated with CpG ODN was not affected in the presence of MEK inhibitors, therefore suggesting that in these cells, MEK-dependent ERK activation is not essential for IL-12 production (Hacker *et al.*, 1999).

Within minutes of CpG ODN uptake in macrophages, MAP kinases are triggered followed by the activation of JNK and p38. Consequently, components of the transcription factor AP-1 are phosphorylated and activated. Triggering of

macrophages/DCs with CpG ODN also induced strong activation of the NF-κB signalling pathway and activation of various inflammatory response genes (Hacker *et al.*, 1999). An intracellular adapter that links the CpG ODN within the endosomes to the initiation of the kinase pathway is not documented. It is speculated that this protein might harbour the recognition sites for CpG DNA motifs (Krieg, 1999; Krieg *et al.*, 1998a).

1.4.4 DNA vaccines

DNA vaccines generally incorporate the encoded antigen of interest within the cDNA, cloned downstream from the eukaryotic promoter of the plasmid. Also present are a bacterial antibiotic resistance gene, an origin of replication allowing for growth in bacteria and the addition of polyadenylation sequences to stabilize mRNA transcripts. The presence of numerous CpG motifs in the plasmid vector also plays a role in the immunogenicity of DNA vaccines by activating APCs at the injection site (Klinman *et al.*, 1997). Bone-marrow-derived DCs and Langerhans cells are important for the priming of antigen-specific immune responses following DNA vaccination (Sparwasser *et al.*, 1998; Ban *et al.*, 2000). Moreover, only small numbers of directly transfected DC are sufficient to present antigen efficiently. Transfection of somatic cells, such as myocytes and keratinocytes, following DNA vaccination is also suggested to participate in the regulation of immune responses by the mechanism of cross-priming (Akbari *et al.*, 1999; Ulmer *et al.*, 1996; Fu *et al.*, 1997). Somatic cells may act as an antigen reservoir permitting the transfer of antigen to bone-marrow-derived DCs in order to maintain $CD8^+$ T-cell immune responses following DNA vaccination (Fu *et al.*, 1997; Corr *et al.*, 1999).

DNA vaccines and CpG ODN have been shown to sustain long-term humoral and cellular immunity in various animal models for preventing or treating pathogenic infection such as *Listeria monocytogenes* and *Leishmania* major (Krieg *et al.*, 1998b; Stacey and Blackwell, 1999). Properties of CpG ODN as an adjuvant are documented to be effective for mucosal immunization generating specific Th1-type immune responses (McCluskie and Davis, 1998). Application of CpG ODN to overcome established Th2-type immune response in diseases such as asthma is also being evaluated (Broide and Raz, 1999). *In vitro*, CpG ODN deviated allergen-

specific human $CD4^+$ T-cells of atopic donors from a Th0/Th2 cytokine profile to a Th1 response (Parronchi *et al.*, 1999). PBMCs, obtained from atopic individuals, cultured with CpG ODN resulted in elevated allergen-specific IgG and IgM levels whereas allergen-specific IgE levels were not significantly affected (Bohle *et al.*, 1999).

Some reports suggest that the immunomodulating properties of certain CpG ODN sequences are species-specific; therefore, identifying human activating CpG ODNs for use in vaccines is essential (Krieg and Wagner, 2000). The occurrence of suppressive motifs in an ODN sequence may alter immunogenicity (Krieg *et al.*, 1998a). Cytosine methylation abolished CpG ODN activity on human PBMCs whereas GpC ODN only partially reduced activation suggesting that the presence of CpG-motifs is not solely responsible for activation of human cells compared to murine models (Parronchi *et al.*, 1999). Therefore, therapeutic use of CpG ODN as adjuvants in humans may need optimizing to eliminate suppressive motifs and including stimulatory sequences.

1.5 *Neisseria meningitidis*

The gram negative encapsulated diplococcus, *N. meningitidis*, is carried in the human nasopharynx of up to 30% of adults, rarely colonizing the proximal airways of infants (Goldschneider *et al.*, 1969a). Movement of meningococci through the mucosal barrier to enter the blood stream results in septicaemia. However, the bacterium may also traverse the blood brain barrier to colonize the meningeal membranes causing in life-threatening meningitis (Brandtzaeg, 1995; Brandtzaeg *et al.*, 1992a). At birth, infants are protected from invasive disease by the presence of maternal antibodies directed at the capsular polysaccharide and outer membrane proteins (OMs) that lie directly beneath the capsule. In addition, the environment of the nasopharynx in infancy does not favour the colonisation by pathogenic meningococci, brought about in part by ongoing innate immune responses and the presence on competing non-pathogeneic *Neisseriae* (eg- *N. lactamica*) (Kim *et al.*, 1989; Sanchez *et al.*, 2001). However, as the levels of maternal-derived antibody decreases, vulnerability to infection increases. This is accompanied by very low specific humoral immunity, poor phagocytic function and low levels of complement proteins. Typically, the highest incidence of disease is in young children between 6-24 months of age. This declines with age, but an increased susceptibility to infection is presented in teenage children (Goldschneider *et al.*, 1969b; Goldschneider *et al.*, 1969a).

Antibody plays a central role in protection against meningococcal infection through binding to bacterial surface structures and activating complement, leading to phagocytosis or direct bactericidal killing (Goldschneider *et al.*, 1969b; Vidarsson *et al.*, 2001). Levels of IgG (IgG1 and IgG3), IgA and IgM antibodies are elevated following systemic infection with *N. meningitidis* (Brandtzaeg, 1992; Pollard *et al.*, 1999a; Vidarsson *et al.*, 2001). Defence against disease requires the induction of both innate and cellular immunity, to provide help for antibody production. The rise in total antibodies following infection is similar in both infants and older children recovering from meningococcal disease, although, this antibody is bactericidal in older children only (Pollard *et al.*, 1999a).

1.5.1 Meningococcal outer membranes

The outer membrane (OM) of meningococci contains lipooligosaccharide (LOS) and numerous outer membrane proteins (OMPs), which enable the bacteria to interact with and adhere to host cells as well as acting as transport proteins (Figure 1.5). LOS is also involved in the pathogenesis of meningococcal disease (Sprong *et al.*, 2001; Waage *et al.*, 1989; Brandtzaeg *et al.*, 1992b). The polysaccharide capsule of the meningococcus surrounds the outer membrane is essential for pathogenicity as it confers resistance to phagocytosis and complement-mediated lysis.

Surface antigens of the meningococcus are highly variable. Serogroups are designated to distinguish between different strains based on the polysaccharide capsule. Five serogroups designated A, B, C, W135 and Y account for the majority of meningococcal disease worldwide. Further classification of meningococci into serotypes and serosubtypes depends on the antigenic structure of the major porins PorB and PorA, respectively (Frasch *et al.*, 1985). In addition, the structure of LOS determines the twelve immunotypes of the organism (Scholten *et al.*, 1994).

During the course of meningococcal disease, growth and lysis of meningococci releases high amounts of outer membrane vesicles (OMV), that are observed microscopically as 'blebs', and are representative of the intact outer membrane proteins of the meningococci that elaborate them (Zollinger *et al.*, 1972). The OM blebs are an accompaniment of spreading organisms for disseminating infection throughout the body and have been observed in the blood and cerebrospinal fluid (CSF) of patients with meningococcal disease (Stephens *et al.*, 1982).

1.5.1.1 Adhesion molecules

Adhesion is an important step in menigococcal pathogenesis: initial adhesion is pilus-mediated followed by stronger adhesion through other structures (Virji *et al.*, 1991). Meningococcal pili are of type IV and are composed of a major subunit called pilin. CD46 is reported to be a receptor for pili and binding through this receptor triggers signal transduction (Kallstrom *et al.*, 1997). Other proteins involved in adhesion and invasion of the bacterium are the opacity family of proteins Opa (class 5 and class 5c), which may interact with several members of the CD66 family and heparan sulfate

proteoglycan receptors on eukaryotic cells (Virji *et al.*, 1996; Chen and Gotschlich, 1996).

1.5.1.2 Porins

Meningococcal porins are the major proteins present in the outer membrane; there are three classes of porins and they have a trimeric β-pleated structure (Nikaido, 1992). They act as channel-forming proteins and function as sieves mediating exchange of nutrients and waste products with the environment (Nikaido, 1992; Jeanteur *et al.*, 1991). Class I porin (PorA) is a 44-47 kDa trimeric cationic protein and its gene is designated *porA* (Tommassen *et al.*, 1990). Class II/III (PorB2 and PorB3) is a 37-42 kDa anion porin with a gene designated *porB* (Tommassen *et al.*, 1990). Class I and class II/III sequences do not vary within an isolate, but antigenic differences amongst strains forms the basis of serotyping (class II/III) and serosubtyping (class I). Class I porin possesses large antigenic variablility on two hypervariable regions VR1 and VR2, which correspond to surface exposed loops I and IV, respectively (Mandrell and Zollinger, 1989).

1.5.1.3 Other surface outer membrane structures

Transferrin binding protein (Tbp) 1 and 2 are responsible for binding human transferrin whereas Ferric binding protein (FbpA) binds iron (Rokbi *et al.*, 1993; Mietzner *et al.*, 1987). The function of neisserial surface protein A (NspA) is unknown to date.

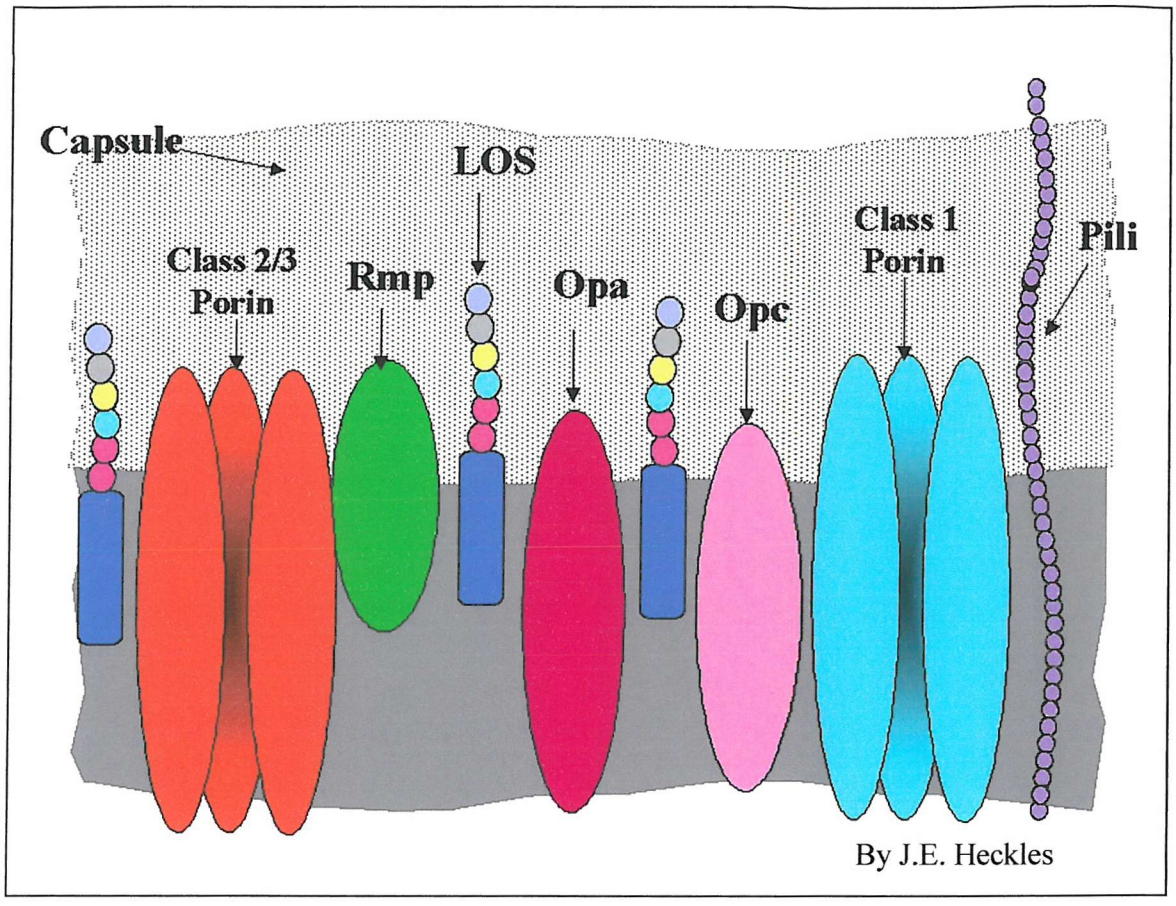


Figure 1.5: ***Neisseria meningitidis*** **outer membrane structures.**

1.5.2 Vaccines against meningococci

Vaccines targeting the production of anti-polysaccharide antibodies for immunity against Serogroup A and C meningococci have demonstrated efficacy against disease (Fairley *et al.*, 1996). However, polysaccharide vaccines are poorly immunogenic in infancy unless conjugated to proteins (Reddin *et al.*, 2001; Fukasawa *et al.*, 1999). Serogroup B polysaccharide is poorly immunogenic in humans due to an antigenically identical structure expressed on human fetal and adult neural cells. The group B capsular polysaccharide is identical to a widely distributed human self-antigen [α(2→8)N-acetyl neuraminic acid or polysialic acid], thus being a poor immunogen in humans (Finne *et al.*, 1983). Thus, immunogenic meningococcal surface structures that may induce protective immune responses from infancy are important for vaccine development (Cadoz, 1998).

Following systemic infection with Serogroup B meningococci, antibodies directed at various outer membrane antigens, such as porins and class 5 Opc, have been detected in convalescent sera. Developments of vaccines have focused on targeting non-capsular meningococcal antigens. Moreover, these therapies have focused on the use of OMPs in the form of membrane vesicles (OMV) (Drabick *et al.*, 1999; Haneberg *et al.*, 1998; Naess *et al.*, 1998). Anti-PorA antibodies, mainly IgG1 or IgG3, produced after infection are predominantly directed against the hypervariable region of loop 4 of the class I protein (Mandrell and Zollinger, 1989; Guttormsen *et al.*, 1994). Vaccine studies have shown the class I porins to be strongly immunogenic in producing serosubtype-specific bactericidal antibody and a level of protection in older children and adults (Bjune *et al.*, 1991; Peeters *et al.*, 1996; van der Voort *et al.*, 1997). However, sequence and antigenic variability amongst the major OM proteins exists limiting the applicability of OMV vaccines. Recently, a hexavalent OMV vaccine containing six different PorA serosubtype proteins has demonstrated the induction of protective antibodies in infants after a course of three doses (Cartwright *et al.*, 1999; van der Ley *et al.*, 1995).

Adult volunteers have lymphoproliferative responses to Opa, Opc and PorA proteins of the meningococcal outer membrane as wells as to OMV after vaccination with Dutch and Norwegian OMV vaccines or following infection. Although OMs from different strains of Serogroup B and C meningococci are antigenically distinct, based

on monoclonal antibody typing, a majority of the amino acid sequence of each of the major OMs is homologous between strains.

There is a need to investigate the effects of the major components of meningococcal OM to assess their potential as immunogens as well as potentiators of immune responses (ie-adjuvants). OM from wild type meningococci contains LOS, and thus these are very toxic for use in humans. However, the LOS component is likely to possess immunostimulatory properties. Therefore, the availability of OM from a mutant strain lacking LOS might offer a useful alternative (Steeghs *et al.*, 1998). Investigation of the effects of OM from wild type or LOS-deficient mutant meningococci on DCs would facilitate a greater understanding of the role these cells play in immunity to meningococci. In addition these studies may allow prediction of future cellular-based vaccine therapies against meningococci.

1.6 AIMS OF PROJECT

The ability of dendritic cells (DCs) to initiate immune responses resides in their specialized capacity to recognize and capture foreign antigen, become mature and efficiently present antigen to T-cells (Steinman, 1991). There is evidence indicating that the type of pathogen encountered by the DC (Signal 0) influences the mode by which DCs mature in terms of co-stimulatory molecules expression and production of cytokines (Kalinski *et al.*, 1999a). The plasticity of DCs that have encountered specific pathogens or their products may differ in their subsequent ability to direct the differentiation of $CD4^+$ Th-cells towards Th1 or Th2 immune responses.

Bacterial components have been investigated as potential adjuvants for their inherent ability to stimulate potent immune responses. Understanding some of the mechanisms by which bacterial components induce maturation of DCs and how this influences subsequent T-cell polarization is critical for the understanding of how they function as adjuvants. The mechanisms by which adjuvants exert their actions largely depends on their nature. The role of the adjuvant is to induce APC maturation by modulating co-stimulatory and adhesion molecules and up-regulating MHC expression on the cell surface, to allow for efficient presentation of antigen to the T-cell. Adjuvants may also induce APCs to produce and secrete cytokines that modulate differentiation of $CD4^+$ Th-cells into Th1 or Th2 subsets, or $CD8^+$ Tc-cells into Tc1 or Tc2 subsets. Production of cytokines by the respective Th and Tc-cells activates host defences directly through the stimulation of innate immunity or indirectly through induction of acquired immunity.

The work presented in this thesis addresses the ability of two distinct forms of bacteria-derived components to behave as adjuvants, demonstrated by their capacity to modulate DC function and subsequently promote T-cell proliferation, *in vitro*. The first of the adjuvants examined are synthetic oligodeoxynucleotides (ODNs), which contain CpG-motifs. Synthetic ODNs are reported to possess immunostimulatory activity in murine models at levels comparable to that seen with bacterial DNA (Quintana *et al.*, 2000). Therefore, the effects of synthetic ODN on human mo-DCs and their monocyte precursors were investigated in this study. The second type of bacterial adjuvant explored in this work are the outer membranes of *Neisseria meningitidis*, with particular emphasis on the LOS and class I porin. The ability of

whole neisserial outer membranes and purified recombinant class I porin to modulate mo-DC antigen presenting function was assessed.

The overall aim of the project was to test the hypothesis that the adjuvanticity of these bacterial products is mediated through modulation of co-stimulatory molecules and mediators expressed by APC. The effects of bacterial components might induce different levels of APC activation that, in turn, could influence the nature of the $CD4^+$ T-helper immune response induced.

Somewhat different questions were explored for each of the adjuvants employed in this study, as follows:

Synthetic ODNs:

1) The effects of different CpG and non-CpG ODNs were examined on mo-DC and their monocyte precursors.
2) The effects of CpG and non-CpG ODNs were examined on monocytes and mo-DCs from non-atopic and atopic subjects.
3) The effects of CpG ODN on mo-DC were examined for their capacity to activate T-cell proliferative responses.

Neisseria meningitidis outer membranes:

1) The activation and maturation of mo-DC following exposure to outer membranes from Group B *N. meningitidis* were examined.
2) Examine the role of meningococcal LOS in mo-DC activation.
3) The effects of purified recombinant class I porin of *N. meningitidis* to modulate the antigen-presenting capacity mo-DCs was examined.

CHAPTER 2

General Materials and Methods

2

2.1 MATERIALS

2.1.1 Standard laboratory chemicals

Chemicals for general laboratory use were purchased either from Sigma Chemical Company (Poole, UK) or Merck Ltd. (UK). All chemicals were of analytical or molecular biology grade.

2.1.2 Cell culture solutions and buffers

All solutions were prepared using sterile pyrogen free water (Southampton University Hospital Pharmacy) or using Ultra High Quality (UHQ) reverse osmosis water purified through an ElgaStat UHQ-PS system. All buffers used for cell cultures were filter-sterilised through 0.22μm filter units (Corning Costar Ltd., UK). Cells were obtained and prepared for culture using sterile 10x stock of phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} (Life, Technologies Ltd. UK) diluted 1:10 in pyrogen free water (Table 2.1). For preparation of FACS buffer or washing of cells by centrifugation, PBS was prepared from tablets (OXOID, UK.) using UHQ water according to the supplier's instructions. Bovine Serum Albumin (BSA) fraction V (Sigma, UK) was generally used in buffers, unless replaced with 10% (w/v) define fetal calf serum (FCS, Hyclone) were indicated.

Buffers used for the purification and culturing of human cells were prepared under sterile conditions and filtered through a 0.22μm filter unit (NUNC, Life technologies).

Table 2.1: PBS-based cell culture buffers.

Buffer	Composition
Elutriation buffer	PBS, 1% BSA fraction V (w/v), 270nM EDTA
MACS buffer	PBS 0.5% BSA fraction V (w/v), 2mM EDTA
FACS buffer	PBS, 1% BSA fraction V (w/v), 0.1% sodium azide (w/v)
Wash buffer	PBS alone

2.1.3 Cell culture media

Reagents used for tissue culture were of tissue culture grade and stored according to manufacturer's recommendations.

2.1.3.1 Media and supplements

Iscoves Modified Dulbecco's medium (IMDM), RPMI 1640 medium with and without phenol red, penicillin/streptomycin (P/S), L-glutamine and sodium pyruvate were purchased from Life Technologies. 2-mercaptoethanol (2-ME) and pooled human AB serum was purchased from Sigma. Defined fetal calf serum (FCS, Hyclone) was purchased from Pierce and Warriner, UK. Pooled human AB serum and FCS was heat inactivated at 56°C for 30 minutes prior to storage at –20°C and filtered-sterilised through 0.22μm filter units before use.

Table 2.2: Medium for cell culture.

Medium:	**Composition**
Monocytes	IMDM, 10% (v/v) heat inactivated Human AB serum, 100units/ml Penicillin and 100μg/ml Streptomycin, 2mM glutamine
Mo-DC and mo-LC	Phenol red-free RPMI, 10% (v/v) FCS (Hyclone), 100units/ml Penicillin and 100μg/ml Streptomycin, 2mM glutamine
CD40-ligand transfected and wild type CHO cell lines	Phenol red-free RPMI, 10% (v/v) FCS (Hyclone), 100units/ml Penicillin and 100μg/ml Streptomycin, 2mM glutamine
THP-1 monocytic cell line	Phenol red RPMI, 10% (v/v) FCS (Hyclone), 100units/ml Penicillin and 100μg/ml Streptomycin, 2mM glutamine

2.1.3.2 Cytokines

Recombinant human (rh) IL-4, rhTGF-β1 and rhTNF-α were obtained from R&D Systems Europe Ltd. (Abingdon, UK). rhGM-CSF (Leucomax ®, Sandoz Pharmaceuticals) was obtained through the hospital pharmacy. rhSCF was obtained through PeproTech EC Ltd. All stock solutions of cytokines were prepared in sterile solutions as per manufacturers' instructions and stored at –80°C.

2.1.4 General tissue culture consumables and reagents

Standard tissue culture plasticware, including flat-bottomed 6, 12, 24, 48 and 96 (plus U-bottomed) well plates, FACS tubes and 15 and 50mL Falcon tubes were purchased from Becton Dickinson (Oxford, UK). Millex-GV filter units (0.22μm) were purchased from Millipore (Watford, UK) and 30mL Universal tubes from Western Laboratories (Aldershot, UK).

2.1.5 Antibodies

Monoclonal antibodies used for flow cytometric analysis are listed in Table 2.3-2.5 shown below. All unconjugated or directly conjugated antibodies were stored at 4^0C. Abbreviations used: Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Southampton General Hospital (SGH).

Table 2.3: Primary antibodies for flow cytometry.

ANTIBODY (clone)	ISOTYPE	FLUORESCENT CONJUGATE	COMPANY
CD1a (NA1/34)	IgG2a	FITC	Dako
CD3 (OKT3)	IgG1	FITC	Professor M. Glennie, SGH
CD3 (B-B11)	IgG1	FITC/PE	Diaclone Research
CD4 (C4120F)	IgG1	FITC	Professor M. Glennie, SGH
CD4 (B-F5)	IgG1	PE	Diaclone Research
CD11b (44)	IgG1	PE	Diaclone Research
CD11c (3.9)	IgG1	PE	Diaclone Research
CD14 (MϕP9)	IgG2b	FITC	Becton Dickinson
CD14 (M5E2)	IgG2a	PE	Pharmingen
CD20 (AT80F)	IgG1	FITC	Professor M. Glennie, SGH
CD40 (LOB7.6)	IgG1	FITC	Professor M. Glennie, SGH
CD40 (mAb89)	IgG1	PE	Immunotech
CD45RA (B-C15)	IgG1	PE	Diaclone Research
CD 45 RO (UCHL1)	IgG2a	FITC	Diaclone Research
CD54 (15.2)	IgG1	FITC	Biogenesis
CD80 (MAB104)	IgG1	PE	Pharmingen
CD83 (HB15A)	IgG2a	PE	Immunotech
CD86 (FUN-1)	IgG1	PE	Pharmingen
CD154 (B-B29)	IgG1	FITC	Diaclone Research
HLA-DR (L243)	IgG1	PE	Becton Dickinson
HLA-DR (L243)	IgG1	FITC	Becton Dickinson
TLR2 (TL2.1)*	IgG2a	Purified	EBiosource, USA
TLR4 (HTA125)*	IgG2a	Purified	EBiosource, USA
TLR9 (26C593)*	IgG1	Purified	Biocarta Europe GmbH

* Biotin-conjugated TLR2, TLR4 and TLR9 mAbs and relevant isotype controls were also used in studies where indicated (purchased from companies described above and conjugated by Dr. Judith Holloway, University of Southampton).

Table 2.4: Intracellular cytokine antibodies for flow cytometry.

ANTIBODY (clone)	ISOTYPE	FLUORESCENT CONJUGATE	COMPANY
IL-12p40/70 (C11.5)	IgG1	PE	Pharmingen
IL-12p70 (20C2)	IgG1	PE	Pharmingen
Langerin CD207 (DCGM4)	IgG1	PE	Immunotech

Table 2.5: Isotype controls for flow cytometry.

ANTIBODY clone	ISOTYPE	FLUORESCENT CONJUGATE	COMPANY
RJDFITC	IgGγ1	FITC	Professor M. Glennie, SGH
X20	IgGγ1	PE	Becton Dickinson
DB718F	IgGγ2a	FITC	Professor M. Glennie, SGH
X39	IgGγ2a	PE	Becton Dickinson
27-35	IgGκ2b	FITC	Pharmingen
R3-34	Rat IgG1	PE	Pharmingen

2.1.6 ELISA kits

Reagents for use in ELISA kits were supplied by individual manufacturers.

ELISA	COMPANY
Human IL-1β kit	IDS, UK.
Human IL-1β matched antibody pairs	Endogen
Human IL-6 kit	IDS, UK
Human IL-6 DuoSet ELISA development system	R&D Systems Ltd, UK.
Human IL-10 kit	IDS, UK.
Human IL-10 matched antibody pairs	Endogen
Human IL-12p40 matched antibody pairs	Biosource
Human IL-12p70 kit	IDS, UK
Human IL-13 kit	IDS, UK
Human IFN-γ kit	IDS, UK
Human TNF-α kit	IDS, UK
Human IL-8 matched antibody pairs	Dr. M. Christodoulides (R&D)
Human RANTES matched antibody pairs	Dr. M. Christodoulides (R&D)
Human MIP-1α matched antibody pairs	Dr. M. Christodoulides (R&D)
Human MIP-1β matched antibody pairs	Dr. M. Christodoulides (R&D)

Solutions for human IL-1β and IL-10 ELISA (Endogen) are described below:

Coating buffer	0.03M sodium carbonate, 0.068M sodium bicarbonate pH(9.4-9.8) for IL-1β and PBS for IL-10
Blocking buffer	PBS with 4% BSA fraction V (w/v), pH 7.2-7.4
Wash buffer	50mM TRIS, 0.2% Tween-20 (v/v), pH 7.9-8.1
Assay Buffer	PBS with 4% BSA fraction V (w/v), pH 7.2-7.4
Stop solution	1M H_2SO_4

Solutions for human IL-6 DuoSet ELISA development system are described below:

Coating buffer	0.05M carbonate-bicarbonate buffer, pH 9.6
Blocking buffer	PBS with 1% BSA fraction V (w/v), pH 7.2-7.4
Wash buffer	PBS with 0.05% Tween-20 (v/v)
Assay buffer	20mM TRIS, 150mM NaCl, 0.1% BSA fraction V (w/v), 0.05% Tween-20 (v/v), pH 7.3
Stop solution	1M H_2SO_4

Solutions for human IL-8, RANTES, MIP-1α and MIP-1β are described below:

Coating buffer	Sodium carbonate buffer, pH 9.6
Blocking buffer	PBS with 1% (w/v) BSA, 5% (w/v) sucrose
Wash buffer	25mM Tris-phosphate buffer, pH 8.0, with 100nM NaCl and 0.05% (v/v) Tween-20

2.1.7 Cell lines

Wild type or human CD40-Ligand transfected Chinese hamster ovary (CHO) cell lines, were obtained from Professor M. Glennie (University of Southampton). THP-1 monocytic cell lines were obtained from American Type Culture Collection (ATCC). Cells lines were free of mycoplasma as tested using the Mycoplasma Plus™ PCR Primer set (Stratagene, UK).

2.1.8 Subjects

Blood was taken by venesection from atopic or normal healthy volunteers after informed consent. Atopic status of the volunteers was assessed on the basis of family history of atopy and positive skin-prick tests for the common allergens: house dust mite, cat dander and/or grass pollen. A positive skin-prick test is determined by a wheal response greater than 3mm in diameter. Healthy subjects on no regular medication were selected on the basis of negative skin-prick tests to the above-mentioned allergens and no symptoms of allergic disease.

2.2 METHODS

All centrifugations were performed in a Mistral 3000i refrigerated centrifuge (Jepson Bolton Laboratory Equipment, UK) unless otherwise stated. All cell incubations were maintained at 37°C, 5% CO_2 in a Heraeus 6000 series incubator (Kendro Laboratory, UK).

2.2.1 Isolation of leukocytes from human peripheral blood

Whole blood (100-150 mL) from healthy non-atopic or atopic volunteers was collected in 10mL vacutainer tubes containing EDTA K_3 (Beckon Dickinson). Blood was overlayed onto density columns of Lymphoprep (Life Technologies), in sterile universals, at a 1:1 ratio of blood to Lymphoprep. Following centrifugation at 2000rpm (900 x *g*), with the brake and acceleration off, for 30 minutes at room temperature, the interface formed between the plasma (top) layer and Lymphoprep (bottom) layer containing peripheral blood mononuclear cells (PBMCs) was collected and placed in sterile 50mL Falcon tubes. Approximately 40mL PBS was added to cells, which were washed three times by centrifuging at 1200rpm (320 x *g*) at 4°C for 10 minutes, discarding the supernatant after each wash. After the last wash cells were re-suspended in 1mL of the appropriate buffer for further enrichments, and a cell count was performed using 0.04% trypan blue (Sigma) to exclude dead cells. Cell concentration was adjusted accordingly for subsequent procedures.

2.2.1.1 Enrichment of human monocytes by plastic adherence

PBMCs were re-suspended in serum-free IMDM supplemented with 100U/mL penicillin and 100μg/mL streptomycin to obtain a final cell density of $10x10^6$ cells/mL. $1x10^6$ PBMCs were kept for determining the percentage of monocytes by flow cytometry (2.2.7). PBMC cell suspension ($10x10^6$ cells/mL) was placed in sterile 60-mm petri dishes and incubated for 1hour. Following incubation, non-adherent cells were removed by repeated thorough washing (3x-5x) with sterile PBS. 2mL IMDM medium containing 10% (v/v) heat-inactivated human AB serum with appropriate treatments was added to the remaining adherent monocytes for varying time points. Following treatment, cell-free supernatants were stored at –40°C pending cytokine analysis (2.2.9). Adherent monocytes were harvested by manual scraping using cell scraper (Triple Red, UK) subsequent to a 15 minute incubation of the

For the isolation of $CD4^+$ T-cells from PBMCs, the T-cell fractions obtained after counter-current elutriation were pooled and resuspended in MACS buffer (80μL / 10^7 cells), to which hapten-antibody cocktail (CD8, CD11b, CD16, CD19, CD36 and CD56) mAbs (20μL / 10^7) was added. Following 10 minute incubation at 4°C, cells were washed twice by centrifugation. Cell pellet was resuspended in ice cold MACS buffer (80μL / 10^7 cells) and MACS anti-hapten microbeads (20μL / 10^7) were added. Following subsequent 15 minute incubation at 4°C cells were washed twice.

After the final wash to remove unbound mAbs, cell pellets were re-suspended in 500μL cold MACS buffer and the cell suspension was loaded onto a Midi MACs VS^+ column that was pre-washed with 3mL cold MACs buffer and placed in a MidiMACs magnet on a MACS MultiStand. After cell loading, the column was flushed 3x with 3mL cold MACS buffer. Unlabelled cells were collected into sterile 15mL tubes. Pelleted cells were re-suspended in mo-DC culturing media (Table 2.2) and a cell count was performed using trypan blue exclusion.

2.2.1.3.2 Direct immunomagnetic depletion

Non-specific binding of immunoglobulins to Fcγ receptors on enriched T-cells or monocytes was blocked as described above. For monocyte purification, cells were labelled with anti-human CD19 Microbeads and anti-CD3 Microbeads by adding 20μL of each bead / 10^7 cells, directly into the cell suspension. For enrichment of $CD45RA^+$ T-cells, from $CD4^+$ purified T-cells, anti-human $CD45RO^+$ Microbeads were used at 30μL / 10^7 cells. In both cases, following 20 minutes incubation in the refrigerator, the cells were re-suspended in 15mL MACS buffer then pelleted by centrifugation at 1200 rpm for 10 minutes at 4°C. The cells resuspended in 500μL cold MACS buffer were separated when passed through a MACS VS^+ column as described above.

2.2.1.3.3 Indirect/direct positive-immunomagnetic selection

Positive magnetic labelling of $CD34^+$ progenitor cells was performed using the indirect Miltenyi $CD34^+$ isolation kit. Here, 100μL of Reagent A1 and Reagent A2 was added per 10^8 cells and left to incubate for 15ninutes at 4°C. Following two wash steps, the cell pellet was re-suspended in 400μL MACS buffer plus 100μL Reagent B

Table 2.6: Elutriation protocol.

Fraction	Tube number	Time	Flow Rate mL/minute	No. Of Tubes to Collect
Cell loading	-	10 min	10	-
T cell 1	1	-	12	1 x 50mls
T cell 2	2	-	14	1 x 50mls
T cell 3	3	-	16	1 x 50mls
T cell 4	4	-	16	1 x 50mls
T cell 5	5	-	18	1 x 50mls
Intermediate 1	6-8	-	18.5mls/min	3 x 50mls
Intermediate 2	9-10	-	20mls/min	2 x 50mls
Intermediate 3	11-13	-	23mls/ml	2 x 50mls
Monocyte	14-17	-	28mls/min	5 x 50mls

2.2.1.3 Enrichment of cells by immunomagnetic depletion

Cells obtained by counter-current elutriation were further purified by depletion of contaminating cells using magnetically conjugated mAbs (Microbeads) and associated materials from Miltenyi Biotec (Bisley, UK), unless otherwise stated. All wash procedures involved resuspension of cells in a large volume of MACS buffer (ie-14mL) and centrifugation at 1200 rpm for 10 minutes at 4°C unless otherwise stated.

2.2.1.3.1 Indirect immunomagnetic depletion

Monocytes were pooled together and centrifuged at 1200rpm for 10 minutes at 4°C in cold MACS buffer (Table 2.1). Pelleted cells were re-suspended in MACS buffer (40μL / 10^7 cells) to which human Fcγ fragments (5μg / 10^6 cells) (Professor M. Glennie, University of Southampton). Cells were left to incubate on ice for 15 minutes, to block non-specific binding of immunoglobulins to Fcγ receptors. Subsequently, cells were labelled with CD2-hapten mAb (10μL / 10^7 cells), by addition of mAb to the cell suspension as directed by the manufacturer. Following 20 minutes incubation on ice, anti-human CD19 microbeads (20μL / 10^7 cells) and MACS anti-hapten microbeads (20μL / 10^7 cells) were added to cell suspension and left to incubate for a further 15 minutes in the refrigerator (3-6°C). Following incubation with antibodies, cells were suspended in 14mL MACS buffer and pelleted by centrifugation at 1200 rpm for 10 minutes at 4°C.

For the isolation of $CD4^+$ T-cells from PBMCs, the T-cell fractions obtained after counter-current elutriation were pooled and resuspended in MACS buffer (80μL / 10^7 cells), to which hapten-antibody cocktail (CD8, CD11b, CD16, CD19, CD36 and CD56) mAbs (20μL / 10^7) was added. Following 10 minute incubation at 4°C, cells were washed twice by centrifugation. Cell pellet was resuspended in ice cold MACS buffer (80μL / 10^7 cells) and MACS anti-hapten microbeads (20μL / 10^7) was added. Following subsequent 15 minute incubation at 4°C cells were washed twice.

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per 10^8 cells, followed by mixing of cells and 15 minute incubation at 4°C. $CD1a^+$ cells were positively selected after a 15 minute incubation at 4°C with mAb CD1a-conjugated with magnetic beads (Miltenyi Biotech) 20μL of each bead / 10^7 cells. After antibody incubations, cells were washed twice, the pellet re-suspended in 500μL cold MACS buffer and passed through a pre-washed MACS VS^+ column. The column was flushed three times with 3mL cold MACs buffer and unlabelled cells were discarded. The column was then removed from the magnetic separator and 3mL cold MACs buffer was added to the column. Using the plunger, the retained cells were eluted. Purity of $CD34^+$ cells was determined by flow cytometry (2.2.7) using FITC-conjugated mAb clone HPCA-2, which recognizes an epitope different from that recognized by the CD34 mAb.

2.2.2 Culturing monocyte-derived dendritic cells (mo-DC) and monocyte-derived Langerhans' like cells (mo-LC)

Leukocyte purification using Lymphoprep followed by further monocyte enrichments by counter-current elutriation (2.2.1.2) and finally by immunomagnetic depletion (2.2.1.3) of $CD2^+$ T-cells/natural killer cells or $CD3^+$ T-cells and $CD19^+$ B-cells yielded between 90-97% purified monocytes as determined by flow cytometry (2.2.7).

2.2.2.1 Culture conditions for conversion of human monocytes to mo-DC

Protocol for culturing mo-DC was adapted from previously described methods (Sallusto and Lanzavecchia, 1994; Suri and Austyn, 1998). Purified monocytes, $1x10^6$ cells/mL, were cultured in sterile flat-bottomed 6- or 12-well plates ($2x10^6$ cell/well). Monocytes were cultured in phenol red-free RPMI 1640 supplemented with 10% (v/v) heat inactivated FCS, 2mM L-glutamine, 100U/mL penicillin and 100μg/mL (complete mo-DC medium). In addition, mo-DC medium contained rh IL-4 (1000 U/mL) and rhGM-CSF (1000 U/mL). On days 2 and 4 of culture, depending on condition of cultures, either 1 mL fresh complete medium was added to the culture or used to replace 1 mL of old culture medium. Fresh complete medium was supplemented with additional rh IL4 and rhGM-CSF to achieve a final concentration of 500U/mL and 1000U/mL, respectively. Cells under these conditions were cultured for 5 days at 37°C, 5% CO_2 during which monocytes differentiated into mo-DCs. On

day 5, $CD1a^+$ $CD14^-$ mo-DCs, were stimulated with appropriate treatments for up to 48hrs.

To obtain mo-DC for subsequent functional assays with T-cells, mo-DCs were differentiated from monocytes for five days in supplemented medium containing 2% (v/v) pooled human AB serum, to minimize high background proliferation. On day 5 of culture, the mo-DCs expressed lower levels of CD1a, compared to cells cultured in FCS, but were negative for CD14.

2.2.2.2 Culture conditions for obtaining mo-LC

Purified monocytes were cultured in sterile 12-well plates, at a density of $2x10^6$ cells/well, initially in complete mo-DC medium (2.2.2.1) containing recombinant cytokines IL-4 (1000U/mL) and GM-CSF (1000U/mL). However on days 2 and 4 of culture, 1mL of old medium was replaced with 1mL fresh complete medium containing recombinant cytokines IL4 (500U/mL) and GM-CSF (1000U/mL) plus rhTGF-β1 (25ng/mL) in the fresh medium. Mo-LC were obtained by culturing monocytes under these conditions at 37°C, 5% CO_2 for 5 days (Geissmann *et al.*, 1998). On day 5, $CD1a^+$ $CD14^-$ mo-LCs were stimulated with appropriate treatments for up to 48hrs.

2.2.3 Human CD40-Ligand-transfected Chinese Hamster Ovary (CHO) cell lines

The adherent cell lines, CD40-Ligand transfected CHO cells and Wild type CHO cells (Professor M. Glennie, Southampton General Hospital) were cultured in complete CHO medium (Table 2.2) at 37% and 5%CO_2. Cells were passaged when confluent (every 2-3 days). Cells were removed off plastic by incubation in 5mL Cell Dissociation Solution (Sigma) for 5-10minutes at 37°C, 5% CO_2. 15mL phenol-free RPMI medium was added to detached cells, which were then harvested and centrifuged once at 1500rpm (500 x g) to remove the cell dissociation solution. Pelleted cells were resuspended in fresh CHO medium and seeded into sterile T75 flasks (Nunc, Life Technologies) at a 1 in 5 CHO cell to medium density. 1mg/mL geneticin G418 (Life Technologies) was added to CD40-Ligand transfected CHO cell cultures for selection of CD40-L positive CHO cells. Expression of CD40-ligand (CD154) was frequently monitored by flow cytometry (2.2.7). Wild type and

CD40L-transfected CHO cells were re-suspended in freezing medium (10% (v/v) Dimethyl sulfoxide (DMSO; Sigma) in FCS) in aliquots (10^6 cells / mL) and cryopreserved by incubation at –80°C, in an isopropanol container for a minimum of 24 hours, followed by transfer of cells into liquid nitrogen for long-term storage. Wild-type and high expressing CD40L-transfected CHO cells were cryopreserved regularly to keep passages low.

2.2.3.1 Co-culture of monocyte-derived dendritic cells and CHO cells

CD40-Ligand transfected or wild type CHO cells were seeded into wells of a 24-well ($2x10^5$ cells) or 48-well ($4x10^5$ cells) plate 2hrs prior to co-culture with mo-DCs to allow cells to adhere. Treated or untreated mo-DCs were harvested and washed twice by centrifuging in 15mL complete mo-DC medium (Table 2.2) at 1500rpm (500 x *g*) for 10 minutes. For measurement of cytokines produced by mo-DCs following CD40 ligation, $1x10^5$ mo-DCs were co-cultured with CD40-Ligand transfected CHO cells (1:2, mo-DC: CHO cell ratio) in wells of a 48-well plate in 500µl final volume of complete mo-DC medium at 37°C and 5%CO_2. Treated or untreated mo-DCs were similarly co-cultured with wild type CHO-cells at the same densities. Supernatants were collected at increasing time points after initial co-culture and stored at –40°C pending cytokine analysis (2.2.9).

For flow cytometric detection of intracellular cytokines expressed by mo-DCs following CD40-ligation, $2x10^5$ treated or untreated mo-DCs were co-cultured CD40-Ligand transfected CHO cells (1:2, mo-DC: CHO cell ratio) in wells of a 24-well plate in 1mL final volume of mo-DC medium at 37°C, 5%CO_2. Treated or untreated mo-DCs were similarly co-cultured with wild type CHO cells at the same densities. 2.5µM monensin (Sigma), a protein transport inhibitor, was added to all wells set up for detection of intracellular cytokines for the last 12 hours of culture. Following 18hr mo-DC – CHO cell co-culture, cells were finally harvested and stained for flow cytometric analysis as described (2.2.7).

2.2.4 Monocyte-derived dendritic cells : T-cells co-cultures

Mo-DCs, cultured for 5 days, were left untreated or exposed for up to 48hrs with appropriate stimuli. These cells were used to assess their ability to drive proliferation of purified allogeneic T-cells.

2.2.4.1 Monocyte-derived dendritic cell driven allogeneic T-cell proliferation assay

Allogeneic $CD4^+$ T-cells or $CD4^+CD45RA^+$ were suspended in RPMI 1640, 5% human AB serum, 2mM L-glutamine, 100U/mL penicillin and 100µg/ml streptomycin (complete T-cell medium) and plated out at 10^5 cells /100µL/well in 96-well U-bottomed plates. Control or treated mo-DCs/mo-LCs were washed thoroughly of stimuli by 3x centrifugation at 1500rpm for 5 minutes prior to irradiation with 25 Gray from a caesium source to prevent any spurious proliferation of possible contaminating T-cells in the mo-DC culture. Subsequently, mo-DCs were re-suspended in complete T-cell medium and added to T-cells in grading numbers per well: 300, 1000, 3000 and 10000 mo-DCs/100µL/well (in triplicates). Control wells were also set up containing T-cells or mo-DCs alone.

Co-cultures were maintained at 37°C, 5%CO_2 for 5 days before 0.5µCi tritiated [H^3] thymidine (specific activity 5 Ci/mmol; Amersham Pharmacia Biotech) was added to each well. Following 18 hours, cells were harvested onto separate glass fibre filters (Camo Ltd. UK) using a semi-automatic cell harvester (Tomtech). The filters were air-dried and placed into individual polyethylene vials (Packard-Canberra, UK) containing 2mL scintillant (Optiscint HiSafe, Wallac). T-cell proliferation was quantified by measuring H^3-thymidine incorporation as counts per minute (CPM) in a 2500 TR liquid scintillation counter (Packard-Canberra, UK). Results are presented as T-cell proliferation in CPM or as stimulation indices (SI) derived using the equation below.

$$SI = \frac{\text{mo-DC treatment CPM} - \text{mo-DC control CPM}}{\text{mo-DC control CPM}}$$

2.2.5 Monocyte-derived dendritic cell driven autologous antigen-specific T-cell proliferation assay

2.2.5.1 Tetanus-toxoid short term T-cell lines

PBMCs, from healthy individuals recently vaccinated against tetanus (within the last five years), were used in these experiments. T-cells and monocytes were obtained from PBMCs by counter current elutriation (2.2.1.2) followed by further enrichment with using MACS separation (2.2.1.3). Monocytes were cryopreserved (10^6 cells /

mL of freezing medium (2.2.3) for future differentiation into mo-DCs. 10^6 purified $CD4^+$ T-cells were co-cultured with 10^6 irradiated autologous PBMCs. Tetanus toxoid, 100ng/ml (Calbiochem, CN Biosciences), was added to cultures and subsequently maintained for 7 days at 37°C and 5% CO_2. On day 7, 10^6 fresh (2^{nd} blood donation) autologous irradiated PBMCs were added to cultures and re-stimulated with 100ng/ml tetanus toxoid. Human rIL-2 (20U/ml) was also added on days 7 and 10 of culture to support T-cell expansion.

2.2.5.2 Co-culture of mo-DCs with tetanus-toxoid $CD4^+$ cells

After 2 weeks, $CD4^+$ cells were harvested and washed thoroughly prior to co-culture with autologous mo-DCs that had been pre-exposed for 24hr to purified recombinant porin of *N. meningitidis* or the relevant control treatments (see Chapter 6). Depending on culture conditions and amount of blood donated by the volunteer, mo-DCs used in these experiments were either differentiated from autologous cryopreserved monocytes (thawed by warming in a 37°C water bath followed by rapid transfer of cells into 2% Human AB complete mo-DC medium) or fresh monocytes obtained in the second blood donation. Five day mo-DC-driven T-cell proliferation assays were set up as described above (2.2.4.1).

2.2.6 $CD34^+$-derived dendritic cells and Langerhans' cells

Anonymised apheresed samples from individuals whose $CD34^+$ stem cells were induced with G-CSF treatment were used in this study to obtain large numbers of $CD34^+$ cells (samples were kindly provided by Professor P. Johnson). Purified $CD34^+$ cells were obtained from mononuclear cells using $CD34^+$ isolation MACS kit (Miltenyi Biotech). Method for culturing $CD34^+$-derived dendritic cells or Langerhans' cells was adapted from published protocols (Gatti *et al.*, 2000; Caux *et al.*, 1999). Purified $CD34^+$ cells were cultured for 14 days at a density of $3x10^5$/mL in complete medium (phenol-free RPMI 1640 medium, 10% (v/v) Hyclone FCS, 2mM L-glutamine, 100U/mL penicillin and 100µg/ml streptomycin) and cytokines 1000U/mL rhGM-CSF, 50ng/mL rhSCF and 20ng/mL TNFα. 0.5mL of medium was replaced with 0.5mL fresh medium containing above-mentioned cytokines, at the same final concentration, every 4 days. To obtain $CD34^+$-derived Langerhans' cells, purified $CD34^+$ cells were initially re-suspended complete medium containing

cytokines as mentioned above. However, on Day 4 of culture, 0.5mL of old medium was replaced with fresh medium containing final concentration of 1000U/mL rhGM-CSF, 50ng/mL rhSCF and 10ng/mL rhTGFβ-1. Cells were maintained for the remaining 10 days for culture in this medium.

After culture, cells were harvested and prepared for flow cytometric analysis of surface phenotype (2.2.7).

2.2.7 Immunostaining for flow cytometry

Cells were labelled directly with antibodies conjugated to FITC or to PE, or indirectly with biotinylated antibodies followed by fluorochrome-conjugated streptavidin. All antibodies were used at optimal concentrations for flow cytometry as determined by frequent titrations, to ensure constant use of optimal concentrations. All antibody incubations were carried out on ice and in the dark. Washing of cells was performed by centrifugation in FACS buffer at 1500rpm (500 x *g*) for 5 minutes at 4°C unless stated otherwise.

2.2.7.1 Pre-blocking cells prior to immunostaining.

Cells were harvested into 15 mL Falcon tubes and pelleted by centrifugation at 1500rpm for 5 minutes at 4°C. The cell pellet was re-suspended in 1ml of ice-cold FACs buffer (Table 2.1) and the cells were subsequently divided equally into the required number of FACs tubes. FACs buffer (1 mL) was added to each FACs tube and the cells were washed a further two times in FACs buffer, discarding the supernatant after each wash. Cell pellets were finally re-suspended in 50µl FACS buffer containing 50µg/ml human Fcγ-fragments, or alternatively 10%(v/v) human AB serum, to block unspecific binding to Fcγ-receptors on cells for 30 minutes on ice.

2.2.7.2 Direct immunostaining for flow cytometry

Following blocking of Fcγ receptors on cells, 100µl of PE or FITC-conjugated mAbs (2.1.5), made up to the appropriate concentration in FACs buffer, was added to cells and left to incubate for a further 30 minutes. In parallel, cells were labelled with appropriate fluorochrome conjugated isotype control antibodies (Table 2.6). After the

incubations, cells were washed twice in 1 mL FACs buffer to remove unbound antibody. Supernatants were discarded after each centrifugation and the cells were finally re-suspended in 250µl FACS buffer and kept on ice and in the dark pending analysis.

2.2.7.3 Indirect immunostaining for flow cytometry

For biotinylated primary antibodies, the pre-blocked cells were incubated for 30 minutes on ice with antibody optimally diluted in 50µL FACs buffer. The cells were washed 3x in 1mL FACs buffer. Cell pellets were re-suspended in 50µL of PE- or FITC or Allophycocyanin (APC)-conjugated streptavidin made up in FACs buffer for 30 minutes on ice. In parallel, cells were labelled with the appropriate primary biotinylated isotype control followed by fluorochrome-conjugated streptavidin. The cells were washed 3x, as above and following the final wash were resuspended in 250µL FACs buffer and kept on ice and in the dark pending analysis.

2.2.7.4 Intracellular cytokine staining for flow cytometry

Following culture of mo-DCs in the presence of monensin (2.2.3.1), cells were harvested and pelleted by centrifugation at 1500rpm (500 x *g*) for 5 minutes at 4°C. Cells were fixed by resuspending in 500µl 4% (w/v) paraformaldehyde in PBS for 15 minutes on ice. Following one wash by centrifugation in 1mL PBS, cells were resuspended in 250µl 0.1M glycine (Sigma) in PBS and left to incubate for 10 minutes on ice. Two washes in 1mL PBS were performed prior to 30 minute permeabilization of cells in 1mL saponin buffer, consisting of PBS, 0.5% saponin (Sigma) and 0.5% BSA, on ice. 10% human AB serum was added to saponin buffer in this step to block unspecific binding to Fc receptors. Following this, cells were washed once in 1mL saponin buffer and incubated for 20minutes with 50µl FITC-conjugated HLA-DR mAb (2.1.5) and PE-conjugated intracellular IL-12p40 or p40/p70 mAb (Table 2.5) made up to appropriate concentration in saponin buffer containing 10% human AB serum. Negative controls (Table 2.6) were also set up by labelling cells with 50µl FITC or PE-matched isotype controls mAbs prepared to correct concentration in saponin buffer containing 10% human AB serum. For all mAb incubations, cells were kept on ice and in the dark. Subsequently, cells were

washed twice in 1mL saponin buffer and once in 1mL FACS buffer (Table 2.1). Finally, cells were resuspended in 200-300µl FACS buffer and kept on ice and in the dark pending analysis.

2.2.7.5 Flow cytometric analysis

Single or double fluorescent-labelled cell suspensions were run through a Fluorescence-Activated Cell Scanner (FACScan, Becton Dickinson) and the analysis was carried out using Lysis II software or Cell Quest software (both Becton Dickinson). Unlabelled cells were used to align the fluorescence detectors to set the histogram peak of cells in the first decade of a four-log scale. To exclude dead cells within the cell population 7 aminoactinomycin D (7-AAD, Sigma), 0.5µg/mL in PBS, was added to cell suspension and a live gate was set on FL3 channel. In addition, a live gate was set on the basis of forward and side scatter dot plot. The fluorescence signals in both FL1 and FL2 channels were compensated by single labelled FITC- and PE conjugated antibodies, respectively, by using the standard compensation circuits on the instrument.

Table 2.7: Settings for reading fluorescence of cell populations.

	Monocyte/ T and B-cells	Mo-DC / Mo-LC / $CD34^+$-derived DC/LC
Compensating tube	HLA-DR-PE HLA-DR- FITC	HLA-DR-PE CD1a- FITC
Forward scatter	E00 Linear 1.0- 1.3	E-01 Linear 8.0 – 8.5
Side scatter	Linear 1.0 – 1.3 310	Log 200
Threshold	200	200
Cell events collected	5000	10,000

Threshold was set in order to eliminate debris within cell suspension from analysis. 5000 – 10,000 cell events were collected for each preparation. For phenotypic analysis of mo-DC, cells were double stained for CD1a and CD14. Cells positive for CD1a and negative for CD14 were collected. T-cells were identified as cells showing strong labelling for CD3 and CD4; monocytes were identified as cells showing strong labelling for CD14. Analysis of $CD34^+$-derived dendritic or Langerhans' cells, was performed by double labelling for $CD1a^+$ cells and phenotypic molecules. During analysis, positive labelled cells within the population were obtained and overlayed with the histogram for the relevant isotype control mAb, typically on the first decade of a four-log scale. The median or geometric mean fluorescence intensity (MFI), where appropriate, of a histogram was obtained from the entire cell population.

2.2.8 Flow cytometric analysis of FITC-dextran uptake

2.2.8.1 Receptor-mediated endocytosis of FITC-dextran

FITC-dextran, 38 200 kDa (Sigma) was suspended in PBS to achieve a stock concentration of 10mg/mL. Receptor-mediated endocytosis of FITC-dextran was performed on 10^6 mo-DCs from various treatments at 37°C for 1 hour in a final

concentration of 250μg/ml FITC-dextran in complete mo-DC medium. In parallel, 10^6 mo-DCs from each condition was incubated with 250μg/ml FITC-dextran on ice (4°C) for 1 hour as a control for surface bound FITC-dextran. Cells were washed by centrifuging twice at 1500rpm (500 x *g*) for 5 minutes at 4°C, prior to re-suspension of cell pellet in 250μL FACs buffer and storing at 4°C in the dark pending flow cytometric analysis.

2.2.8.2 Flow cytometric analysis of FITC-dextran uptake

Mo-DCs incubated with FITC-dextran were passed through a FACScan and typically detected as described (2.2.7.5). Cell debris was eliminated using a forward scatter threshold. Mo-DCs incubated at 4°C were used to set the sensitivity of the fluorescence detectors to appropriate levels, such that on a histogram the peak of cells lay in the first decade of a four-log scale.

2.2.9 Methods for detecting cytokines and chemokines by sandwich ELISA

Production of cytokines IL-6, IL-10, IL-1β, IL-12p70 and TNF-α in supernatants following treatment of monocytes or mo-DC was measured by ELISA according to manufacturer's instructions using reagents supplied with the kits or prepared in house (2.1.6).

Maxisorp ELISA plates (NUNC, Life Technologies) were coated with capture mAb diluted in the recommended coating buffer at mid-range concentration recommended by manufacturer, for 18-24hrs at room temperature. 200μL ELISA blocking buffer was added to each well and plates were incubated for a further 1 – 2hr in order to block non-specific mAb binding. This was followed by three washes with 300μL/well of ELISA wash buffer. Standard curves were made in duplicate, using recombinant cytokines at dilutions suggested by the manufacturer ranging between 15 – 1500pg/ml in assay buffer. Test supernatants were added to wells in duplicate and left to incubate at room temperature. Where necessary, test supernatants were diluted 1:10, 1:20 or 1:40 in assay buffer in order for cytokine concentration to fall within the standard curve. Recommended length of supernatant incubation by individual manufacturers varied between 1 – 3hr. Optimally diluted biotin-conjugated detection mAb at recommended concentrations was added during supernatant incubation or following a wash step according to manufacturers instructions. In all cytokine

ELISAs, streptavidin-conjugated horseradish peroxidase (HRP) diluted 1:8000 – 1:16,000 in assay buffer, was added to each well and incubated for 30 minutes at room temperature followed by three washes as above. Tetramethylbenzidine free base (TMB) substrate (Sigma, or supplied with kit) was added and incubated for 15 – 20 minutes at room temperature in the dark. The colour reaction was stopped with sulphuric acid (0.18M – 2M) and the plates were read at a wavelength of 450nM on a SpectraMAX 340PC plate reader (Molecular Devices, UK).

The levels of chemokines IL-8, RANTES, MIP-1α and MIP-1β in supernatants were quantified by sandwich immuno-assays using matched pairs of specific antibodies (mAbs and reagents were kindly provided by Dr. M. Christodoulides). 96-well FluoroNunc™ Maxisorp plates were coated with capture mAb (2-4μg/mL) overnight at room temperature. After through washing (2.1.6) the wells were blocked (2.1.6) for 1hr at room temperature. Standards were prepared by serial dilutions (12500 – 20pg/mL) of purified recombinant chemokine proteins obtained from PeproTech EC Ltd. Chemokine standards and culture supernatants were diluted with Delfia® Assay buffer (Wallac) and added to wells, in duplicate, for 2hr incubation at room temperature. Matched biotinylated detecting antibodies, diluted in Delfia® Assay buffer (2μg/mL), were added to wells after thorough washing of the plate and left to incubate for 2hrs at room temperature followed by a final thorough washing of the plates. Measurement of bound biotin-labelled antibodies was achieved using a time resolved fluorimetry system (Delfia®, Wallac). Europium (Eu)-labelled streptavidin (100ng/mL) was added to each well and after 1hr incubation, levels of chemokines were detected by the addition of Delfia® Enhancement Solution (Wallac) followed by measurement of emitted fluorescence on a 1234 Delfia fluorimeter (Christodoulides *et al.*, 2000). The concentration of each chemokine in supernatants was extrapolated from the corresponding standard curve.

2.2.10 Nitric oxide analysis

Nitric oxide in supernatants was analysed using a nitric oxide analyser NOA™ 280 (Sievers, Boulder, CO, USA) with a high-sensitivity detector for measuring nitric oxide based on a gas-phase chemiluminescent reaction between nitric oxide and ozone according to the following equation:

$$NO + 0_3 \rightarrow NO_2^{\bullet}$$

$$NO_2^{\bullet} \rightarrow NO_2^{-} + h\nu$$

NO released into liquid, such as cell culture media, reacts with dissolved oxygen to form nitrite (NO_2^-). All samples were therefore reduced to NO in order to be measured using this technique. In brief, the supernatants were injected into an acidic NaI (Acros Organics) solution (0.05 g NaI in 1 mL PBS and 5 mL acetic acid) prepared in the vessel of the NOA apparatus where the NO_2^- was reduced to NO according to the following equation:

$$I^- + NO_2^- + 2H^+ \rightarrow NO + \frac{1}{2} I_2 + H_2O$$

The resulting NO was pumped into a small volume chemiluminescent reaction chamber where it was mixed with ozone generated from oxygen in a connected electrostatic ozone generator. The emission from electronically excited nitrogen dioxide ($NO_2^{\bullet}$) resulting from the chemiluminscent reaction of NO with O_3, is in the red and near infrared spectrum (>600 nm), and was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube. Each sample was run in duplicate.

A calibration curve was constructed before any samples were analysed, by stepwise addition of increasing concentrations of $NaNO_2$ prepared in PBS. The linear fit correlates the emission (mV) generated by the chemiluminescent reaction of NO with O_3, with the corresponding NO concentration. The slope of the curve was then used to calculate the concentration of NO measured in the cell culture samples. The sensitivity for measurement of NO and its reaction products in liquid samples is approximately 1 pmol.

2.2.11 TaqMan reverse-transcription polymerase chain reaction

2.2.11.1 Isolation of total RNA

Total RNA was extracted from cells following various treatments using QIAShredder columns, RNeasy Mini Kit (including columns, 1.5 mL and 2.0 mL collection tubes, RNase Free water, RLT buffer, RW1 buffer, RPE buffer) and RNase-Free DNase Set Kit (including RNase Free DNase I, Buffer RDD) purchased from QIAGEN Ltd.

(Crawley, UK). RNA purification was carried out according to the supplier's instructions at room temperature using sterile Aerosol resistant tips (Molecular Bio-Products). With the exception of cell harvest, all centrifuges were performed in a MSE MicroCentaur centrifuge.

Cultured monocytes, mo-DCs or mo-LCs were harvested into 15 mL Falcon tubes and spun at 1500rpm (500 × g) for 5 minutes at 4°C. The pellet, containing up to 5×10^6 cells, was resuspended in 350 µL RLT buffer, containing 0.145 M 2-mercaptoethanol, followed by thorough mixing. The cell lysate was loaded onto a QIAShredder column placed in a collection tube and spun at 13000 rpm (>8000 x g) for 2 minutes. 70 % ethanol was prepared by diluting 7 parts absolute ethanol (LAB3) with 3 parts DNase/RNase free water (Life Technologies). One volume 70 % ethanol, approximately 350 µL, was added to the homogenised cell lysate in the collection tube and was mixed by repeated pipetting. The ethanol : lysate mix was loaded onto a RNeasy mini spin column placed in a new collection tube and spun at > 8000 × g for 15 seconds. The flow-through was discarded. The spin column was washed once, reusing the collection tube, by adding 350 µL RW1 buffer followed by centrifugation at >8000 × g for 15 seconds. The flow-through was discarded and the collection tube reused. 70 µL RDD buffer was added to 10 µL DNase I (2.7 U/µL) and without further handling added to the spin columns followed by 15 minutes incubation. DNA degradation was stopped by adding 350 µL RW1 buffer followed by centrifugation at 8000 × g for 15 seconds. The flow-through was discarded. The spin column was placed in a new 2 mL collection tube and washed by adding 500 µL RPE buffer, containing 4 volumes of absolute ethanol, followed by centrifugation at 8000 × g for 15 seconds. The flow-through was discarded and the collection tube reused. The spin columns were washed once more with 500 µL working RPE buffer and spun at 13000 for 2 minutes. The spin column was placed in a new collection tube and the total RNA was eluted by adding 30 µL pre-warmed RNase free water followed by centrifugation at >8000 × g for 1 minute. The eluted RNA was stored on ice pending measurement of RNA content.

2.2.11.2 Quantification of RNA concentration

RiboGreen® RNA kit (Molecular Probes, used as directed), containing RiboGreen RNA quantitation reagent, 20x TE buffer and ribosomal RNA standard, was used to quantitate purified total RNA from fresh or cultured monocytes as wells as cultured mo-DCs and mo-LCs. This method uses a sensitive (>1ng/mL RNA) fluorescent nucleic acid stain for determining the concentration of RNA in solution and was performed as directed by the manufacturer. Nuclease-free plasticware was used to prepare samples and the RNA standard curve.

In brief, four-fold serial dilutions of the RNA standards (final concentration 200μg/mL-3.125μg/mL) were prepared in TE-buffer. 100μL of each concentration was placed into wells of 96-well Maxisorp plate (Nunc, Life Technologies), in duplicate. Into the same plate, 100μL of test RNA sample was also placed into separate wells in duplicate. When appropriate, the test RNA was typically diluted in TE buffer at 1:1000 – 1:5000, prior to addition into the 96-well plate. Blank control wells were set up containing 100μL or TE buffer only. Immediately prior to use the RiboGreen reagent was prepared by diluting 1:2000 in TE buffer of which 100μL was added to each well containing standard curve or test samples. Wells were mixed by gently shaking of the plate, in the dark. Measurement of sample fluorescence was determined with a fluorescent spectrophotometer (wavelengths: excitation 480nm and emission 520nm) using CytofluorII software. Concentration of total RNA was extrapolated from the RNA standard curve after correcting the fluorescence values by subtracting those of blank wells from the sample wells. RNA samples were stored at –80°C.

2.2.11.3 Random hexamer primer reverse transcription

Reverse transcription of the total RNA was performed using the Omniscript™ Reverse Transcription kit (QIAGEN Ltd., Crawley, UK) that includes Omniscript Reverse Transcriptase, 10x buffer RT, 5mM dNTP mix and RNase-free water. Random hexamer primers were purchased from Promega, UK. RNase inhibitor (ANTI-Rnase, 20U/μl) was purchased from Ambicon. Reaction mixes were set up in 0.2 mL thin-walled tubes (Advanced Biotechnologies Ltd.). For each RNA sample one positive reverse transcription reaction (+RT) containing reverse transcriptase

enzyme, and one negative reverse transcription reaction (-RT) without enzyme was set up.

Each +RT reaction (20μL total volume), prepared on ice, consisted of a maximum of 0.5 μg RNA in a total volume of 14.1 μL RNase and DNase free water, 0.5 μL random hexamer primers (50 ng/mL), 2μL buffer, 2μL dNTP and 0.4μL RNase inhibitor. The Omniscript reverse transcriptase enzyme, 1μL, was finally added to the +RT mixture. The RNA was reverse transcribed at 37°C for 60 minutes in a GeneAmp 9600 thermal cycle (PE Biosystems, Warrington, UK). The resulting cDNA was stored at - 80 °C. –RT reaction mixtures were prepared and treated as the +RT reaction mixtures with the exception of Omniscript enzyme being replaced by RNase and DNase free water.

2.2.11.4 RNA Standard curve

Human purified total PBMC RNA was diluted into concentrations ranging from 400ng - 3.125ng, and converted into cDNA using Omniscript reverse transcription enzyme. Individual RTs were performed for the different RNA concentrations, rather than preparation of curve by serially diluting stock cDNA, to take into account possibly inefficiencies within the reaction. These cDNA were prepared for optimisation of probes and primers for use in TaqMan PCR (2.2.11.5) as well as to generate standard curves for semi-quantitive analysis of mRNA in test samples.

2.2.11.5 TaqMan multiplex polymerase chain reaction

Relative quantification of human TLR2, TLR4 and TLR9 normalised to 18S ribosomal RNA (rRNA) was performed. TLR probes (Biosource International) and primers (Genosys, Sigma) were designed by Dr. Judith Holloway (Southampton University, Table 2.8). Pre-developed (2x) TaqMan Universal PCR Master mix and 20x 18S rRNA primer and probes were purchased from PE Biosystems (Warringnnton, UK). Multiplex TaqMan polymerase chain reaction (multiplex TaqMan PCR) was performed using TLR and 18S rRNA probes carrying different fluorescent dyes (FAM and VIC respectively) so both targets can be measured in the

same tube. The samples were placed in an ABI PRISM 7700 sequence detection system (PE Biosystems) where amplification of the target cDNAs was recorded over 40 cycles of amplification.

2.2.11.5.1 Optimisation of TLR probes and primers

Concentration of TLR2, TLR4 and TLR9 probes and primers were established using cDNA from human PBMCs. Upper and lower TLR primers were prepared in UHQ at concentrations ranging 600nM - 200nM. Initially, different possible combinations of upper and lower primers were added to the PCR Reaction mix containing a high concentration of the respective TLR probe. ΔRn is an indicator of the magnitude of the signal generated by the given set of PCR conditions. Whereas, the threshold cycle (C_T) is the cycle number at which the exponential amplification curve crossed the threshold line. The lowest primer concentrations giving the maximum ΔRn value were used in subsequent experiments (see Table 2.9). Optimal TLR probe concentrations were determined, by diluting in UHQ into concentrations ranging 200nM – 25nM and then set up in the PCR reaction with the appropriate optimum primer concentrations. Lowest concentrations that gave the minimum C_T value for each probe target determined was optimal (see Table 2.9).

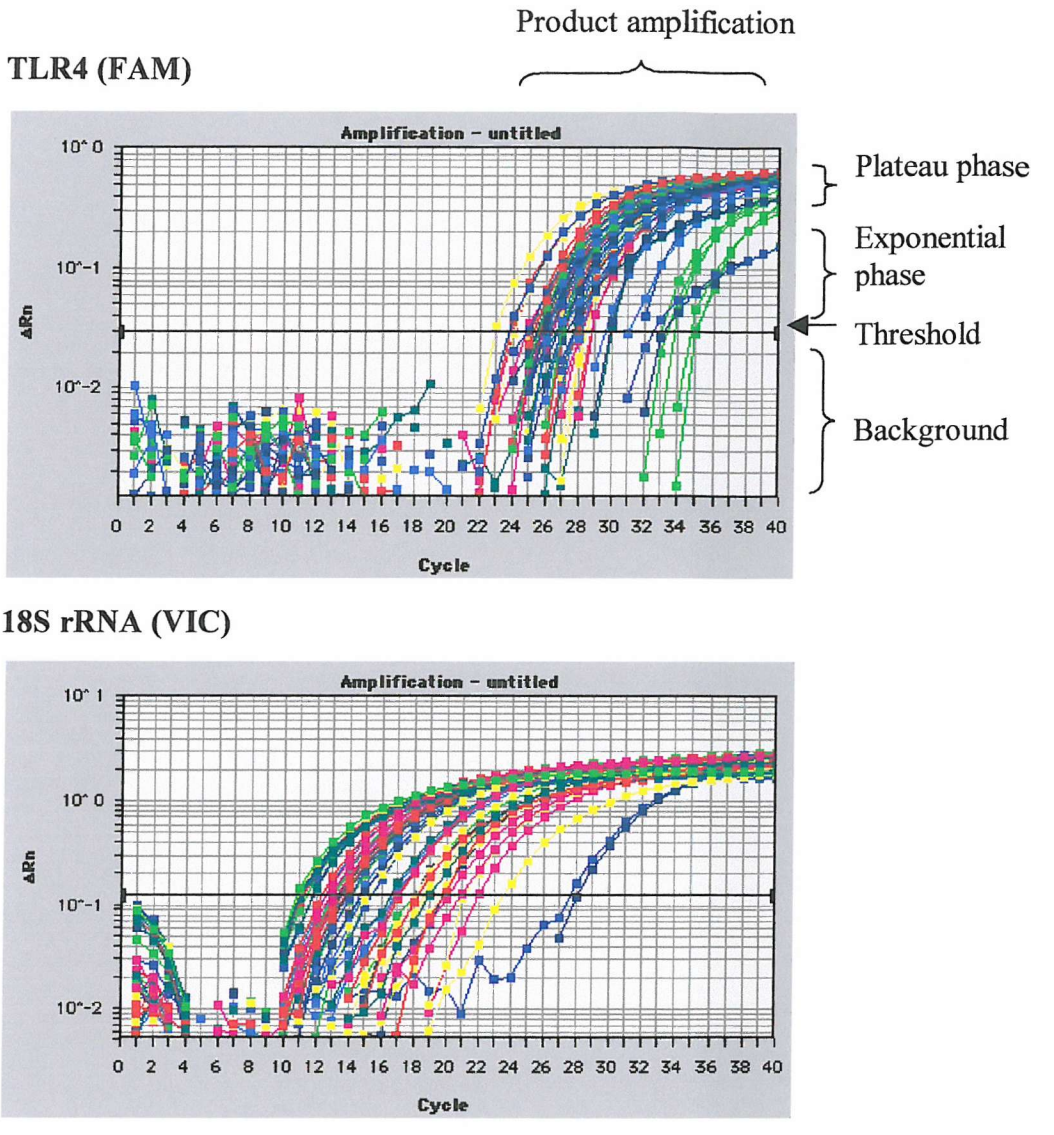

Figure 2.1: Interpreting TaqMan data.

Detection of Toll-like receptor (TLR) 4 and endogenous 18s rRNA expression in test samples. Data obtained from TaqMan sequence detection systems software was adjusted to give accurate C_T values by setting up the baseline and threshold values according to manufacturer's directions. Data was subsequently exported into Microsoft Excel to view the results and determine the relative quantification.

Table 2.8: TLR primer and probe sequences

TLR2 primers Sigma-Genosys Ltd.	UPPER 5′-CTACTGGGTGGAGAACCTTATGGT-3′ LOWER 5′-CCGCTTATGAAGACACAACTTGA-3′
TLR2 probe Biosource International	5′-FAM-AGGAGCTGGAGAACTTCAATCCCCCC-TAMRA-3′
TLR4 primers Sigma-Genosys Ltd.	UPPER: 5′-TCCATGAAGGTTTCCATAAAAGC-3′ LOWER: 5′-CTGCCAGGTCTGAGCAA TCTC-3′
TLR4 probe Biosource International	5′-FAM-ATTGTTGTGGTGTCCCAGCACTTCATCC-TAMRA-3′
TLR9 primers Sigma-Genosys Ltd.	UPPER: 5′-CTCTGAAGACTTCAGGCCCAACT-3′ LOWER: 5′-TGCACGGTCACCAGGTTGT-3′
TLR9 probe Biosource International	5′-FAM-AGCACCCTCAACTTCACCTTGGATCTGTC-TAMRA-3′

Table 2.9: TLR primer and probe concentrations

	Probe and Upper /Lower primer stock conc. (μM)	Upper primer conc. (μM)	Lower primer conc. (μM)	Upper/Lower primer to add per reaction	Probe conc. to add per reaction
TLR2	10μM	0.6	0.6	3.3μL/tube	1.1μL / tube
TLR4	10μM	0.5	0.5	2.75μL/tube	0.7μL / tube
TLR9	10μM	0.5	0.5	2.75μL/tube	1.1μL / tube

2.2.11.5.2 PCR

Duplicate reaction mixes for each cDNA preparation were prepared in 0.2mL MicroAmp optical tubes (PE Biosystems). For each 55μL reaction mix, 4μL of the cDNA, 27.5μL 2x TaqMan Universal PCR Master mix, 2.7μL 20x 18S rRNA pre-developed primer and probes were added. Appropriate primers and probes (see Table 2.9) were also added to each tube. Finally, the total volume was made up with UHQ. To control for contamination within the reagents (negative controls), the reaction mix was prepared replacing the cDNA with UHQ.

25μL of each duplicate reaction mix was placed into optical density tubes (ABI prism optical tubes and caps; Applied Biosystems, UK). After amplification, the C_T was determined for each cDNA preparation. In order to accurately set the threshold, the baseline was first set such that the amplification curve growth on a linear scale begins at a cycle number greater than the highest baseline cycle number. The threshold value was then set above the background within the exponential phase of the logarithmic scale amplification plot (Figure 2.1).

PBMC RNA standard curves were generated by plotting the average C_T values for the TLR targets against the Log RNA concentration. In addition, the standard curves for the endogenous control 18S rRNA for each TLR target were plotted. For each test cDNA preparation, the relative RNA concentration was extrapolated from the TLR and 18s RNA standard curves. In addition, the relative RNA concentration for each cDNA sample was then normalised to that for the endogenous internal standard 18S rRNA by subtracting the 18S rRNA from the TLR target.

2.2.12 Confocal microscopy

Images were acquired using a SP2 Confocal Laser Scanning Microscope (Leica, UK) equipped with argon, blue helium neon and red helium neon lasers with a spectral head. In all cases, mo-DCs were viewed using x40 and x63 oil-immersion objectives. A series of confocal sections were taken at 0.5μm intervals.

2.2.12.1 Immunostaining sections of human skin

Immunostaining for confocal microscopy was performed at room temperature. Human epidermal skin sections obtained by suction blistering protocols (Chapter 5) were incubated in PBS containing 50mM NH_4Cl for 10 minutes, to block free amino acid groups, followed by extensive washing with PBS. Individual skin sections were placed into separate wells of a 48-well plate and incubated for 30 minutes in 500μL FACS buffer (2.1.2) containing 10% Human AB serum. Subsequently, the sections were transferred into fresh wells containing appropriate antibodies made up in 200μL FACS buffer (TLR2- or TLR4-biotin conjugated mAbs 20μg/ml plus FITC-conjugated CD1a 10μg/ml; TLR9 10μg/ml). In addition, separate skin sections were incubated with the appropriate isotype control mAbs. Following 30 minute incubation, the sections were rinsed thoroughly in FACS buffer prior to a further 30 minute incubation in the respective secondary antibodies made up in 200μL FACS buffer (for TLR2 and TLR4 staining, streptavidin-conjugated PE (Sigma) was used; for TLR9 staining PE secondary antibody (Jackson Laboratories) was used). Sections stained with isotype control mAbs were also incubated with the corresponding secondary mAbs. The sections were rinsed thoroughly with FACS buffer prior to mounting onto polylysine-coated microscope slides (LAB3). A small amount of Moviol (Agar Scientific, UK) was placed on the stained cells and covered with a covered slip. The slides were stored at 4°C in the dark pending analysis by confocal microscopy.

2.2.13 Plasmid purification

Endotoxin-free purification of plasmid DNA was performed using QIAGEN EndoFree™ Plasmid Maxi kit (QIAGEN Ltd., UK), which includes: QIAGEN-tip 500, QIAfilter maxi cartridges, Buffers (P1, P2, P3, QBT, QC, ER and TE), endotoxin-free water and RNase A. Protocol was followed according to the manufacturer instruction.

In brief, cultured ampicillin-resistant *E. coli* bacteria harbouring pcDNA3 vector (kindly provided by Dr. S. Thirdborough, University of Southampton) were harvested and pelleted at 6000 x *g* at 4°C. The pellet was lysed by resuspension in 10mL of

Buffer P1 containing RNase A (final concentration 100μg/ml). Buffer P2 (10mL) was subsequently added, gently inverting the mixture to avoid shearing of genomic DNA, and left to incubate at room temperature for 5 minutes. Pre-chilled Buffer P3 (10mL) was added to the lysate and the tube inverted gently. The lysate was poured into the QIAfilter cartridge and incubated for 10minutes at room temperature, during which the QIAGEN-tip was equilibrated with 10mL QBT buffer, and allowed to empty by gravity flow. The lysate was expelled from the QIAfilter using a plunger, 2.5mL of buffer ER was added to the filtered lysate and left to incubate on ice for 30 minutes after gentle inversion of the solution. The lysate was subsequently added into the QIAGEN-tip and allowed to drain into the resin by gravity. The QIAGEN-tip resin was then washed twice with 30mL QC buffer, and the DNA eluted with 15mL QF buffer. The DNA was precipitated with 10.5mL isopropanol, and centrifuged at 15000 x *g* for 30 minutes at 4°C. The DNA was washed with 5mL 70% ethanol (made up in endotoxin-free water) and centrifuged at 15000 x *g* for 10 minutes at 4°C. The supernatant was removed, the pellet allowed to air dry and then redissolved in 400μL endotoxin-free TE buffer. The concentration of DNA was determined by UV spectrophotometry (absorbance at 260nm).

2.2.14 Limulus amebocyte lysate assay for detection of endotoxin

Detection and semi-quantitation of endotoxin in the *N. meningitidis* class I porin preparation was performed using an E-Toxate multiple test vial kit (Sigma) according to the suppliers instruction. In brief, an E-Toxate® working solution was prepared by adding endotoxin-free water (supplied with the kit) to the lyophilised E-Toxate® using a pyrogen-free graduated plastic pipette. The E-Toxate® was dissolved by gentle swirling, then chilled on ice. The lyophilised endotoxin standard was dissolved in endotoxin-free water by vigorous shaking for 2 minutes followed by 30 seconds shaking at 10 minutes intervals over a 30-minute period. A standard curve ranging from 400 endotoxin units (EU)/ mL to 0.015 EU/mL was prepared in baked borosilicate tubes (Sigma) by serially diluting the endotoxin standard provided in endotoxin-free water. Dilution of the sample was made in endotoxin-free water. 0.1 mL sample, diluted standard or endotoxin-free water (negative control) was added to the baked tubes. An equal volume of E-Toxate® working solution was added to the sample, diluted standard or endotoxin-free water, then gently mixed and covered with

Parafilm. Following 1hour incubation at 37°C, the tubes were gently inverted to detect gelation. A positive test was defined as formation of a hard gel, whereas all other gel consistencies were considered negative. The level of endotoxin in the sample was calculated by multiplying the inverse of the highest dilution of sample found positive (e.g. 1/64) by the lowest dilution of standard found positive (e.g. 0.03125 EU/mL). Under the above conditions the endotoxin level in stock amounts of purified *N. meningococcal* class I porin (2.3mg/ml) would equal 2 EU/mL according to the following equation. (5 EU = 1 ng endotoxin.)

Endotoxin (EU/mL) = (1/(1/64)) × 0.03125 = 2 EU/mL

Therefore, 1μg/ml meningococcal class I porin = 0.00087 EU/ml = 0.174pg/ml of endotoxin.

2.2.15 Statistical analysis

Using Arcus Quick Stat program (Research Solutions), the non-parametric Wilcoxon signed rank test or Mann Whitney U test was used where appropriate to compare responses within a subject group or between the atopic and non-atopic subject group, respectively. In addition, Students t-test was used where appropriate and Analysis of variance (either one- or two-way ANOVA) was employed to determine the overall difference within an experiment possessing a number of variables. A *p*-value of less than 0.05 was considered statistically significant.

CHAPTER 3

Effects of Immunostimulatory Oligodeoxynucleotides on Human Monocytes

3

3.1 INTRODUCTION

The discovery of specific sequences present in bacterial DNA that are capable of exerting potent effects on a variety of murine immune cells has led to wide investigations of their use in novel therapeutic strategies. The immunostimulatory properties of bacterial DNA have been attributed, in part, to the presence of unmethylated CpG-motifs (Krieg, 2000). Synthetic oligodeoxynucleotides (ODN) containing CpG-motifs are also stimulatory. In mice, CpG ODN exhibit adjuvant properties and when given by a number of immunization routes, including mucosal immunization, are potent inducers of IL-12 production and hence of Th1- and CTL responses for multiple antigens (Brazolot Millan *et al.*, 1998; Klinman *et al.*, 1999). In non-human primates, CpG ODN have also been reported to act as potent adjuvants (Jones *et al.*, 1999). One observation, consistent in both human and murine studies, is the requirement for unmethylated CpG ODN sequences in order to exert immunostimulatory effects on cells. Whilst inversion of 'CpG' to read 'GpC' eliminates the stimulatory capacity of the ODN in murine models, in humans this inversion may only result in a partial reduction of activity (Parronchi *et al.*, 1999). An additional difficulty in comparing the effects of CpG ODN in mice and humans is brought about by conflicting data suggesting human and murine cells respond to different CpG-sequences.

At the beginning of this project there were only few publications on the effects of CpG ODN on various human cells. Therefore, the initial objectives of this study were to characterize and compare the effects of synthetic immunostimulatory CpG ODN on monocytes obtained from non-atopic and atopic individuals, *in vitro*. Published findings have described the ability of synthetic CpG ODN to promote Th1 response, suggesting that these agents could be applied for the treatment of diseases such as allergy or asthma which are associated with a strong Th2 response (Kovarik *et al.*, 1999; Parronchi *et al.*, 1999; Kohama *et al.*, 1999). APC express a variety of cell surface molecules that are known to participate in T-cell activation. In addition to soluble mediators produced by APCs, co-stimulatory signals are important factors that influence the differentiation of naïve T-helper cells towards Th1 or Th2 phenotypes (van Gool *et al.*, 1996; Cella *et al.*, 1996). However, in atopic individuals, APC

function is reported to be altered compared to non-atopic individuals (Kapsenberg *et al.*, 1998). The role of CD80 and CD86 in provision of co-stimulation to T-cells is suggested by some investigators to differ in monocytes from atopics and non-atopics (Creery *et al.*, 1996; Hofer *et al.*, 1998). Moreover, monocytes from atopic asthma patients show lower production of IL-12 in response to bacterial components compared to control subjects (Pouw Kraan *et al.*, 1997).

The effects of CpG- and non-CpG ODN on monocytes from both subject groups were evaluated by measuring changes in surface expression of HLA-DR and co-stimulatory molecules CD80, CD86 and CD40. Bacterial DNA but not vertebrate DNA is reported to induce cellular activation (Sparwasser *et al.*, 1998; Krieg, 2002). Similar to ODN, the immunostimulatory property of bacterial-derived plasmid DNA is partially due to the presence of high numbers of CpG-motifs within the backbone (Klinman *et al.*, 1997). Therefore, the effect of plasmid or genomic DNA on inducing changes in monocyte phenotype is also examined as a positive and negative control stimulus, respectively. The activities of CpG and non-CpG on the differential cytokine (IL-1β, IL-6, IL-10, IL-12p40 and IL-12p70) production by monocytes from both subject groups were also investigated.

The results presented in the second section of this chapter address the expression and regulation of Toll-like receptor (TLR) 2, TLR4 and TLR9 on human monocytes, following exposure to specific or non-specific bacterial ligands. TLRs are highly conserved family of pattern recognition receptors that are critical for instigating immune responses to bacterial products (Krutzik *et al.*, 2001). The physiological roles of TLR2 and TLR4 are the best characterized. As described in the Introduction (1.2.2.2) TLR2 is involved in responses to Gram-positive bacteria, mycobacteria and yeast, whereas TLR4 is required for the responses to LPS derived from Gram-negative bacteria. Recently, TLR9 was identified to play critical roles in the recognition of unmethylated CpG motifs in murine cells (Hemmi *et al.*, 2000). Therefore, it was important to determine whether human monocytes express TLR9.

3.2 MATERIALS AND METHODS

3.2.1 Synthetic Oligodeoxynucleotides (ODN):

Unmethylated nuclease-resistant phosphorothioate-modified ODN were purchased from MWG (Munich, Germany). Lyophilized synthetic ODN were dissolved in endotoxin-free TE (10mM Tris, 1mM EDTA) buffer at a stock concentration of 100μM.

Sequences of ODNs investigated:

CpG ODN '1668' : 5′-TCC ATG ACG TTC CTG ATG CT-3′

Non-CpG ODN '1720': 5′-TCC ATG AGC TTC CTG ATG CT-3′

Non-CpG ODN '1745': 5′-TCC ATG AGC TTC CGT AGT CT-3′

Non-CpG ODN '100' : 5′-TCC AGT AGC TTC AGT AGT CT-3′

CpG ODN '2006' : 5′-TCG TCG TTT TGT CG T TTT GTC G TT-3′

'CpG' motifs in sequences are underlined and presented in bold black letters. Inverted 'CpG' motifs to read 'GpC' are underlined and presented in green. 'TpG' and 'GpT' motifs in sequences are presented in red and blue, respectively.

The CpG ODN '1668' 20-mer sequence was used in this study due to reports on its stimulatory effects on murine bone-marrow dendritic cells (Lipford *et al.*, 1997) The CpG ODN '2006' 24-mer sequence was chosen for use in this study because of reported stimulatory properties on human B-cells and peripheral blood dendritic cells (Hartmann *et al.*, 1999; Krieg *et al.*, 1995) The non-CpG ODN '1720' and '1745' were chosen as control ODNs containing inverted CpG motifs reported to lack stimulatory properties in murine cells (Lipford *et al.*, 1997; Yi *et al.*, 1996). The non-CpG ODN '100' was designed by modification of CpG ODN 1668 by inverting the 'CpG' motif as well as inverting the 'TpG' motif. Inversion of 'TpG' was carried out to eliminate potential contribution to immunostimulatory effects (Yi *et al.*, 1996).

3.2.2 Treatment of human monocytes with ODN

Monocytes from atopic or normal donors, obtained by plastic adherence (2.2.1.1) were cultured in supplemented IMDM containing 10% (v/v) human AB serum. Number of monocytes in each treatment was determined by measuring % $CD14^+$ cells in $1x10^6$ PBMCs from respective donors.

CpG '1668' and non-CpG '100', '1720', '1745' were prepared in supplemented IMDM containing 10% heat inactivated human AB serum. Following preliminary dose-response experiments, optimal concentration for stimulating monocytes with ODN was 1μM. Adherent monocytes were exposed to 2mL of the respective treatments and left to culture at 37°C and 5% CO_2 for 20hrs, 40hrs or 60hrs. Control untreated cells were stimulated with endotoxin free TE-buffer (vehicle control) at the same volume the ODN was used.

3.2.3 Measurement of cytokine production

Following monocyte treatments, cell-free supernatants were stored at –70°C pending analysis. Cytokine secretion into supernatants was measured by ELISA using monoclonal matched pair antibodies, according to manufacturers instructions (2.2.9) Where necessary, supernatants were diluted 1:10, 1:20 or 1:40 in the appropriate assay buffer in order for cytokine concentration to fall within the standard curve of the ELISA assay. Concentration of cytokines was calculated by relating the absorbance values of duplicate wells to the standard curve. Absorbance was read at 450nm on an ELISA plate reader. The concentration of cytokines was converted to pg / $1x10^6$ cells.

3.2.4 Phenotypic analysis by flow cytometry following ODN treatment

Treated adherent monocytes were manually scraped off the plastic following a 20 min pre-incubation of culture dishes on ice to aid detachment of cells. Fc-receptors on cells were blocked (2.2.7.1) prior to staining cell surface molecules using fluorescent conjugated antibodies (2.1.5). Surface expression of CD80 (PE), CD86 (PE), CD40 (FITC) was measured on HLA-DR (FITC or PE)-gated monocytes. Analysis of CD14 expression was performed by single mAb staining of cells or double staining with HLA-DR mAb where indicated. For analysis of TLR expression on $CD14^+$-gated monocytes mAbs directed to TLR2 biotin-conjugated (2^y streptavidin-PE),

TLR4 (directly conjugated-PE) and TLR9 (indirectly conjugated-PE) were used. Appropriate fluorochrome conjugated isotype control antibodies were used in parallel (Table 2.6). During flow cytometric analysis cells were collected within a viable forward side scatter gate together with the exclusion of dead cells using 7-AAD. Representative flow cytometric profiles (FACS) are shown and where appropriate data is presented as the summary of the mean or median fluorescence intensities of mAb staining (indicated in the figure legends).

3.2.5 Quantification of TLR mRNA levels by TaqMan RT-PCR

Following treatment of monocytes with synthetic ODN, *E. coli* LPS and/or plasmid DNA (pcDNA3 vector), total RNA was extracted using the RNeasy kit method (Qiagen) and treated with DNAse I (RNAse-free DNAse, Promega) according to the manufacturers' instructions. RNA was quantified using RiboGreen® RNA kit (Molecular Probes, used as directed) with a fluorescence spectrophotometer and CytofluorII software. cDNA was prepared from 400ng of total RNA using the Omniscript reverse transcriptase preamplification system (Promega) with random hexamer primers (Promega). The cDNA levels of TLR2, TLR4 and TLR9 were quantified by TaqMan PCR using an ABI prism 7700 sequence detector according to the manufacturer's instructions (Applied Biosystems). The cDNA levels during the linear phase of amplification were normalized against ribosomal 18s (Pre-Developed Assay Reagent, PE Applied Biosystems). Relative RNA concentrations were extrapolated using a human PBMC standard curve. Probes and primers used are listed in section 2.2.11.5.1.

CHAPTER 3

Results: Section I

Effect of Immunostimulatory Oligodeoxynucleotides Phenotype and Cytokine production on Human Monocytes

3.3 PHENOTYPE AND CYTOKINE PRODUCTION BY MONOCYTES STIMULATED WITH OLIGODEOXYNUCLEOTIDES

3.3.1 Expression of surface antigens on monocytes following ODN treatment

3.3.1.1 HLA-DR expression on monocytes from non-atopic and atopic donors

Adherent monocytes obtained from non-atopic or atopic donors were treated with ODN for 20hrs. Harvested monocytes were analysed for changes in HLA-DR expression by flow cytometry. Figure 3.1A and 3.1B shows representative FACs profiles from experiments performed on monocytes from non-atopic and atopic donors, respectively. Stimulation of monocytes with 1μM CpG '1668' for 20hrs caused a small decrease in HLA-DR expression, compared with control monocytes treated with endotoxin-free TE buffer alone. Similarly, treatment of monocytes with 1μM non-CpG '100' decreased HLA-DR expression compared with control cells. No difference in response was observed between non-atopic and atopic monocytes following exposure to ODNs.

Effects of ODN on expression of HLA-DR by monocytes derived from non-atopic and atopic donors are shown in Figure 3.2 and 3.3, respectively. Although a small decrease in HLA-DR expression on CpG '1668'-treated compared with control monocytes was observed this was not found to be statistically significant in either of the subject groups. Patterns of HLA-DR expression on monocytes observed following treatment with non-CpG '1745' and '1720' compared with control cells are inconsistent in all subjects tested. Non-CpG '100' had no or insignificant effect in decreasing HLA-DR expression on both non-atopic and atopic monocytes.

The average of absolute HLA-DR fluorescence intensities following 20hr treatment of monocytes with ODN from both subject groups are summarised in Figure 3.4. The basal expression of surface HLA-DR in non-atopics was found to be two-fold higher compared with atopics ($p<0.05$, Mann-Whitney test).

A) Non-atopic

B) Atopic

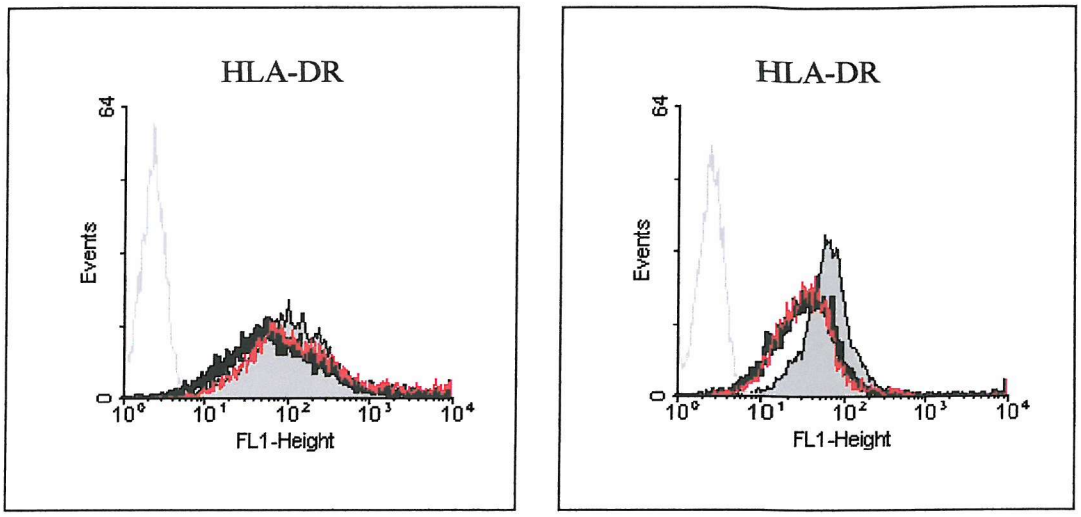

Figure 3.1: Flow cytometric analysis of HLA-DR on monocytes following ODN treatment.

Monocytes from non-atopic (A, n=6) and atopic donors (B, n=9) were treated with CpG '1668' 1μM (**thick black line**) or non-CpG '100' 1μM (red line) for 20hrs. Solid grey histograms are control monocytes treated with TE-buffer only. Clear grey histograms represents cells stained with appropriate isotype control mAb. FACs profiles are derived from one representative donor.

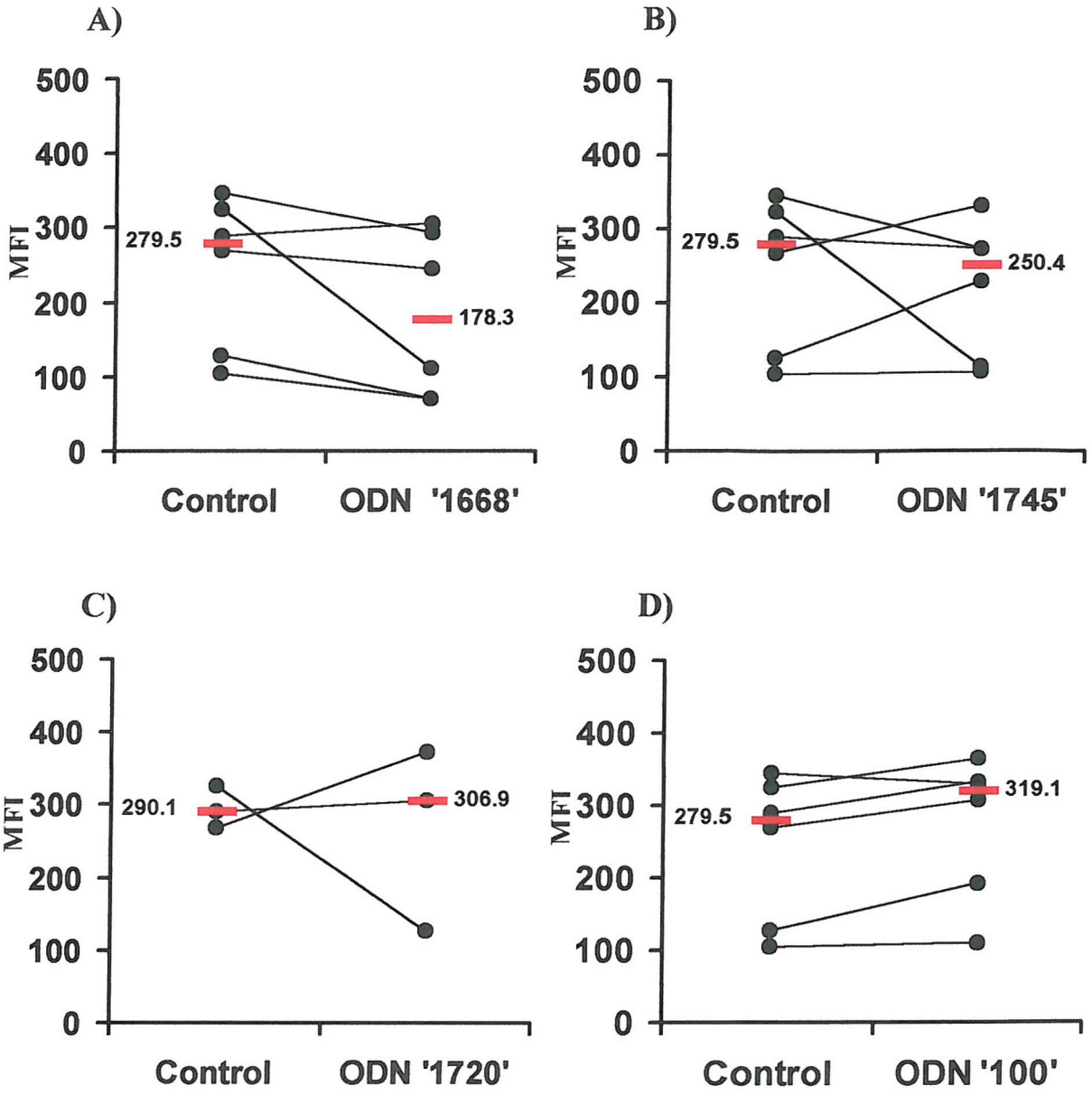

Figure 3.2: Absolute individual HLA-DR MFI values of monocytes from non-atopic donors.

HLA-DR surface expression on monocytes from non-atopic donors following stimulation for 20hrs with 1μM ODN; A) CpG '1668' (n=6), B) non-CpG '1745' (n=6), C) non-CpG '1720' (n=3) and D) non-CpG '100' (n=6). Control monocytes were treated with endotoxin free TE-buffer. Expression of HLA-DR was determined by flow cytometry. Graphs show absolute individual HLA-DR median fluorescence intensity (MFI) after appropriate treatments as indicated. Red bars represent the median with values shown alongside.

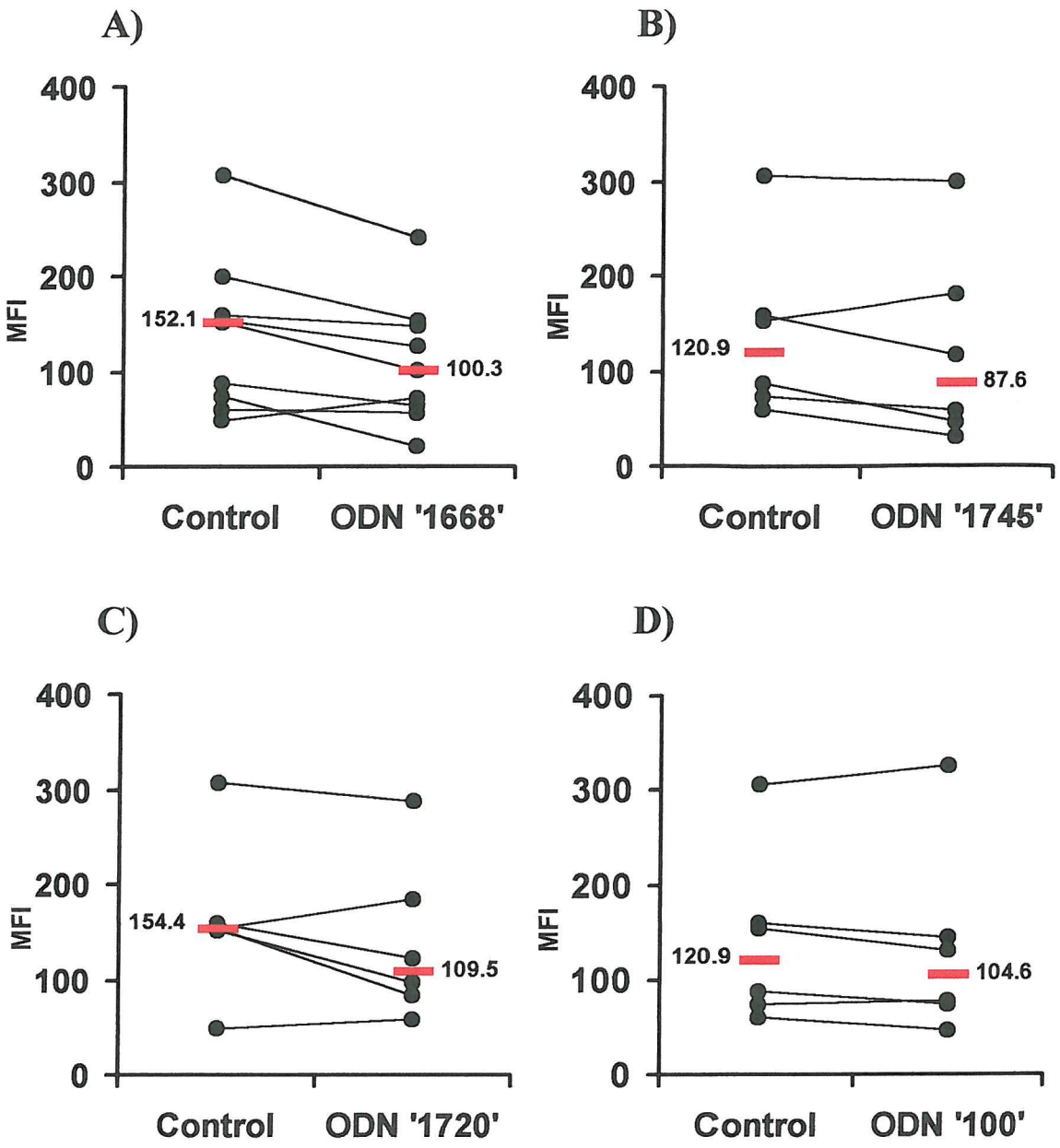

Figure 3.3: Absolute individual HLA-DR values of monocytes from atopic donors.

HLA-DR surface expression on monocytes from atopic donors following 20hr stimulation with 1μM ODN; A) CpG '1668' (n=9), B) non-CpG '1745' (n=6), C) non-CpG '1720' (n=6) and D) non-CpG '100' (n=6). Control monocytes were exposed to endotoxin free TE-buffer alone. Expression of HLA-DR was determined by flow cytometry. Graphs show absolute individual HLA-DR median fluorescence intensities (MFI) after appropriate treatments as indicated. Red bars represent the median with values shown alongside.

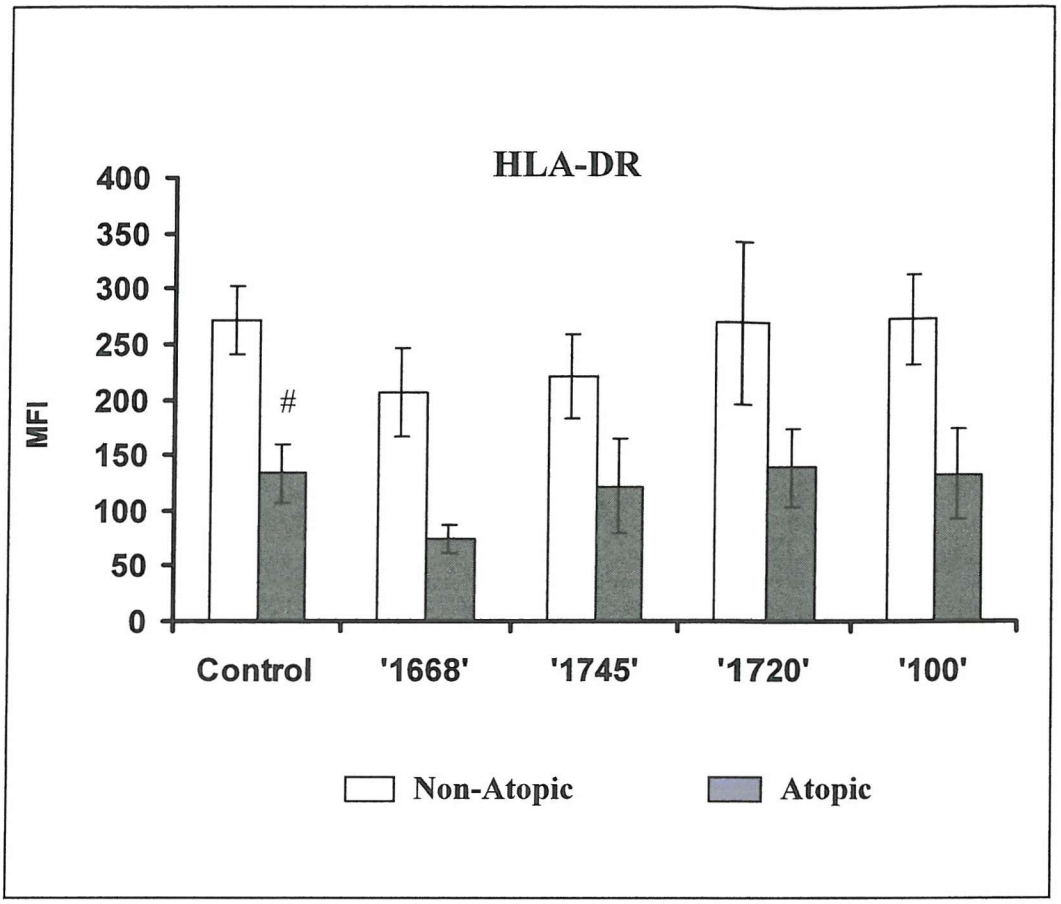

Figure 3.4: Summary of HLA-DR expression on monocytes from non-atopic and atopic donors following treatment with ODN.

Monocytes were treated with TE-buffer only (control) or treated with 1μM ODN (CpG '1668', non-CpG '1745', non-CpG '1720' 1μM or non-CpG '100') for 20hrs. Data represents the average of all the individual absolute mean fluorescence intensities (MFI) of cells single stained with HLA-DR mAb, as determined by flow cytometry, for the respective treatments. Bars represent standard error of the mean.

= Significance ($p<0.05$) observed between non-atopic and atopic individuals.

Sample numbers:

Non-Atopic: Control n=6; '1668' n=6; '1745' n=6; '1720' n=3; '100' n=6

Atopic: Control n=9; '1668' n=9; '1745' n=6; '1720' n=6; '100' n=6

3.3.1.2 CD80 expression on monocytes from non-atopic and atopic donors

ODN-treated monocytes were analysed for changes in CD80 expression by flow cytometry. Figure 3.5A and 3.5B shows representative FACs profiles for monocytes from non-atopic and atopic donors, respectively. Treatment of monocytes with CpG '1668' did not markedly affect CD80 expression after 20hrs compared to non-CpG '100' and vehicle treated monocytes.

Expression of CD80 on monocytes from non-atopic and atopic donors is summarised in Table 3.1. Exposure to CpG '1668' for 20hr resulted in minor increases in CD80 fluorescence intensity on atopic monocytes compared to control atopic monocytes exposed to vehicle control. This increase was not observed following treatment with non-CpG '100'. However, using Wilcoxon's test, this CpG-induced increase in CD80 MFI was not found to be significant. Changes in CD80 expression on monocytes from non-atopic subjects were not observed following ODN treatment. Non-CpG '1720' or '1745' similarly resulted in no or insignificant change in CD80 expression in atopic monocytes compared cells treated with TE buffer alone.

Time course experiments were performed to examine the kinetics of CD80 expression on monocytes following treatment with ODN. FACs profiles of CD80 surface expression following treatment of adherent monocytes from non-atopic subjects with CpG '1668', CpG '2006' or non-CpG '100' are shown in Figure 3.6. After 40hr of exposure, a small increase in fluorescence intensity of CD80 mAb staining was observed on monocytes treated with CpG '1668' and '2006' compared to vehicle and non-CpG '100' treated monocytes (data not shown). However, following 60hr treatment with CpG '1668' or '2006' a more visible increase in CD80 was observed (Fig. 3.6). Non-CpG ODN '100' did not affect CD80 expression at any time points analysed compared to control monocytes. *E. coli* LPS was employed in these experiments as a positive stimulus for the induction of CD80 up-regulation. *E. coli LPS*–mediated increase in CD80 expression on monocytes was observed after 20, 40 and 60hr stimulation compared to control monocytes. Due to low sample numbers (n=3) statistical significance of changes in CD80 expression over varying lengths of treatment was not achieved.

A) Non-atopic B) Atopic

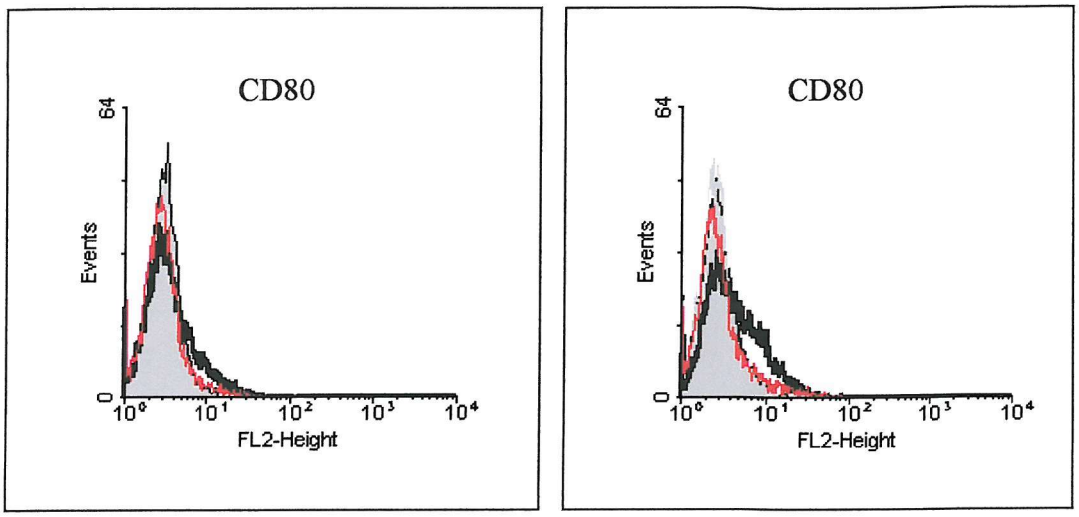

Figure 3.5: Flow cytometric analysis of CD80 on monocytes following ODN treatment.

Monocytes from non-atopic (A, n=6) and atopic donors (B, n=10) donors were treated for 20hr with CpG '1668' 1μM (**thick black line**) and non-CpG '100' 1μM (red line). Solid grey histograms are control monocytes treated with TE-buffer only. Clear grey histograms represents cells stained with appropriate isotype control mAb. FACs profiles are shown from one representative donor.

Table 3.1	Non-Atopic	Atopic
Control	0.4 ± 0.2, n=6	1.9 ± 0.7, n=10
CpG '1668'	0.4 ± 0.4, n=6	3.1 ± 1.1, n=10
Non-CpG '1745'	0.3 ± 0.3, n=4	2.8 ± 1.3, n=7
Non-CpG '1720'	0.2 ± 0.1, n=6	1.8 ± 0.8, n=6
Non-CpG '100'	0.5 ± 0.1, n=6	1.8 ± 0.8, n=6

Table 3.1: Summary of CD80 expression on monocytes from non-atopic and atopic donors following ODN treatment.

Monocytes were treated with 1μM ODN (CpG '1668', non-CpG '1720', non-CpG '1745' or non-CpG '100') for 20hrs. Control monocytes were treated with TE-buffer only. Cells were harvested and analysed for CD80 surface expression on HLA-DR^+-gated monocytes. Data presented are the average of absolute individual mean fluorescence intensities (MFI) following treatment of monocytes with ODN ± standard errors (n= sample numbers for each treatment).

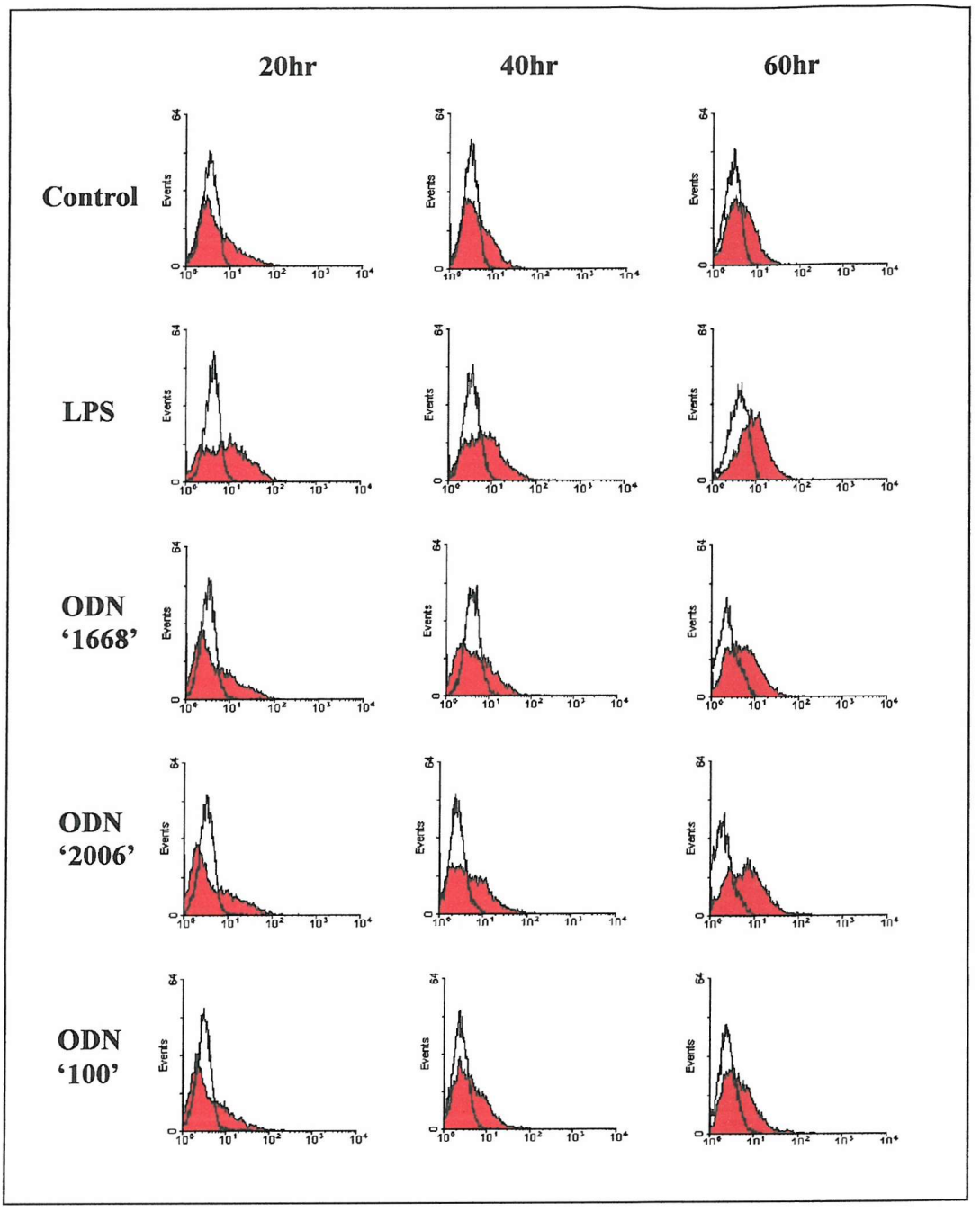

Figure 3.6: Analysis of CD80 expression on monocytes following treatment with ODN or LPS over different time points.

Monocytes from non-atopic donors were treated with *E. coli* LPS 1μg/ml and 1μM CpG '1668', CpG '2006' or non-CpG '100' for 20hr, 40hr and 60hr. Control monocytes were treated with TE-buffer alone. Subsequently, cells were harvested and analysed for CD80 expression on HLA-DR^+-gated monocytes by flow cytometry. Cells stained with relevant isotype controls are shown as clear histograms. Data is representative of three experiments.

3.3.1.3 CD86 expression on monocytes from non-atopic and atopic donors

Changes in CD86 expression on monocytes treated with ODN were examined by flow cytometry. Exposure of adherent monocytes to 1μM CpG '1668' for 20hrs resulted in a marked increase in CD86 surface expression compared to control monocytes treated with TE-buffer only. In contrast, monocytes stimulated with 1μM non-CpG '100' showed no change of CD86 expression. Figures 3.7A and 3.7B illustrate representative FACs profiles from experiments performed on monocytes from non-atopic and atopic donors, respectively. The effect of CpG '1668' was similar on monocytes from non-atopic and atopic donors (Figure 3.7-3.10).

Individual absolute median fluorescence intensities of CD86 mAb staining from monocytes obtained from non-atopic and atopic donors are shown in Figure 3.8 and 3.9, respectively. Significant increases in CD86 expression were observed after exposure of non-atopic ($p<0.05$ Wilcoxon's test) and atopic ($p<0.01$ Wilcoxon's test) monocytes to CpG '1668'. Similarly treatment with non-CpG '1745' resulted in increased CD86 expression in both non-atopic ($p<0.05$ Wilcoxon's test) and atopic monocytes ($p<0.05$ Wilcoxon's test) compared with vehicle control-treated monocytes. Non-CpG '1720' resulted in moderate increase in CD86 expression in certain donors of both subject groups compared to control, however overall, this was not significant. Non-CpG '100' did not affect CD86 expression. The effects of ODN in expression of CD86 by monocytes of both subject groups are summarised in figure 3.10.

Kinetics of CD86 expression was investigated on monocytes from non-atopic donors following treatment with ODN. CD86 surface expression following 20hr, 40hr and 60hr treatment with CpG '1668', CpG '2006' or non-CpG '100' is represented in Figure 3.11 and Table 3.2. *E. coli* LPS was used as a positive control stimulus. CpG '1668' and CpG '2006' increased CD86 expression compared to vehicle treated-monocytes at all time points analysed. Whereas, non-CpG '100' did not affect CD86 expression. LPS markedly increased CD86 expression on monocytes after 20hr treatment; at later time points this increase was less pronounced. Due to low sample numbers statistical significance of changes in CD86 expression following CpG ODN treatment at 40 and 60hrs was not achieved.

A) Non-atopic B) Atopic

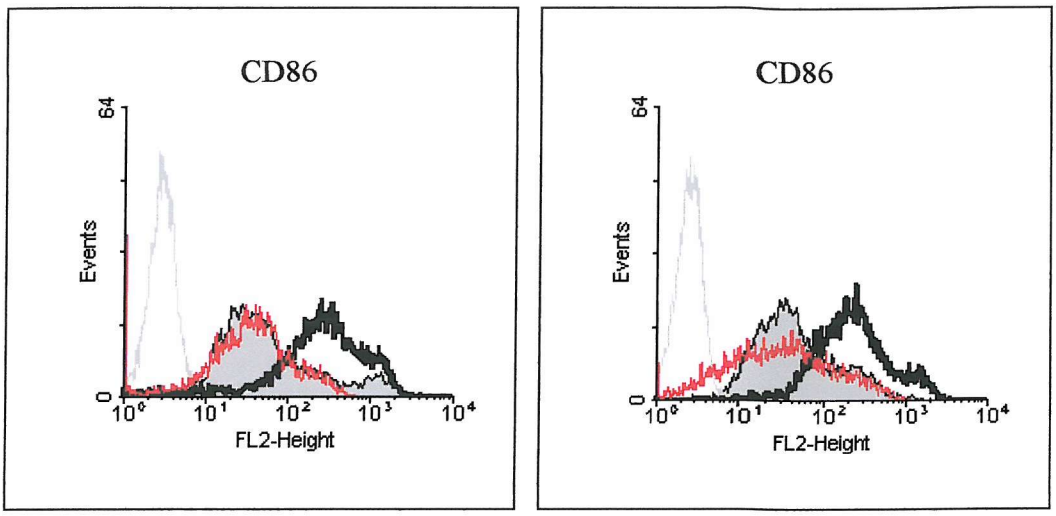

Figure 3.7: Flow cytometric analysis of CD86 on monocytes following ODN treatment.

Monocytes from non-atopic and (A, n=6) and atopic donors (B, n=10) were analysed for surface expression of CD86 after 20hr treatment with CpG '1668' 1μM (**thick black line**) and non-CpG '100' 1μM (red line). Solid grey histograms represent control monocytes treated with vehicle alone. Clear grey histograms represent cells stained with appropriate isotype control mAbs. FACs profiles shown are representative of one donor.

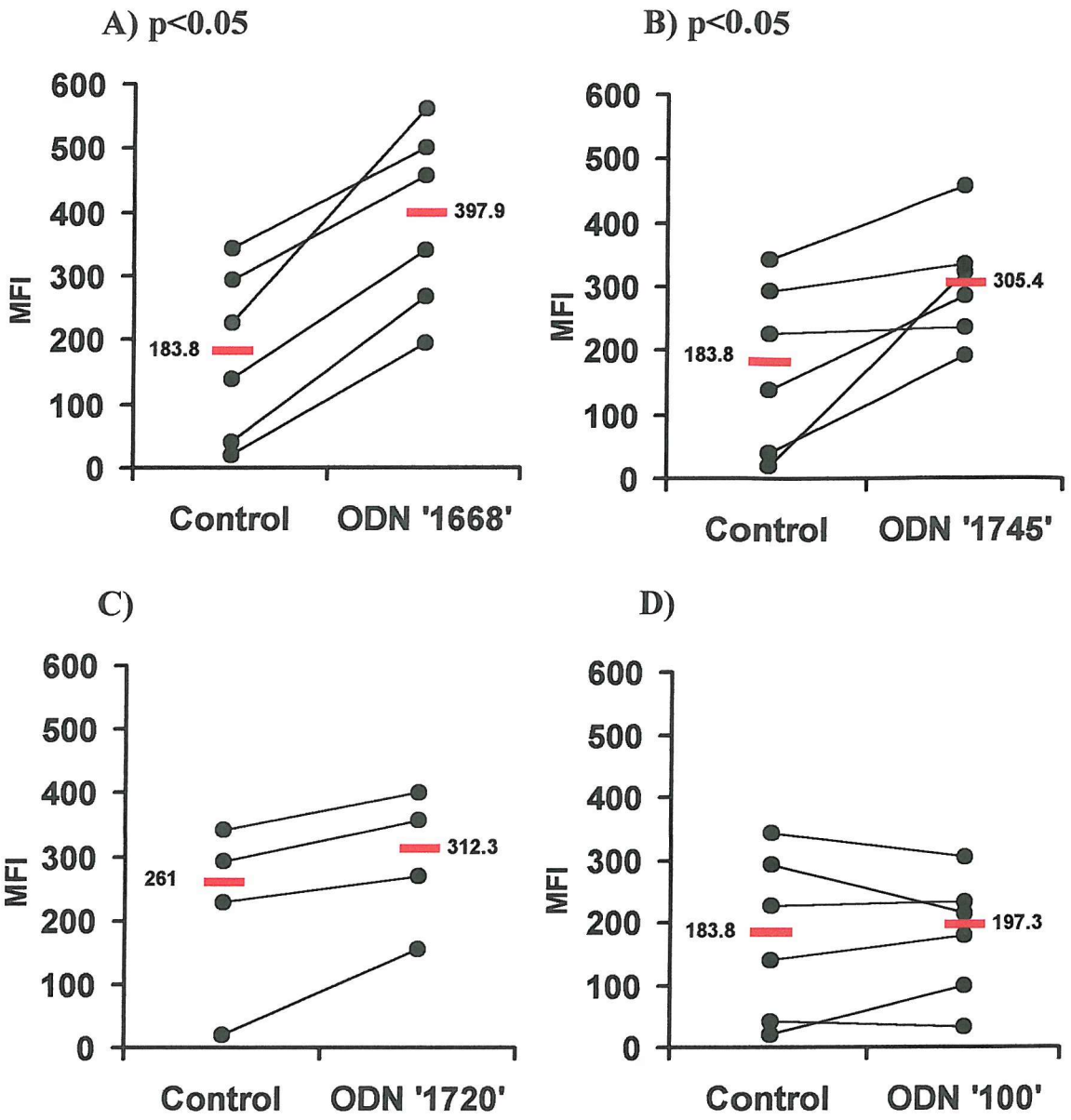

Figure 3.8: Absolute individual CD86 MFI values from monocytes of non-atopic donors

Surface CD86 expression on monocytes from non-atopic donors following 20hr stimulation with 1μM ODN; A) CpG '1668' (n=6, $p<0.05$), B) non-CpG '1745' (n=6, $p<0.05$), C) non-CpG '1720' (n=4) and D) non-CpG '100' (n=6). Control monocytes were exposed to TE-buffer alone. Expression of CD86 was determined by flow cytometry. Graphs show absolute individual CD86 median fluorescence intensity (MFI) after appropriate treatments as indicated. Red bars represent the median with values shown alongside.

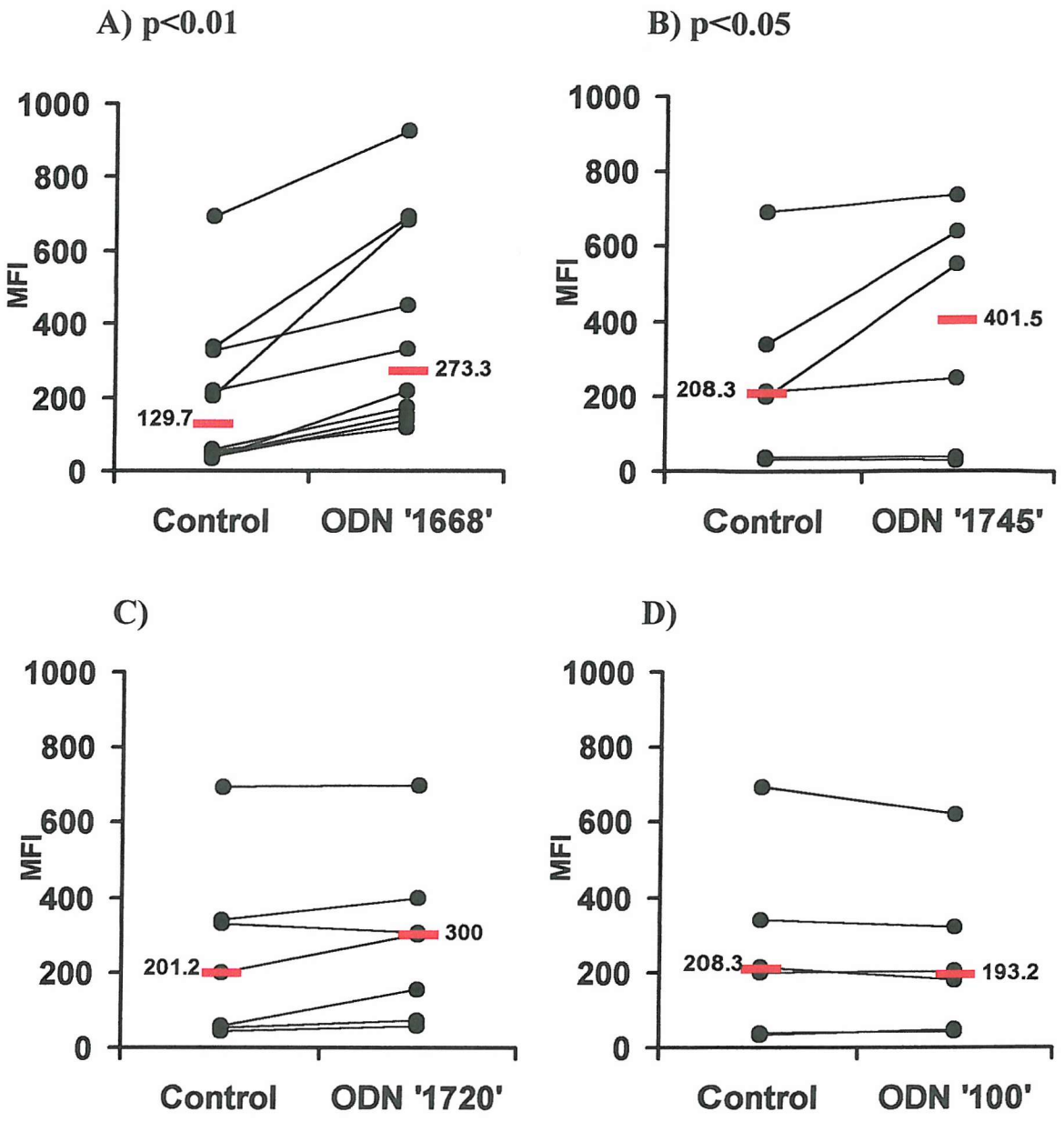

Figure 3.9: Absolute individual CD86 MFI values from monocytes of atopic donors.

Monocytes from atopic donors were treated for 20hr with 1μM ODN; A) CpG '1668' (n=10, p<0.01), B) non-CpG '1745' (n=6, p<0.05), C) non-CpG '1720' (n=7) and D) non-CpG '100' (n=6). Control monocytes were exposed to TE-buffer alone. Expression of surface CD86 was determined by flow cytometry. Graphs show individual absolute CD86 median fluorescence intensities (MFI) after appropriate treatments as indicated. Red bars represent the median with values shown alongside.

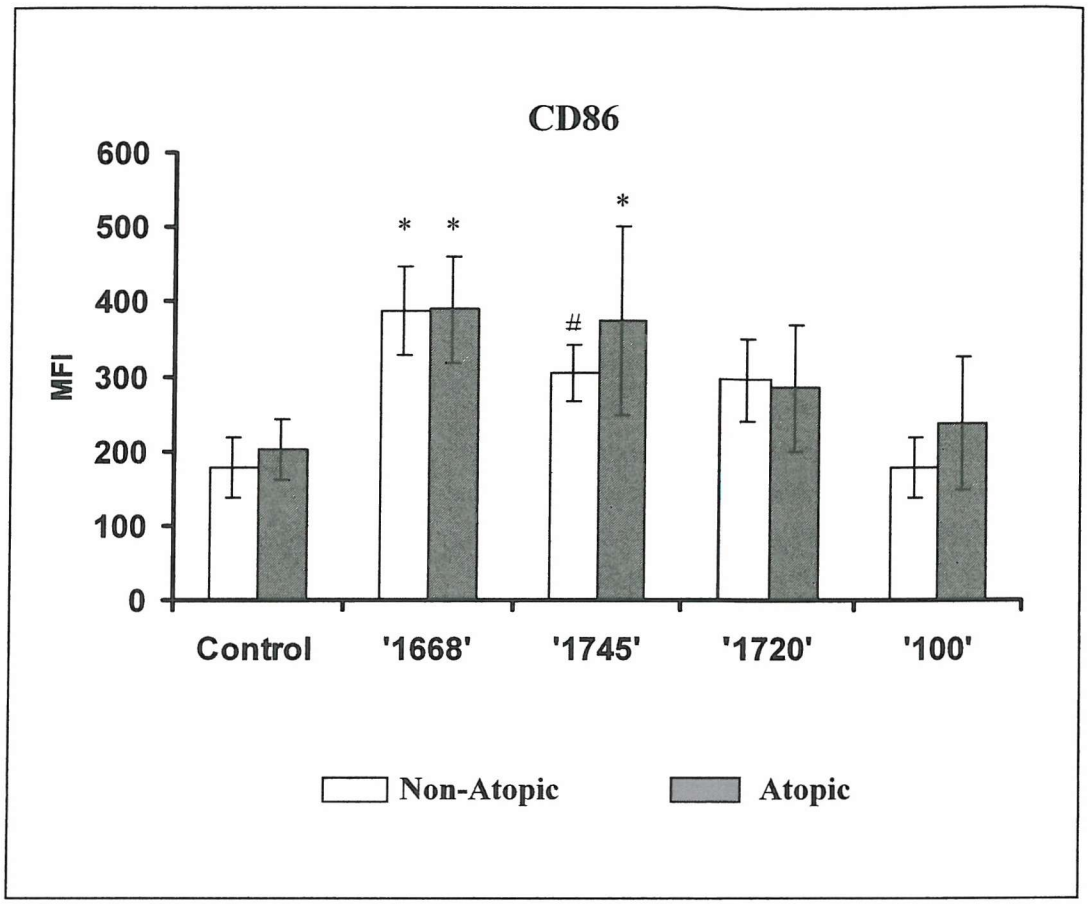

Figure 3.10: Summary of CD86 expression on monocytes obtained from non-atopic and atopic subjects following treatment with ODN.

Monocytes were treated with TE-buffer only (control) or treated with 1μM ODN (CpG '1668', non-CpG '1745', non-CpG '1720' or non-CpG '100') for 20hrs. Data in columns represents the average of all the individual absolute mean fluorescence intensities (MFI) of CD86 mAb staining on HLA-DR$^+$-gated monocytes, as determined by flow cytometry, for the respective treatments. Bars represent standard error of the mean. *= significance ($p<0.05$) compared to control, # = significance ($p<0.01$) compared to control.

Sample numbers:

Non-Atopic: Control n=6; '1668' n=6; '1745' n=6; '1720' n=4; '100' n=6

Atopic: Control n=10; '1668' n=10; '1745' n=6; '1720' n=7; '100' n=6

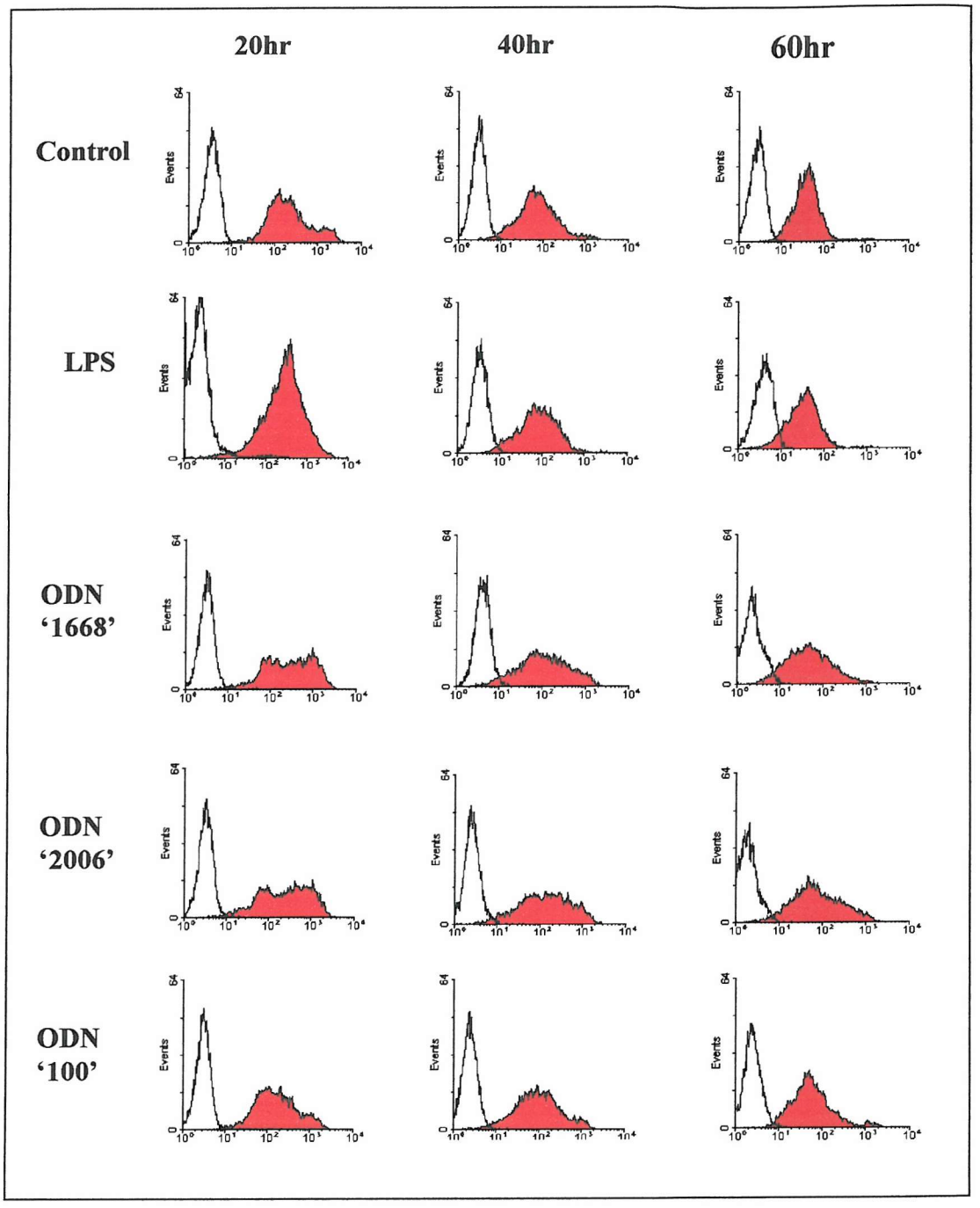

Figure 3.11: Analysis of CD86 expression on monocytes following treatment with ODN or LPS over different time points.

Monocytes from non-atopic donors were treated with *E. coli* LPS 1μg/ml and 1μM CpG '1668', CpG '2006' or non-CpG '100' for 20hr, 40hr and 60hr. Subsequently cells were harvested and analysed for CD86 expression by flow cytometry. Cells stained with relevant isotype controls are shown as clear histograms. Data is representative of three experiments.

Table 3.2	20hr	40hr	60hr
Control	131.5 ± 32.5	75.9 ± 21.2	56.7 ± 14.7
***E. coli* LPS**	359.4 ± 52.6	207.9 ± 45.2	98.6 ± 22.9
CpG '1668'	469.5 ± 145.2	359.4 ± 34.1	196.3 ± 12.2
CpG '2006	503.7 ± 75.9	399.9 ± 44.1	208.9 ± 23.3
Non-CpG '100	154.3 ± 38.6	89.1 ± 5.6	75.4 ± 12.6

Table 3.2: Summary of CD86 expression on monocytes following prolonged treatment with ODN or *E. coli* LPS.

Monocytes from non-atopic donors were treated with 1μg/ml *E. coli* LPS or 1μM ODN (CpG '1668', CpG '2006' or non-CpG '100') for 20hr, 40hr and 60hr. Cells were harvested and analysed for CD86 expression on HLA-DR^+-gated monocytes by flow cytometry. Data represents the average of absolute individual mean fluorescence intensity of CD86 mAb staining following treatment ± standard error (n=3).

3.3.1.4 CD40 expression on monocytes from non-atopic and atopic donors

The ability of ODN to affect CD40 expression on monocytes was assessed by flow cytometry. CD40 expression on monocytes from non-atopic or atopic donors was largely unaffected after 20hr exposure with either CpG '1668' or non-CpG '100' (Figures 3.12A/B). Minor increases in the intensity of CD40 mAb staining were observed following treatment of monocytes from certain non-atopic donors with CpG '1668' compared to control monocytes exposed to vehicle alone (Figures 3.13). However, these changes are comparable to the changes in CD40 expression observed following treatment with non-CpG '1745', '1720' and '100' after 20hrs which failed to reach statistical significance (Wilcoxon's test). For monocytes derived from atopic donors, no conclusive pattern of CD40 expression was observed following treatment with CpG '1668', non-CpG '1745' and non-CpG '1720' (Figure 3.14).

The effect of ODNs on CD40 expression by monocytes are summarised in Figure 3.15. Preliminary experiments (n=2) examining the effects of longer exposure to CpG ODN '1668' and '2006' revealed no major differences in CD40 expression on monocytes at 40hr and 60hr compared to non-CpG ODN '100' and vehicle treated-monocytes (data not shown). This time-course experiment was only done with cells from non-atopic donors.

A) Non-Atopic B) Atopic

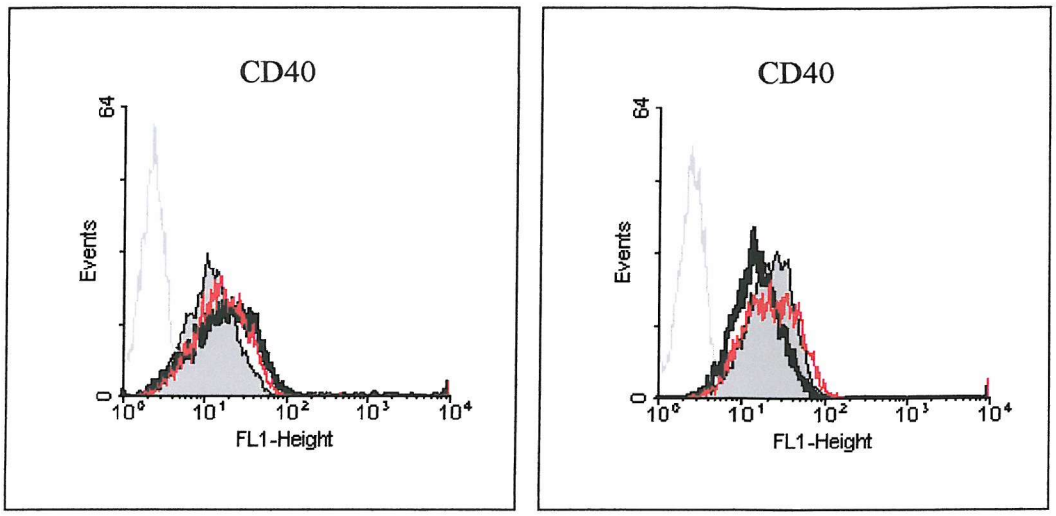

Figure 3.12: Flow cytometric analysis of CD40 expression on monocytes following ODN treatment.

Monocytes from non-atopic (A) and atopic donors (B) were analysed for surface CD40 expression after 20hr treatment with CpG '1668' 1μM (**thick black line**) and non-CpG '100' 1μM (red line). Solid grey histograms are control monocytes treated with TE-buffer alone. Clear grey histograms represents cells stained with appropriate isotype control mAb. FACs profiles shown are representative of one donor.

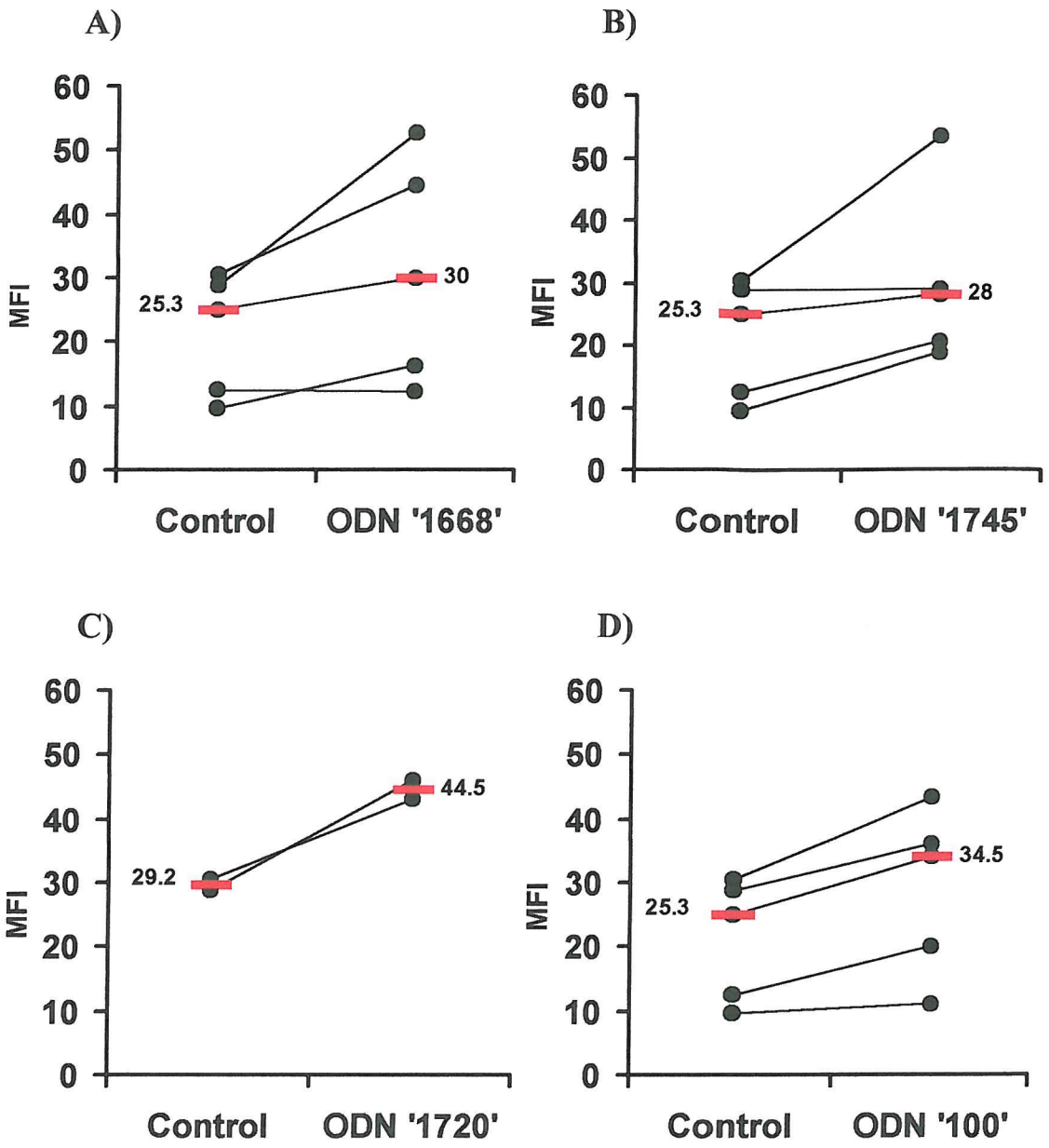

Figure 3.13: Absolute individual CD40 MFI values of monocytes from non-atopic donors.

CD40 surface expression on monocytes was determined by flow cytometry following 20hr stimulation with 1μM ODN; A) CpG '1668' (n=5), B) non-CpG '1745' (n=5), C) non-CpG '1720' (n=2) and D) non-CpG '100' (n=5). Control monocytes were treated with endotoxin free TE-buffer. Graphs show absolute individual CD40 median fluorescence intensities (MFI) for each donor after respective treatments. Red bars represent the median with value shown alongside.

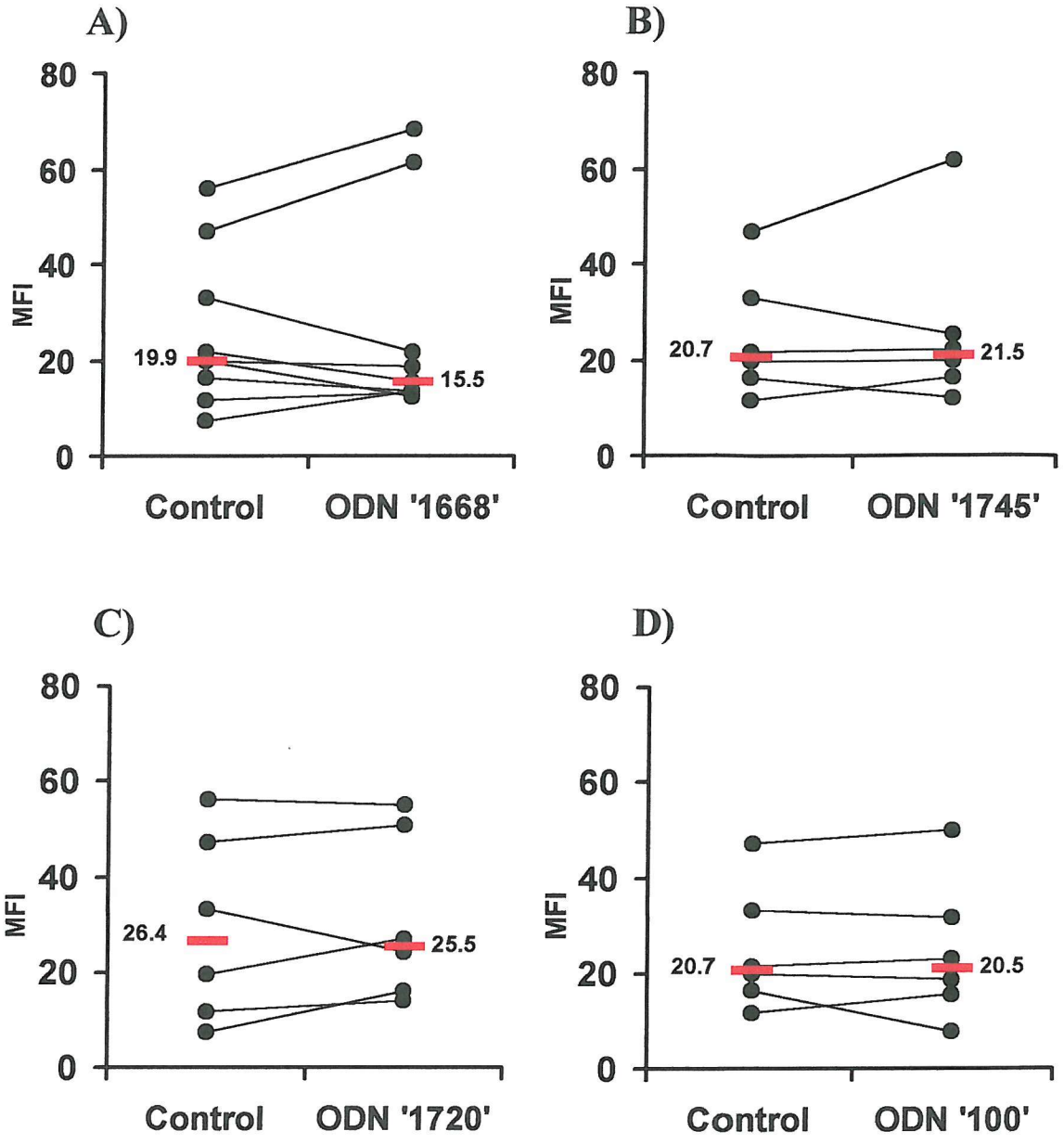

Figure 3.14: Absolute individual CD40 MFI values of monocytes from atopic donors.

CD40 surface expression on monocytes was determined by flow cytometry following 20hr stimulation with 1μM ODN; A) CpG '1668' (n=9), B) non-CpG '1745' (n=6), C) non-CpG '1720' (n=6) and D) non-CpG '100' (n=6). Control monocytes were treated with vehicle control alone. Graphs show absolute individual CD40 median fluorescence intensities (MFI) after appropriate treatments as indicated. Red bar represents the median with values shown alongside.

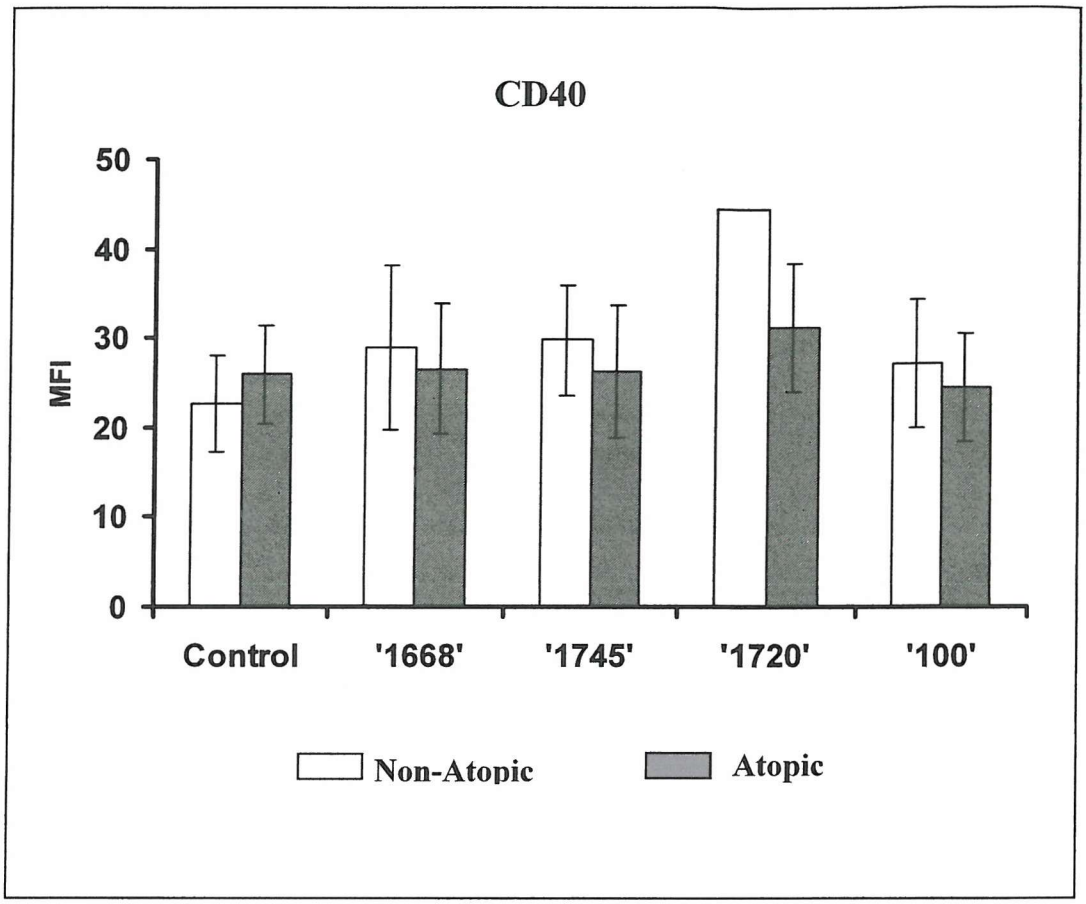

Figure 3.15: Summary of CD40 expression on monocytes obtained from non-atopic and atopic subjects following treatment with ODN.

Monocytes were treated with TE-buffer alone (control) or treated with 1μM ODN (CpG '1668', non-CpG '1745', non-CpG '1720' or non-CpG '100') for 20hrs. Data in columns represents the average of all the individual absolute mean fluorescence intensities (MFI) of CD40 mAb staining on HLA-DR$^+$-gated monocytes, as determined by flow cytometry, for the respective treatments with error bars.

Sample numbers:

Non-Atopic: Control n=5; '1668' n=5; '1745' n=5; '1720' n=2; '100' n=5

Atopic: Control n=9; '1668' n=9; '1745' n=6; '1720' n=6; '100' n=6

3.3.1.5 Expression of CD14 on monocytes following treatment with ODN

CD14 expression was unaffected by treatment of monocytes with *E. coli* LPS. By contrast, all ODNs tested induced a marked decrease in CD14 expression compared to vehicle control (for *p* values see legend for Table 3.3). There were no differences between the effects on cells from non-atopic and atopic donors. The data in Table 3.3 and Figure 3.16 represents percentage $CD14^+$ monocytes and the average of the individual CD14 MFI values, respectively, before and after ODN treatment. Figure 3.17 shows a representative FACs profile of CD14 expression on non-atopic monocytes treated with 1μg/ml *E. coli* LPS or 1μM ODN (CpG '1668', CpG '2006' or non-CpG '100') over different time points. Percentage of $CD14^+$ monocytes treated with vehicle control remained unaffected throughout culture. Similar FACS profiles were observed following treatment of monocytes derived from atopic subjects (data not shown).

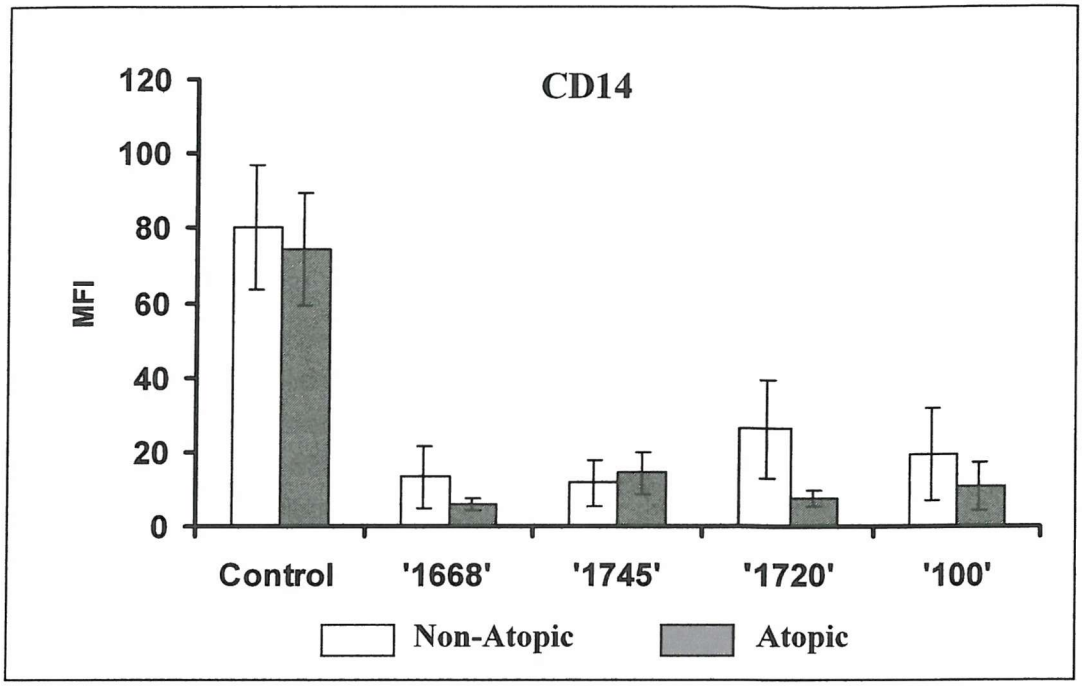

Figure 3.16: Summary of CD14 expression on monocytes obtained from non-atopic and atopic subjects following treatment with ODN.

Monocytes were treated with TE-buffer only (control) or with 1μM ODN (CpG '1668' (n=6), non-CpG '1745' (n=5), non-CpG '1720' (n=6) or non-CpG '100' (n=6)) for 20hrs. Data represents the average of the individual absolute mean fluorescence intensity (MFI) of mAb CD14 staining determined by flow cytometry for the appropriate treatments with standard error bars.

Table 3.3	Non-Atopic	Atopic
Control	87 ± 5.4, n=6	92.3 ± 5.7, n=10
CpG '1668'	38 ± 8.3, n=6 *	27.3 ± 2.3, n=10 *
Non-CpG '1745'	27.6 ± 5.3, n=5 *	27.3 ± 3.3, n=6 *
Non-CpG '1720'	32.2 ± 4.1, n=6 *	36.2 ± 3.3, n=6 *
Non-CpG '100'	41.5 ± 8.2, n=6 *	39.8 ± 2.8, n=6 *

Table 3.3: Percentage of $CD14^+$ monocytes following treatment with ODN determined by flow cytometry.

Monocytes from non-atopic or atopic donors were treated with 1μM ODN (CpG '1668', non-CpG '1720', non-CpG '1745' or non-CpG '100') for 20hrs. Control monocytes were exposed to TE-buffer alone. Data shows the average percentage of monocytes expressing surface CD14 following treatment ± standard errors. n = sample numbers for each treatment, * = significant change ($p<0.05$) compared to control.

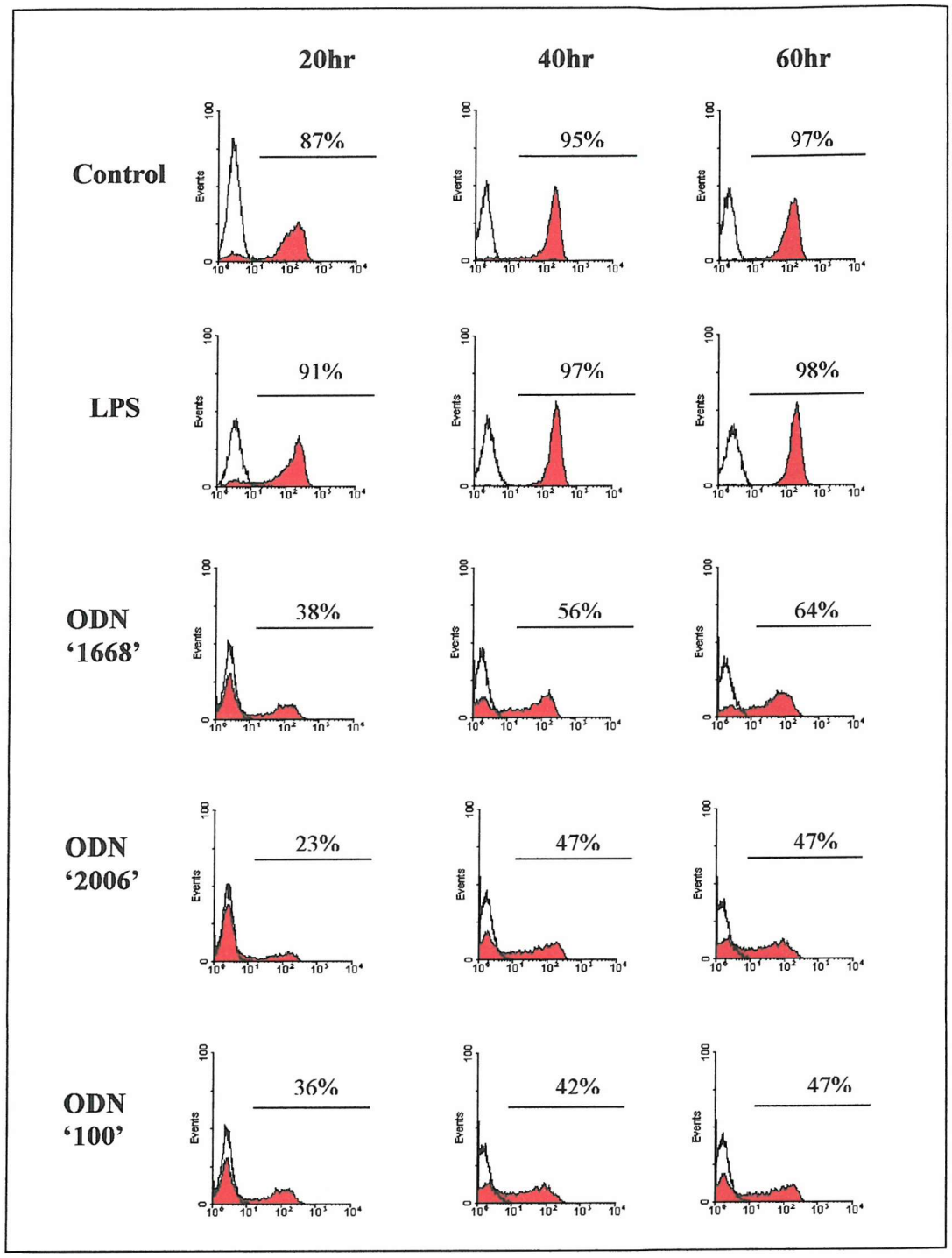

Figure 3.17: Surface CD14 expression on monocytes following treatment with LPS and ODN over increasing time points.

Monocytes from non-atopic subjects were treated with 1μg/ml *E. coli* LPS or 1μM ODN (CpG '1668', CpG '2006' or non-CpG '100') for 20hr, 40hr and 60hr. Control monocytes were exposed to TE-buffer alone. Cells were subsequently harvested and single stained using mAb CD14 for FACS analysis. Relevant isotype controls are shown as clear histograms. Percentages shown on each profile represents $CD14^+$ monocytes. Data is one representative from at least three experiments.

3.3.2 Cytokine production by monocytes treated with ODN

The ability of ODN to stimulate the production of cytokines by monocytes was examined. Cells from healthy non-atopic and atopic individuals were stimulated for 20hrs with CpG '1668' or non-CpG '100' and the production of IL-1β, IL-6, IL-10 and IL-12p40 were quantified by ELISA (Figure 3.18 and 3.19). CpG '1668' induced elevated production of all cytokines examined. The increased production of IL-1β, IL-10 and IL-12p40 was significant in monocytes from non-atopic donors ($p<0.05$, Wilcoxon's test). Monocytes from atopic donors showed a significant increase in IL-1β and IL-6 production ($p<0.05$ Wilcoxon's test). Treatment with non-CpG '100' had no significant effect on secretion of IL-1β, IL-6, IL-10 and IL-12p40 by monocytes from both subject groups. Baseline production of IL-6 and IL-12p40 by control monocytes from atopic donors was moderately higher than that observed by non-atopic donors, although this did not achieve statistical significance. Biologically active IL-12p70 was not detected in culture supernatants after any of the treatments (sensitivity of assay >15pg/ml).

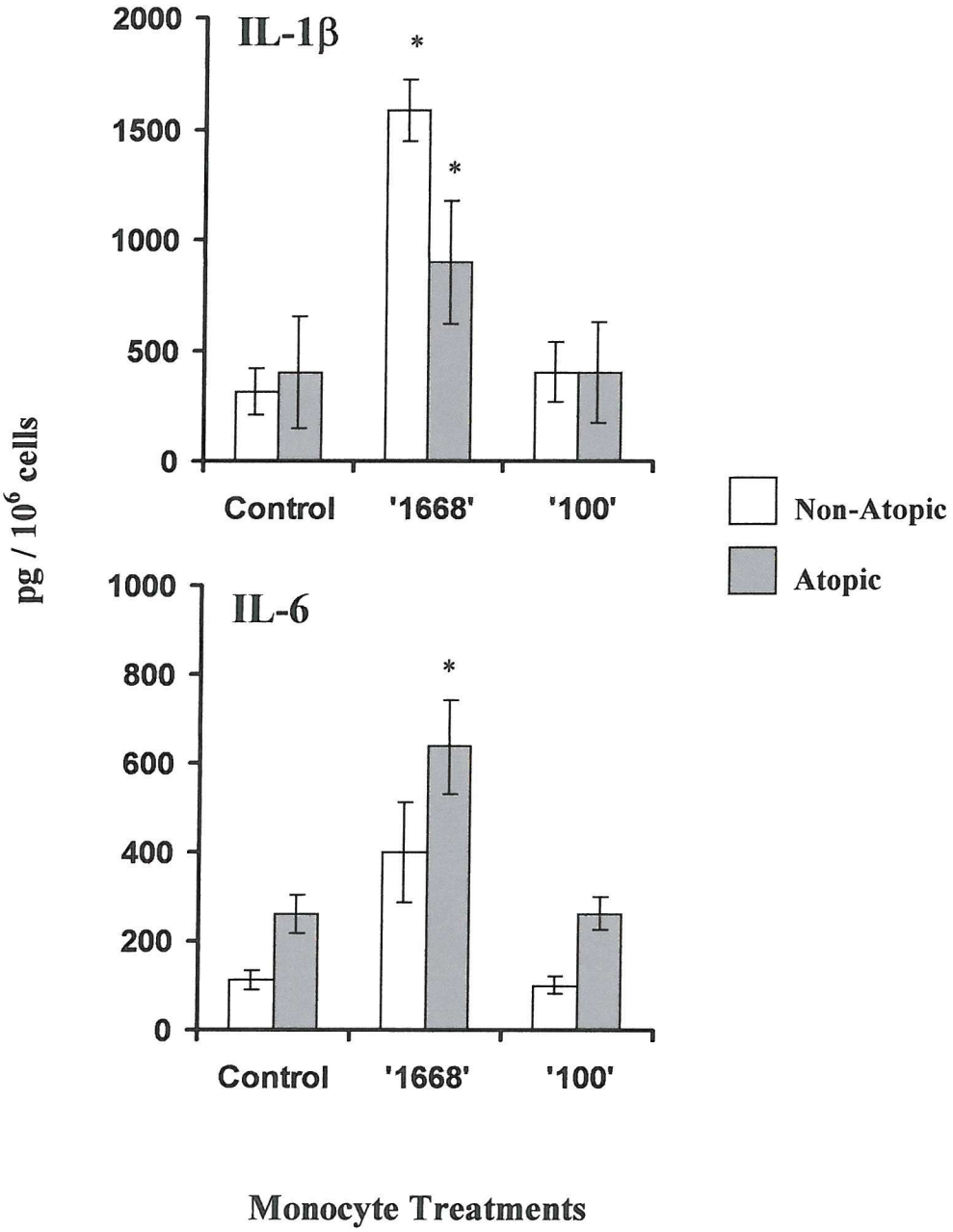

Figure 3.18: Production of IL-1β and IL-6 by monocytes treated with ODN.

Monocytes from non-atopic and atopic subjects were stimulated with CpG '1668' 1μM or non-CpG '100' 1μM for 20hr. Control monocytes were exposed to TE-buffer only. Secretion of IL-1β (non-atopic; n=5, atopic; n=5) and IL-6 (non-atopic; n=4, atopic; n=5) into supernatants was detected by ELISA. * = Significant change ($p<0.05$) compared to control.

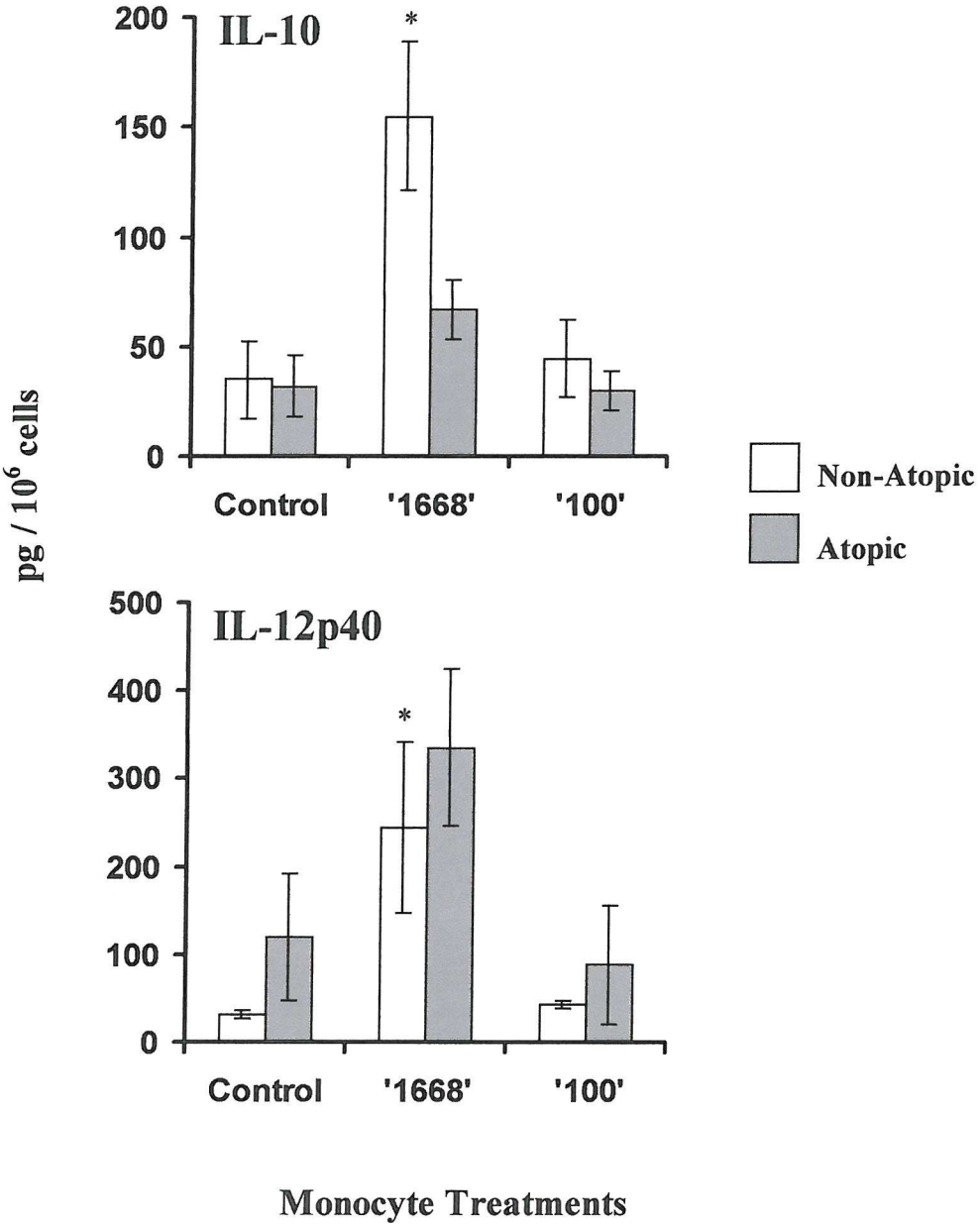

Figure 3.19: Production of IL-6 and IL-12p40 by monocytes treated with ODN.

Monocytes from non-atopic and atopic subjects were stimulated with CpG '1668' 1μM or non-CpG '100' 1μM for 20hr. Control monocytes were exposed to TE-buffer only. Secretion of IL-10 (non-atopic; n=5, atopic; n=4) and IL-12p40 (non-atopic; n=5, atopic; n=4) into supernatants was detected by ELISA. * = Significant change ($p<0.05$) compared to control.

3.3.3 Effect of Plasmid DNA on Monocyte Phenotype

DNA vaccines employ recombinant bacterial plasmids that encode the desired foreign antigen (Donnelly *et al.*, 1997). However, non-coding plasmids vectors have also been demonstrated to stimulate antigen-specific immune responses (Boccaccio *et al.*, 1999; Quintana *et al.*, 2000). Bacterial DNA but not eukaryotic DNA is widely reported to cause activation of human cells due to the presence of repeated CpG-motifs within the plasmid backbone. Therefore, the results presented Figure 3.20 and 3.21 examine the effects of bacterial plasmid pcDNA3 vector on co-stimulatory molecule and HLA-DR expression on human monocytes. In addition, the activity of genomic DNA, obtained from the human monocytic cell line THP-1 on monocyte phenotype was assessed.

Expression of CD80, CD86, CD40 and HLA-DR on monocytes from a non-atopic donor after 20hr exposure to 10μg/ml plasmid DNA (pcDNA3 vector) or 10μg/ml genomic DNA as determined by flow cytometry is illustrated in Figure 3.20. In parallel, monocytes were stimulated with 1μM (2μg/ml) ODN (CpG '1668', CpG '2006' or non-CpG '100') to compare the relative changes in surface molecule expression after treatments. Control cells were treated with TE-buffer alone. Monocytes treated with plasmid DNA up-regulated CD86 expression with MFIs comparable to those shown by monocytes treated with CpG '1668' or '2006'. Of note, pcDNA3 possess greater numbers of CpG-motifs in the plasmid backbone than those present in the '1668' or '2006' ODNs used in this work. Similarly HLA-DR expression was moderately decreased in monocytes treated with either CpG ODN ('1668' or '2006') or plasmid DNA. In contrast, only plasmid DNA-treated monocytes exhibited an up-regulation in CD40 expression. Expression of surface molecules in monocytes treated with non-CpG '100' or THP-1 genomic DNA were comparable to control monocytes treated with vehicle only. Flow cytometric profiles shown in figure 3.20 are representative of three experiments performed using monocytes from non-atopic subjects. In all three experiments similar changes in expression of surface molecules were observed. This study was not performed using monocytes from atopic individuals.

The ability of plasmid DNA to affect CD14 expression on monocytes was also investigated. In these experiments, monocytes were treated with 1μM ODN (CpG '1668', CpG '2006' or non-CpG '100') or 10μg/ml plasmid DNA for 20hrs. Monocytes were also treated with 1μg/ml *E. coli* LPS in parallel cultures to control for the actions of ODN on CD14 expression. Following treatment, cells were harvested and doubly labelled with mAbs directed against HLA-DR and CD14 to demonstrate that the stimuli were solely affecting CD14 expression on the surface of cells. Figure 3.21A shows histograms of HLA-DR and CD14 mAb staining on control monocytes. In figure 3.21B, dot plots illustrate the percentage of HLA-DR^+CD14^+ cells following treatment. Manual scraping of adherent monocytes from culture wells results in damage to a proportion of cells. However, during flow cytometric analysis dead cells were excluded using 7-AAD. Control monocytes stimulated with vehicle alone were 94% HLA-DR^+CD14^+ (92% ± 7.2, n=4). A decrease in percentage of double positive cells was observed following treatment of monocytes with CpG '2006' (32% ± 10.2, n=4), CpG '1668' (28% ± 5.5, n=4) or non-CpG '100' (52% ± 18.9, n=4). In contrast, percentage of HLA-DR^+CD14^+ cells following treatment with plasmid DNA (94% ± 7.5, n=4) or LPS (91% ± 3.3, n=4) was largely unaffected compared to control monocytes. THP-1 vertebrate DNA did not affect CD14 expression on monocytes (HLA-DR^+CD14^+ 98% ± 1.5, n=4; dot plot not shown in Fig 3.21B).

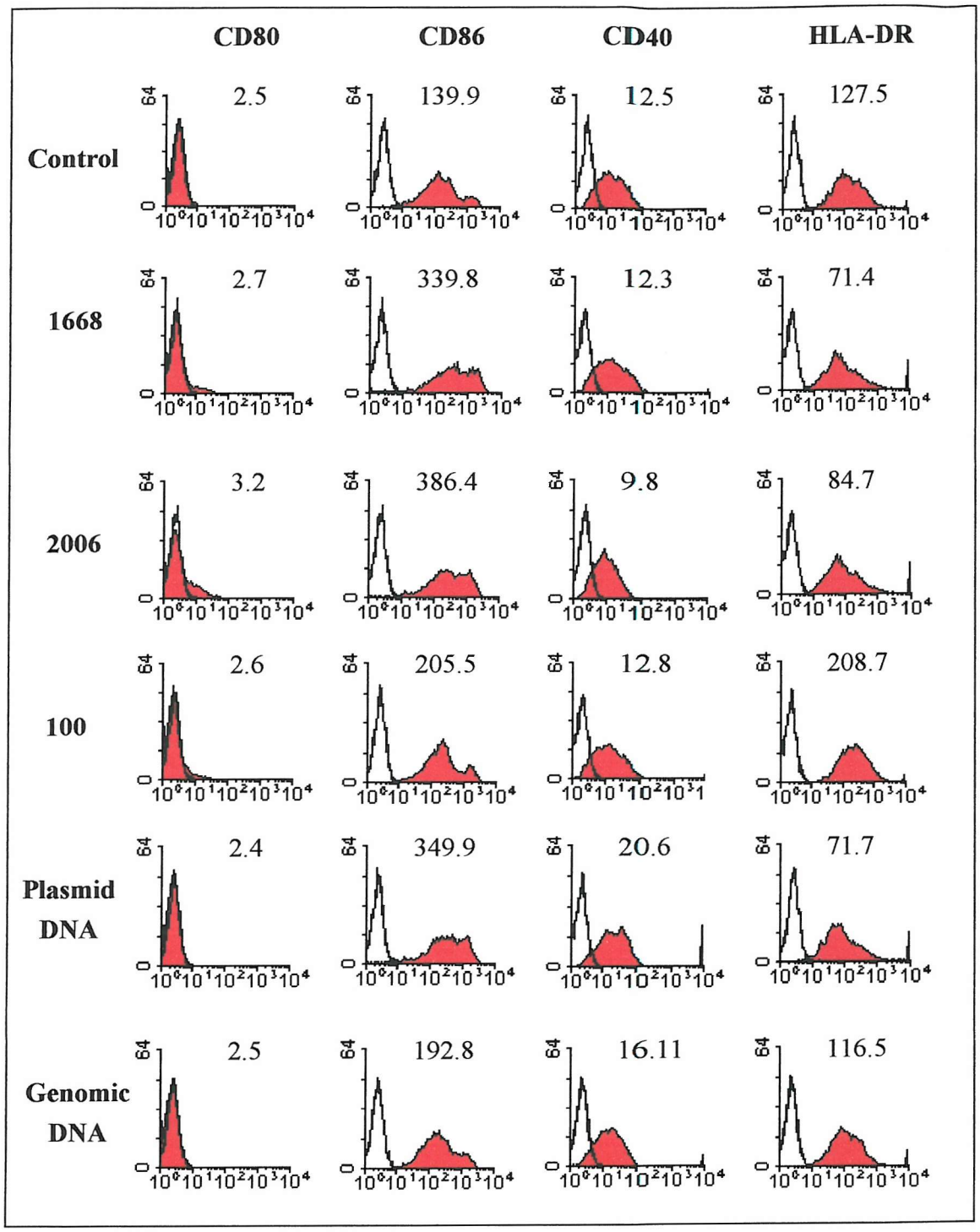

Figure 3.20: Phenotypic expression of monocytes following treatment with ODN, plasmid DNA or genomic DNA.

Monocytes were stimulated with 1μM ODN ('1668', '2006', '100'), 10μg/ml pcDNA3 plasmid DNA, 10μg/ml genomic DNA (monocytic THP-1 cell line) or vehicle control for 20hrs. Surface expression of CD80, CD86, CD40 and HLA-DR was determined by flow cytometry (red histogram). In parallel, cells were stained with relevant isotype control mAbs (clear histogram). MFI of mAb staining is shown on each histogram. Data is representative of one out of three experiments using different non-atopic donors (similar results were obtained in all three experiments).

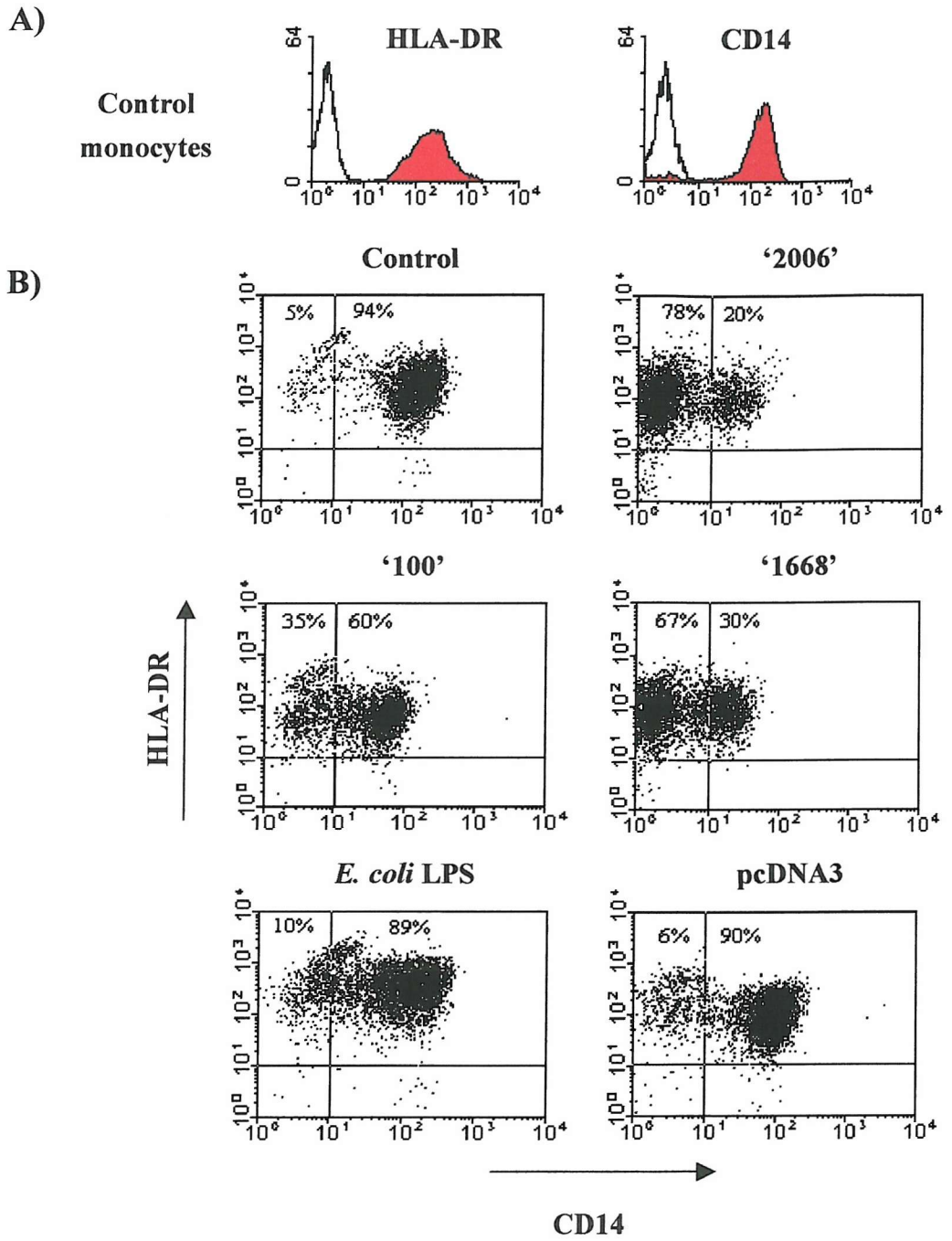

Figure 3.21: HLA-DR and CD14 double staining of monocytes treated with ODN, LPS or plasmid DNA.

Monocytes from non-atopic subjects were treated with 1μM ODN (CpG '1668', CpG '2006' or non-CpG '100'), 1μg/ml *E. coli* LPS or 1μg/ml pcDNA3 plasmid DNA vector for 20hr. Control monocytes were exposed to vehicle control only. Cells were subsequently harvested and double stained for CD14 and HLA-DR for FACS analysis. A) Expression of CD14 and HLA-DR on control monocytes is shown as red histograms. Relevant isotype controls are shown as clear histograms. B) Dot plots illustrating percentage of cells expressing $CD14^{+}HLA\text{-}DR^{+}$ cells. Data is one representative from four experiments.

CHAPTER 3

Results: Section II

Expression of Toll-like Receptors on Human Monocytes

3.4 EXPRESSION OF SURFACE TOLL-LIKE RECEPTORS ON HUMAN MONOCYTES

During study for this thesis, TLR9 was identified as the receptor responsible for mediating responses by CpG ODN (Hemmi *et al.*, 2000). Therefore, it was important to define whether the surface expression TLR9 on human monocytes correlates with their responses to CpG ODN. Until recently, commercial mAbs directed against TLR9 were not available and analysis of TLR9 mRNA expression was possible by RT-PCR. In the subsequent results presented in this chapter only monocytes from non-atopic donors were used.

Expression of TLR2, TLR4 and TLR9 on human monocytes was examined by flow cytometry (figure 3.22). Fresh uncultured monocytes are positive for surface expression of TLR2, TLR4 and TLR9. However, the intensity of mAb staining is low in comparison to the intensity of surface CD14 staining on monocytes. Data shown are representative of five separate analyses on monocytes from non-atopic individuals.

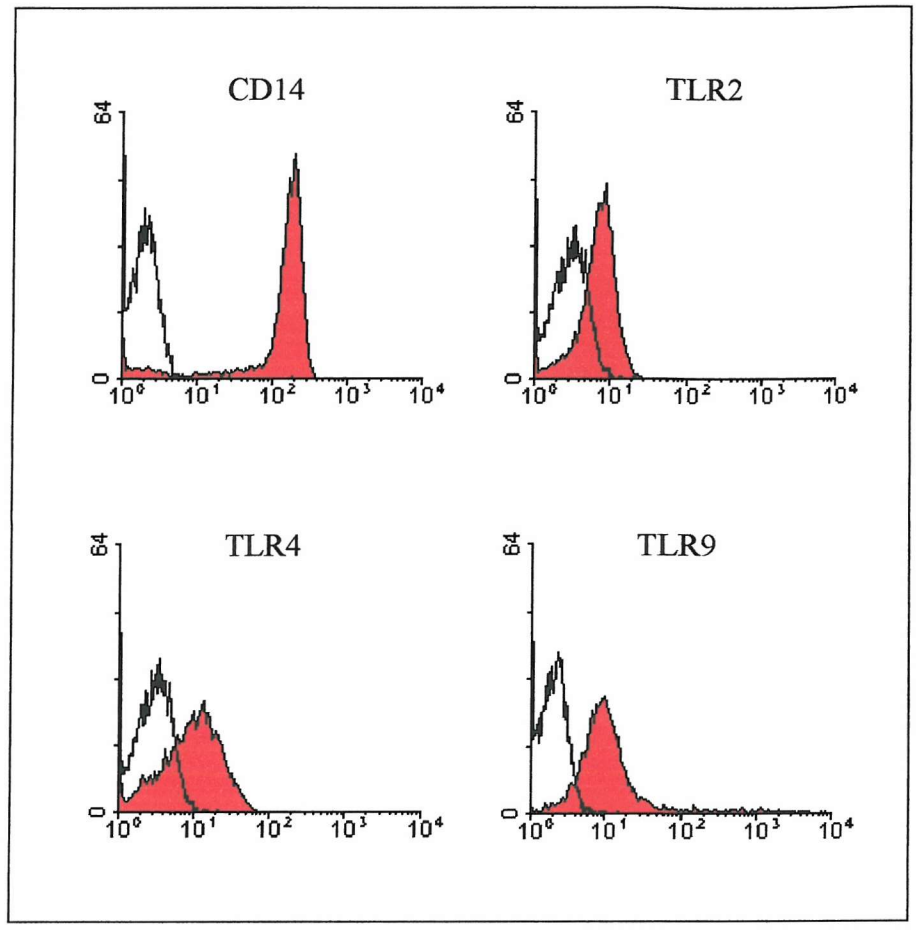

Figure 3.22: Expression of TLR2, TLR4 and TLR9 on monocytes.

$CD14^+$-gated monocytes were analysed for surface expression of TLRs by flow cytometry. Uncultured monocytes were obtained from non-atopic subjects and immunostained for surface expression of TLR2, TLR4 and TLR9. FACs profiles shown are representative of five separate experiments.

3.5 CHANGES IN TOLL-LIKE RECEPTOR EXPRESSION ON MONOCYTES

Different microbial products are reported to activate different TLRs (Re and Strominger, 2001). The studies described here aimed to determine whether the expression of any or all TLRs was altered in response to particular stimuli encountered by monocytes.

Semi quantitive TaqMan RT-PCR was employed to assess the regulation of TLR2, TLR4 and TLR9 mRNA expression on monocytes following exposure to varying stimuli (Figure 3.23). Monocytes were cultured for 20hrs with either of the following treatments: 1) CpG '2006', 2) non-CpG '100', 3) CpG '2006' plus 1μM non-CpG '100', 4) pcDNA3 plasmid DNA, 5) *E. coli* LPS, 6) LPS plus '2006' or 7) LPS plus non-CpG '100'. Control monocytes received TE-buffer vehicle alone. Total RNA was extracted from treated cells and reverse transcribed into cDNA. Specific probes and primers directed against TLR2, TLR4 and TLR9 were used to assess mRNA expression following the respective treatments. The cDNA levels during the linear phase of amplification were normalized against 18s ribosomal RNA. The results presented in the Figure 3.23 shows relative fold changes in TLR mRNA in treated monocytes compared to control cells. Relative concentrations were extrapolated from human PBMC standard curve.

Treatment of monocytes with ODN (2006, 100 or 2006+100) did not significantly affect TLR2 expression compared to control monocytes. However, pcDNA3 treatment resulted in a moderate increase in TLR2 expression, although, this did not achieve statistical significance ($p>0.05$). Stimulation of monocytes with LPS and similarly with LPS plus ODN (2006 or 100) did result in enhanced TLR2 mRNA expression. Although there was variation amongst the different donors, a moderate synergistic increase in TLR2 mRNA was observed in monocytes exposed to CpG '2006' plus LPS that was greater than the sum of the respective treatments. On the other hand, an apparent additive increase in TLR2 mRNA following treatment of monocytes with LPS and non-CpG '100' was observed. Results from these experiments suggest that LPS induces TLR2 mRNA expression in monocytes. Moreover, the presence of LPS together with CpG ODN regulates the expression of TLR2 in monocytes.

Similar to TLR2 mRNA, treatment of monocytes with '2006', '100' or '2006' plus '100' did not affect expression of TLR4 mRNA (figure 3.23). However, an increase in TLR4 mRNA was observed following 20hr exposure of monocytes to pcDNA3 or LPS. Combination of '2006' and LPS resulted in moderate elevation of TLR4 mRNA compared to control or '2006'-treated monocytes, yet, this increase was less than that observed by LPS treatment alone. TLR4 mRNA levels in LPS and non-CpG '100' stimulated monocytes were comparable to levels in cells treated with LPS alone. These results indicate that TLR4 mRNA levels in monocytes is induced in the presence of LPS or pcDNA3 but is unaffected by synthetic CpG ODN.

Expression of TLR9 mRNA in monocytes increased subsequent to treatment with CpG '2006' (figure 3.23). This increase was not observed after stimulation with non-CpG '100'. On the other hand, stimulation of monocytes with both '2006' and '100' did not markedly affect TLR9 mRNA expression compared to control cells. Whilst an increase in TLR9 mRNA was noted following treatment with plasmid vector pcDNA3, no change was observed after treatment of monocytes with LPS. An additive increase in mRNA levels is shown by '2006' plus LPS treatment of monocytes. Whereas, LPS plus '100' treatment did not affect levels of TLR9 mRNA expression compared to levels following either stimulus alone or to control monocytes. These findings suggest it's the presence of 'CpG' in pcDNA3 and ODN '2006' that induces up-regulation of TLR9 mRNA in monocytes. However, the expression of TLR9 mRNA on monocytes remains unaffected in the presence of LPS.

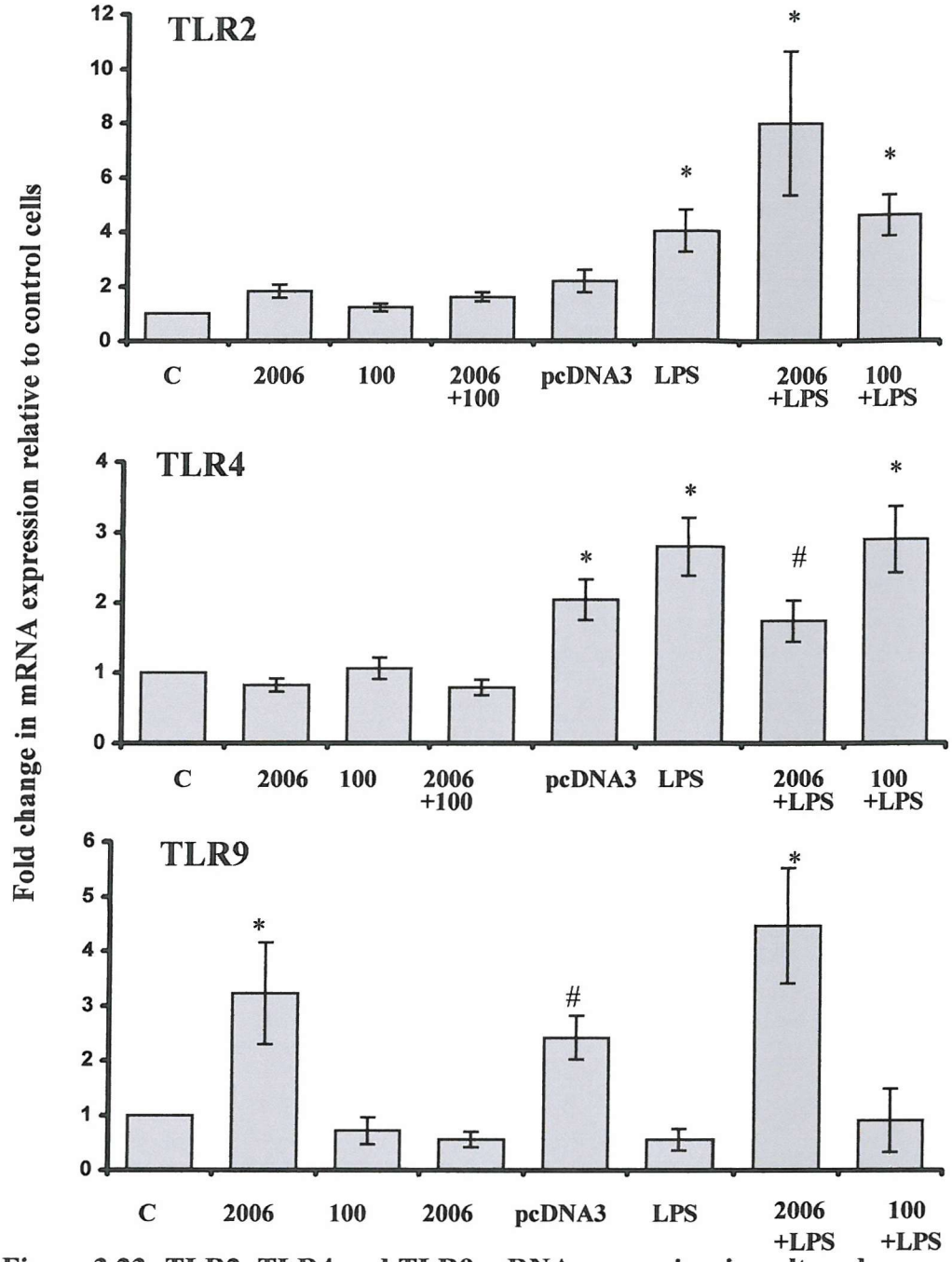

Figure 3.23: TLR2, TLR4 and TLR9 mRNA expression in cultured monocytes.

$CD14^+$ monocytes enriched by magnetic depletion of $CD2^+$ and $CD19^+$ cells were treated for 20hrs with 1μM ODN (CpG '2006 and/or non-CpG '100'), 10μg/ml plasmid vector pcDNA3, 1μg/ml *E. coli* LPS, 1μM '2006'+ 1μg/ml LPS or 1μM '100'+ 1μg/ml LPS. Monocytes were also stimulated with vehicle control (C). Total RNA was extracted and reverse transcribed into cDNA. Serial dilutions of total PBMC RNA were converted into cDNA to create a standard curve. The relative amounts of TLR2, TLR4 and TLR9 mRNA were quantitated by TaqMan RT-PCR. Concentration of TLR mRNA on test samples, extrapolated from PBMC standard curve, are normalised to endogenous ribosomal 18S RNA. Data are expressed as fold change in mRNA in test samples relative to control cells ± standard error of five experiments. * $p<0.05$ compared to control, # $p=0.05$ compared to control.

CHAPTER 3

Discussion of Results

3.6 DISCUSSION

3.6.1 Summary of results

Exposure of isolated human monocytes to CpG '1668' for 20hrs resulted in strikingly increased expression of co-stimulatory molecule CD86, a minimal increase in CD80, no change in CD40 and a moderate decreased expression of HLA-DR. CpG '2006' exhibited similar actions on monocytes. Up-regulation of CD80 was observed following prolonged treatment of monocytes with CpG '1668' and '2006'. Furthermore, these changes were observed in the majority of donors tested from both non-atopic and atopic subject groups. Non-CpG '100' did not significantly alter expression of any of the surface molecules examined. The observed actions of plasmid DNA on monocyte phenotype are comparable with that of CpG ODN. By contrast, vertebrate genomic DNA had no effect on any of the surface molecules analysed.

Findings from this study demonstrate that human $CD14^+$ monocytes express surface TLR2, TLR4 and TLR9. Moreover, these results show that expression of TLR2, TLR4 and TLR9 on monocytes is modulated by different bacterial components. CpG ODN stimulation solely enhanced the expression of TLR9 mRNA; in contrast, expression of TLR2 and TLR4 was unaffected. pcDNA3 plasmid vector enhanced TLR4 and TLR9 mRNA expression. LPS stimulation of monocytes affected TLR2 and TLR4 mRNA expression but had no effect on TLR9 mRNA.

3.6.2 Effects of ODN on Phenotype of Monocytes

3.6.2.1 Aim of screening immunostimulatory ODN

In order to identify ODN sequences that possessed strong immunomodulating properties a primary screen of ODN containing 'CpG' or 'GpC' motifs on surface antigen expression on monocytes was performed. To determine whether the observed increases in CD86 expression were attributed to 'CpG' motifs present in the '1668' sequence, the effects of non-CpG ODN '1720' were initially investigated. The '1720' sequence, identical to '1668' except for the inversion of the 'CpG' motif, was chosen for use in these studies as a control non-CpG ODN due to its published lack of stimulatory activity on murine bone marrow-derived dendritic cells (Sparwasser *et al.*,

1998). In certain donors of both non-atopic and atopic subject groups, treatment with non-CpG ODN '1720' did in fact result in increased staining of CD86 but overall this failed to achieve statistical significance. However, it suggested that inversion of 'CpG' motifs was not sufficient to eliminate the stimulatory properties of the ODN '1720'. Moreover, this finding prompted the suggestion that additional motifs other than 'CpG' present in ODN '1668' and '1720' may be responsible for up-regulating CD86 expression. 'TpG'-motifs are reported to contribute to the immunostimulatory properties of ODN in murine B-cells (Yi *et al.*, 1996). Therefore in light of this, non-CpG ODN '1745' and non-CpG ODN '100' were designed based on modifications of ODN '1720' and '1668', respectively. ODN '1745' is similar in structure to ODN '1720' with the exception of inverting two out of three 'TpG' motifs. The ODN '100' sequence is similar in structure to ODN '1668' but contains no 'CpG' or 'TpG' motifs. Comparable to '1720', ODN '1745' did not completely prevent CD86 up-regulation in monocytes obtained from some atopic and non-atopic donors. However, the effect of non-CpG ODN '100' on CD86 expression was comparable to vehicle-treatment and it was therefore chosen as an appropriate control ODN for subsequent studies. These results suggest that the activating properties of ODN on human monocytes may not depend solely on the presence of 'CpG' motifs and agrees with the report suggesting that 'TpG' motifs may also contribute to the immunostimulatory properties of ODN.

3.6.2.2 Kinetics of CD80 and CD86 expression on ODN-treated monocytes

Examining the kinetics of surface molecule expression revealed that CpG '1668' induced early up-regulation of CD86 although lesser increases were seen after 40hr and 60hr stimulation. Treatment with non-CpG ODN '100' had no effect on CD86 expression at all time points tested. CpG '2006' is reported to potently induce activation of human B-cells and peripheral DCs (Hartmann *et al.*, 1999; Krieg *et al.*, 1995), and in this study its effects were examined on expression of CD86 on monocytes. Similar trends in CD86 up-regulation were observed on monocytes treated with CpG '2006' or CpG '1668'. Potent CpG ODN usually have two or three CpG motifs. However, the addition of more than three motifs does not necessarily increase the activity of the ODN further (Yamamoto *et al.*, 1994). Moreover, it has

been suggested that for optimal stimulatory capacity, CpG motifs within the ODN should be spaced with at least two bases, preferably T's (Hartmann *et al.*, 2000; Hartmann and Krieg, 2000). Although the sample sizes for these time course experiments are low (n=3) the degree of CD86 increase in '2006'-treated monocytes was not greater than in cells stimulated with ODN '1668', considering that ODN '2006' contains three relatively equally spaced 'CpG' motifs compared with the one 'CpG' motif in '1668'.

Exposure of monocytes from either non-atopic or atopic donors to CpG ODN '1668' for 20hrs produced no significant differences in CD80 compared to non-CpG ODN '100' treated monocytes. However, 60hr following initial exposure, CpG '1668' induced CD80 up-regulation on monocytes from non-atopic donors. Interestingly, a slight but more visually apparent increase in CD80 was observed in response to CpG ODN '2006' at 40hr and 60hr after initial exposure (n=3). Whether this earlier induction of CD80 is the result of stronger activation by ODN '2006' due to the presence of more than one 'CpG' motifs in the ODN remains to be established. However, following CpG ODN treatment the delayed increases in CD80 expression in monocytes from both subject groups may be due to the differential kinetics of CD80 regulation. It is also noteworthy that extended culture of monocytes results in their differentiation into cells with macrophage morphology (preliminary findings, data not shown). The observed changes in CD80 expression subsequent to 60hr treatment with CpG '1668' or '2006' may be the result of enhanced responsiveness of macrophages to these ODN.

3.6.2.3 Effect of ODN on HLA-DR expression

CpG ODN is reported to down-regulate MHC class II expression on murine macrophages and reduces antigen-processing function after 18hr exposure (Chu *et al.*, 1999). The results presented here show that monocytes from some non-atopic and atopic donors, treated with ODN, particularly CpG-'1668', showed decreased HLA-DR expression compared to vehicle-treated monocytes. The non-CpG '100' had no significant effect on HLA-DR expression. Of note, the ODN sequence reported to decrease MHC class II expression on murine macrophages is different from that used in this investigation but contains two sets of 'CpG' motifs (Chu *et al.*, 1999). CpG

ODN '2006' –induced decreases in HLA-DR expression on human monocytes were comparable to those observed after treatment with CpG '1668'. It would appear that the presence of more that one set of CpG motifs in ODN, as in the case of ODN '2006', does not result in a more pronounced effects of the ODN on surface expression of HLA-DR in monocytes. Also, the ability of *E. coli* LPS to increase staining of HLA-DR contrasts with the effects of CpG ODN (Figure 3.21).

3.6.2.4 Expression of ODN on CD14 expression

CD14 is a PRR that plays a role in innate immunity to bacteria. A putative ligand for CD14 is LPS of Gram-negative bacteria (Wright, 1995). CD14 is found as a plasma-membrane-anchored molecule (mCD14) on the surface of monocytes and macrophages as well as in soluble form (sCD14) in plasma (Wright, 1995). On unstimulated monocytes mCD14 is markedly reduced during the first 10hrs of culture (early findings in this study). This reduction is suggested to be the result of mCD14 shedding due to its susceptibility to be cleaved. Monocytes cultured *in vitro* for 20hrs without stimulation express high levels of CD14. Upon stimulation of monocytes with *E. coli* LPS for 20hrs, surface CD14 expression remained largely unaffected. However, treatment with ODN, regardless of the presence or inversion of 'CpG' and 'TpG'-motifs, caused a marked reduction in the percentage of monocytes expressing CD14. This reduction in CD14 was not the result of cells dying during culture as flow cytometric analysis was performed using a gate on live cells. Moreover, after ODN treatment, monocytes double labelled for HLA-DR and CD14 showed a greater percentage of cells expressing surface bound HLA-DR compared to CD14. It may be possible that the presence of ODN throughout the 20hr culture affects re-generation of mCD14 to the plasma membrane following its initial cleavage. Longer duration of monocyte cultures with ODN increased the percentage of $CD14^{+}$ monocytes (approximately up to 50% monocytes expressed CD14). This may be a reflection of the gradual breakdown of ODN in the cultures.

3.6.2.5 Effect of plasmid DNA on monocyte phenotype

The findings in this study demonstrated that plasmid vector pcDNA3 had rather similar effects to synthetic ODN on monocyte phenotype. These included the marked

up-regulation of CD86 and decrease in HLA-DR expression. However, differences noted following pcDNA3 treatment include moderate increases in CD40 expression as well as no observed changes in percentage of HLA-DR^+CD14^+ monocytes. The pcDNA3 vector contains multiple copies of the immunostimulatory sequences AACGTT and AGCGCT in its ampicillin resistance gene (Boccaccio *et al.*, 1999). It is feasible that the CpG-motifs within the plasmid are responsible for the observed effects on monocytes in this study. However, in addition to varying copy numbers, the presence of other stimulatory or inhibitory sequences within the plasmid backbone may account for the observed differences in monocyte phenotype compared with synthetic ODN.

In contrast to pcDNA3 or CpG ODN, it was observed in this study that treatment of monocytes with genomic DNA had no effect on their phenotype. This is likely to be the result of the relatively lower frequency of CpG motifs in mammalian DNA compared with bacterial DNA. Ishii and colleagues demonstrated the ability of murine genomic DNA to induce maturation of bone marrow-derived DCs (Ishii *et al.*, 2001). However, the authors reported that this stimulatory activity is mediated by factors other than the rare numbers of CpG sequences present in the double stranded genomic DNA (Ishii *et al.*, 2001).

3.6.3 Differences in ODN-treated monocytes from non-atopic and atopic donors

In terms of differences in constitutive surface antigen expression between non-atopic and atopic monocytes, expression of HLA-DR was 2-fold higher in non-atopics ($p<0.05$). This may suggest monocytes from atopic subjects exhibit a more immature phenotype than monocytes from non-atopic individuals. Of the other surface molecules analysed similar changes in phenotype were seen in monocytes from non-atopic and atopic subjects in response to CpG ODN, with CD86 expression showing the bigger effect.

Production of various cytokines was enhanced in both subject groups following CpG ODN treatment compared to non-CpG ODN highlighting the immunostimulatory potential of 'CpG'-containing ODN. Although not statistically significant, the increase in IL-1β and IL-10 produced following exposure to CpG '1668' was greater in monocytes from non-atopics compared to atopic subjects. The increase of IL-6 production by CpG '1668'-treated monocytes was similar in both subject groups.

However, constitutive production of IL-6 was higher in monocytes from atopic subjects. Larger sample numbers are needed to determine whether the apparent increase in IL-12p40 production by atopic monocytes is statistically significant. Production of bioactive IL-12p70 by CpG '1668'-treated monocytes was not detectable in supernatants by ELISA (production below detection limit of the assay).

3.6.4 Regulation of Toll-like receptors

TLRs are highly conserved microbial pattern recognition receptors that play important roles in alerting the immune system to invading pathogens (Anderson, 2000). In this study, flow cytometric analysis using recently available commercial mAbs revealed the surface expression of TLR2, TLR4 and TLR9 expression on human monocytes.

In addition, using TaqMan RT-PCR it was possible to examine the modulation of TLR2, TLR4 and TLR9 mRNA expression by human monocytes following stimulation with distinct microbial components. It was observed that *E. coli* LPS increased TLR2 and TLR4 mRNA expression in monocytes. TLR4 together with MyD88 adaptor protein and/or CD14 has been documented to play critical roles in LPS-mediated immune responses (Akashi *et al.*, 2000; Kawai *et al.*, 1999). TLR2 is known to principally mediate responses from components of Gram-positive bacteria (Lien *et al.*, 1999). Nonetheless, there are reports illustrating the ability LPS to transduce signals via TLR2 as a possible default pathway when TLR4 is absent (Yang *et al.*, 1998; Kirschning *et al.*, 1998). Surface expression of TLR2 and TLR4 on monocytes was observed in this study. It is possible that the observed increase in TLR2 mRNA following LPS stimulation of monocytes can be attributed in part to contaminating lipoproteins present in the commercial LPS preparation (Hirschfeld *et al.*, 2000). TLR2 mRNA expression was not significantly affected in pcDNA3-treated monocytes, whereas a marked increase in TLR4 mRNA was observed. This was not due to endotoxin contamination because purification of plasmid pcDNA3 was performed using endotoxin-removal columns and, therefore, may be the result of other factors present within the vector.

The ability of CpG '2006' ODN to increase TLR9 mRNA in human monocytes is shown in this study to be dependent on the presence of 'CpG' motifs as no change in expression was observed after treatment with non-CpG '100'. Moreover, TLR9 mRNA increases after treatment with pcDNA3 plasmid, CpG ODN but not *E. coli*

LPS suggests that this up-regulation is due to the presence of 'CpG' sequences. TLR9 has been identified as the receptor responsible for the recognition of CpG ODN in murine and increasing evidences supports this finding in human models (Hemmi *et al.*, 2000; Bauer *et al.*, 2001a; Bauer *et al.*, 2001b). To date it is not yet known whether TLR9 or DNA-PK (an intracytoplasmic protein linked to CpG-mediated immune activation) is responsible for activation by dsDNA (Chu *et al.*, 2000). Unexpectedly, findings in this report observed an inhibition in TLR9 mRNA up-regulation after combined treatment of monocytes with CpG '2006' and non-CpG '100'. TLR2 and TLR4 mRNA levels were unaffected in CpG ODN-treated monocytes suggesting that these receptors have little involvement in CpG-mediated responses after 20hrs exposure.

Future experiments will be necessary to determine whether the kinetics of TLR2 and TLR4 mRNA, and indeed TLR9 mRNA expression, is modified at earlier time points following the initial stimulation of monocytes with CpG ODN. It is feasible that early after infection, the initial activation of monocytes results in the up-regulation of a majority of TLRs. Subsequently, expression of TLRs may be regulated depending on the duration and the nature of the pathogen or stimuli encountered. Exposure of monocytes to CpG '2006' and LPS showed a moderate synergistic increase in TLR2 mRNA levels after 20hrs. Up-regulation of TLR9 mRNA was also noted after '2006' and LPS treatment. On the other hand, the presence of CpG '2006' together with LPS moderately down-regulated TLR4 mRNA expression on monocytes compared to LPS only or LPS and non-CpG '100' treatments. LPS-mediated responses are observed in monocytes and macrophages as early as three hours post-exposure and are maximal at 24hrs (Hailman *et al.*, 1996). Whilst CpG ODN activation is also reported to take place early after treatment, CpG-mediated responses may still be observed 48hrs following initial stimulation (Hacker *et al.*, 1999). Intact bacterial pathogens do not display their DNA. Therefore, detection of CpG DNA following bacterial challenge may imply that the host has successfully destroyed the invading organism.

In this study, the down-regulated expression of TLR4 mRNA following the combined treatment of monocytes with '2006' and LPS compared to LPS alone may be resultant of continued requirement for signalling through TLR9, shown by the increase in TLR9 mRNA, 20hrs after treatment. It is feasible that LPS signalling through TLR4

dominated at earlier time points. The increase in TLR2 mRNA following '2006' and LPS treatment of monocytes at present is unclear. If contaminating lipoproteins are partially responsible for the LPS-mediated increase in TLR2 mRNA, these may also be involved in the observed increases after '2006' and LPS stimulation.

To conclude, the results presented in this study concur with recent published observations that human monocytes are activated by CpG ODN and plasmid DNA (Bauer *et al.*, 2001b; Bauer *et al.*, 1999). The results showing differential changes in TLR mRNA expression suggests that these receptors may be regulated *in vivo* following microbial infection. Expression of TLRs on monocytes from atopic individuals was not assessed in this work. However, further investigation on whether atopics monocytes exhibit altered TLR expression or differences in regulation may contribute to understanding of the responses mounted by these cells during microbial infection.

CHAPTER 4

Effects of Immunostimulatory Oligodeoxynucleotides on Human Monocyte-derived Dendritic cells and Monocyte-derived Langerhans' cells

4

4.1 INTRODUCTION

The ability of human monocytes to respond to CpG ODN prompted the suggestion that, ODN are also capable of inducing activation of monocyte-derived DCs (mo-DC). Therefore, the observations in Chapter 3 were extended to determine whether CpG ODN affected surface phenotype and cytokine production by mo-DC, and also by monocyte-derived Langerhans' cells (mo-LC). In these studies, the ODN sequences CpG '2006' or '1668' and non-CpG '100' were examined for their effects on these cells. The activities of CpG '2006' as opposed to '1668' were primarily investigated in subsequent studies as a result of observations in Chapter 3 that '2006' potently modifies monocyte phenotype, in addition to reports stating that '2006' preferentially activates human cells (Krieg and Wagner, 2000; Krieg *et al.*, 1995).

Following from the observation in Chapter 3 that monocytes express TLR9, in addition to TLR2 and TLR4, surface expression of these receptors was characterized on human DCs. The different DC subsets used in this study include mo-DCs/LC, $CD34^+$-derived DCs/LCs and skin Langerhans' cells.

In murine models of allergic asthma, CpG ODN have been explored as an effective adjuvant for treatment of established Th2–diseases. Administration of CpG ODN prevents sensitization to an allergen but also reduces established Th2-mediated in the airways by promoting the induction of Th1-like cytokines in response to the allergen (Kline *et al.*, 1999; Sur *et al.*, 1999). In addition, CpG ODN are reported to prevent the development of eosinophilic airway inflammation and bronchial hyperreactivity in wild-type mice as well as IL-12 and IFN-γ knock-out mice suggesting that CpG ODN may prevent pre-existing Th2 responses by multiple mechanisms (Kline *et al.*, 1999). The present investigation aimed to determine whether activities of CpG ODN on mo-DC serves as useful model to direct the *in vitro* differentiation of allergen-specific $CD4^+$ T-cells from atopic donors from Th2 to Th1 effector cells.

4.2 MATERIALS AND METHODS

4.2.1 ODN sequences

CpG ODN '1668', CpG ODN '2006' and non-CpG '100' were used (sequences as described in Chapter 3 section 3.2.1).

4.2.2 Treatment of Mo-DCs and Mo-LCs with ODN

Mo-DCs were obtained by culturing purified monocytes (2.2.2.1) for five days in phenol red-free RPMI 1640 medium supplemented with 10% FCS and recombinant cytokines IL-4 and GM-CSF. To obtain mo-LC (2.2.2.2.), monocytes were cultured for five days in phenol red-free RPMI 1640 medium as described above but with addition of TGF-β1 (25ng/ml) on Day 4 of culture. 1μM CpG '1668', CpG '2006' or non-CpG '100' were added to cells. Vehicle control-treated cells received the same volume of endotoxin free TE-buffer. Mo-DCs and mo-LCs were also stimulated with 10μg/ml plasmid (pcDNA3) or genomic (THP-1) DNA. *E. coli* LPS (1μg/ml) or TNF-α (25ng/ml) was used as a positive stimulus activation of mo-DC and mo-LC. Following preliminary time-course experiments with TNF-α, as the positive control, the optimal duration of treatment for induction of changes in surface molecule phenotype of mo-DC was 40hrs.

4.2.3 Allogeneic T-cell proliferation

Medium used for differentiating purified monocytes into mo-DCs or mo-LCs for subsequent co-culture with purified allogeneic $CD4^+$ T-cells consists of phenol red-free RPMI 1640 medium supplemented with 2% human AB and rhIL-4 and rhGM-CSF as previously described (2.2.4). Following treatment of mo-DC or mo-LC, cells were prepared for co-culture with allogeneic purified $CD4^+$ T-cells. T-cell proliferation was assessed by ^{3}H-thymidine incorporation described in section 2.2.4.1.

4.2.4 Culturing Human $CD34^+$-derived Dendritic or Langerhans' cells

Anonymised apheresed samples from individuals whose $CD34^+$ stem cells were induced with G-CSF treatment were used in this study to obtain large numbers of $CD34^+$ cells. Purified $CD34^+$ cells were obtained from mononuclear cells using $CD34^+$ isolation MACS kit (Miltenyi Biotech). To obtain $CD34^+$-derived dendritic cells ($CD34^+$-DC), $CD34^+$ cells were cultured for 14 days in the presence of rhGM-

CSF, rhSCF and TNFα (2.2.6). Medium was replaced with fresh medium and cytokines every 4 days. $CD34^+$-derived Langerhans' cells, ($CD34^+$-LC), were obtained by maintaining cells for 14 days with the mentioned cytokines in addition to rhTGFβ-1 (2.2.6). After culture, cells were harvested and phenotypic analysis was determined by flow cytometry.

4.2.5 Epidermal $CD1a^+$ Langerhans' cells from human skin

Human epidermis was obtained using the suction blister method (Friedmann *et al*, 1987). Plastic chambers containing two holes (6mm diameter) were placed on the volar forearm of two volunteers. A vacuum of 250mm Hg was applied with and electric suction pump for approximately 90 minutes. During this period the epidermis separated from the dermis, the split occurring through the basement membrane. The blister roofs were carefully removed using sterile scissors. The epidermal blister roofs were disaggregated into a single cell suspension by incubation for 15 minutes in 1x Trypsin/EDTA (Sigma) containing 50U/mL DNaseI (Life Technologies) at 37°C (water bath). Regular gentle mixing of the tubes was performed to encourage cell dissociation. After incubation, 500μL heat inactivated FCS was added per 1mL of total solution to inactivate the Trypsin. Cells in suspension were harvested and washed twice in ice-cold PBS by centrifugation at 1200rpm for 10minutes at 4°C. The cell pellet was re-suspended in 60μL (per 10^7 cells) ice-cold MACS buffer to which 20μL (per 10^7 cell) magnetically conjugated-CD1a mAbs were added. Following 15 minute incubation at 4-6°C (in the fridge), cells were washed twice in MACS buffer by centrifugation as described above. The resuspended cell pellet was loaded onto a MACS column and CD1a cells were obtained by positive selection using CD1a mAbs (2.2.1.3.3).

4.2.6 Flow cytometric analysis of surface molecules

Non-adherent mo-DC and mo-LC were harvested from culture dishes by gentle pipetting to avoid cellular activation. Fc-receptors on cells were blocked (2.2.7.1) prior to staining for cell surface molecules. Expression of CD1a (FITC), CD14 (PE), CD83 (PE), CD80 (PE), CD86 (PE), CD40 (PE) and HLA-DR (PE) were measured. For phenotyping $CD34^+$-DC or -LC, surface expression of CD11b (PE) and CD11c (PE) and intracellular staining against Langerin (PE) were included in the mAb panel mentioned above. Surface TLR expression on mo-DCs, mo-LCs, $CD34^+$-DC or

$CD34^+$-LC was assessed using mAbs directed to TLR2 (biotin-conjugated; 10μg/mL), TLR4 (PE; 5μg/mL) and TLR9 (PE; 10μg/mL). Biotin-conjugated TLR2, 4, 9 mAbs (all at 10μg/mL) followed by streptavidin-conjugated PE (Biosource International; 1:1000) secondary antibodies were used for staining purified $CD1a^+$ Langerhans' cells. Appropriate fluorochrome conjugated isotype control antibodies were used in parallel. During flow cytometric analysis, viable cells were collected within a forward / side scatter gate together with the exclusion of dead cells using 7-AAD.

4.2.7 Measurement of cytokine production

After 40hr treatment, mo-DCs were harvested and the volume of supernatant measured. Cytokine secretion into supernatants was measured by ELISA using monoclonal matched-pair antibodies, according to manufacturers instructions (2.2.9) Where necessary, supernatants were diluted 1:10 in the appropriate assay buffer in order for cytokine concentration to fall within the range of the standard curve. Concentration of cytokines was calculated by relating the absorbance values of duplicate wells to the standard curve. Absorbance was read at 450nm on an ELISA plate reader. The concentration of cytokines was converted to pg / 10^6 cells.

4.2.8 TaqMan RT-PCR

Total RNA was extracted using the RNeasy kit method (Qiagen) and treated with DNAse I (RNAse-free DNAse, Promega) according to the manufacturers' instructions. RNA was quantified using RiboGreen® RNA kit (Molecular Probes, used as directed) and quantified with a fluorescence spectrophotometer using CytofluorII software. cDNA was prepared from 400ng of total RNA using the Omniscript reverse transcriptase preamplification system (Promega) with random hexamer primers (Promega). The cDNA levels of TLR2, TLR4 and TLR9 were quantified by TaqMan PCR using an ABI prism 7700 sequence detector according to the manufacturer's instructions (Applied Biosystems). The cDNA levels during the linear phase of amplification were normalized against ribosomal 18s (Pre-Developed Assay Reagent, PE Applied Biosystems). Relative RNA concentrations were interpreted from a PBMC standard curve.

CHAPTER 4

Results: Section I

Effect of Immunostimulatory Oligodeoxynucleotides on Phenotype and Function of Human Monocyte-derived Dendritic Cells and Monocyte-derived Langerhans' Cells

4.3 PHENOTYPE OF Mo-DC and Mo-LC

4.3.1 Expression of CD1a, CD14 and CD83 on mo-DC and mo-LC

Mo-DC and mo-LC were obtained by culturing purified monocytes in the presence of rhIL-4 and rhGM-CSF. For the differentiation of mo-LC, the 4-day cultures were supplemented with rhTGF-β1 for an additional 24hrs. At day 5, mo-DC cell populations (n=5) comprised on average, 92% $CD1a^+$ (± 3.6) and 1% $CD14^+$ (± 0.8) cells as determined by flow cytometry. Mo-LC cell populations (n=3) consisted on average 94% $CD1a^+$ (± 3.6) and 1.5% $CD14^+$ (± 3.2) cells as determined by flow cytometry. The remaining cell populations comprised $CD3^+$ T-cells. Initial observations were used using TNF-α as a positive stimulus to show cell maturation. Following 40hr exposure to TNF-α, expression of CD1a and CD14 was not altered on mo-DC and mo-LC (Figure 4.1 and 4.2, respectively). However, a significant increase in CD83, an indicator of DC maturation, was observed in TNF-α treated mo-DC (average % change relative to control mo-DC, 3013% ± 800, n=5; $p<0.05$ Wilcoxon's test) and TNF-α treated mo-LC (average % change relative to control mo-LC, 313% ± 38.5, n=3). However, changes in CD83 in TNF-α treated mo-LC were not as pronounced as those observed in TNF-α treated mo-DC.

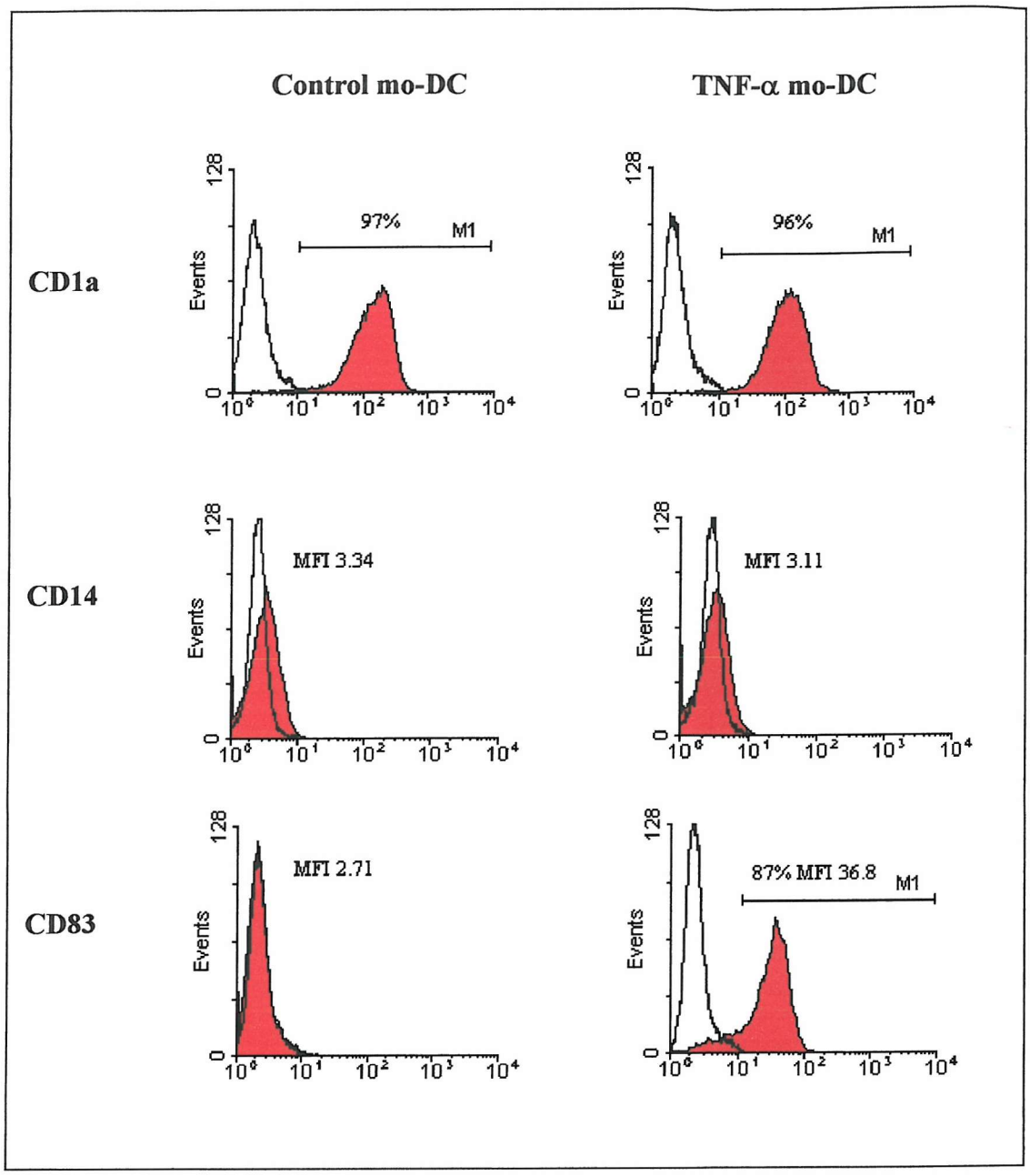

Figure 4.1: Phenotype of immature and TNF-α stimulated mo-DCs.

Surface expression of CD1a, CD14 and CD83 on control and TNF-α treated mo-DC. 5 day mo-DC cultured in RPMI medium containing 10% FCS supplemented with rhIL-4 and rhGM-CSF express high levels of CD1a and very low levels of CD14 and CD83 (red histograms). 40hr treatment with 25ng/ml TNF-α results in up-regulation of CD83. Clear histograms represent cells stained with appropriate isotype controls. % positive cells and MFI values are shown. FACS profiles are representative of one donor (n=5).

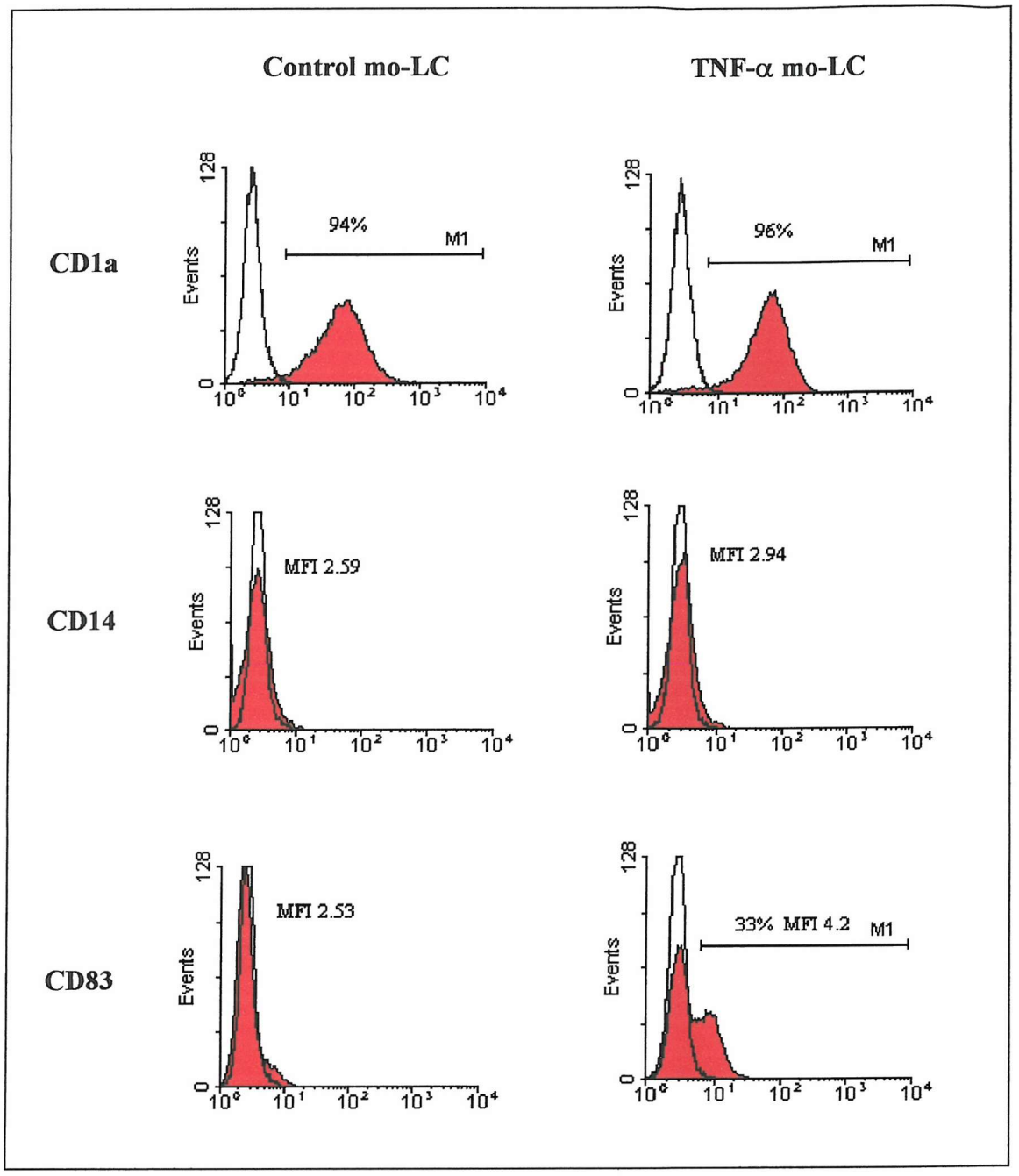

Figure 4.2: Phenotype of immature and TNF-α stimulated mo-LCs

Surface expression of CD1a, CD14 and CD83 on control and TNF-α treated mo-LC. 5 day mo-DC cultured in RPMI medium containing 10% FCS supplemented with rhIL-4 and rhGM-CSF and rhTGF-β1 express high levels of CD1a and very low levels of CD14 and CD83 (red histograms). 40hr treatment with 25ng/ml TNF-α results in up-regulation of CD83. Clear histograms represent cells stained with appropriate isotype controls. % positive cells and MFI values are shown. FACS profiles are representative of one donor (n=3).

4.3.2 Effects of LPS on mo-DC phenotype

The ability of *E. coli* LPS to induce maturation of mo-DC phenotype was also examined. Treatment of mo-DC with *E. coli* LPS 1μg/ml for 40hr did not affect expression of either CD1a or CD14 (Figure 4.3). However, LPS treatment markedly up-regulated surface expression of HLA-DR, CD83 and co-stimulatory molecules CD80, CD86 and CD40, as determine by flow cytometry. Similar patterns of surface molecule expression were also observed after shorter exposure (20hrs) of LPS to mo-DCs (data not shown). LPS induced activation of mo-LCs, but the up-regulation of surface molecules was not as prominent as that seen in LPS activated-mo-DCs (Table 4.1). Summary data in Table 4.1 illustrates the average mean fluorescence intensity of mAb staining following exposure of LPS to mo-DCs or mo-LCS.

Table 4.1	Control mo-DC	LPS mo-DC	Control mo-LC	LPS mo-LC
CD1a	95.2 ± 13.5	102.6 ± 5.5	113.7 ± 21.6	120.1 ± 32.2
CD14	1.9 ± 0.2	2.2 ± 1.1	2.1 ± 0.8	1.5 ± 0.2
CD83	1.5 ± 0.1	13.6 ± 2.3*	3.1 ± 0.7	6.3 ± 2.2
CD80	5.1 ± 1.8	25.9 ± 5.4*	3.8 ± 1.9	15.5 ± 3.1*
CD86	3.2 ± 1.7	185.9 ± 24.9*	2.7 ± 0.2	32.8 ± 4.4*
CD40	76.7 ± 11.2	255.9 ± 32.2*	38.9 ± 5.1	52.9 ± 2.6
HLA-DR	30.6 ± 5.5	199.5 ± 55.4*	7.8 ± 2.3	13.5 ± 0.3*

Table 4.1: Summary of mo-DC and mo-LC phenotypic changes following exposure to *E. coli* LPS.

Mo-DC and mo-LC were treated with 1μg/ml *E. coli* LPS for 40hrs. Control cells were left untreated. Surface marker expression was determined by flow cytometry. Data shows average of absolute individual HLA-DR mean fluorescence intensity values ± standard errors (n=5). * = Significance observed ($p<0.05$, Wilcoxon's test) compared to control.

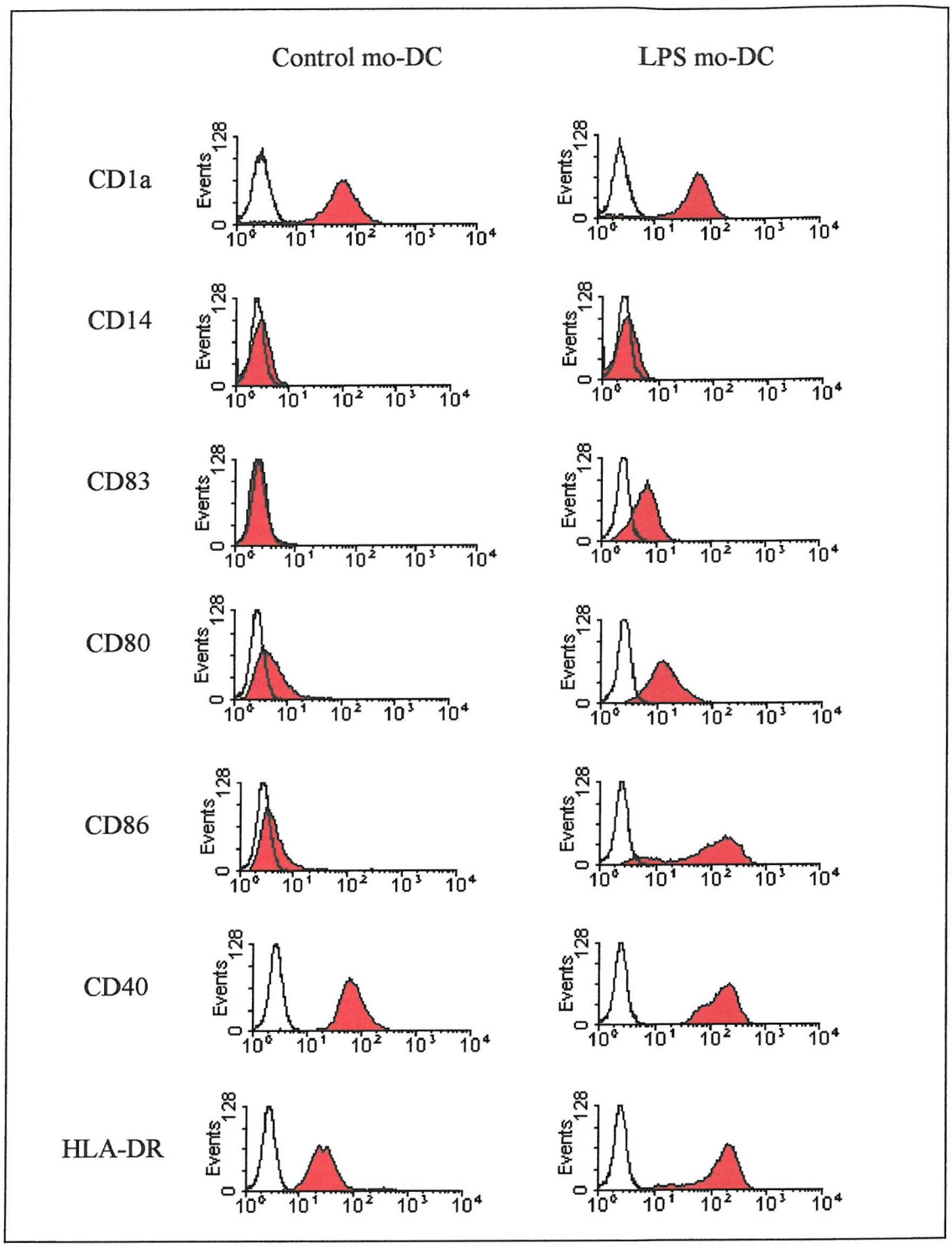

Figure 4.3: Phenotype of mo-DC stimulated with LPS

Mo-DC were exposed to 1μg/ml *E. coli* LPS for 40hrs. Expression of surface CD1a, CD14, CD80, CD83, CD86, CD40 and HLA-DR before and after treatment was determined by flow cytometry (red histograms). Profiles are representative of one of five separate experiments. Cells stained with appropriate isotype controls mAbs are represented by clear histograms.

4.3.3 Effects of ODN treatment on mo-DC and mo-LC phenotype

Mo-DC and mo-LC were treated with ODN and changes in surface antigens CD80, CD86, CD40 and HLA-DR were analysed by flow cytometry. The ODNs used in these investigations include CpG '1668', CpG '2006' and non-CpG '100'. Control cells were stimulated with vehicle only. Treatment of mo-DC and mo-LC with TNF-α served as a positive control stimulus of cell activation. The mo-DC and mo-LC FACS profiles shown are representative of one non-atopic donor. The summary data represents the average of absolute individual mean fluorescence intensity values for each sample tested.

Expression of HLA-DR (Figure 4.4 and summary Table 4.2), CD80 (Figure 4.5 and summary Table 4.3), CD86 (Figure 4.6 and summary Table 4.4) and CD40 (Figure 4.7 and summary Table 4.5) on mo-DC and mo-LC did not change following treatment with CpG '1668' and '2006' or non-CpG '100'compared to control cells.

TNF-α treated mo-DC show a significant ($p<0.05$) increase in HLA-DR expression (Table 4.2). Whereas, mo-LCs treated with TNFα shown only a small increase in $HLA\text{-}DR^{+}$ cells (20% ± 3.4 n=3; Figure 4.4B). However, due to a low sample size at present, statistical significance of changes in HLA-DR expression on TNF-α treated mo-LC was not possible. CD80 expression increased significantly ($p<0.05$) in TNF-α treated mo-DC (Table 4.3) but not in TNF-α treated mo-LC (Table 4.3). Similarly, CD86 expression was markedly increased ($p<0.05$) in TNF-α treated mo-DC (Table 4.4). While on mo-LCs, there was a small non-significant increase in percentage of cells expressing CD86 (27% ± 4.2, n=3; Figure 4.6B). CD40 expression was slightly up-regulated on TNF-α treated mo-DC and to a lesser extent on mo-LC compared to their respective control cells (Table 4.5). These increases in CD40 expression failed to achieve statistical significance.

Mo-DC and mo-LC derived from monocytes obtained from atopic subjects did not demonstrate changes in co-stimulatory molecules or HLA-DR in response to ODN treatment (n=2, data not shown).

A) Mo-DC B) Mo-LC

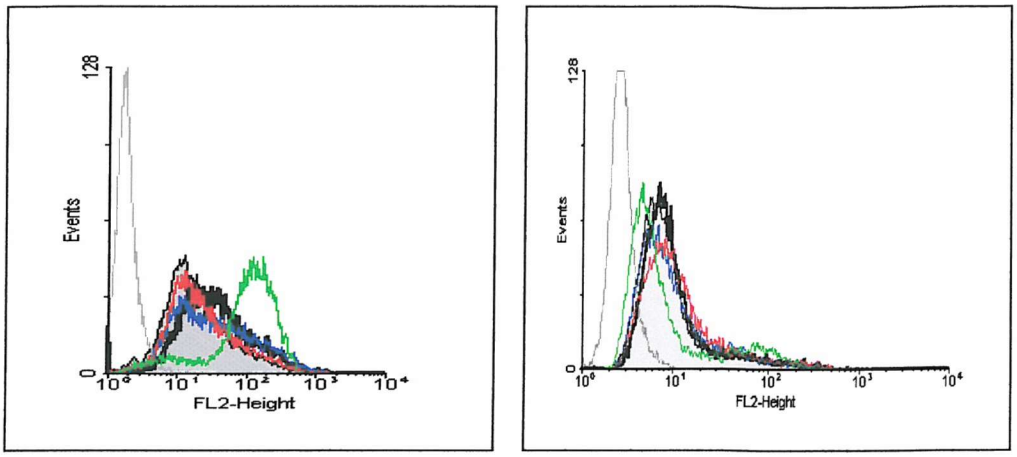

Figure 4.4: Expression of HLA-DR on mo-DC and mo-LC after ODN treatment.

Flow cytometric analysis of HLA-DR expression on A) mo-DC and B) mo-LC after 40hr treatment with ODN: CpG'1668' 1μM (thick black line), CpG '2006' 1μM (blue line) or non-CpG '100' 1μM (red line). TNF-α 25ng/ml (green line) was used as a positive control. Solid grey histograms are control cells treated with vehicle alone. Open grey histograms represent cells stained with appropriate isotype controls. FACS profiles are derived from one representative donor.

HLA-DR	Mo-DC	Mo-LC
Control	18.5 ± 7.7, n=5	5.9 ± 2.3, n=3
CpG '1668'	22.9 ± 8.4, n=5	5.3 ± 0.9, n=3
CpG '2006'	12.4 ± 5.2, n=4	4.9 ± 1.3, n=3
Non-CpG '100'	15.4 ± 5.3, n=5	5.0 ± 1.1, n=3
TNF-α	179.4 ± 15.4, n=5 *	7.9 ± 2.1, n=3

Table 4.2: Summary data of HLA-DR expression on ODN-treated mo-DC and mo-LC.

Mo-DC and mo-LC were treated with CpG '1668' 1μM, CpG '2006' 1μM or non-CpG '100' 1μM for 40hrs. Control cells were treated with TE-buffer alone. HLA-DR expression was determined by flow cytometry. Data shows average of absolute individual HLA-DR mean fluorescence intensity values ± standard errors. n = sample numbers for each treatment. * = Significant ($p<0.05$) compared to control.

A) Mo-DC B) Mo-LC

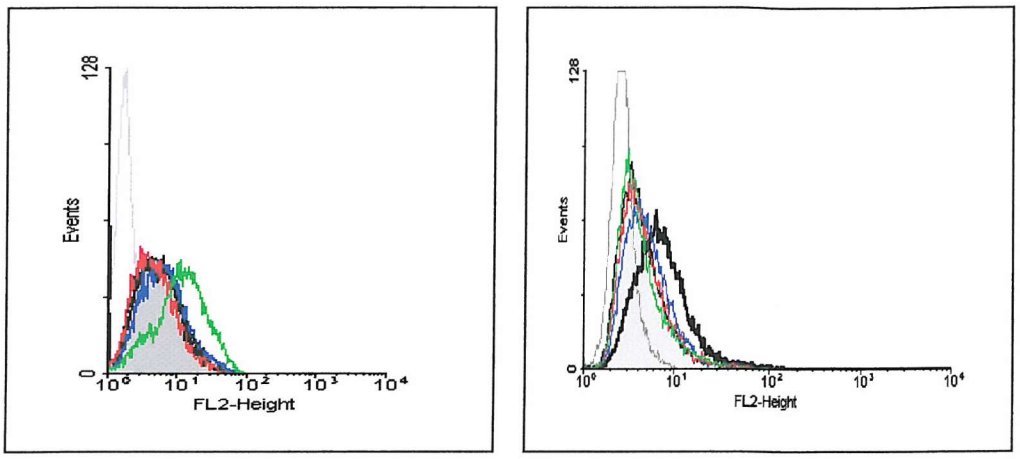

Figure 4.5: Expression of CD80 on mo-DC and mo-LC after ODN treatment.

Flow cytometric analysis of CD80 expression on A) mo-DC and B) mo-LC after 40hr treatment with ODN: CpG '1668' 1μM (**thick black line**), CpG '2006' 1μM (blue line) or non-CpG '100' 1μM (red line). TNF-α 25ng/ml (green line) was used as a positive control. Solid grey histograms are control cells treated with vehicle alone. Open grey histograms represent cells stained with appropriate isotype controls. FACS profiles are derived from one representative experiment.

CD80	Mo-DC	Mo-LC
Control	2.1 ± 0.5, n=5	1.9 ± 0.1, n=3
CpG '1668'	3.6 ± 1.5, n=5	2.4 ± 0.7, n=3
CpG '2006'	2.6 ± 1.0, n=4	2.2 ± 0.1, n=3
Non-CpG '100'	3.6 ± 1.1, n=5	2.1 ± 0.3, n=3
TNF-α	5.9 ± 1.2, n=5 *	2.9 ± 0.7, n=3

Table 4.3: Summary data of CD80 expression on ODN-treated mo-DC and mo-LC.

Mo-DC and mo-LC were treated with CpG '1668' 1μM, CpG '2006' 1μM or non-CpG '100' 1μM for 40hrs. Control cells were treated with TE-buffer alone. Expression of CD80 was determined by flow cytometry. Data shows average of absolute individual CD80 mean fluorescence intensity values ± standard errors. n = sample numbers for each treatment. * = Significant ($p<0.05$) compared to control.

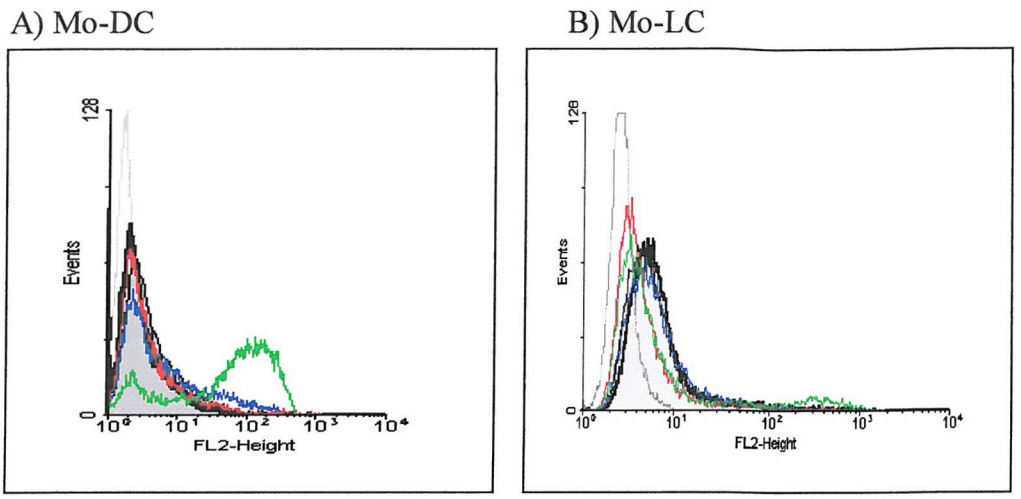

Figure 4.6: Expression of CD86 on mo-DC and mo-LC after ODN treatment.

Flow cytometric analysis of CD86 expression on A) mo-DC and B) mo-LC after 40hr treatment with ODN: CpG '1668' 1μM (thick black line), CpG '2006' 1μM (blue line) or non-CpG '100' 1μM (red line). TNF-α 25ng/ml (green line) was used as a positive control. Solid grey histograms are control cells treated with vehicle alone. Open grey histograms represent cells stained with appropriate isotype controls. FACS profiles are derived from one representative experiment.

CD86	Mo-DC	Mo-LC
Control	1.8 ± 0.4, n=5	1.9 ± 0.6, n=3
CpG '1668'	2.3 ± 0.5, n=5	1.6 ± 0.7, n=3
CpG '2006'	2.6 ± 0.5, n=4	1.7 ± 0.6, n=3
Non-CpG '100'	1.5 ± 0.3 n=5	1.4 ± 0.5, n=3
TNF-α	96.4 ± 11.0 n=5 *	2.1 ± 0.7, n=3

Table 4.4: Summary data of CD86 expression on ODN-treated mo-DC and mo-LC.

Mo-DC and mo-LC were treated with CpG '1668' 1μM, CpG '2006' 1μM or non-CpG '100' 1μM for 40hrs. Control cells were treated with TE-buffer alone. Expression of CD86 was determined by flow cytometry. Data shows average of absolute individual CD86 mean fluorescence intensity values ± standard errors. n = sample numbers for each treatment. * = Significant ($p<0.05$) compared to control.

A) Mo-DC B) Mo-LC

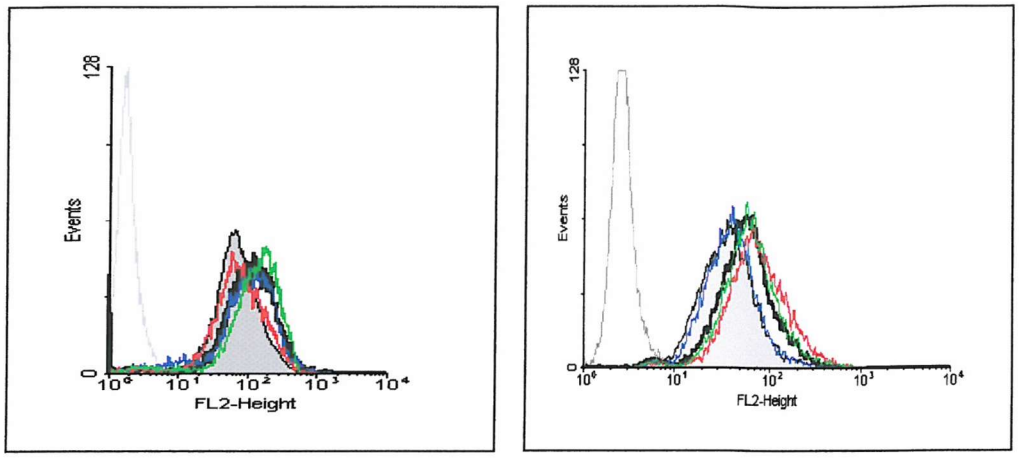

Figure 4.7: Expression of CD40 on mo-DC and mo-LC after ODN treatment.

Flow cytometric analysis of CD40 expression on A) mo-DC and B) mo-LC after 40hr treatment with ODN: CpG '1668' 1μM (thick black line), CpG '2006' 1μM (blue line) or non-CpG '100' 1μM (red line). TNF-α 25ng/ml (green line) was used as a positive control. Solid grey histograms are control cells treated with vehicle alone. Open histograms show cells stained with appropriate isotype controls. FACS profiles are derived from one representative donor.

CD40	Mo-DC	Mo-LC
Control	81.9 ± 26.0, n=4	51.8 ± 11.2, n=3
CpG '1668'	64.3 ± 23.1, n=5	47.4 ± 7.2, n=3
CpG '2006'	49.9 ± 18.0, n=4	49.0 ± 9.8, n=3
Non-CpG '100'	86.4 ± 15.3, n=5	61.6 ± 8.6, n=3
TNF-α	131.7 ± 14.7, n=4	75.8 ± 11.6, n=3

Table 4.5: Summary data of CD40 expression on ODN-treated mo-DC and mo-LC.

Mo-DC and mo-LC were treated with CpG '1668' 1μM, CpG '2006' 1μM or non-CpG '100' 1μM for 40hrs. Control cells were treated with TE-buffer alone. Expression of CD40 was determined by flow cytometry. Data shows average of absolute individual CD40 mean fluorescence intensity values ± standard errors. n = sample numbers for each treatment. * = Significant ($p<0.05$) compared to control.

4.3.4 Effect of plasmid DNA on Mo-DC phenotype expression

Treatment of monocytes with plasmid DNA had been seen to trigger a coordinate series of changes in their phenotype (Chapter 3). In mo-DCs (from non-atopic donors) however, similar to results observed after CpG ODN treatment, plasmid DNA failed to induce marked activation of mo-DC (Figure 4.8). Minor increases in HLA-DR expression on mo-DCs were noted after plasmid DNA treatment although; this was also seen after treatment with THP-1 genomic DNA (Figure 4.8) and did not achieve statistical significance ($n=5$, $p>0.05$). Expression of CD80 and CD86 on mo-DCs was not altered after stimulation with plasmid or THP-1 genomic DNA. Moreover, inability of plasmid or genomic DNA to induce maturation of mo-LC was also observed (data not shown, $n=3$).

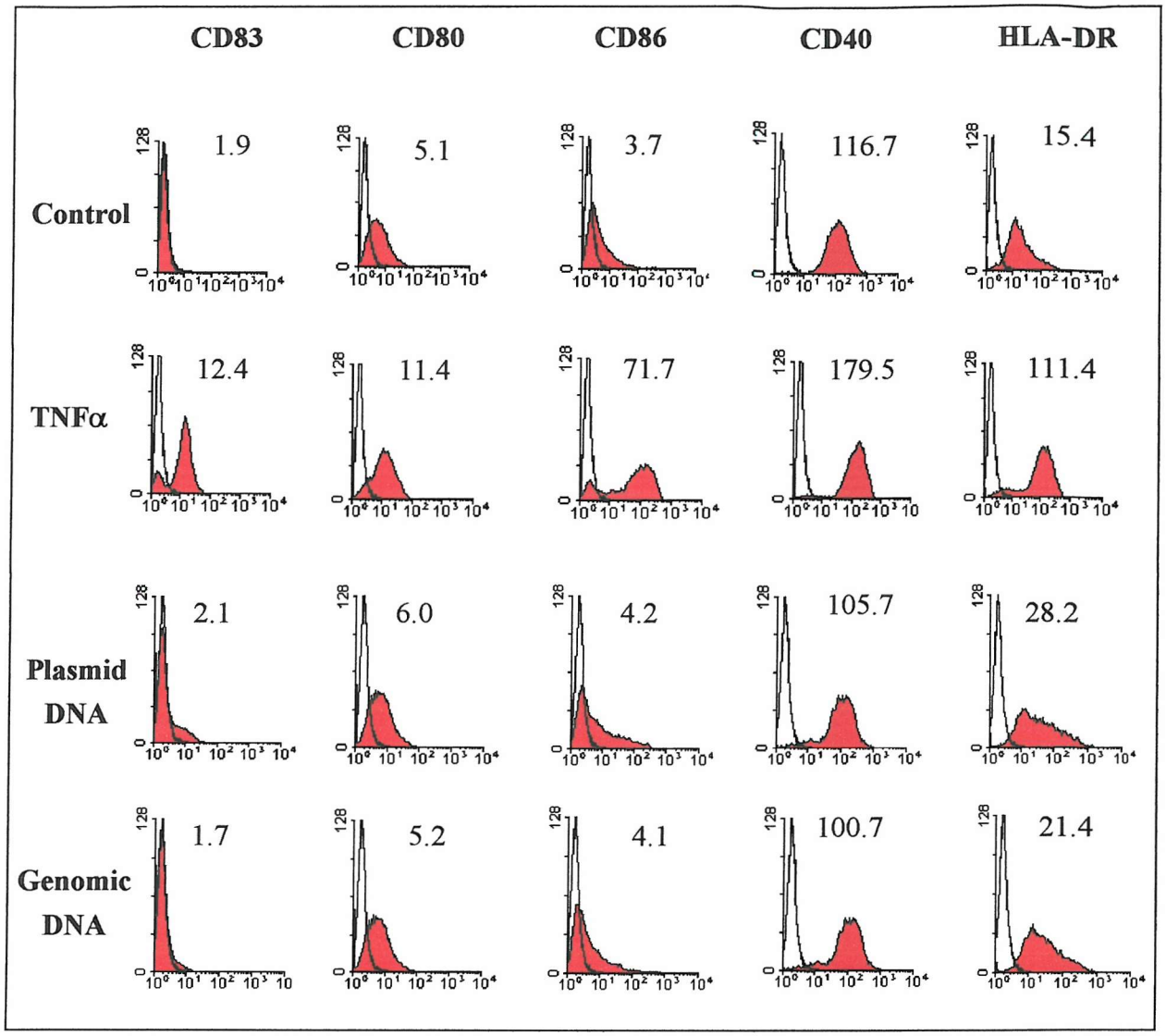

Figure 4.8: Phenotypic expression of mo-DCs treated with plasmid or genomic DNA.

Mo-DCs were exposed to 10μg/ml pcDNA3 plasmid DNA, 10μg/ml genomic DNA (THP-1 monocytic cell line) or 25ng/ml TNFα for 40hrs. Control cells were treated with vehicle only (TE buffer). Surface expression of CD83, CD80, CD86, CD40 and HLA-DR was determined by flow cytometry. Red histograms represent cells strained with mAb and clear histograms represent cells stained with relevant isotype control mAbs. Numbers on each histogram corresponds to the median fluorescence intensity for surface molecule staining. Results shown are representative of one out of five separate experiments.

4.4 PRODUCTION OF CYTOKINES BY Mo-DC AND Mo-LC AFTER STIMULATION WITH OLIGODEOXYNUCLEOTIDES

In addition to analysis of the effects of ODN on expression of co-stimulatory molecules by mo-DC and mo-LC, their effects were examined on production of IL-1β, IL-6, IL-10, IL-12p40 and TNFα. Cells from non-atopic donors were treated with 1μM CpG ODN '1668' or '2006', 1μM non-CpG ODN '100', 1μg/ml *E. coli* LPS or 25ng/ml TNFα for 40hrs. Cytokines were quantified by ELISA and results are presented as average production (pg/10^6 cells) with standard errors of three separate experiments (Table 4.6). Treatment with either CpG or non-CpG ODN did not evoke the production of any cytokines by mo-DCs (Table 4.6A) or mo-LCs (Table 4.6B). Also, cytokine production was not detected in supernatants of cells treated with ODN for 20hrs (data not shown, n=3). However, treatment of mo-DCs or mo-LCs with LPS or TNFα did result in cytokine secretion (these did not achieve statistical significance due to low sample numbers).

Mo-DCs and mo-LCs from atopic donors also failed to produce IL-1β, IL-6, IL-10, IL-12p40 and TNFα in response to ODN (n=2, preliminary data not shown).

A) Mo-DC

	IL-1β	IL-6	IL-10	IL-12p40	TNFα
Control	nd*	nd	nd	nd	nd
'1668'	nd	15.7 ± 1.6	21.3± 2.6	nd	nd
'2006'	nd	nd	18.3 ± 4.9	nd	nd
'100'	nd	16.1 ± 0.8	16.3 ± 1.7	nd	nd
'LPS'	116.7 ± 15.6	213.1 ± 63.3	128 ± 23.5	566.9 ± 89.4	396.1 ± 38.2
TNFα	192.5 ± 23.2	132.9 ± 3.5	51.3± 16.2	98.1 ± 13.6	#

nd* = not detected

B) Mo-LC

	IL-1β	IL-6	IL-10	IL-12p40	TNFα
Control	nd*	nd	nd	nd	nd
'1668'	nd	nd	nd	nd	nd
'2006'	nd	nd	nd	nd	nd
'100'	nd	nd	nd	nd	nd
LPS	58.8 ± 13.5	153.5 ± 1.4	94.8 ± 0.9	120.9 ± 6.1	133.7 ± 33.8
TNFα	50.9 ± 6.5	78.9 ± 6.7	32.7 ± 5.8	77.1 ± 2.4	#

nd* = not detected

Table 4.6: Effect of ODN or LPS on cytokine production by mo-DCs and mo-LCs

Mo-DCs (A) and mo-LCs (B) were treated with 1μM CpG '1668', 1μM CpG '2006', 1μM non-CpG '100', 1μg/ml *E. coli* LPS or vehicle control for 40hrs. Cell-free supernatants were assayed for the presence of IL-1β, IL-6, IL-10, IL-12p40 or TNFα by ELISA. Results are presented as mean production (pg/10^6 cells) ± SE; n=3 for mo-DC and mo-LC. nd = not detected; levels below detection limits of the assay (<15pg/ml), # = not performed.

4.5 Mo-DC/Mo-LC DRIVEN T-CELL PROLIFERATION

Although mo-DCs and mo-LCs failed to exhibit changes in co-stimulatory molecule expression and cytokine production following exposure to CpG ODN, their capacity to stimulate T-cells was examined. In these experiments, mo-DCs and mo-LCs were obtained by culturing monocytes from non-atopic donors in rhIL-4 and rhGM-CSF supplemented medium containing 2% human AB serum instead of 10% FCS to reduce background proliferation. Expression of CD1a on untreated mo-DCs or mo-LCs cultured in human AB serum were markedly lower compared to cells cultured in 10% FCS (data not shown, n=3). Nonetheless, as for FCS cultured- mo-DCs or mo-LCs, no changes in phenotype or production of cytokines were observed following treatment with CpG ODN (data not shown, n=3). Proliferation assays were performed using allogeneic purified $CD4^+$ T-cells (<98% $CD4^+$ cells, purity determined by flow cytometry). Mo-DCs (Figure 4.9) or mo-LCs (Figure 4.10) were stimulated for 40hrs with 1μM CpG ODN ('1668' or '2006'), 1μM non-CpG ODN '100', 25ng/ml TNFα or 1μg/ml *E. coli* LPS. After treatment, cells were washed thoroughly prior to co-culture with purified allogeneic $CD4^+$ T-cells. ODN treated mo-DCs or mo-LCs did not induce T-cell proliferation compared to vehicle-treated cells. On the other hand, T-cell proliferation was markedly enhanced when cells were matured by pre-incubation with either TNF-α (stimulation index at 1:10 DC/LC: T-cell ratios, mo-DC: 11.9 ± 6.6 n=3; mo-LC: 5 ± 2.8 n=3) or LPS (stimulation index at 1:10 DC/LC: T-cell ratios, mo-DC: 5.5 ± 2.2 n=3; mo-LC: 3.7 ± 0.5 n=3).

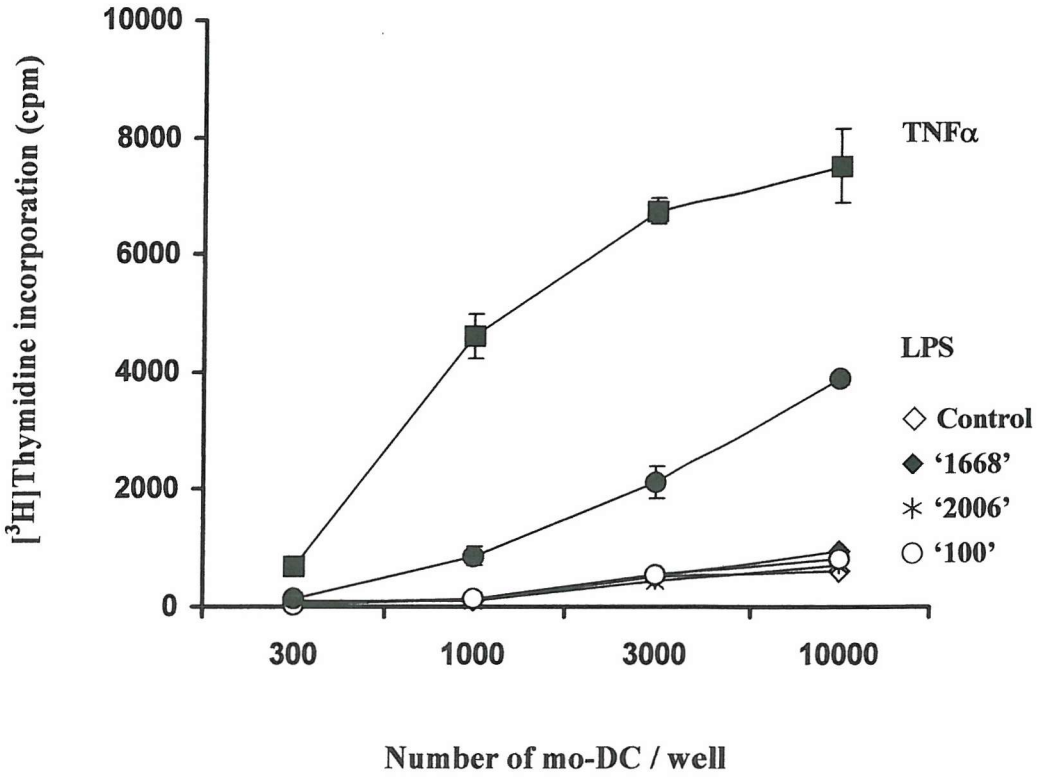

Figure 4.9: Induction of allogeneic T-cell proliferation by mo-DCs.

Mo-DCs were treated for 40hrs with 1μM ODN: CpG '1668', CpG '2006' or non-CpG '100'. Control cells received vehicle alone. As a positive stimulus for induction of proliferation, mo-DCs were pre-incubated for 40hrs with 25ng/ml TNFα or 1μg/ml *E. coli* LPS. Mo-DCs were washed extensively following treatment. Graded numbers of mo-DCs were co-cultured with 10^5 purified allogeneic $CD4^+$ T-cells per well. After 5 days, proliferation was assessed by thymidine incorporation. Results are presented as mean counts per minute (cpm, of triplicate cultures) ± standard error. Data shown is representative of one out of three separate experiments.

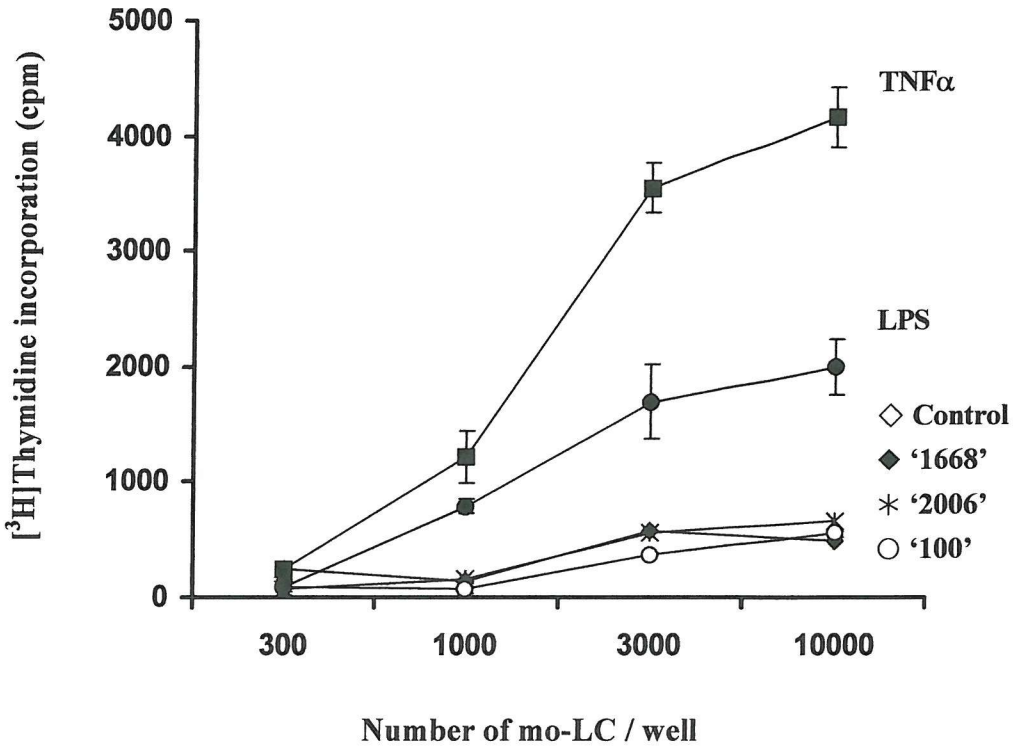

Figure 4.10: Induction of allogeneic T-cell proliferation by mo-LCs.

Mo-LCs were treated for 40hrs with 1μM ODN: CpG '1668', CpG '2006' or non-CpG '100'. Control cells received vehicle alone. As a positive stimulus for induction of proliferation, mo-LCs were pre-incubated for 40hrs with 25ng/ml TNFα or 1μg/ml *E. coli* LPS. Mo-LCs were washed extensively following treatment. Graded numbers of mo-LCs were co-cultured with 10^5 purified allogeneic $CD4^+$ T-cells per well. After 5 days, proliferation was assessed by thymidine incorporation. Results are presented as mean counts per minute (cpm, of triplicate cultures) ± standard error. Data shown is representative of one out of three separate experiments.

CHAPTER 4

Results: Section II

Toll-like Receptor Expression on Human Dendritic cells and Langerhans' cells

4.6 SURFACE EXPRESSION OF TOLL-LIKE RECEPTORS ON Mo-DC AND Mo-LC

Differential expression of TLR4 and TLR9 on mo-DCs was assessed to identify whether differences in receptor expression account for the observed responses to LPS and CpG ODN, respectively. Moreover, expression of TLR2, TLR4 and TLR9 on mo-DCs and mo-LCs was compared by flow cytometric analysis (Figure 4.11). $CD1a^+$ mo-DC and mo-LCs exhibit low staining of TLR2 and TLR4. However, intensity of TLR4 staining was greater in mo-DCs than mo-LCs. In contrast, neither mo-DCs nor mo-LCs expressed surface TLR9. These observations suggest that the lack of TLR9 expression on mo-DCs and mo-LCs may account for the inability of these cells to respond to CpG ODN. On the other hand surface TLR4 is present on these cells, albeit at low levels, but this may be sufficient for mo-DCs to mediate LPS signalling.

This study was only performed using mo-DCs and mo-LCs from non-atopic donors. Results shown are representative of three separate experiments, with similar expression observed in all experiments.

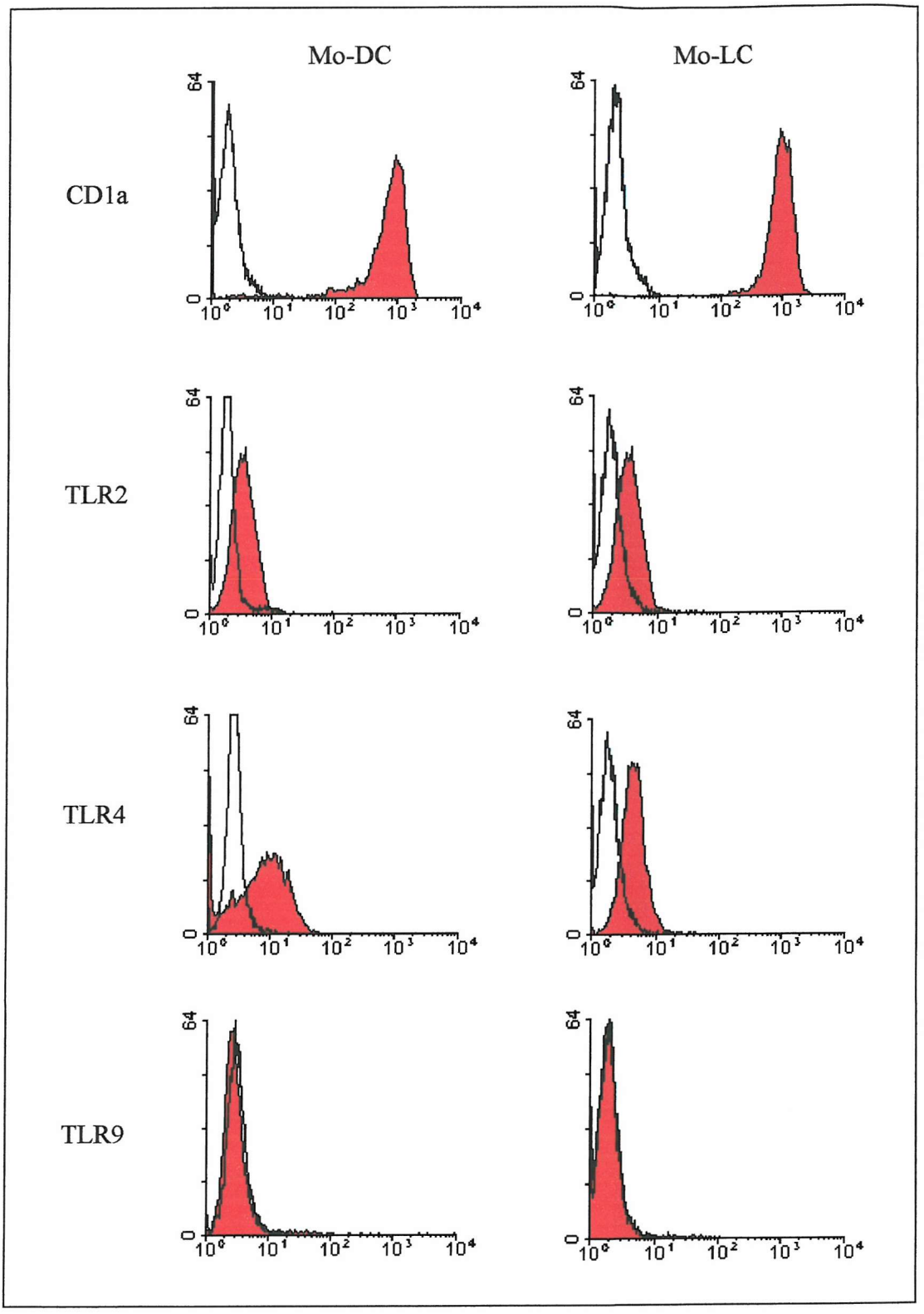

Figure 4.11: Expression of Toll-like receptors on mo-DC and mo-LC.

$CD1a^+$ mo-DCs and mo-LCs were analysed for surface expression of TLR2, TLR4 and TLR9 by flow cytometry. Immunostaining with mAbs is shown as red histograms. Relevant isotype mAbs controls are shown as clear histograms. Data is representative of one of three donors tested.

4.7 TOLL-LIKE RECEPTOR mRNA EXPRESSION ON Mo-DC AND Mo-LC

Messenger RNA transcripts of TLR2, TLR4 and TLR9 in mo-DCs and mo-LCs were measured by TaqMan RT-PCR. Expression of TLR mRNA was compared between monocytes, mo-DC and mo-LC obtained from non-atopic donors. The data in Figure 4.12 shows the relative fold change in TLR mRNA levels in mo-DCs and mo-LC compared to uncultured monocytes from the same donors. TLR2 mRNA levels were comparable in monocytes and mo-DCs although a decreased pattern of expression was observed in mo-LCs. TLR4 mRNA levels were higher in monocytes than mo-DCs and mo-LCs. No major difference in TLR4 expression was seen between mo-DCs and mo-DCs. However, only monocytes were observed to express TLR9 mRNA.

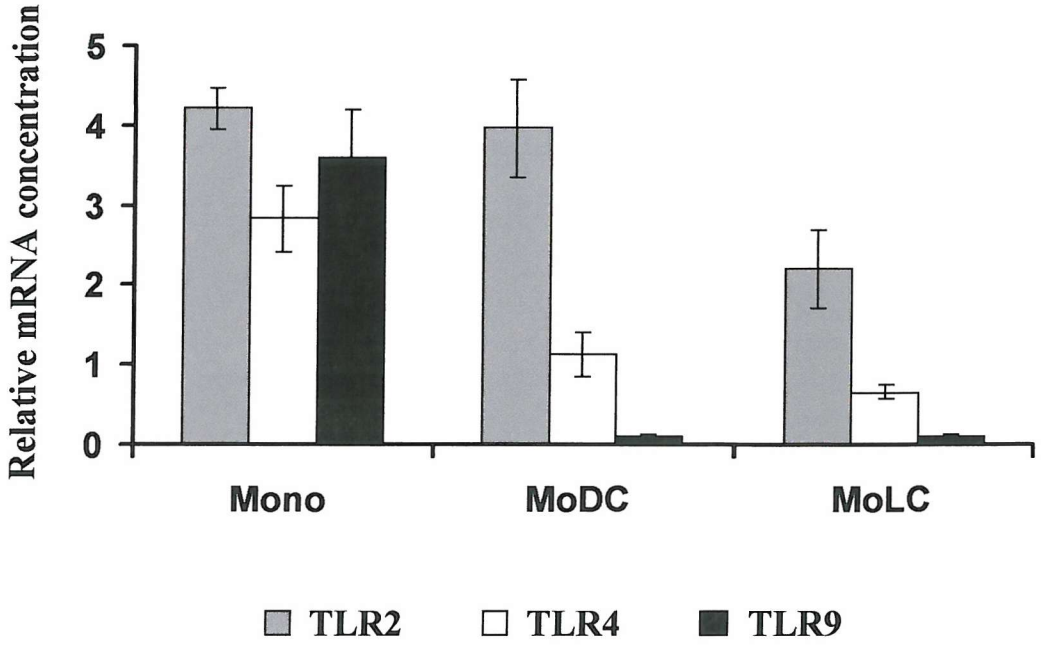

Figure 4.12: Comparison of TLR mRNA in monocytes, mo-DC and mo-LC.

Purified monocytes (mono) from healthy donors were differentiated, *in vitro,* into mo-DC or mo-LC. Total RNA was extracted from cells and 400ng was reverse transcribed into cDNA. Serial dilutions of PBMC Total RNA (ng) were converted into cDNA to create a standard curve. Levels of TLR2, TLR4 and TLR9 mRNA in monocytes, mo-DCs and mo-LC were determined by TaqMan RT-PCR and normalised to endogenous ribosomal 18S RNA prior to relative concentrations being extrapolated from the PBMC standard curve. Data is expressed as the relative mRNA concentration in monocytes, mo-DCs and mo-LCs ± standard error of five experiments.

4.8 EXPRESSION OF TOLL-LIKE RECEPTOR mRNA DURING CULTURE OF MONOCYTES TO Mo-DCs

As monocytes differentiated to mo-DCs in the presence of rhIL-4 and rhGM-CSF TLR mRNA expression (as measured by TaqMan RT-PCR) underwent significant changes (Figure 4.13). TLR2 mRNA was markedly decreased after 24hrs (Day 1) in culture compared to fresh monocytes, however expression steadily increased so that by Day 5 levels were comparable with those in fresh monocytes. TLR4 mRNA levels were moderately decreased during the first three days of culture but increased on days 4 and 5. In contrast, TLR9 mRNA was markedly reduced after 24hrs in culture and declined further over the five-day culture period ($p<0.05$, Student t-test) (Figure 4.13). Monocytes from healthy donors were used in these experiments.

Analysis of TLR mRNA expression throughout culture of monocytes to mo-LCs was not performed.

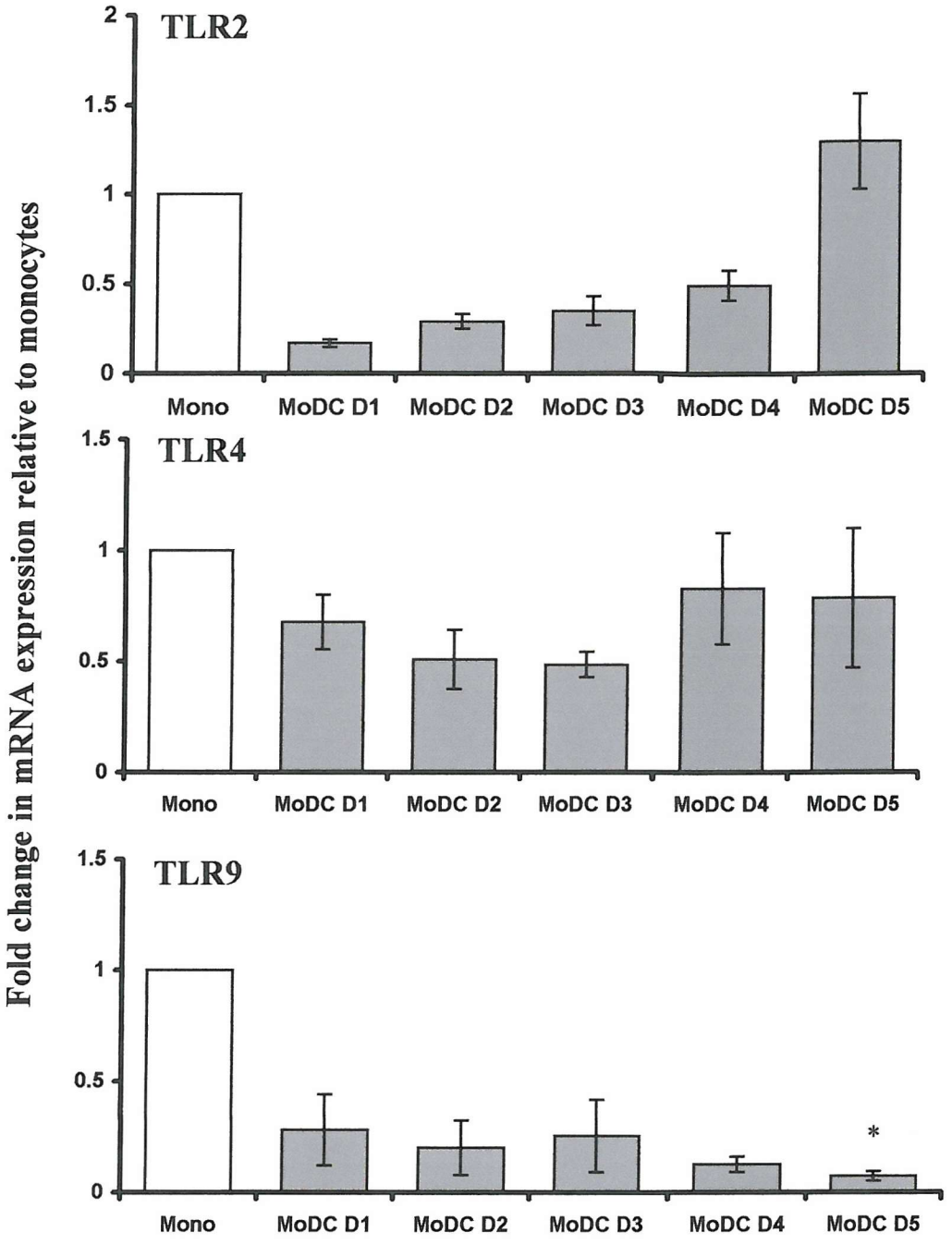

Figure 4.13: Expression of TLR2, TLR4 and TLR9 mRNA during differentiation of monocytes to mo-DCs.

Purified monocytes from non-atopic donors were cultured for five days in the presence of rhIL-4 and rhGM-CSF to induce differentiation into mo-DCs. Total RNA was extracted from freshly isolated monocytes (D0, white column) and from cultured cells over the next five days (D1-5, grey columns). Levels of TLR2, TLR4 and TLR9 mRNA were determined by TaqMan RT-PCR and normalised to endogenous ribosomal 18S RNA prior to concentrations being extrapolated from the PBMC standard curve. Data is expressed as fold change in mRNA in mo-DCs (D1-D5) relative to uncultured monocytes ± standard error of five experiments. * Significance observed ($p<0.05$) compared to monocytes.

4.9 CD34$^+$-DERIVED DENDRITIC CELLS AND CD34$^+$-DERIVED LANGERHANS' CELLS

Following from the observations that human mo-DCs and mo-LCs do not express surface TLR9, preliminary experiments were subsequently performed to differentiate purified human CD34$^+$ cells into cells exhibiting dendritic (CD34$^+$-DC) or Langerhans' cells (CD34$^+$-LC) characteristics. These investigations were carried out to determine whether expression of surface TLR2, TLR4 and in particular TLR9 was exhibited on these CD34$^+$-derived cells.

4.9.1 Phenotype of cells

In order to obtain dendritic cells, purified human CD34$^+$ cells were maintained for 14 days in the presence of rhGM-CSF, rhSCF and rhTNFα (Caux *et al.*, 1996). rhTGF-β1 was included in culture medium to drive the development of Langerhans' cells (Gatti *et al.*, 2000; Caux *et al.*, 1999). On day 14 mixed cell populations were obtained in the cultures. Therefore, using two-colour flow cytometry the phenotype of CD1a$^+$ CD14$^-$ cells was initially examined.

Figure 4.14 and 4.15 illustrate expression of surface molecules on CD34$^+$-DCs and CD34$^+$-LCs. Moderately different phenotypes of CD1a$^+$ cells were observed following the different culture methods. CD34$^+$-DCs expressed very low levels of CD11b and CD80, moderate levels of CD86 and HLA-DR and comparatively higher levels of CD11c and CD40. There was no surface expression of CD14 and CD83 on these cells. CD1a$^+$ CD34$^+$-LCs also expressed low levels of CD11b however, some staining of CD83 was observed. In addition, these cells showed high levels of CD11c. In contrast to CD34$^+$-DCs, a proportion of CD1a$^+$ CD34$^+$-LCs cells stained brightly for CD40, CD86 and HLA-DR.

Langerin is described as a marker of LCs (Valladeau *et al.*, 2000). Therefore, expression of intracellular Langerin was examined in CD34$^+$-DCs and –LCs (Figure 4.14). TGF-β1 supplemented cultures induced moderate intracellular expression of Langerin in CD34$^+$-LCs but not CD34$^+$-DCs.

Phenotypic analysis presented in Figure 4.14 and 4.15 is representative of $CD34^+$-derived DC or LCs obtained from one apheresed sample. Morphology of $CD34^+$-DCs and –LCs from two separate donors were analysed showing similar results.

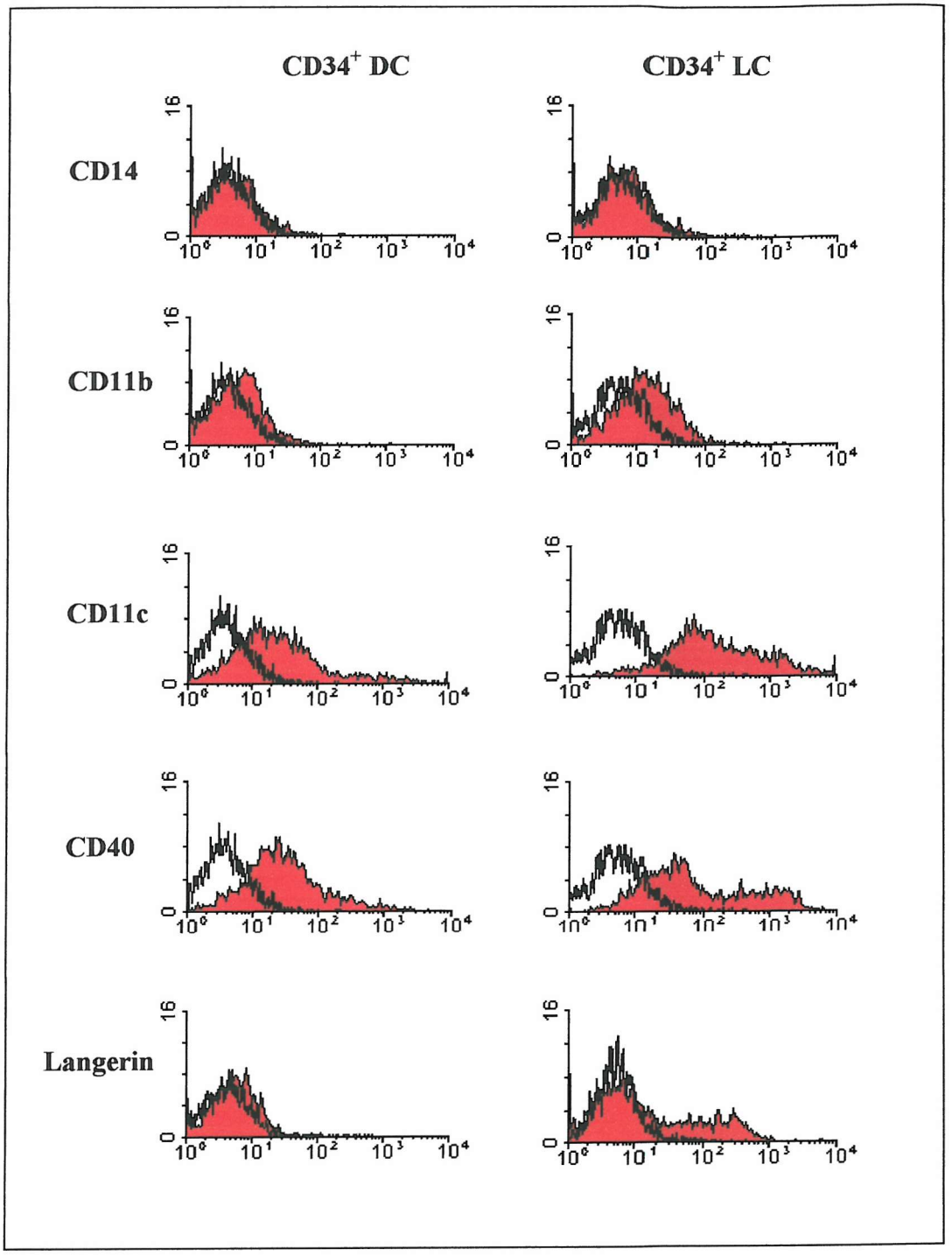

Figure 4.14: Phenotype of CD34$^+$-derived dendritic and Langerhans' cells I.

Purified CD34$^+$ cells were cultured for 14 days in the presence of rhSCF, rhGM-CSF and rhTNFα to obtain CD34$^+$-derived dendritic cells (DC). rhTGFβ1 was added to cultures for the development of CD34$^+$-derived Langerhans' cells (LC). Using flow cytometry, CD1a$^+$ cells (2000 cells) were gated and analysed for surface expression of CD14, CD11b, CD11c, CD40 and intracellular expression of Langerin. mAb staining of molecules is shown as red histograms. Clear histograms represent cells stained with relevant isotype controls. Flow cytometric profiles are representative of two individual experiments.

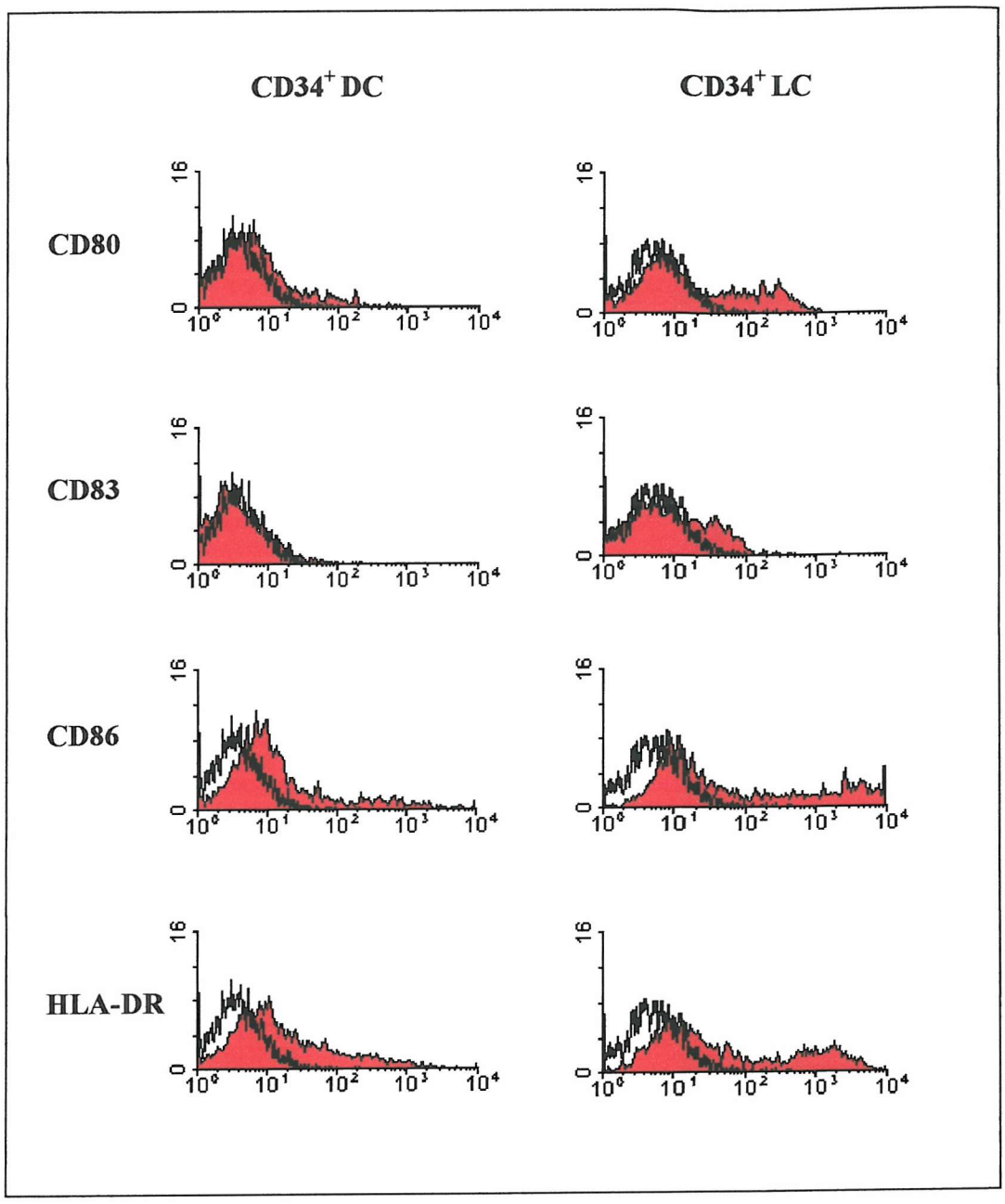

Figure 4.15: Phenotype of CD34$^+$-derived Dendritic and Langerhans' cells II.

Purified CD34$^+$ cells were cultured for 14 days in the presence of rhSCF, rhGM-CSF and rhTNFα to obtain CD34$^+$-derived dendritic cells (DC). rhTGFβ1 was added to cultures for the development of CD34$^+$-derived Langerhans' cells (LC). Using flow cytometry, CD1a$^+$ cells (2000 cells) were gated and analysed for surface expression of CD80, CD83, CD86 and HLA-DR. mAb staining of molecules is shown as red histograms. Clear histograms represent cells stained with relevant isotype controls. Flow cytometric profiles are representative of two individual experiments.

4.9.2 Expression of Toll-like receptors

Surface TLR2, TLR4 and TLR9 expression was examined on $CD34^+$-DCs and –LCs using two-colour flow cytometry analysis (Figure 4.16). $CD1a^+$ $CD34^+$-DCs exhibited low expression of TLR2 and TLR4. In contrast, $CD1a^+$ $CD34^+$-LCs were negative for TLR2 staining but low staining for TLR4 was noted. Similar to observations on mo-DCs and mo-LCs, $CD1a^+$ $CD34^+$-DCs and –LCs were negative for surface expression of TLR9.

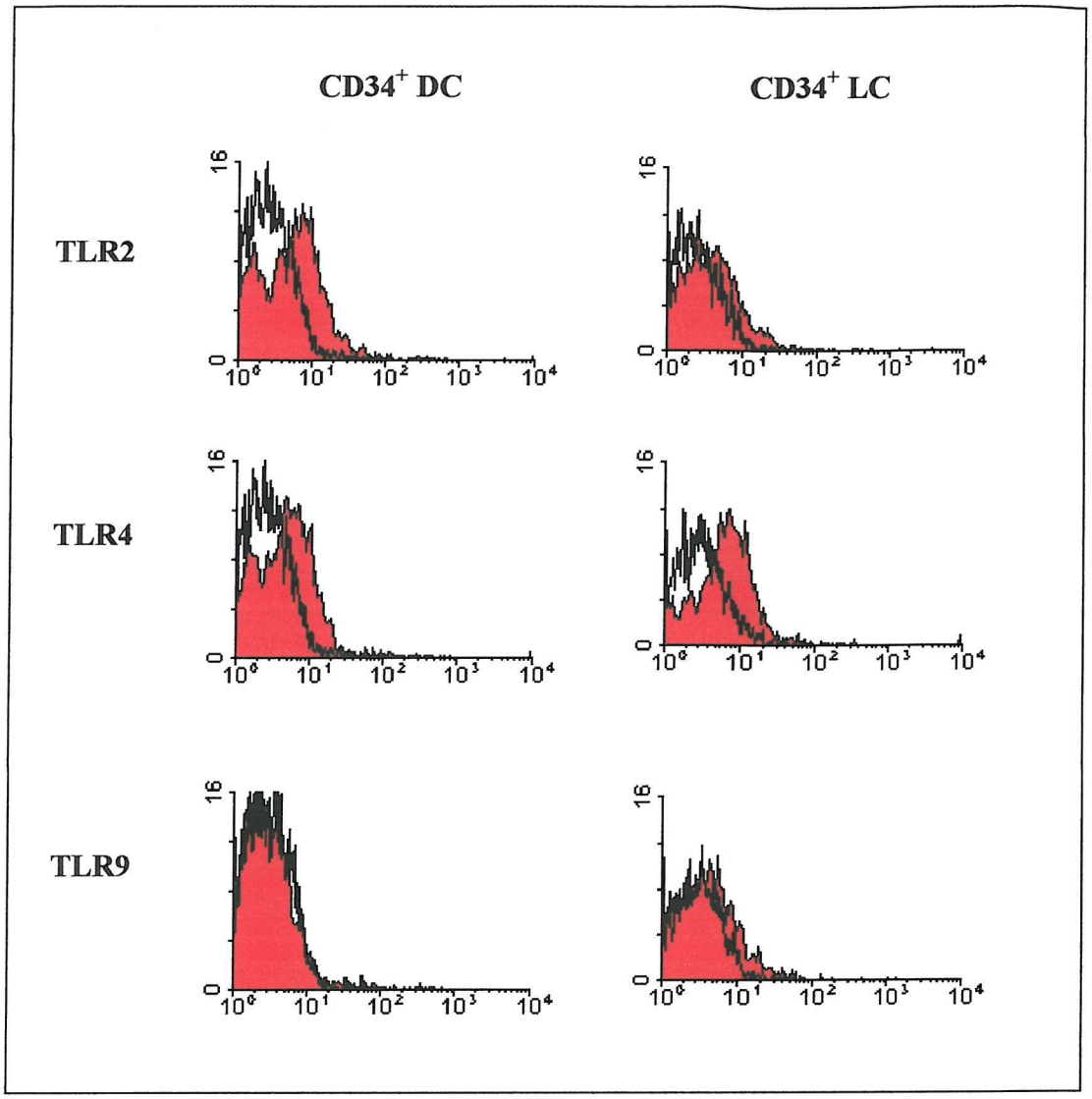

Figure 4.16: Toll-like receptor expression on CD34$^+$-derived dendritic and Langerhans' cells.

Purified CD34$^+$ cells were cultured for 14 days in the presence of cytokines for differentiation into CD34$^+$-derived dendritic cells (DC) or Langerhans' cells (LC). Surface expression of TLR2, TLR4 and TLR9 on CD1a$^+$ cells was determined by flow cytometry. mAb staining is shown as red histograms. Clear histograms represent cells stained with relevant isotype controls. Flow cytometric profiles are representative of one out of three separate experiments.

4.10 HUMAN SKIN LANGERHANS' CELLS

Preliminary experiments were performed to analyse TLR expression on human primary Langerhans' cells (LC). Epidermal layers of human skin were obtained by suction blistering. Enrichment of Langerhans' cells from the epidermal samples was possible following enzymatic dissagregation and selection of $CD1a^+$ cells, using magnetic beads.

4.10.1 Confocal microscopy of epidermal skin samples

LCs were readily identifiable by confocal microscopy after mAb staining for CD1a due to their distinct morphology (Figure 4.17A). Double labelling $CD1a^+$ and $TLR4^+$ cells in skin sections illustrates a population of $CD1a^-$ $TLR4^+$ cells. However, it was not possible to ascertain whether $CD1a^+$ cells expressed TLR4 (Figure 4.17B). Double labelling for CD1a and TLR9 or similarly TLR2 was not possible due to experimental difficulties. Nonetheless, TLR9 expression was demonstrated on cells within the skin samples after single staining only (Figure 4.17C). Sections labelled with fluorescent-conjugated matched isotype controls were negative (Figure 4.17A; data not shown for TLR4 mAb isotype control). Confocal images of skin samples, shown in Figure 4.17, were performed on one donor only.

4.10.2 Phenotypic analysis of purified Langerhans' cells

$CD1a^+$ LCs enriched from epidermal skin samples was negative for CD14, CD80 or CD86 expression but do express moderate levels of HLA-DR and high levels of CD40 as determined by flow cytometry. In addition, $CD1a^+$ LCs expressed intracellular Langerin. Flow cytometric profiles from two separate donors are shown in Figure 4.18. $CD1a^+$ LC did not express the DC maturation marker CD83 (data not shown, n=2).

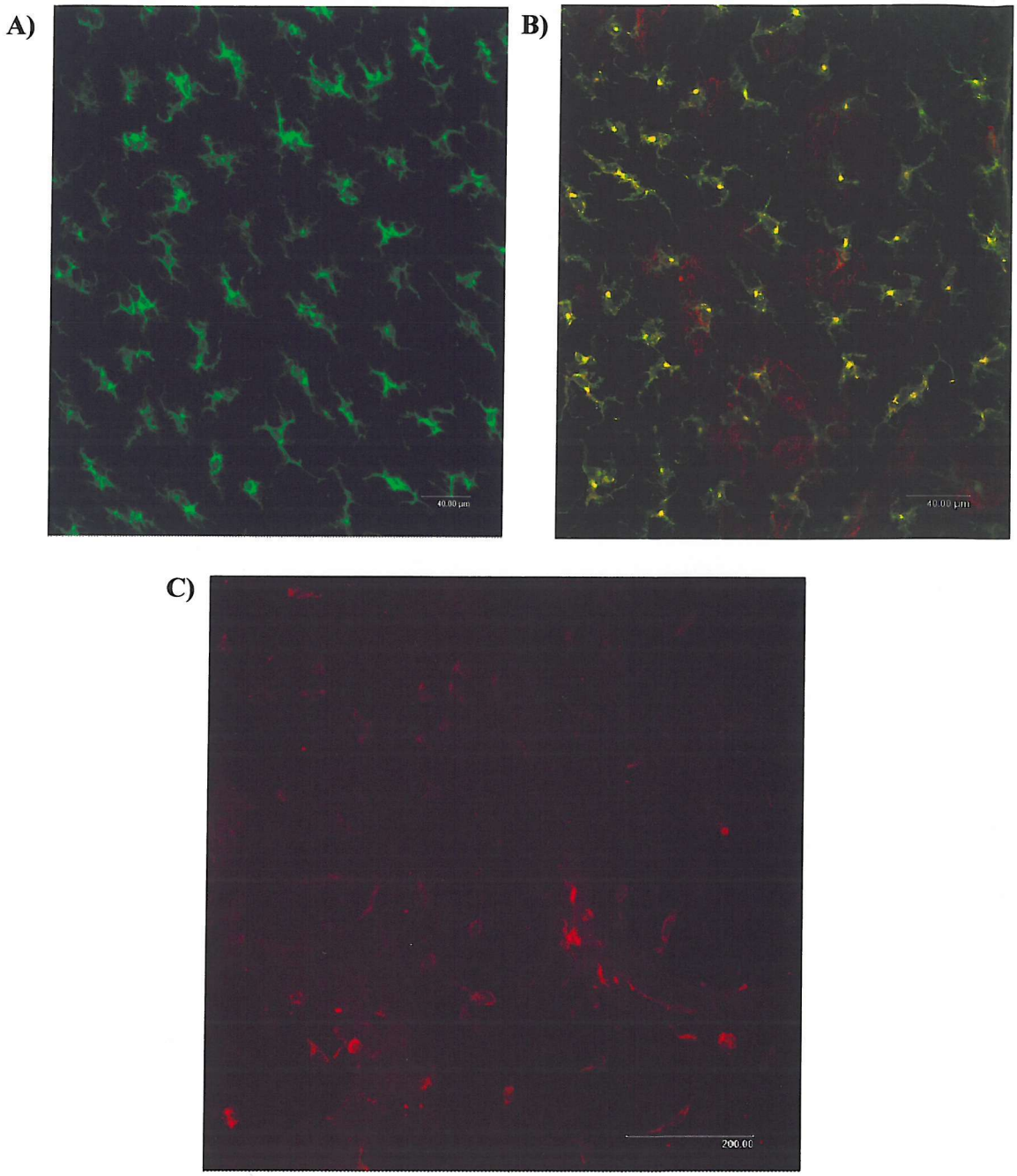

Figure 4.17: Confocal microscopy of human epidermal skin sections.

Epidermal skin sections obtained by suction blistering were single or double stained with mAbs: **A)** FITC-conjugated CD1a (10µg/ml) and PE-conjugated isotype IgG1, **B)** FITC-conjugated CD1a (10µg/ml) and PE-conjugated TLR4 (20µg/ml) or **C)** purified TLR9 (10µg/ml) followed by PE-secondary mAb (1:1000) only. Following mAb incubations and repeated washes with FACS buffer, stained sections were mounted onto microscope slides and viewed by confocal microscopy (A and B = x 175 magnification; C = x 95 magnification).

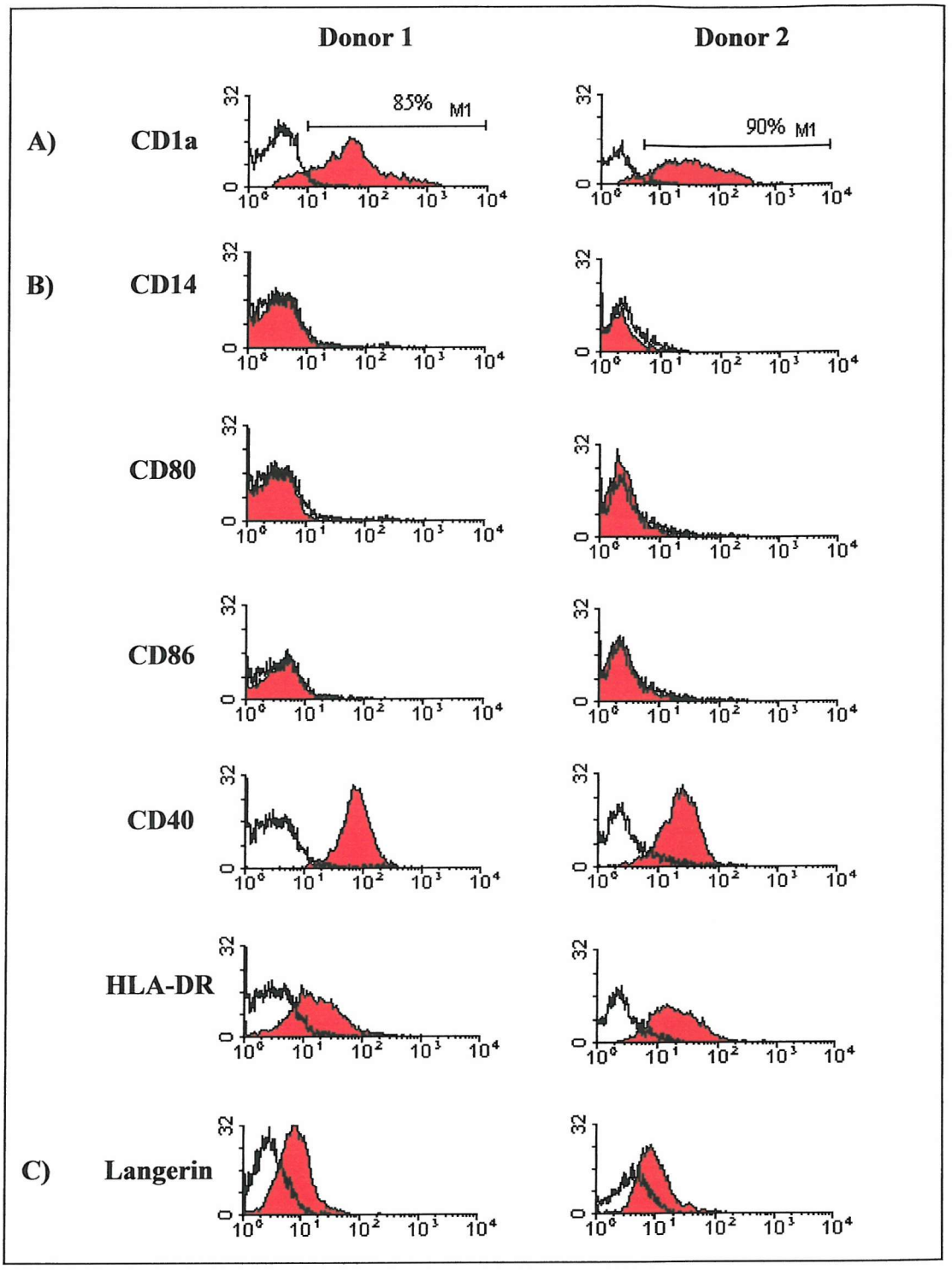

Figure 4.18: Phenotype of human skin Langerhans' cells.

Expression of molecules on LCs was determined by flow cytometry (3000 events collected). **A)** Purity of $CD1a^+$ LCs following dissociation from epidermal skin sections followed by enrichment using $CD1a^+$ magnetic beads. **B)** $CD1a^+$ LCs were gated and analysed for surface expression of CD14 (PE) CD80 (PE), CD86 (PE), CD40 (PE) or HLA-DR (PE) shown as red histograms. **C)** Intracellular expression of Langerin (PE) on $CD1a^+$ LC. Clear histograms represent cells stained with relevant isotype matched controls.

4.10.3 Expression of Toll-like receptors on human Langerhans' cells

Surface expression of TLR2, TLR4 and TLR9 was analysed by flow cytometry on $CD1a^+$ LC purified from epidermal samples (Figure 4.19). Preliminary data from two separate donors demonstrate low level TLR2 expression on $CD1a^+$ LC. TLR4 mAb staining was only evident on $CD1a^+$LC of Donor 2. Interestingly, TLR9 expression was demonstrated on $CD1a^+$LC of both donors. CD1a-lacking cells obtained from the dissociated skin sections also demonstrated TLR2, TLR4 and TLR9 expression (Donor 2 only, n=1). Characterizations of these CD1a-negative cells were not achieved but are most probably keratinocytes.

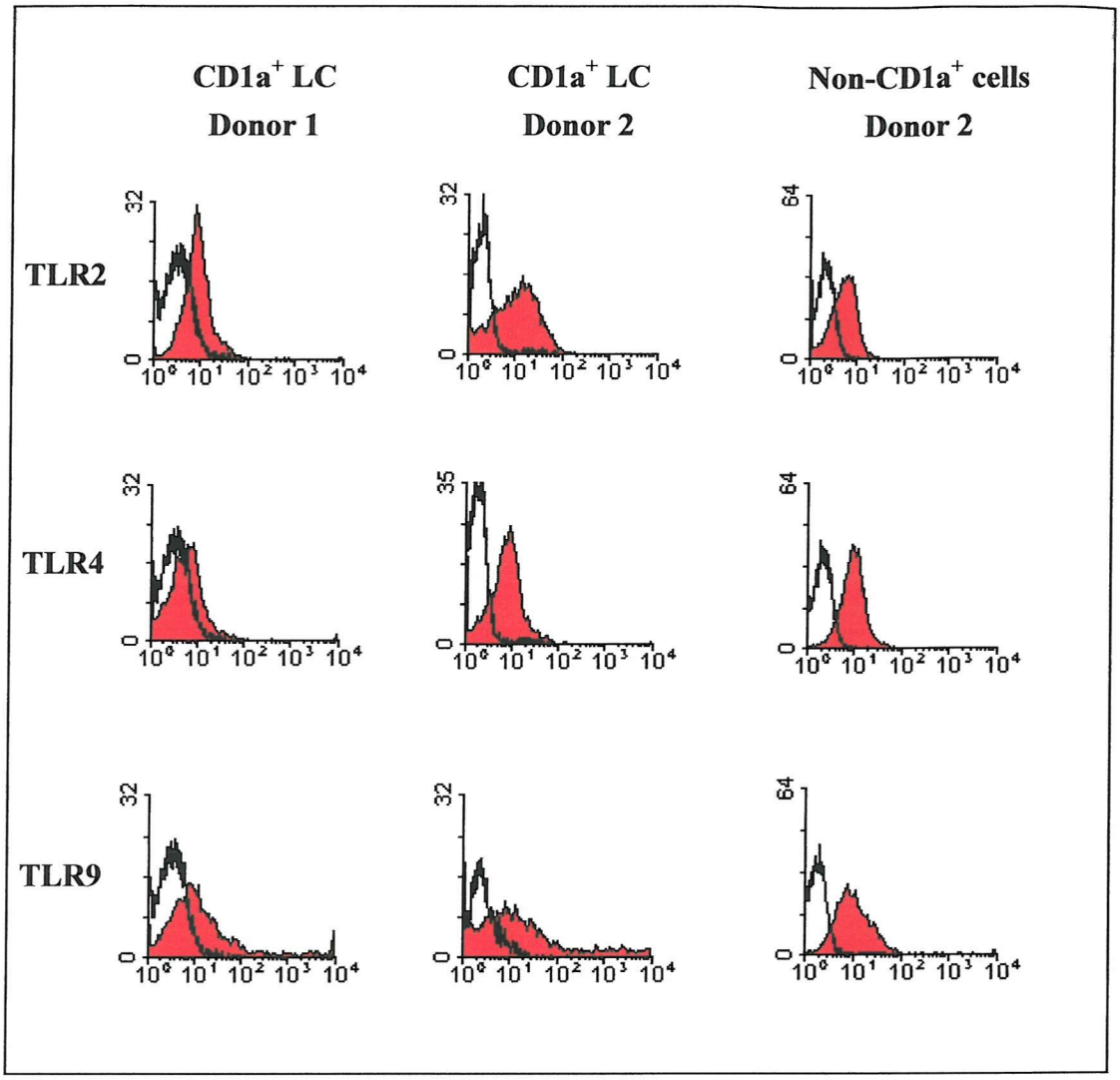

Figure 4.19: Toll-like receptor expression on human skin Langerhans' cells.

Purified LCs enriched from human epidermal skin sections by positive selection using CD1a conjugated-magnetic beads (CD1a$^+$ LC) were double labelled for CD1a (FITC) and TLR2, TLR4 or TLR9 (biotin-PE) mAbs. Dissociated epidermal skin cells lacking CD1a expression (Non CD1a$^+$ cells) were also labelled for TLR2, TLR4 or TLR9. Solid red histograms represent cells stained with mAbs. Clear histograms represent cells stained with relevant isotype matched control.

CHAPTER 4

Discussion of Results

4.11 DISCUSSION

4.11.1 Summary of Results

In contrast to findings on monocytes, human mo-DCs and mo-LCs failed to respond to CpG ODN '1668' and '2006' in terms of changes in co-stimulatory expression, production of cytokines and induction of T-cell proliferation. However, mo-DCs exposed to *E. coli* LPS or TNFα exhibited a markedly mature phenotype. Mo-DCs or mo-LCs exposed to CpG ODN failed to promote allogeneic T-cell proliferation.

Analyses of Toll-like receptor by immunostaining or TaqMan RT-PCR revealed that mo-DCs and mo-LCs clearly expressed TLR2 and TLR4 but are devoid of TLR9. In addition, $CD34^+$-derived DCs or LCs lacked surface TLR9 expression. However, preliminary results indicate the expression of surface TLR9 on $CD1a^+$ skin Langerhans' cells.

4.11.2 Effects of CpG ODN on Monocyte-derived Dendritic Cells

Murine macrophages and bone marrow-derived DCs are strongly activated by CpG ODN, undergoing maturation as reflected by the up-regulation of surface molecule expression and ability to produce high levels of various pro-inflammatory cytokines (Lipford *et al.*, 1997; Sparwasser *et al.*, 1998). However, results presented in this thesis demonstrate important differences in the responsiveness of human mo-DCs to CpG ODN in terms of phenotypic maturation and cytokine production.

Since human monocytes clearly did respond to CpG ODN (Chapter 3) but mo-DCs did not, preliminary experiments aimed to identify reagents added during the culture of mo-DCs that may reduce or impair the responses of these cells to ODN. $CD1a^+CD14^-$ mo-DCs, derived from monocytes by culturing in the presence of rhIL-13, instead of rhIL-4, and rhGM-CSF (n=3, data not shown), did not respond to CpG ODN. Omitting GM-CSF from the cultures affected differentiation into mo-DC. Furthermore, stimulating immature mo-DC and mo-LC with suboptimal doses of TNF-α (0.25ng/ml), to induce a slightly more mature phenotype (Kalinski *et al.*, 1997), did not affect the subsequent response to CpG ODN (data not shown).

Whilst no changes in surface molecule expression or cytokine production by ODN-treated mo-DCs were observed, attempts were made to see if there were changes that

may indicate active signal transduction. Flow cytometric measurement of intracellular calcium ion $[Ca^{2+}]i$ using the fluorescent indicator dye, Fluo-3, did not reveal differences in fluctuation between ODN-treated and immature mo-DCs (data not shown). Moreover, no changes in tyrosine phosphorylation following treatment with CpG ODN were detected by western blotting (data not shown).

Collectively, the lack of phenotypic changes and inability to produce cytokines following exposure to CpG ODN suggested that mo-DC/LC function was not modulated by ODN. This was further confirmed by the inability of CpG ODN treated-mo-DCs or mo-LCs to stimulate allogeneic T-cell proliferation. Results presented in this study also demonstrated that plasmid DNA had no effect on mo-DC phenotype. However, Schattenberg and colleagues have reported that human mo-DCs are potently activated by plasmid DNA (Schattenberg *et al.*, 2000). The reason for the differences between the present findings and those in Schattenberg's report are presently unclear. The plasmid vectors reported to induce mo-DC maturation and acquisition of functional capacity are different from that used in this study. Nonetheless, activation of monocytes by plasmid DNA was demonstrated in Chapter 3 of this thesis, thus showing it does possess stimulatory properties. Very low concentrations of LPS (<10pg/ml, preliminary findings) are sufficient to modify mo-DC phenotype, therefore, there is little likelihood that contamination with LPS was the cause of the observed changes in monocytes since mo-DCs were largely unaffected. In addition, preparation of plasmid DNA involved removal of free-endotoxin as directed by the manufacturers'.

The findings presented in this chapter suggest that the responsiveness to CpG ODN is altered during *in vitro* differentiation of monocytes to mo-DC. During the performance of this study, the inability of mo-DCs to respond to CpG ODN has been reported by various investigators (Krug *et al.*, 2001a; Hartmann *et al.*, 1999). The lack of mo-LC activation by CpG ODN, as demonstrated in this study, has not been described to date.

4.11.3 TLR9: Receptor for CpG DNA

The identification of TLR9 as the receptor involved in CpG ODN-mediated responses was recently published (Hemmi *et al.*, 2000). Therefore, it was crucial to determine whether the inability of mo-DCs to respond to CpG ODN was indeed due to the lack

of TLR9 receptor expression. As shown in the results, monocytes express TLR9 mRNA transcripts. However, throughout the five-day culture during which they differentiate into mo-DCs, TLR9 mRNA was markedly diminished. Absence of functional TLR9 on mo-DCs was also demonstrated by the lack of surface TLR9 expression determined by flow cytometry. The observation that TLR9-expressing monocytes (Chapter 3) are capable of responding to CpG ODN, but mo-DCs lacking TLR9 do not, does indicate the requirement for surface TLR9 in order to confer responsiveness to CpG ODN. Indeed, the expression of TLR9 in human cells has been demonstrated to confer responsiveness to CpG-containing motifs (Bauer *et al.*, 2001a; Takeshita *et al.*, 2001). It would be interesting to determine whether transfection of TLR9 into mo-DCs would subsequently result in cytokine production and changes in surface molecule expression following stimulation with CpG ODN. A recently described mRNA electroporation protocol is capable of efficiently transfecting mo-DCs with the desired antigen without modifying the cells maturation state (Van Tendeloo *et al.*, 2001). Therefore, examining changes in phenotype following CpG ODN treatment could readily assess mo-DC activation.

Although results from this study demonstrate that CpG ODN were not able to activate mo-DCs, CpG ODN can act as a strong signal for the activation and maturation of peripheral blood $CD4^+$ DC (Hartmann *et al.*, 1999). $CD4^+$ DC comprise $CD123^+$ plasmacytoid DCs. Recently, purified BDCA-4 positively-selected human plasmacytoid DCs, expressing TLR9 mRNA, have been shown to respond to CpG ODN stimulation (Hornung *et al.*, 2002). CpG ODN '2006' have been reported to promote survival, maturation and homing of plasmacytoid DC to the lymph nodes but are weak at inducing IFN-α by the plasmacytoid DCs. Hornung and colleagues have recently reported that CpG ODN does not directly activate purified monocytes (Hornung *et al.*, 2002). The authors suggested that indirect activation of monocytes is brought about by the production of cytokines from low numbers of plasmacytoid DCs, present within the monocyte populations, following CpG ODN treatment (Hornung *et al.*, 2002). In this project monocytes were obtained by plastic adherence, therefore, it is feasible that low numbers of contaminating plasmacytoid DCs were present in the cultures during treatment contributing to the production of cytokines detected by ELISA. A separate report demonstrated increased TNFα, IL-12 and IL-6 production by purified monocytes treated with CpG ODN (Bauer *et al.*, 2001b).

Nonetheless, in this thesis assessment of monocyte phenotype following CpG treatment was performed by flow cytometry on HLA-DR^+ -gated monocytes. Use of CD14 as a marker for monocytes was not favourable in this thesis due to the marked decrease in CD14 expression following treatment with ODN (Chapter 3). BDCA-4 is expressed on fresh plasmacytoid $CD123^{bright}$ $CD11c^-$ DCs but not on monocytes (Dzionek *et al.*, 2000). Although Hornung and colleagues report that CpG ODN does not activate purified monocytes, they have demonstrated basal levels of TLR9 mRNA in these cells (Hornung *et al.*, 2002). Purified myeloid DCs are not directly activated by CpG ODN, which fits with the described lack of TLR9 mRNA transcripts in these cells (Hartmann *et al.*, 1999; Kadowaki *et al.*, 2001b). Although, DCs grown from $CD34^+$ progenitors in the absence of IL-4 are suggested to be responsive to CpG ODN (Bauer *et al.*, 2001b). The lack of TLR9 surface expression on $CD1a^+$ $CD34^+$-DCs as shown from results in this study would suggest that these cells do not become activated in the presence of CpG ODN.

Actions of CpG ODN in murine models are well described. However, there are clear differences in how murine and human dendritic cells respond to CpG ODN. These appear to relate to the expression of TLR9 on these cells. Moreover, the distinct expression patterns of TLR9 on different DC lineages and on other APCs suggests different roles played by these cells in the recognition and responses to CpG DNA present within the environment.

4.11.4 TLR expression on Langerhans' cells

Langerhans' cells play a significant role in the skin and form the first line of defence against invading pathogens. As a result, it is conceivable that epidermal LCs express TLRs, although this is not thoroughly documented. TLR3, involved in the recognition of dsRNA, was initially described as being exclusively expressed by human DC (Alexopoulou *et al.*, 2001; Muzio *et al.*, 2000b). However, skin LCs are reported not to express TLR3 mRNA transcripts (Muzio *et al.*, 2000b). More recently TLR3 mRNA was reported to be expressed in human natural killer cells and absent in plasmacytoid DCs, thus, challenging the data suggesting sole expression of TLR3 on DCs (Hornung *et al.*, 2002).

Results observed in this study on the differential expression of TLR2, TLR4 and TLR9 on mo-DC and $CD34^+$-DCs were extended to examine whether mo-LCs and

$CD34^+$-LCs express these receptors. Mo-LCs expressed low levels of TLR2 and TLR4. In contrast, moderate expression of TLR4 only was observed on $CD34^+$-LCs. As demonstrated for mo-DCs and $CD34^+$-DCs, TLR9 was lacking on both subtypes of cultured-LCs suggesting that these cells would not be responsive to CpG ODN. Preliminary data presented in this thesis demonstrate TLR9 expression on purified human skin LCs by flow cytometry, thus suggesting that these cells might respond to CpG ODN. TLR2 was also expressed on epidermal LCs whereas TLR4 expression remains inconclusive at present due to low sample numbers. Further work will be needed for confirmation of TLR9 expression on LCs. It is conceivable that suction blistering might result in the undesired activation of LCs that in turn affects TLR expression. It would be expected that LC activation would result in increased expression of HLA-DR and co-stimulatory molecules CD80 and CD86. However, CD80/CD86 expression was not detected on $CD1a^+$ LCs, and moderate levels of HLA-DR were demonstrated suggesting a relatively immature phenotype.

Distinct levels of maturation were exhibited by mo-DCs and mo-LCs in response to LPS or TNF-α. Increased expression of co-stimulatory molecules and HLA-DR and cytokine production were less pronounced in LPS or TNFα-treated mo-LCS. TGF-β1 is reported to inhibit LC maturation in response to TNF-α, IL-1 and LPS, but does not affect CD40-mediated maturation (Geissmann *et al.*, 1999). Whilst CpG ODN exposed mo-LCs were not able to drive proliferation of allogeneic T-cells, activation of mo-LCs by TNF-α and LPS moderately enhanced T-cell proliferation (n=2).

4.11.5 Activation of mo-DC by lipopolysaccharide

LPS of Gram-negative bacteria is a potent maturation stimulus for DCs (Verhasselt *et al.*, 1997; Langenkamp *et al.*, 2000). The results presented in this study illustrate differences in the activation status of human mo-DC induced by LPS compared to CpG ODN, *in vitro*. Changes in mo-DC phenotype were observed following exposure to LPS, which included enhanced surface expression of HLA-DR and co-stimulatory molecules. Increased expression of the DC maturation marker CD83 was also observed following LPS treatment. Absence of surface CD14 is characteristic of mo-DCs differentiated from monocytes, *in vitro,* in medium containing FCS supplemented with rhIL4 and rhGM-CSF. IL-4 mediates down-regulation of CD14 expression via decreased transcription of CD14 mRNA. LPS is a putative ligand for

CD14 on monocytes (Wright, 1995). However, the lack of CD14 expression does not affect LPS-mediated responses on mo-DCs suggesting the involvement of other receptors capable of initiating signalling. TLR4 is reported to play a principal role for LPS-recognition by human and murine macrophages (Beutler, 2000; Weinstein *et al.*, 1993). Additionally, human mo-DCs are reported to respond to LPS by a soluble CD14-dependent pathway (Verhasselt *et al.*, 1997).

Results from this study agree with recent published observations that mo-DCs express mRNA transcripts for TLR4 (Visintin *et al.*, 2001; Jarrossay *et al.*, 2001). Moreover, data presented in this thesis reveals surface expression of functional TLR4 on $CD1a^+$ mo-DCs as determined by flow cytometry. Visintin and colleagues have reported that immature mo-DCs do not express very high levels of TLR4, shown by the lack of mAb staining, but do express TLR4 mRNA transcripts (Visintin *et al.*, 2001). Those authors also suggest that TLR4 expression on mo-DCs may be regulated during activation with LPS demonstrated by transient increase in TLR4 mRNA levels observed early after maturation which decreases following longer culture periods (Visintin *et al.*, 2001). Interestingly, similar down-regulated TLR2 levels were also observed after LPS stimulation of mo-DCs. In addition, these patterns of TLR2 and TLR4 expression were also noted in mo-DCs matured with non-microbial stimuli, namely TNFα (Visintin *et al.*, 2001). The TLR4 mAb used in this study is different to that used in Visintin's study, and showed low intensity of staining on immature mo-DCs.

The observed absence of surface CD14 suggests that low levels of functional TLR4 expression correlates with the activation of immature mo-DCs by LPS. However, the presence of TLR2 on immature mo-DCs, as shown in the results, cannot exclude the possibility of this receptor also participating in the activation of mo-DCs by LPS. Involvement of TLR2 together with lipopolysaccharide binding protein has been implicated in transducing LPS-mediated activation (Kirschning *et al.*, 1998; Yang *et al.*, 1998). However, this role of TLR2 remains controversial due to contaminating lipoproteins within commercially available LPS preparations probably signalling through TLR2 (Hirschfeld *et al.*, 2000). Nonetheless, structural variants of LPS from *Leptospiral interrogans* or *Porphyromonas gingivalis* have been suggested to activate cells through a TLR2 dependent mechanism (Werts *et al.*, 2001; Hirschfeld *et al.*, 2001). In addition, studies using macrophages have suggested the possibility that

TLR2 co-operates with TLR4 when the latter receptor is underexpressed to strengthen LPS-derived signals (Beutler *et al.*, 2001; Sato *et al.*, 2000).

Differences in TLR expression on mo-DCs appear to dictate the nature of the immune response instigated by this cell in response to different microbial products. Only, mo-DCs from normal volunteers were used in this study. Thus, future evaluations on TLR patterns and intensity of expression on mo-DCs from atopic donors may disclose differences in the manner in which these cells respond to various stimuli when allergen is present in the environment.

In summary, as monocytes differentiate into mo-DCs or mo-LCs there is a loss of expression of TLR9 at the message and protein levels. This is associated with a loss of responsiveness of these cells to CpG ODN, reflected by the lack of phenotypic maturation and inability to produce cytokines as well as to drive T-cell responses. As a result of the present observations it is clear that mo-DCs do not serve as a useful model for the study of CpG ODN action. In turn, the attempts to examine whether CpG ODN could modulate mo-DC function to influence the *in vitro* differentiation of allergen-specific $CD4^+$ T-cells from Th2 to Th1 cells could not be performed. Preliminary finding in this thesis that human skin LCs expresses surface TLR9 disputes the current belief that CpG responsive-DCs arise from lymphoid progenitors.

CHAPTER 5

Effects of *Neisseria meningitidis* on Human Monocyte-derived Dendritic Cells: Stimulatory Properties of Meningococcal Outer Membranes

5

5.1 INTRODUCTION

Serogroup B *N. meningitidis* is responsible for the majority of meningococcal disease occurring worldwide for which currently there is no effective vaccine (van Deuren *et al.*, 2000). Vaccines based on the capsular polysaccharides of serogroups, A, C,Y and W-135 meningococci have been developed and are able to confer protection against disease. However, the polysaccharide of serogroup B meningococci is poorly immunogenic and is cross-reactive with components found in human neural tissue and fetal antigens (Finne *et al.*, 1983).

During the course of meningococcal disease, the growth and lysis of meningococci releases outer membrane (OM) vesicles, which contain a significant amount of membrane proteins as well as lipo-oligosaccharide (LOS). These OM vesicles have been observed in the blood and cerebrospinal fluid (CSF) of patients with meningococcal disease (Stephens *et al.*, 1982). Development of therapeutic strategies against serogroup B meningococci have focused on the immunogenicity of purified OM (Fischer *et al.*, 1999; Fukasawa *et al.*, 1999; Haneberg *et al.*, 1998; Naess *et al.*, 1998). LOS is known to possess potent adjuvant effects that would strengthen the immune response directed against OMs (Quakyi *et al.*, 1997). Yet, the LOS present within the OM would be likely to result in unacceptable side effects and toxicity in humans, (Fischer *et al.*, 1999; Fukasawa *et al.*, 1999; Haneberg *et al.*, 1998; Naess *et al.*, 1998). Various approaches have been attempted, such as detergent extraction to remove LOS from the OM for the development of more favourable vaccines. However, use of detergent is not completely efficient and is also likely to alter the composition and balance of protein antigens within the OM. The recent identification of a viable, LOS-deficient mutant of *N. meningitidis*, generated by targeted deletion of the genes involved in LOS biosynthesis (Steeghs *et al.*, 1998), offers the possibility of preparing new OM vaccines without resort to modification with detergents.

Immature DCs reside in various organs, including the respiratory tract where they encounter microbial organisms (Julia *et al.*, 2002; McWilliam *et al.*, 1996). It is feasible that early during infection, meningococci and surplus OM released into the environment interact with DC resident in the nasopharyngeal mucosa. Therefore, the objectives of this study were to examine the effects of purified OM from wild type

H44/76 and LOS-deficient mutant of serogroup B *N. meningitidis* on their ability to initiate specific immune responses by human monocyte-derived dendritic cells (mo-DC).

The consequences of co-culturing mo-DCs with viable wild type or LOS-deficient mutant of *N. meningitidis,* in terms of cytokine, chemokine and nitric oxide (NO) production by the cells in response to bacterial challenge, was determined. The results presented in the second half of this chapter examines and compares the effects of OM from wild type H44/76 and LOS-deficient mutant of *N. meningitidis* serogroup B on the response of mo-DCs. Following recognition of microbial antigens, DCs undergo maturation resulting in an augmented capacity to prime T-cells (Langenkamp *et al.*, 2000; Cella *et al.*, 1997b; de Jong *et al.*, 2002). Differential changes in expression of MHC class II and co-stimulatory molecule expressions on mo-DCs stimulated with OM from either of these strains was determined. In addition, cytokine and chemokine production, modulation of TLR mRNA expression and receptor-mediated endocytosis were used as markers of activation and maturation of mo-DCs. Finally, the ability of OMs from either of these strains to modulate mo-DC function in order to promote the induction of allogeneic $CD4^+$ or primary T-cell responses was investigated.

5.2 MATERIALS AND METHODS

5.2.1 *N. meningitidis* outer membranes

Culture of bacteria and purification of outer membranes from either variant or meningococcal lipo-oligosaccharide was performed by Dr. M. Christodoulides (University of Southampton). The *N. meningitidis* strain H44/76 (B:15:P1.7,16: $Cap^+Pil^+LPS^+Opa^+Opc^+$), subtype P1.7,16 reference strain, and the LOS-deficient mutant *N. meningitidis* strain H44/76 pLAK33 ($Cap^+Pil^+LPS^-$) were used in these studies (Steeghs *et al.*, 1998; Frasch *et al.*, 1985). All strains were grown on proteose-peptone agar at 37°C for 18h in an atmosphere of 5%(v/v) CO_2. Outer membranes (OM) were prepared from both strains by extraction of whole cells with lithium acetate as described previously (Tinsley and Heckels, 1986). LOS was purified from *N. meningitidis* strain MC58 (B:15:P1.7,16b: $Cap^+Pil^+LPS^+Opa^+Opc^+$) by extraction with hot phenol as described previously (Lambden and Heckels, 1982).

5.2.2 Challenge of mo-DCs with viable *N. meningitidis*

Mo-DC were cultured in phenol-free RPMI 1640 medium containing 10% (v/v) FCS and 2mM glutamine, but without antibiotics. After five days of culture, approximately 1×10^4 colony forming units (cfu) of either wild type H44/76 or LOS-deficient mutant pLAK33 were added to the wells. Challenge experiments were maintained for up to 48hr at 37 °C and 5% (v/v) CO_2. Viability of each bacterial strain in the presence of mo-DCs at different intervals was determined by centrifuging (13,000 rpm, 5 minute) the cultures and lysing the mo-DC/bacteria pellets by the addition of saponin followed by incubation for 15 minute at 37 °C (Virji *et al.*, 1991). The total bacterial colony forming units (cfu) was then quantified by colony counting.

5.2.3 Nitric oxide production

Cell and bacteria-free supernatants obtained following challenge of mo-DCs with viable bacteria were stored at –80°C. Nitric oxide (NO) secretion by mo-DCs into supernatants was detected, in duplicate, using a nitric oxide analyser NOA™ 280 as described (2.2.10). Concentration of NO (μM) was extrapolated from a standard curve as described (2.2.10).

5.2.4 Treatment of mo-DCs with *N. meningitidis* outer membrane

Mo-DCs used in studies examining changes in surface phenotype or receptor-mediated endocytosis were obtained by culturing purified monocytes (2.2.1.2 and 2.2.1.3) for five days in phenol red-free RPMI 1640 medium supplemented with 10% FCS, 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin and recombinant cytokines IL-4 and GM-CSF (2.2.2). To obtain mo-DC for subsequent functional assays with T-cells, mo-DCs were differentiated from purified monocytes for five days in supplemented medium containing 2% (v/v) pooled human AB serum, to minimize high background proliferation.

Immature 5-day $CD1a^{+}CD14^{-}$ mo-DCs were exposed to outer membranes from wild type H44/76 strain (OM) or LOS-deficient mutant pLAK33 H44/76 strain (pLAK-OM). The activity of pure LOS, isolated from MC58, on mo-DCs was also assessed. The dose of treatment varied within studies (maximum concentration 1μg/ml). Optimal length of treatment to observe changes in mo-DC phenotype and production of soluble mediators was 24hrs.

5.2.5 Surface phenotype and receptor-mediated endocytosis

Mo-DCs were harvested from culture dishes by gentle pippeting to avoid cellular activation. Certain treatments resulted in mo-DCs adhering strongly to plastic. In these cases, culture dishes were incubated on ice for up to 20 minutes to aid detachment of cells. Fc-receptors on cells were blocked (2.2.7.1) prior to staining for cell surface molecules using fluorescent conjugated antibodies (2.1.5). Expression of CD1a (FITC), CD14 (PE), CD83 (PE), CD80 (PE), CD86 (PE), CD40 (PE), HLA-DR (PE) and CD54 (FITC) was assessed by flow cytometry (2.2.7.5). Appropriate fluorochrome conjugated isotype control antibodies were also used in parallel (2.1.5).

For analysis of receptor-mediated endocytosis of FITC-dextran, 5-day mo-DCs were left untreated or exposed for a further 24hrs to *N. meningitidis* outer membranes (OM or pLAK-OM) or pure LOS. Cells were harvested, washed by centrifugation and incubated with 10% FCS supplemented medium containing 250μg/ml FITC-dextran for 1hr at 37°C or 4°C. The level of FITC-dextran uptake was assessed by flow cytometry (2.2.8.2).

During flow cytometric analysis cells were collected within a viable forward side scatter gate together with the exclusion of dead cells using 7-AAD.

5.2.6 Measurement of Toll-like receptor mRNA expression on mo-DCs

Following treatment of mo-DCs, total RNA was extracted using the RNeasy kit method (Qiagen) and treated with DNAse I (RNAse-free DNAse, Promega) according to the manufacturers' instructions. cDNA was prepared from 400ng of total RNA using the Omniscript reverse transcriptase preamplification system (Promega) with random hexamer primers (Promega). The cDNA levels of TLR2 and TLR4 were quantified by TaqMan PCR using an ABI prism 7700 sequence detector according to the manufacturer's instructions. The cDNA levels during the linear phase of amplification were normalized against ribosomal 18s (Pre-Developed Assay Reagent, PE Applied Biosystems). Relative RNA concentrations were extrapolated using a PBMC standard curve. Probes and primers used are listed in Table 2.8 and 2.9. Results are presented as fold increase in relative mRNA levels in treated cells compared to control cells.

5.2.7 Measurement of cytokine or chemokine production by Mo-DCs

Following treatment of mo-DCs with viable whole *N. meningitidis,* purified outer membranes or pure LOS supernatants were harvested and stored at –80°C pending analysis. Cytokine or chemokine secretion into supernatants was assessed by specific immunoassay using monoclonal matched pair antibodies, according to manufacturers instructions (2.1.6). Where necessary, supernatants were diluted 1:10 – 1:50 in the appropriate assay buffer in order for the concentration to fall within the standard curve. Production of soluble mediators was calculated by relating the absorbance values of duplicate wells to the appropriate standard curve.

5.2.8 Mo-DC driven T-cell proliferation assays

T-cells were obtained from human peripheral blood by counter-current elutriation (2.2.1.2). Purified $CD4^+$ or naïve $CD4^+CD45RA^+$ T-cells were used in allogeneic mixed leukocyte reactions (MLR) experiments. Purity of $CD4^+$ or naïve T-cells was assessed by flow cytometry for every experiment. The mo-DC/T-cell proliferation assay medium consisted of phenol-red RPMI 1640 medium supplemented with 5% (v/v) pooled human AB serum, 2mM glutamine, 100U/ml penicillin and 100μg/ml

streptomycin, 1%(v/v) sodium pyruvate, 0.05 M 2-mercaptoethanol and 2mM HEPES. Purified $CD4^+$ or naïve T-cells ($1x10^5$ cells) were co-cultured with graded numbers of irradiated treated or control mo-DCs in 96-well plates. Proliferation assays were maintained at 37°C in humidified atmosphere containing 5% CO_2. After five day co-culture, proliferation was assessed by incorporation of H^3-thymidine (0.5μCi / well) for the last 18hrs (2.2.4). Results are presented as T-cell proliferation in counts per minute (CPM) or as stimulation indices (SI) derived using the equation below.

$$SI = \frac{\text{mo-DC treatment CPM} - \text{mo-DC control CPM}}{\text{mo-DC control CPM}}$$

5.2.9 Polarization of naïve T-cells

Wells of a 48-well plate were coated overnight with 1μg/mL anti-CD3 (clone OKT3, in house) made up in carbonate/bicarbonate buffer (Sigma). Treated or control untreated irradiated mo-DCs were co-cultured with purified allogeneic naïve T-cells (1:10 ratio of DCs to T-cells) in 48-well plates in a total of 1mL proliferation assay medium (see above). After four days, cells were harvested and $1x10^5$ cells/mL were plated onto anti-CD3 pre-coated wells for a further 24hrs. Supernatants were harvested and stored at –80°C pending analysis of IFN-γ and IL-13 production by ELISA.

CHAPTER 5

Results: Section I

Effect of *N. meningitidis* on Production of Soluble Mediators by Mo-DCs

5.3 INTERACTION OF Mo-DCs WITH VIABLE *N. meningitidis*

5.3.1 Viability of mo-DC or bacteria after co-culture

Mo-DCs were challenged with a wild type *N. meningitidis* strain H44/76 or with a LOS-deficient mutant derived from this strain, H44/76 pLAK33. The survival of the two meningococcal strains in the presence of mo-DC, over time, was assessed. The wild type H44/76 strain grew in the mo-DC cultures (Figure 5.2A) and analysis by confocal microscopy confirmed the presence of large numbers of bacteria in association with the cells (Figure 5.1). In contrast, decreasing numbers of viable pLAK33 LOS-deficient mutant bacteria were found in culture supernatants after challenge (Figure 5.2A). Confocal microscopy confirmed the absence of the mutant bacteria in these cultures (data not shown). Following challenge with either bacterial strain the viability of mo-DCs was investigated using a cell viability assay (LIVE/DEAD Molecular Probes) and confocal microscopy (data not shown). Twenty four hours after challenge with either variant, the viability of activated mo-DC, which had become adherent to the culture well, was greater than 95%.

5.3.2 Nitric oxide production by mo-DCs

One of the important anti-bacterial cytotoxic mechanisms used by leukocytes is the production of nitric oxide (NO) (MacMicking *et al.*, 1997). Therefore, the ability of mo-DCs to produce NO in response to challenge with *N. meningitidis* was investigated. Constitutive production of NO by untreated immature mo-DCs increased about 4-fold over the 48 hr culture period (Figure 5.2B). Following challenge of mo-DCs with the LOS-deficient mutant, NO release was similar to that of control cells during the course of the experiments. However, 9 hr after challenge with wild type H44/76 meningococci, there was significant reduction of NO release ($p<0.05$, Student t-test) (Figure 5.2B).

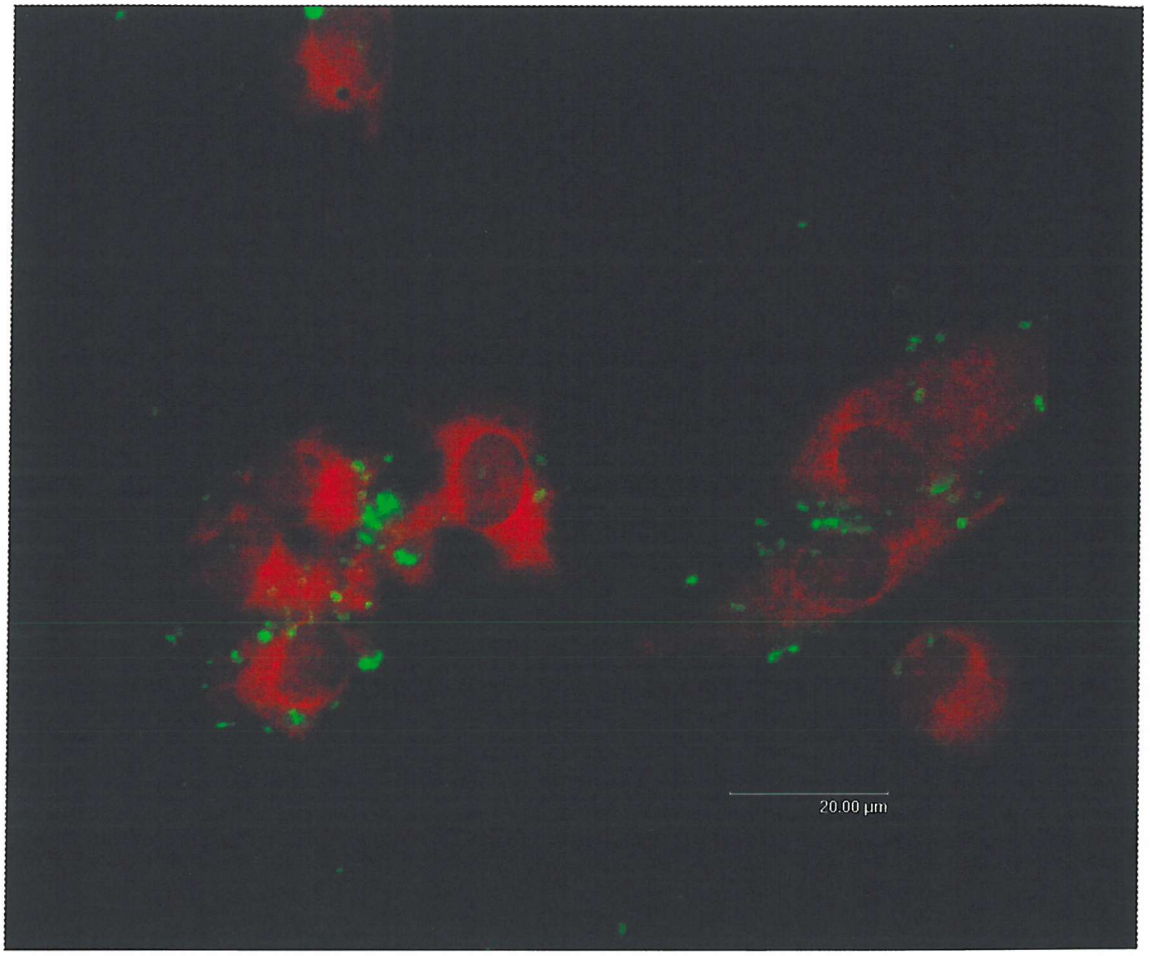

Figure 5.1: Confocal microscopy illustrating contact between *N. meningitidis* and human mo-DCs.

5-day cultured mo-DCs, grown in the absence of antibiotics, were challenged with 10^4 CFU viable bacteria for 24hrs. After treatment, bacteria in suspension were washed away with PBS. Cells were fixed with acetone for 10 minutes, followed by a 1hr block with 10% (v/v) normal rabbit serum (NRS) in PBS. Bacteria were labelled with polyclonal mouse-anti-outer membrane antibody (1:1000 in 10% (v/v) NRS/PBS) for 1hr at 37°C. Following thorough washing, rabbit anti-mouse FITC-conjugate (1:100 in 10% (v/v) NRS/PBS) was added for 1hr at room temperature in the dark. Mo-DCs were counterstained with Evans blue (0.0025 % (v/v) in PBS) for 20 minutes at 37°C. Cultures were washed thoroughly, mounted and viewed by confocal microscopy (n=3). Apart from culture of mo-DCs, Dr. M. Christodoulides performed these experiments (in collaboration).

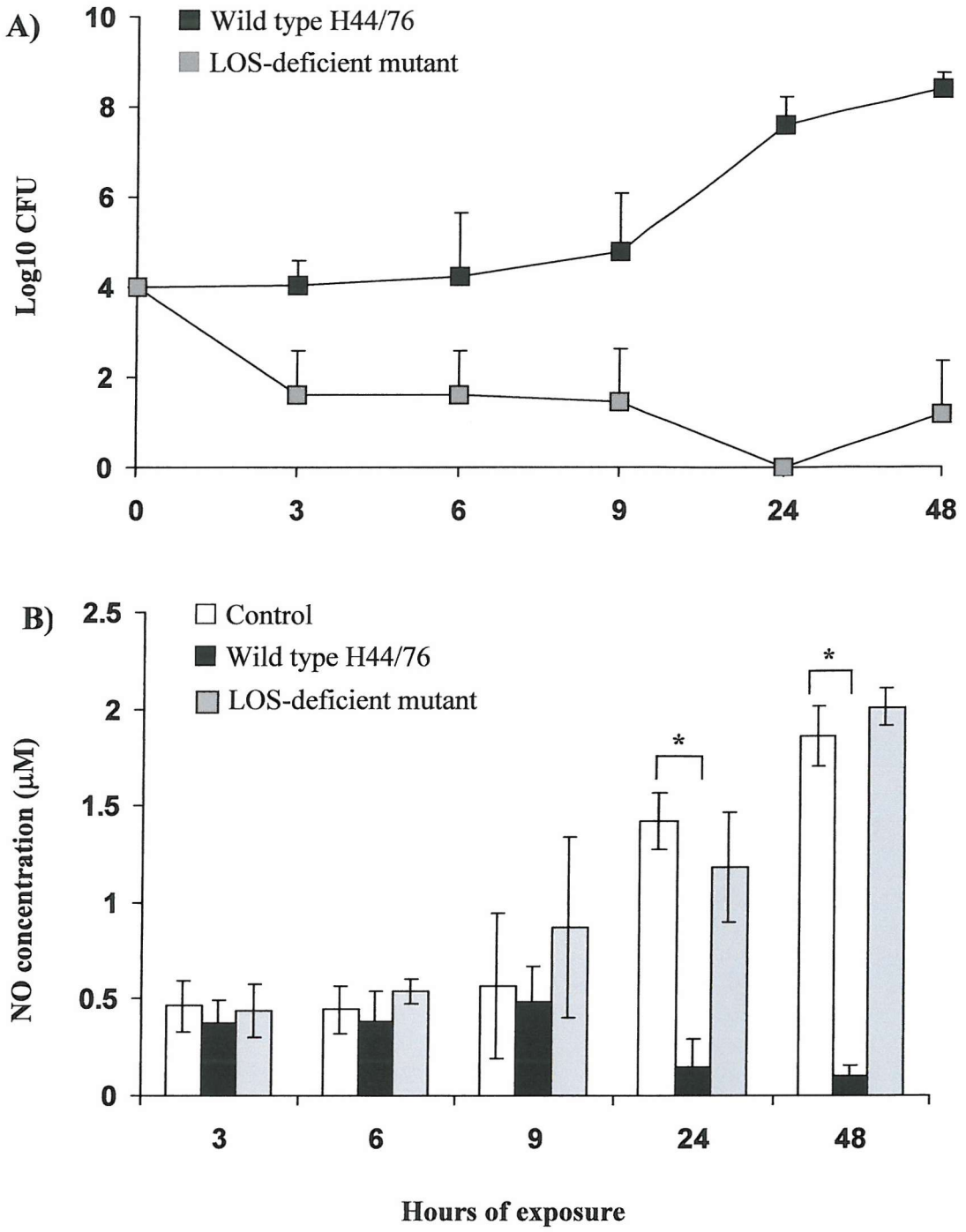

Figure 5.2: Interactions of mo-DC with *N. meningitidis*.

A) Mo-DCs were challenged with 10^4 viable wild type H44/76 bacteria or LOS-deficient mutant pLAK33 bacteria for varying lengths of time. Data represents average bacteria numbers in supernatants ± standard errors (n=3).

B) Concentration of NO released into supernatants by mo-DC throughout culture with viable wild type H44/76 or LOS-deficient mutant bacteria. Data represents average production ± standard error bars (n=3). *=Significance observed ($p<0.05$, Student t-test) compared to control.

5.3.3 Production of cytokines and chemokine by mo-DCs challenged with *N. meningitidis*.

The production levels of IL-1β, IL-6, IL-10, TNFα, IL-12p40 and IL-12p70 (Figure 5.3) by mo-DCs was assessed following challenge with the viable parental H44/76 and LOS-deficient mutant pLAK33. Variation in cytokine production was observed by mo-DCs derived from different donors in response to the bacteria. Following treatment with wild type H44/76 strain, there was a time-dependent increase in production of all cytokines measured. Significant levels ($p<0.05$, Student t-test) of IL-6 and TNFα are observed as early as 9hrs post-infection, and maximal levels of IL-6, IL-10, IL-12p40, IL-12p70 and TNFα were seen 24hrs after treatment, reaching plateau levels by 48hrs. However, highest concentrations of IL-1β were detected after 48hrs. In contrast to the wild type strain, challenge with the LOS-deficient mutant induced production of significantly lower levels of cytokines by mo-DC (Figure 5.3). After 24hrs of exposure, mo-DCs treated with the LOS-deficient strain produced 12-fold less TNFα, 4-fold less IL-6 and 5-fold less IL-12p40. Biologically active IL-12p70 was undetectable in supernatants from mo-DCs treated with the LOS-deficient mutant bacteria.

Following challenge, supernatants were also assayed for production of IL-8, MIP1α, MIP1β and RANTES (Figure 5.4). Chemokines were released by mo-DCs cultured with the wild type strain in a time-dependent fashion. Significant levels of MIP-1β were detectable as early as 9hrs with maximal levels observed 48hrs post-infection. However, significant production of RANTES and MIP-1α occurred at 24hrs and 48hrs, respectively. Treatment of mo-DCs with LOS-deficient bacteria induced significant ($p<0.05$, Student t-test) levels of RANTES and MIP-1α after 48hrs, whereas moderate ($p>0.05$) increases in MIP-1β were observed at 24hrs compared to untreated control mo-DCs. Markedly lower levels of RANTES, MIP-1α and MIP-1β were produced by mo-DC following exposure to LOS-deficient bacteria compared to cells treated with the wild type strain. In contrast, there was no significant difference in secretion of IL-8 over time from mo-DCs challenged with either meningococcal strain (Figure 5.4).

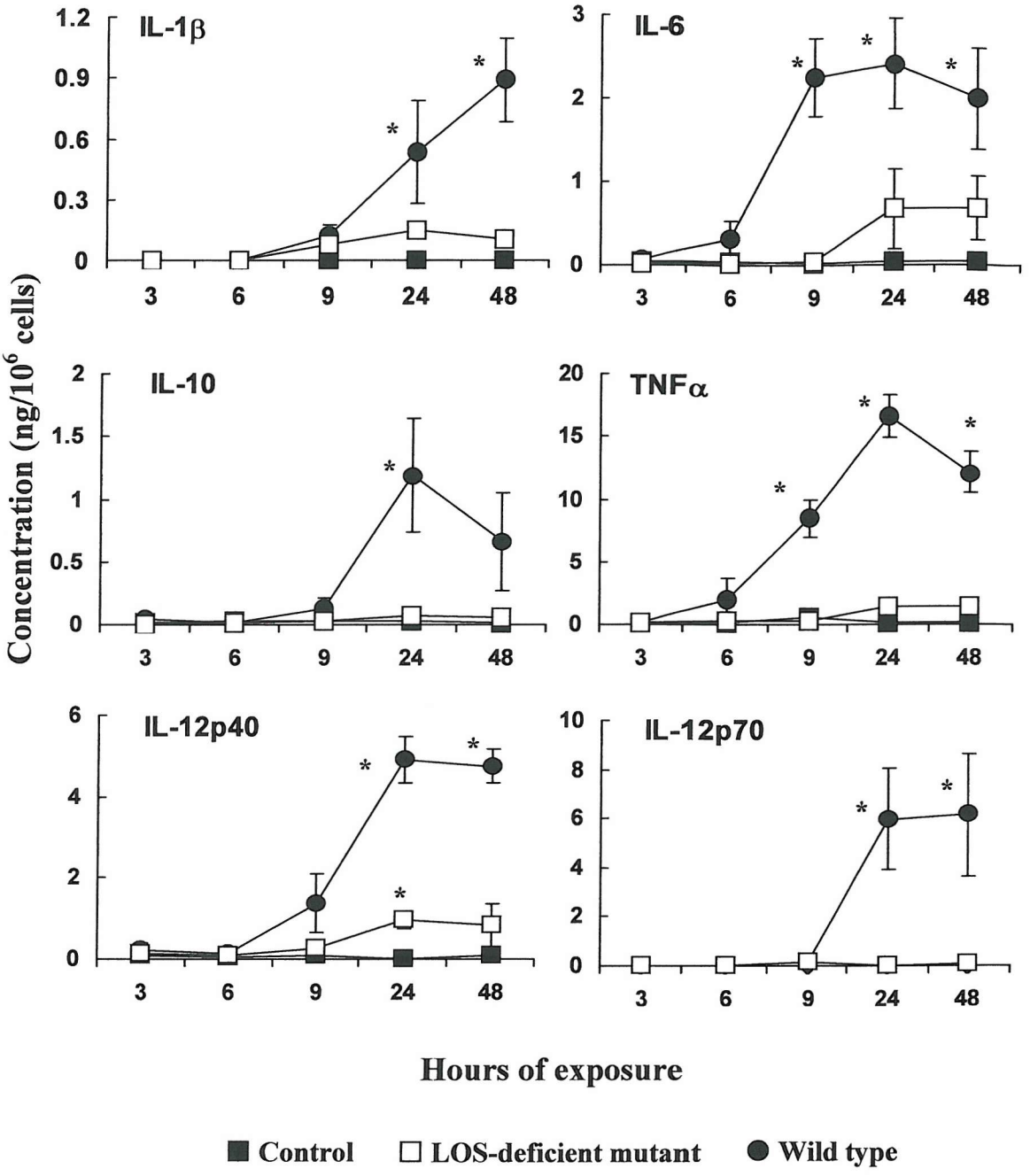

Figure 5.3: Cytokine production by mo-DC following challenge with viable *N. meningitidis.*

Mo-DCs exposed to live serogroup B *Neisseria meningitidis*: H44/76 wild type strain or LOS-deficient mutant. Control mo-DCs were left untreated. $1x10^5$ cells were inoculated with bacteria (starting seeding number 10^4 CFU) for indicated times. Cytokines were detected in supernatants by specific immunoassay. Results are presented as mean ± standard error (n=4). * = Significant change ($p<0.05$, Student t-test) compared to control cells.

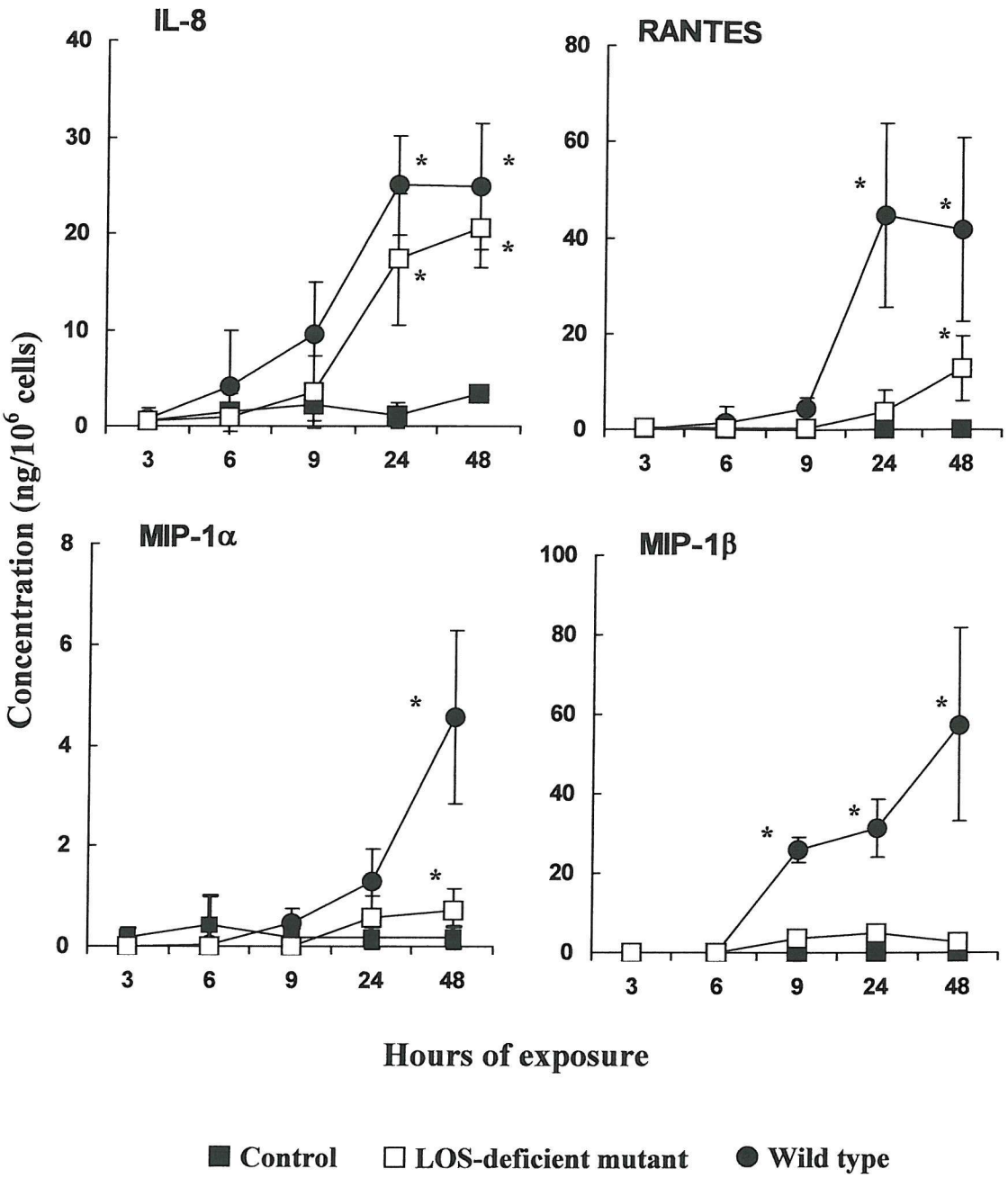

Figure 5.4: Chemokine production by mo-DC following challenge with viable *N. meningitidis.*

Mo-DCs exposed to live serogroup B *Neisseria meningitidis*: H44/76 wild type strain or LOS-deficient mutant. Control mo-DCs were left untreated. $1x10^5$ cells were inoculated with bacteria (starting seeding number 10^4 CFU) for indicated times. Chemokines were detected in supernatants by specific immunoassay. Results are presented as mean ± standard error (n=4). * = Significant change ($p<0.05$, Student t-test) compared to control cells.

CHAPTER 5

Results: Section II

Effect of *N. meningitidis* Outer Membranes on Phenotype and Function of Mo-DCs

5.4 EFFECT OF OUTER MEMBRANES ON Mo-DC ACTIVATION

5.4.1 Activity of outer membranes on Mo-DC phenotype

The ability of *N. meningitidis* outer membranes to induce activation of mo-DCs was assessed by examining phenotypic changes following treatment. Mo-DCs incubated for 24hrs with purified outer membranes from *N. meningitidis* wild type H44/76 strain (Figure 5.5C) acquired distinct morphological changes shown by pronounced dendrite formation and increased cellular clumping, as observed by phase contrast microscopy, reflecting an activated phenotype compared to cells treated with vehicle alone (Figure 5.5A). Similar changes were observed for mo-DCs stimulated for 24hrs with purified LOS (Figure 5.5B). The morphology of mo-DCs stimulated with outer membranes from LOS-deficient mutant *N. meningitidis* H44/76 pLAK33 (pLAK-OM, Figure 5.5D) was comparable to vehicle-treated mo-DCs (Figure 5.5A), although slightly more veiled cells were observed.

After 24hrs of exposure, mo-DCs were collected and analyzed by flow cytometry for expression of cell surface molecules (Figure 5.6). Exposure of mo-DCs to 1μg/ml wild type OM or pure LOS resulted in marked elevation in expression DC maturation marker, CD83, co-stimulatory molecules CD80, CD86 and CD40, HLA-DR, and adhesion molecule CD54 (ICAM-1). In contrast, comparable amounts of pLAK-OM only induced minor up-regulation of CD86 and MHC class II molecules. Summary of phenotypic changes following treatment with outer membranes or pure LOS is presented as median fluorescence intensity in Figure 5.7.

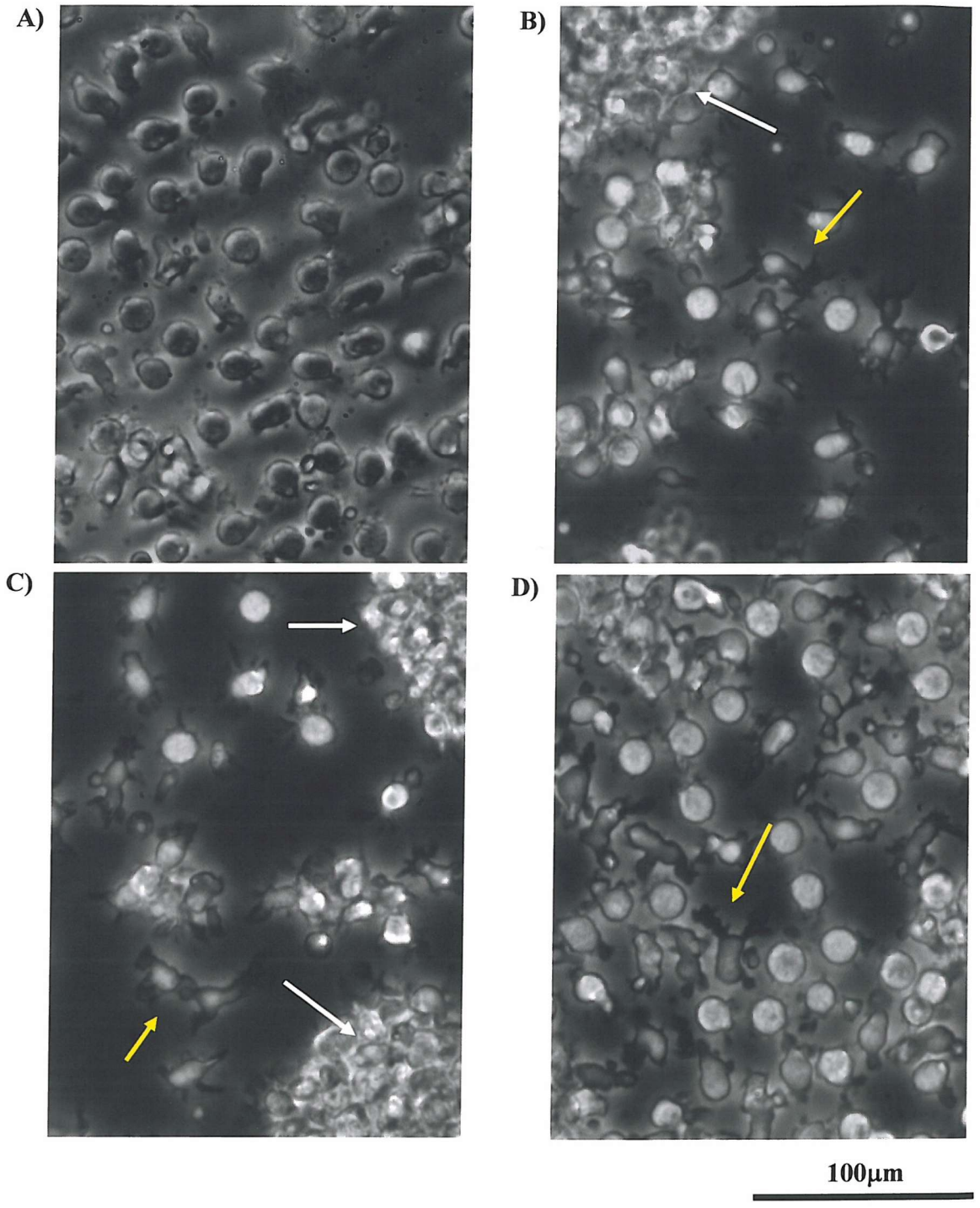

Figure 5.5: Morphology of mo-DCs following treatment with outer membranes from wild type or LOS-deficient mutant *N. meningitidis*.

Five-day cultured mo-DCs were A) left untreated (control), or stimulated for 24hrs with 1µg/ml of B) pure LOS, C) wild type OM or D) LOS-deficient OM. Cells were viewed by phase contrast at x20 magnification. White arrows indicate clustering of mo-DCs, yellow arrow show greater veiled dendrites (compared to control cells).

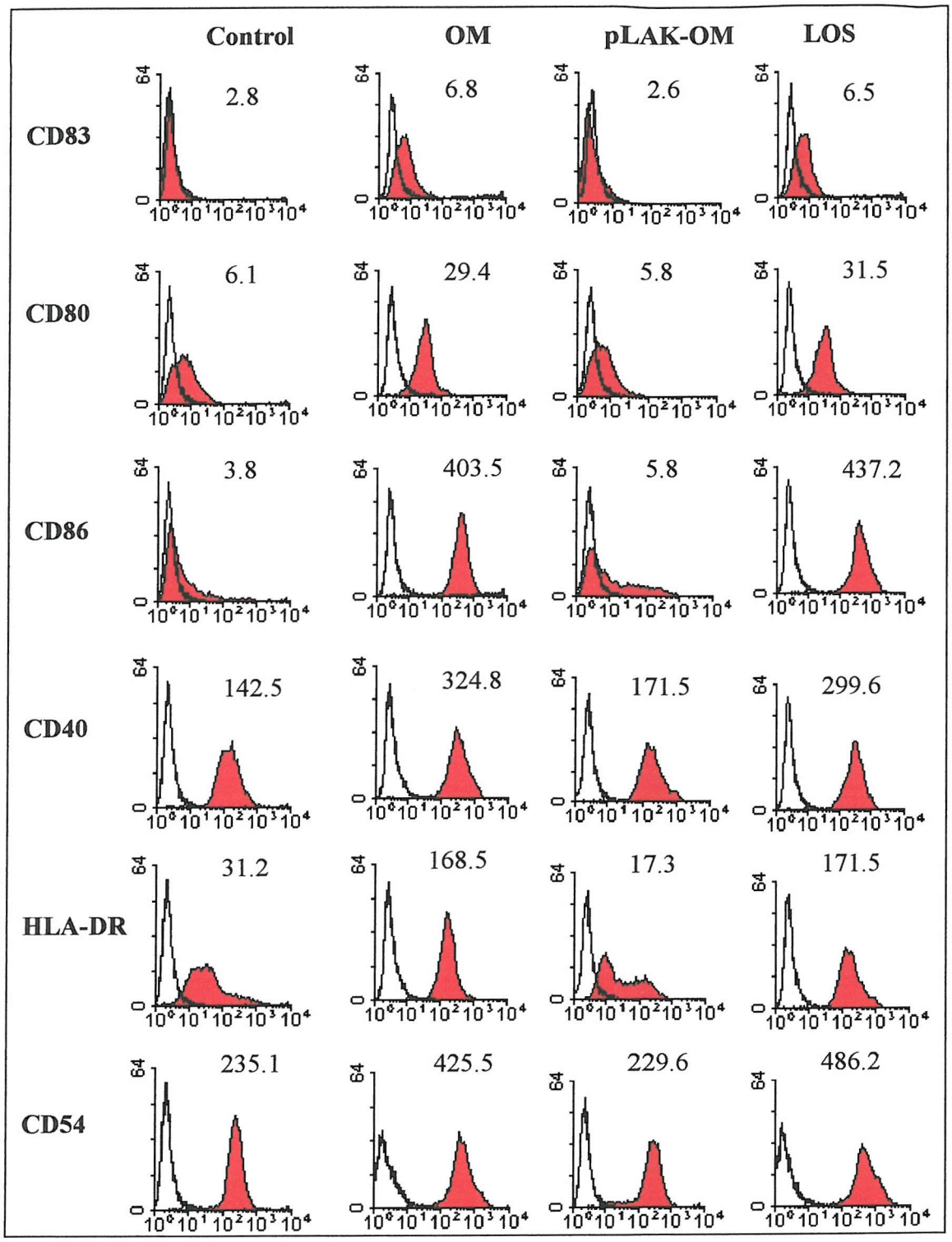

Figure 5.6: Phenotypic analysis of mo-DC exposed to *N. meningitidis* outer membranes or pure LOS.

Cell surface expression of CD83, CD80, CD86, CD40, HLA-DR and CD54 on control untreated or $CD1a^+$ cells stimulated for 24hr with 1μg/ml outer membranes from wild type H44/76 strain (OM), outer membranes from LOS-deficient mutant (pLAK-OM) or pure meningococcal LOS. mAb staining of surface molecules is shown as the red histograms. Cells stained with relevant isotype mAb is shown as clear histograms. Numbers on each histogram corresponds to the median fluorescence intensity of mAb staining. Data shown is a representative of eight experiments.

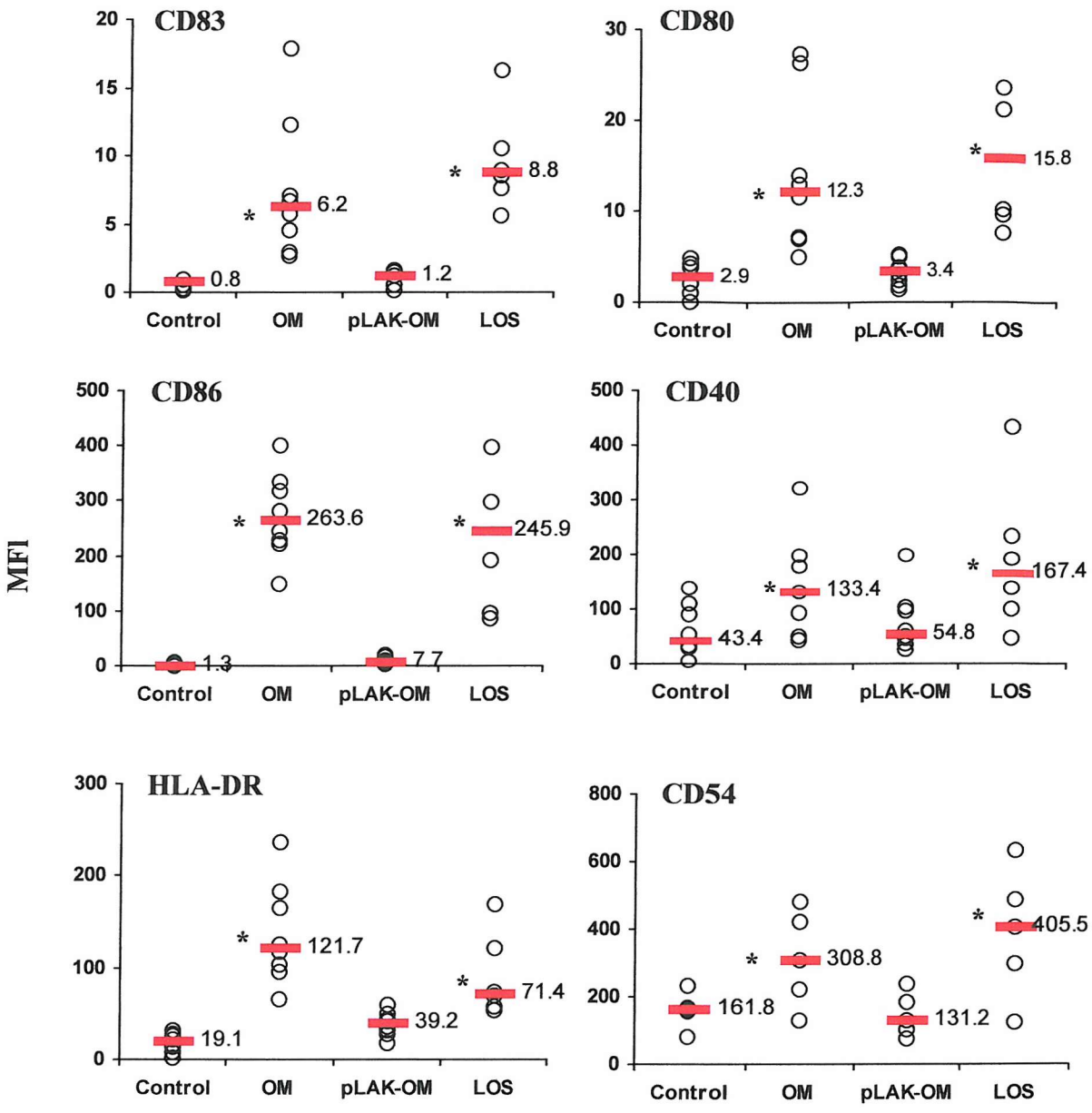

Figure 5.7: Summary data illustrating changes in mo-DC phenotype following treatment with *N. meningitidis* outer membranes or pure LOS.

Mo-DCs were left untreated or treated for 24hrs with 1µg/ml wild type outer membranes (OM, n=8), 1µg/ml LOS-deficient OM (pLAK-OM, n=8) or 1µg/ml pure LOS (n=5). Expression of CD83, CD80, CD86, CD40, HLA-DR and CD54 was analysed by flow cytometry. Data shows all the absolute individual median fluorescence intensity (MFI) after the respective treatments (○). Median for each treatment is shown as red bar with the value shown alongside. *=Significance observed ($p<0.05$, Wilcoxon's test) compared to control cells.

5.4.2 Effects of *N. meningitidis* outer membranes on receptor-mediated endocytosis by mo-DC

An early event during DC maturation is a reduction in the ability to capture exogenous antigens and this can be observed by diminished receptor-mediated endocytosis (Sallusto *et al.*, 1995). Flow cytometric analysis (Figure 5.8) revealed that immature mo-DCs are capable of taking up FITC-labelled dextran at 37°C (median fluorescence intensity 41.0 ± 52, n=5). This uptake was markedly inhibited following 24hr treatment of mo-DCs with outer membranes from the wild type H44/76 strain (median fluorescence intensity 2.3 ± 1.2, $p<0.05$ n=5) or pure meningococcal LOS (median fluorescence intensity 3.4 ± 0.5, $p<0.05$ n=5). However, FITC-dextran uptake was partially reduced in mo-DCs treated with outer membranes from the LOS-deficient mutant strain (median fluorescence intensity 15.2 ± 23, $p<0.05$ n=5) indicating a degree of maturation exhibited by these cells. Figure 5.8 shows representative flow cytometric profiles of FITC-dextran uptake by control and treated mo-DCs. Mo-DCs were incubated 4°C to reveal the level of non-specific binding of FITC-dextran to these cells.

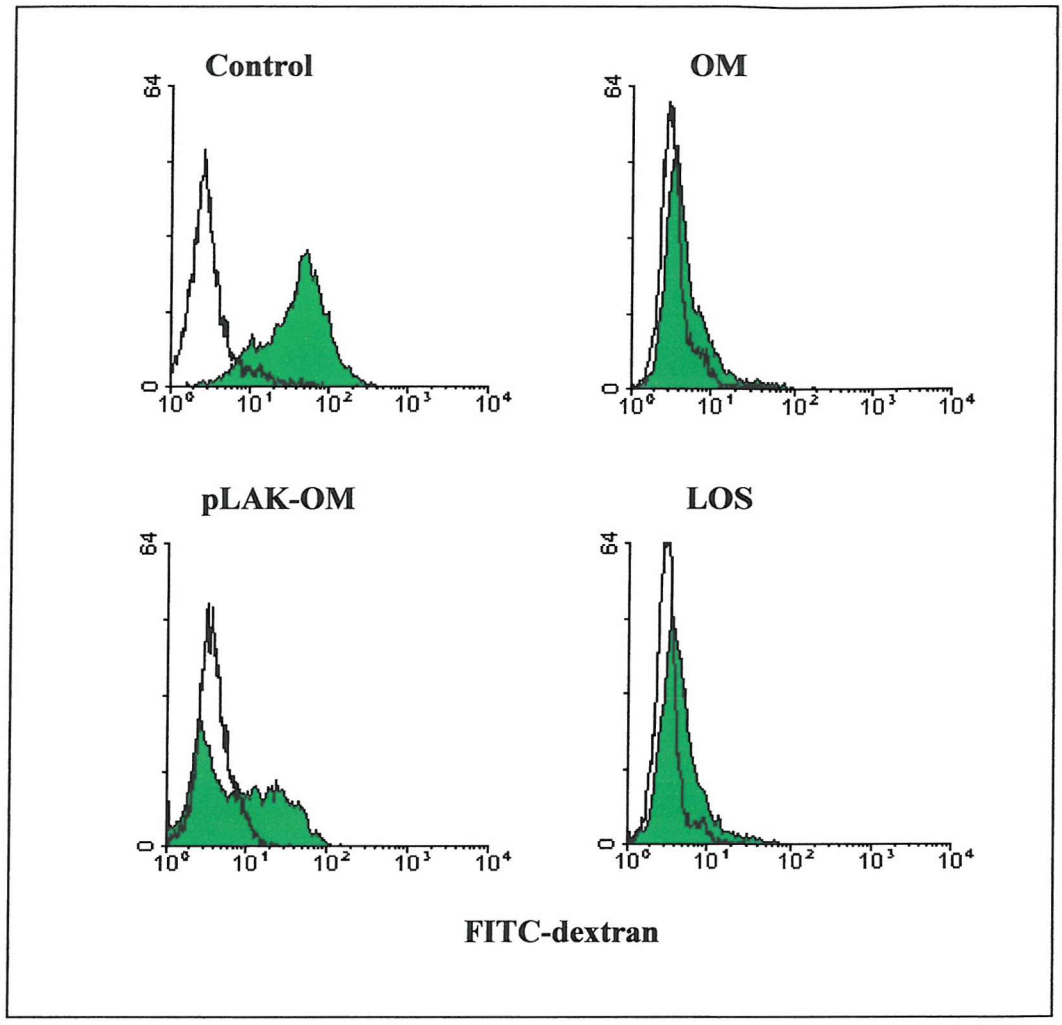

Figure 5.8: Actions of *N. meningitidis* outer membranes or pure LOS on mo-DCs receptor-mediated endocytosis.

Mo-DCs were left untreated (control) or exposed to 1μg/ml outer membranes from wild type H44/76 strain (OM) or LOS-deficient mutant strain (pLAK-OM) or 1μg/ml pure LOS for 24hrs. Following treatment, mo-DC were harvested and washed thoroughly prior to incubation with 250 μg/ml FITC-dextran for 1hr. Antigen uptake was assessed by flow cytometric analysis using a FACScan. Solid histograms represent cells incubated with FITC-dextran at 37°C, 5% CO_2. Clear histograms represent cells incubated with FITC-dextran at 4°C. Results are a representative of five separate experiments.

5.4.3 Production of soluble mediators by mo-DCs following exposure to outer membranes of *N. meningitidis.*

The ability of *N. meningitidis* outer membranes to induce cytokine and chemokine production by mo-DCs was assessed. Effects of outer membranes obtained from the wild type H44/76 strain (OM) or LOS-deficient mutant strain (pLAK-OM) as well as pure meningococcal LOS were compared.

Dose-dependent increases in the production of IL-6, IL-10, IL-12p40 and TNFα by mo-DCs following exposure OM, pLAK-OM or pure LOS were observed (Figure 5.9). Marked increases in levels of IL-12p40 (n=4) and TNFα (n=4) were induced in mo-DCs treated for 24hrs with OM at doses as low as 0.01μg/ml. Production of IL-6 (n=3) and IL-12p40 (n=4) by LOS-treated mo-DCs reached significance at concentrations of 0.1μg/ml. Generally, secretion of cytokines was greatest from mo-DCs pre-exposed to wild type OM; with the exception of IL-6, where higher concentrations were induced by mo-DCs treated with pure LOS. pLAK-OM-treated mo-DCs were markedly less potent than mo-DCs pre-exposed to OM or pure LOS for production of all cytokines measured. Sample numbers for these time-course experiments are low. Nonetheless, production of cytokines achieved statistical significance at varying concentrations of treatment (see Figure 5.9, $p<0.05$ Students t-test).

The production of cytokines and chemokines by mo-DCs treated with the top concentration (1μg/ml) of meningococcal outer membranes are illustrated in Table 5.1. Wild type OM induced mo-DCs to release significantly ($p<0.05$) high levels of IL-1β, IL-6, IL-10, TNFα, IL-12p40 and IL-12p70 (Figure 5.6). In contrast, pLAK-OM failed to induce significant levels of cytokine production except for IL-6 and IL-10. Stimulation of mo-DCs for 24hrs with 1μg/ml pure LOS resulted in the production of IL-6 (2.1 ng/10^6 cells ± 0.5, n=5, $p<0.05$), IL-10 (0.1 ng/10^6 cells ± 0.03, n=5), IL-12p70 (0.4 ng/10^6 cells ± 0.05, n=5, $p<0.05$) and TNFα (1 ng/10^6 cells ± 0.3, n=5, $p<0.05$).

A significant ($p<0.05$) increase in IL-8, MIP-1α, MIP-1β and RANTES production by mo-DCs was observed following treatment with wild type OM (Table 5.1). Significant ($p<0.05$) levels of IL-8 and MIP-1β were released by mo-DCs stimulated

for 24hr with pLAK-OM (Table 5.1). In addition, secretion of MIP-1α and RANTES were also noted in pLAK-OM treated mo-DCs, although these failed to achieve significance. Detection of chemokine production by LOS-treated mo-DCs was not performed in this study.

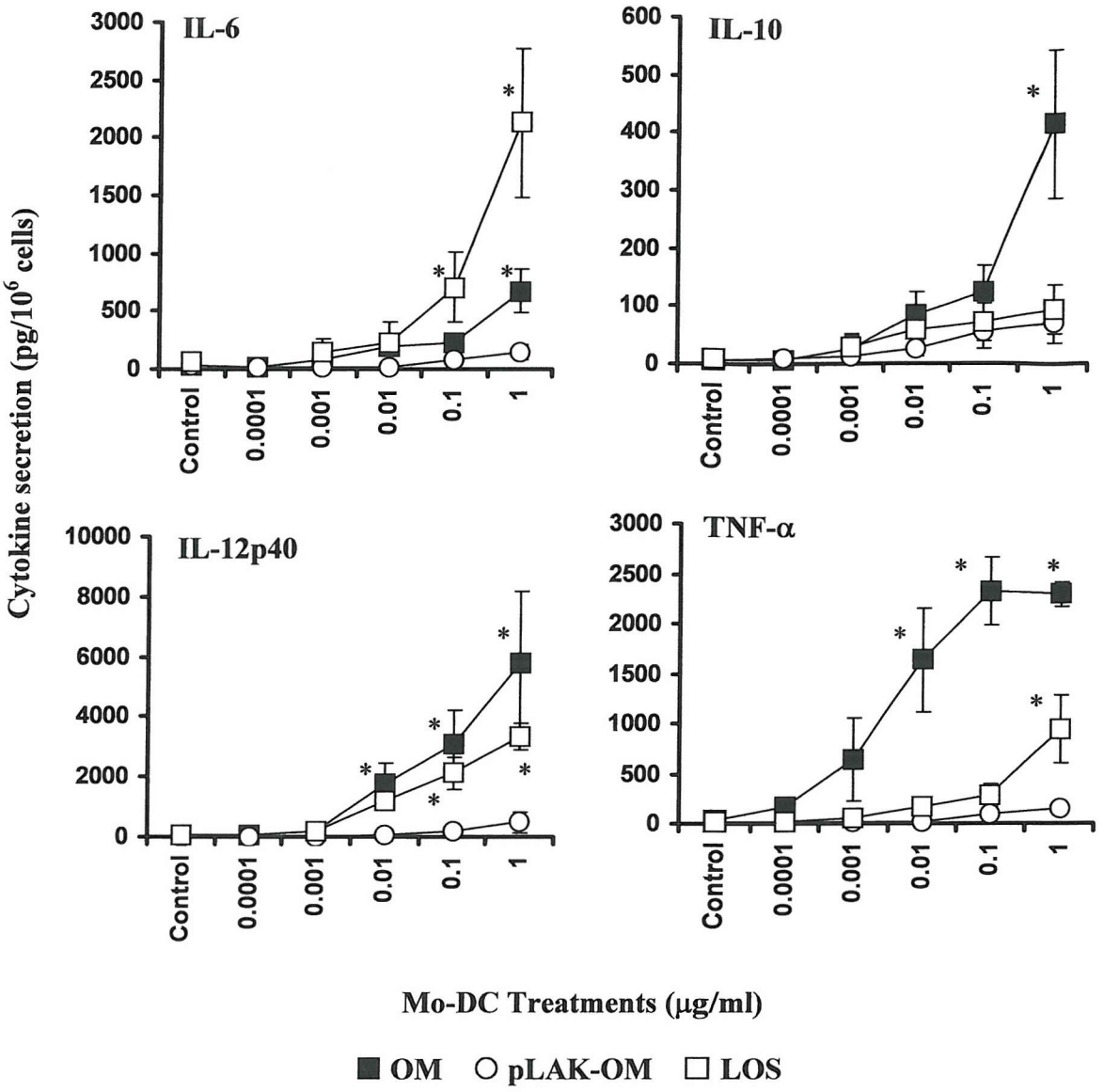

Figure 5.9: Dose-dependent production of cytokines by mo-DCs following treatment with *N. meningitidis* outer membranes or LOS.

Cells were left untreated or stimulated for 24hrs with varying concentrations (0.0001-1μg/ml) of outer membranes from wild type strain (OM) or LOS-deficient mutant (pLAK-OM) or pure meningococcal LOS. Supernatants were assayed for IL-6 (n=3), IL-10 (n=3), TNFα (n=5) and IL-12p40 (n=4) by ELISA. Results are presented as average cytokine production pg/10^6 cells with standard error bars. *= Significance observed ($p<0.05$, Student t-test) compared to control cells.

Table 5.1: Effects of *N. meningitidis* outer membranes on cytokine and chemokine production by mo-DCs.

Cells were left untreated (control) or stimulated for 24hrs with 1μg/ml of outer membranes from H44/74 strain (OM) or LPS-deficient mutant (pLAK-OM). Supernatants were assayed for IL-1β (n=3), IL-6 (n=8), IL-10 (n=8), TNFα (n=8), IL12p40 (n=8), IL12p70 (n=8), IL-8 (n=6), MIP-1α (n=5), MIP-1β (n=5) and RANTES (n=6) by ELISA. Results are presented as mean (ng/10^6)± SE. * Denotes significance observed ($p<0.05$, Student t-test) compared to control cells.

	Control	OM	pLAK-OM
IL-1β	0.02 ± 0.004	0.4 ± 0.01 *	0.05 ± 0.01
IL-6	0.02 ± 0.01	1.1 ± 0.2 *	0.4 ± 0.06 *
IL-10	0.02 ± 0.02	0.2 ± 0.01 *	0.1 ± 0.03 *
IL-12p40	0.1 ± 0.02	5.5 ± 1.2 *	0.7 ± 0.2
IL-12p70	<0.015	0.3 ± 0.04 *	<0.015
TNFα	0.06 ± 0.01	2.8 ± 0.7 *	0.3 ± 0.1
IL-8	2.3 ± 1.0	45.6 ± 14.0 *	8.2 ± 2.0 *
RANTES	0.04 ± 0.002	26.1 ± 6.0 *	1.1 ± 0.4
MIP-1α	0.02 ± 0.002	4.8 ± 0.5 *	0.7 ± 0.3
MIP-1β	0.07 ± 0.004	15.3 ± 5.0 *	1.7 ± 0.6 *

5.4.4 Effect of outer membranes on Toll-like receptor mRNA expression by Mo-DCs.

The effects of outer membranes from wild type or LOS-deficient mutant strains, or pure LOS, on the expression of TLR2 and TLR4 mRNA transcripts on mo-DCs were investigated with TaqMan RT-PCR (Figure 5.10). Exposure of mo-DCs for 24hrs to either wild type OM or pure LOS significantly down-regulated TLR2 mRNA expression ($p<0.05$, Wilcoxon's test). While interestingly, TLR4 mRNA expression was increased ($p<0.05$, Wilcoxon's test). These differential regulation patterns of TLR2 and TLR4 mRNA expression were also observed in mo-DCs treated with *E. coli* LPS (see chapter 6). Outer membranes from the LOS-deficient-mutant induced a significant trend towards an increase in mo-DC TLR2 mRNA expression ($p<0.05$, Wilcoxon's test), while TLR4 mRNA expression was unaffected ($p>0.05$, Wilcoxon's test).

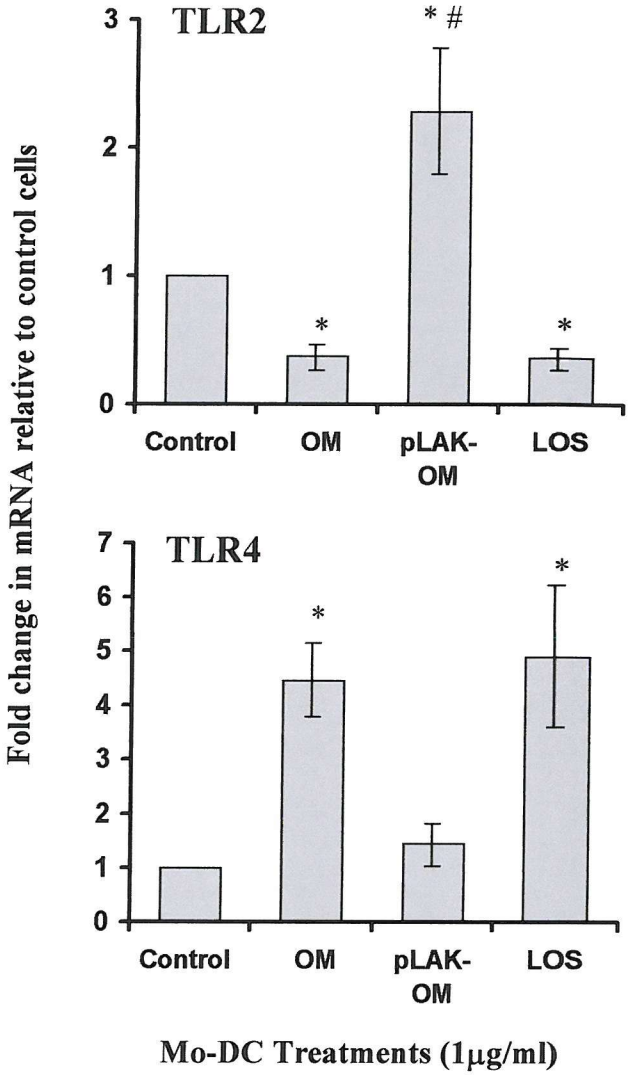

Figure 5.10: Expression of TLR2 and TLR4 mRNA on mo-DCs following treatment with *N. meningitidis* outer membranes or pure LOS.

Cells were left untreated (Control) or exposed for 24hrs to 1μg/ml outer membranes of wild type H44/76 strain (OM) or LOS-deficient mutant (pLAK-OM) or 1μg/ml pure LOS. After treatment, extracted RNA was reverse transcribed into cDNA. Expression of TLR2 and TLR4 mRNA on mo-DCs extrapolated from a PBMC standard curve following semi-quantitation by TaqMan PCR. TLR mRNA levels were normalized to the amount of ribosomal 18s RNA. Results are presented as average fold change in expression following treatment relative to control cells ± standard error (TLR2 n=5 except for pLAK-OM treatment where n=4; TLR4 n=5 for all treatments). * = Significance ($p<0.05$) observed. # = One additional donor (data not shown) did not show change in TLR2 mRNA expression following treatment.

5.5 EFFECTS OF OUTER MEMBRANES ON Mo-DC DRIVEN T-CELL PROLIFERATION

5.5.1 Allogeneic $CD4^+$ T-cell proliferation

The results presented so far in this chapter demonstrate that outer membranes from wild type *N. meningitidis* or pure meningococcal LOS result in marked maturation of mo-DCs shown by up-regulation of MHC class II and co-stimulatory molecules and production of a variety of cytokines and chemokines. However, marked differences were noted in the response of mo-DC to outer membranes of LOS-deficient mutant. Thus, the functional consequences of these effects on the capacity of mo-DCs to stimulate allogeneic T-cell proliferation were assessed (Figure 5.11). Mo-DCs were pre-exposed for 24hrs to 1μg/ml of wild type outer membranes (OM) or LOS-deficient outer membranes (pLAK-OM) or 1μg/ml pure LOS. Following treatment, mo-DCs were washed thoroughly of stimulus and subsequently co-cultured with $1x10^5$ purified allogeneic $CD4^+$ T-cells. Purity of $CD4^+$ cells (>95%, n=6) was determined by flow cytometry for every experiment (Figure 5.11A). In the absence of any activation, immature mo-DCs induced only modest allogeneic proliferation, seen at high mo-DC to T-cell ratios. Mo-DCs pre-exposed to wild type OM induced strong $CD4^+$ proliferation (Figure 5.11B). Whilst pLAK-OM was shown to induce only moderate changes in mo-DC phenotype and production of soluble mediators (Figure 5.6 and Table 5.1), pLAK-OM-treated mo-DCs also stimulated allogeneic $CD4^+$ proliferation but at a lower level compared to mo-DCs stimulated with wild type OM (Figure 5.11B). In contrast, mo-DCs treated with purified LOS showed a lower ability to induce $CD4^+$ proliferation in comparison with mo-DCs treated with OM or LOS-deficient OM.

Summary of allogeneic T-cell proliferation driven by OM, pLAK-OM or pure LOS-treated mo-DCs is presented as the average CPM for treated cells minus the CPM for the appropriate control cells or as stimulation indices at the 1000 mo-DC / well only (Figure 5.12A and B, respectively).

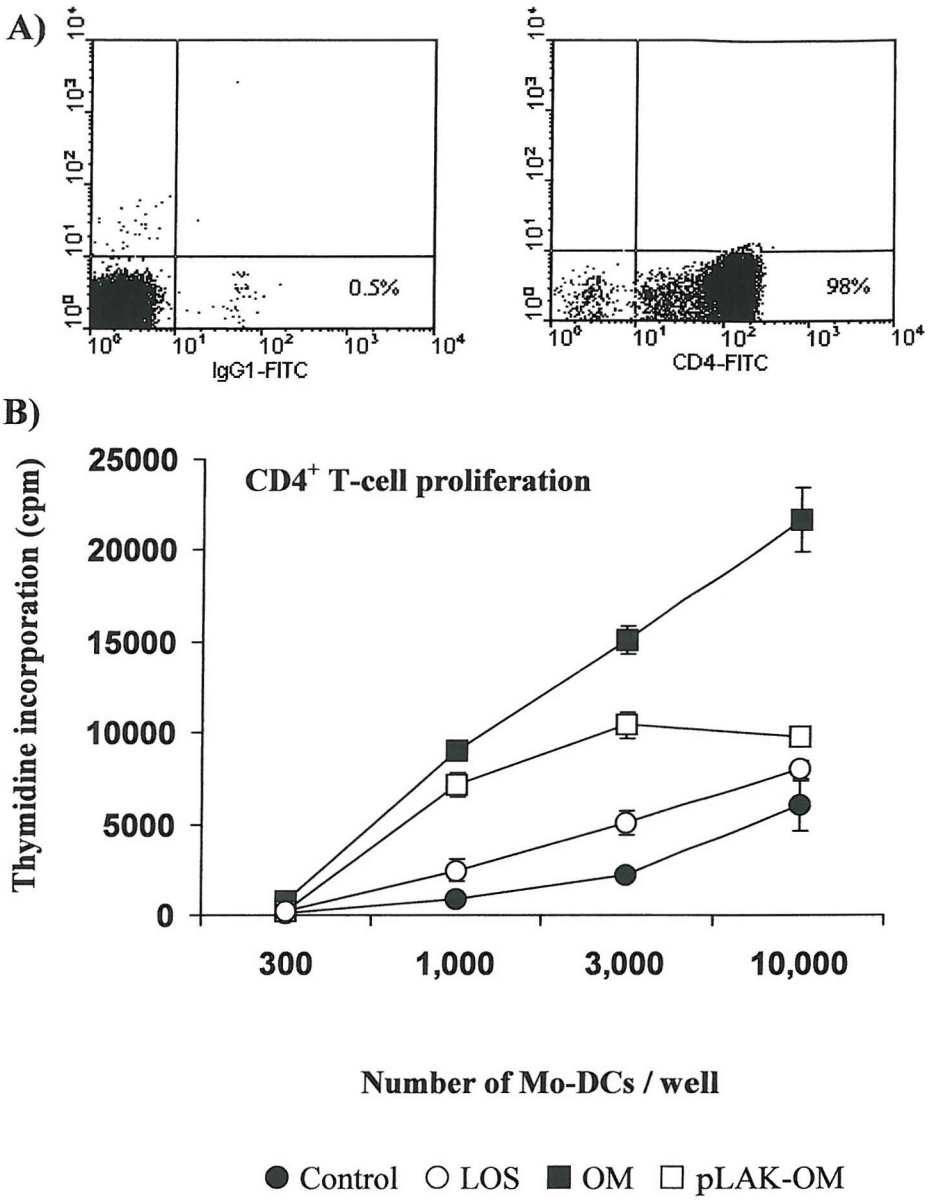

Figure 5.11: Effects of *N. meningitidis* outer membranes or LOS on Mo-DC allogeneic T-cell proliferation.

Mo-DCs were left untreated or exposed to 1μg/ml wild type outer membranes (OM), 1μg/ml LOS-deficient outer membranes (pLAK-OM) or 1μg/ml pure LOS for 24hrs. Mo-DCs were washed thoroughly following treatment. **A)** $CD4^+$ T-cells were obtained from allogeneic donors and purity was assessed by flow cytometry. **B)** Graded numbers of mo-DCs were co-cultured with $1x10^5$ purified allogeneic $CD4^+$ T-cells. After 5 days, T-cell proliferation was assessed by H^3 thymidine incorporation over the last 18hrs. Data is representative of one donor ± standard error of triplicate cultures for each treatment.

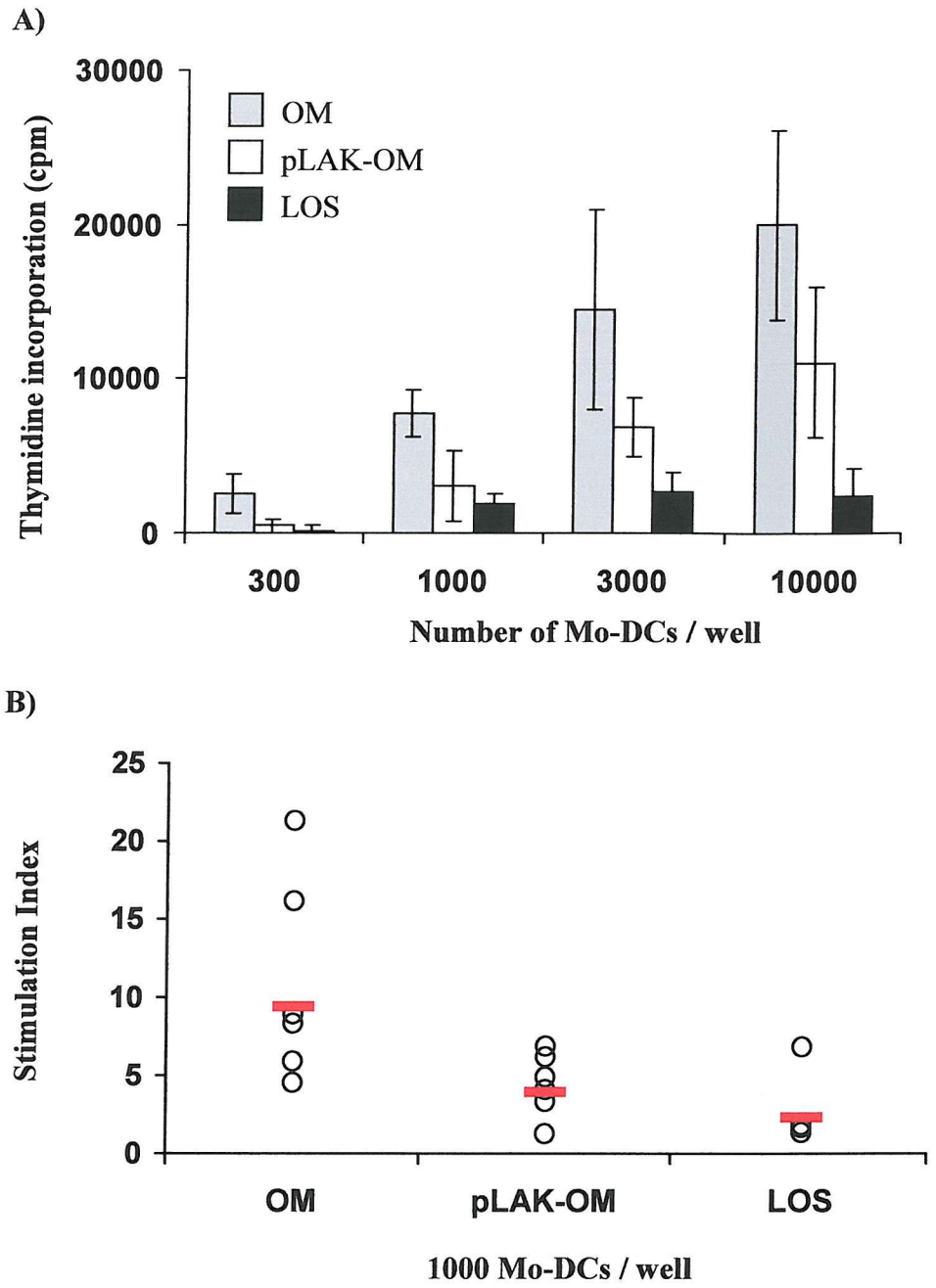

Figure 5.12: Summary of effects of *N. meningitidis* outer membranes or LOS on mo-DC driven allogeneic T-cell proliferation.

Graded numbers of mo-DCs, pretreated for 24hrs with 1μg/ml wild type outer membranes (OM, n=6), 1μg/ml LOS-deficient outer membranes (pLAK-OM, n=6) or 1μg/ml pure LOS (n=4), were co-cultured with $1x10^5$ allogeneic $CD4^+$ T-cells, in triplicate. After 5 days, T-cell proliferation was assessed by H^3 thymidine incorporation over 18hrs. **A)** Data is presented as average of CPM for each treatment minus CPM for respective control cells with standard error bars. **B)** Data is presented as stimulation index (SI) for each treatment at the 1000 mo-DC/well concentration only (mo-DC:T-cell, 1:100). Individual SI is presented as open circles and the median is shown as a red bar.

5.5.2 Allogeneic naïve T-cell proliferation

The capacity of mo-DC to activate primary T-cell responses was subsequently investigated (Figure 5.13). Allogeneic naïve $CD4^+CD45RA^+$ T-cells were enriched from purified $CD4^+$ T-cell fractions by depletion of $CD45RO^+$ cells. Purity (>97% n=7) was assessed by flow cytometry for each experiment (Figure 5.13A). Mo-DCs pre-pulsed with wild type outer membranes (OM) or LOS-deficient outer membranes (pLAK-OM) were able to stimulate naïve $CD4^+$ T-cell proliferation (Figure 5.13B). However, only minor increases in naïve T-cell proliferation were observed by mo-DCs pre-exposed to pure LOS compared to untreated control mo-DCs.

Summary of allogeneic naïve T-cell proliferation driven by OM, pLAK-OM or pure LOS-treated mo-DCs is presented as the average of CPM for treated cells minus the CPM for the appropriate control cells or as stimulation indices at the 1000 mo-DC / well concentration only (Figure 5.14A and B, respectively). Similar to the effects of LOS on mo-DC driven $CD4^+$ proliferation (Figure 5.12), LOS-treated mo-DCs incurred minor changes in naïve T-cell proliferation (Figure 5.14).

The ability of the activation stimulus delivered by meningococcal outer membranes to influence mo-DC driven polarised T-cell responses was examined. Mo-DCs pre-exposed for 24hrs to wild type OM, pLAK-OM or purified LOS were co-cultured for four days with naïve T-cells followed by a further 24hr stimulation of proliferating cells with anti-CD3 mAb. Treated mo-DCs encouraged allogeneic naïve T-cell differentiation into effector cells producing both Th1 (IFN-γ)- and Th2 (IL-13)-favouring cytokines (Figure 5.15). Only low levels of IFN-γ were detected in supernatants of naïve T-cells co-cultured with immature control mo-DCs (21.2 $pg/10^5$ cells ± 3.6, n=3). However, mo-DCs pre-exposed to OM or pure LOS promoted the development of T-cells secreting higher levels of IFN-γ than IL-13. Conversely, mo-DCs treated with pLAK-OM encouraged the development of effector cells producing higher levels of IL-13 than IFN-γ (Figure 5.15). Due to low sample numbers, statistical significance was not achieved.

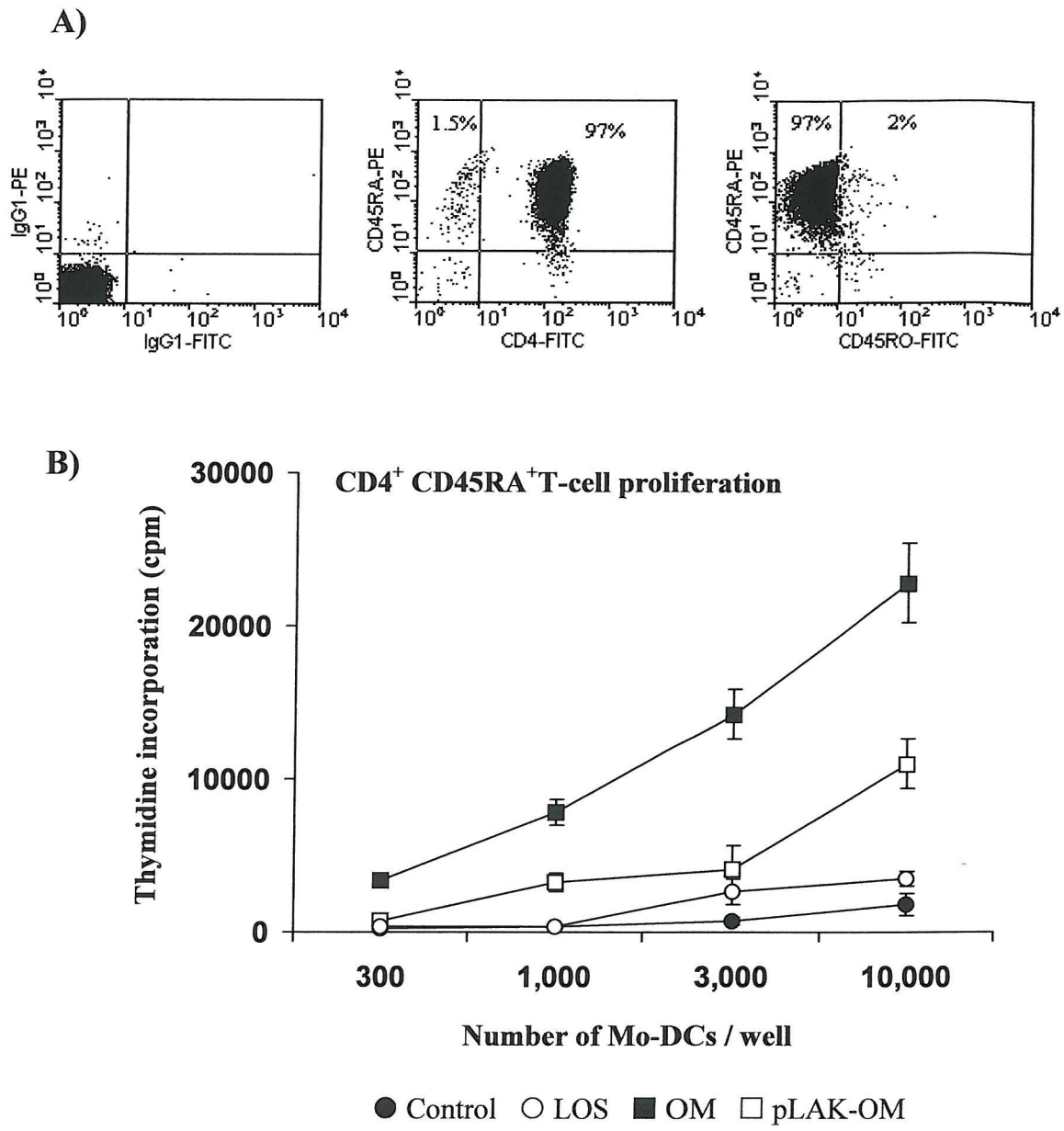

Figure 5.13: Effects of *N. meningitidis* outer membranes or LOS on Mo-DC allogeneic naïve T-cell proliferation.

A) Naïve CD45RA$^+$ T-cells were enriched from CD4$^+$ T-cells. Purity of cells was assessed by flow cytometry. **B)** Mo-DCs were left untreated or exposed to 1μg/ml wild type outer membranes (OM), 1μg/ml LOS-deficient outer membranes (pLAK-OM) or 1μg/ml pure LOS for 24hrs. Mo-DCs were washed thoroughly following treatment. Graded numbers of mo-DCs were co-cultured with $1x10^5$ purified allogeneic CD4$^+$ T-cells. After 5 days, T-cell proliferation was assessed by H^3 thymidine incorporation over 18hrs. Data is representative of one donor ± standard error of triplicate cultures for each treatment.

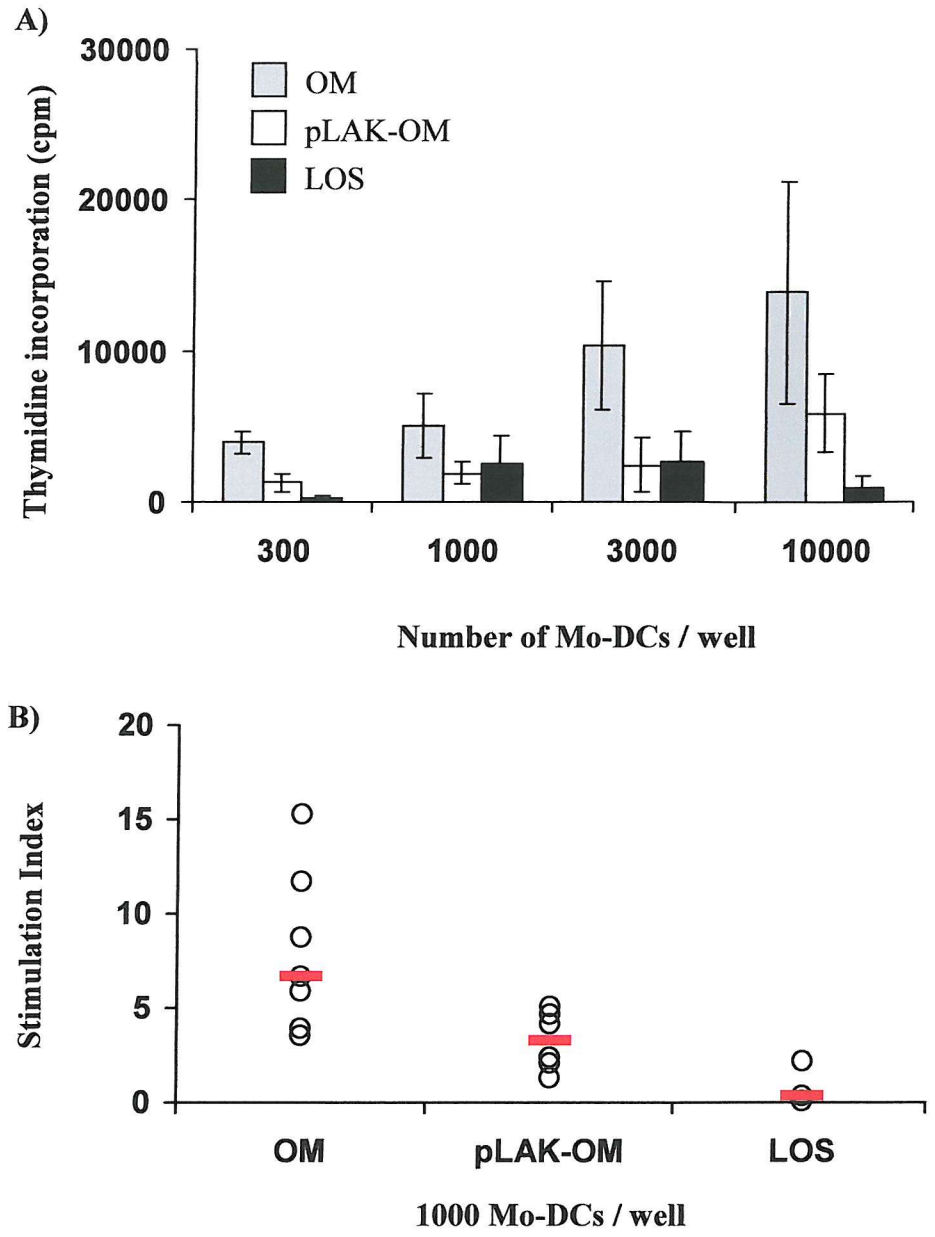

Figure 5.14: Summary data showing effects of *N. meningitidis* outer membranes or LOS on mo-DC driven allogeneic naïve T-cell proliferation.

Graded numbers of mo-DCs, pre-treated for 24hrs with 1μg/ml wild type outer membranes (OM, n=7), 1μg/ml LOS-deficient outer membranes (pLAK-OM, n=6) or 1μg/ml pure LOS (n=3), were co-cultured with $1x10^5$ allogeneic naïve $CD4^+CD45RA^+$ T-cells in triplicate. After 5 days, T-cell proliferation was assessed by H^3 thymidine incorporation over 18hrs. **A)** Data is presented as average of CPM for each treatment minus CPM for respective control cells with standard error bars. **B)** Data is presented as stimulation index (SI) for each treatment at the 1000 mo-DC/well concentration only (mo-DC: T cell, 1:100). Individual SI is presented as open circles and the median SI is shown as a red bar.

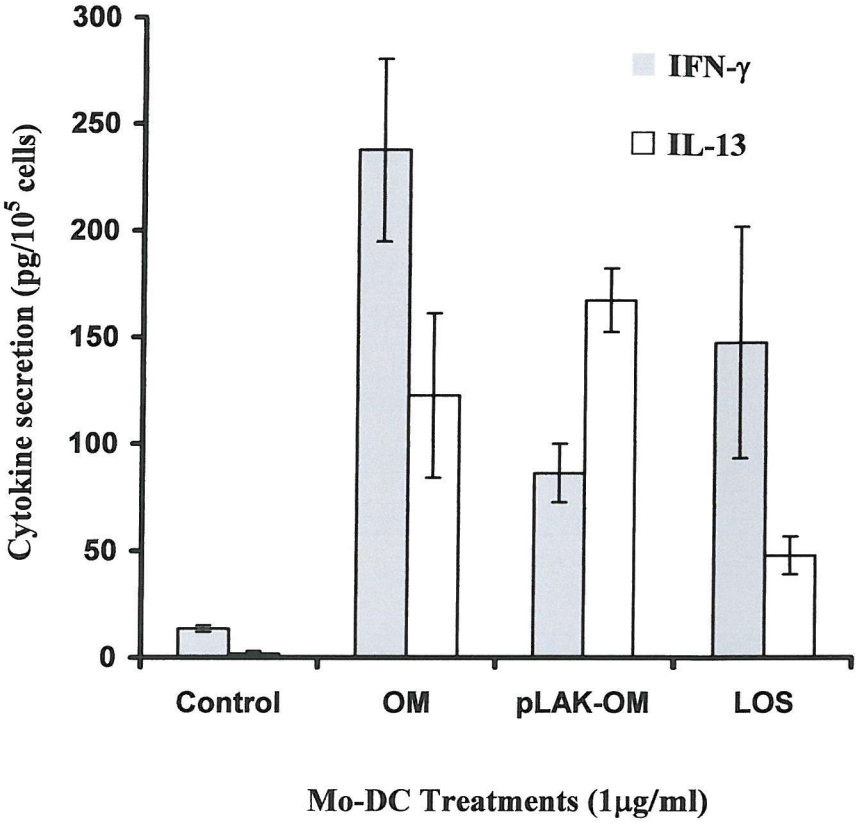

Figure 5.15: Activity of *N. meningitidis* outer membranes or LOS on mo-DCs to influence IFN-γ and IL-13 production by naïve T-cells.

Allogeneic $CD4^+CD45RA^+$ naïve T-cells were co-cultured with mo-DCs pre-exposed for 24hrs to 1μg/ml of wild type outer membrane (OM), LOS-deficient outer membrane (pLAK-OM) or pure LOS. After four-days culture, naïve cells were harvested and re-stimulated with membrane-bound anti-CD3 (OKT3) for a further 24hrs. Production of IFN-γ and IL-13, by naïve cells, was assessed by ELISA. Results are presented as mean cytokine production ± SE, from three separate experiments.

CHAPTER 5

Discussion of Results

5.6 DISCUSSION

5.6.1 Summary of Results

Wild type bacteria were able to survive and grow in the presence of mo-DCs, whereas LOS-deficient mutant meningococci appeared to die in these cultures. The LOS-deficient mutant did not affect NO secretion by mo-DCs. Co-culture with wild type meningococci was accompanied by a significant reduction in production of NO by mo-DCs. High concentrations of soluble mediators were also released by mo-DCs after challenge with viable wild type meningococci compared to LOS-deficient bacteria.

Outer membranes from H44/76 wild type and LOS-deficient (pLAK-OM) mutant meningococci exert distinct effects on activation and maturation of mo-DCs. Differential regulation of TLR2 and TLR4 mRNA was observed on mo-DCs exposed to wild type OM or pLAK-OM. Exposure of mo-DCs with wild type OM resulted in marked phenotypic changes showing up-regulation of all surface molecules analysed and reduced capacity to take up FITC-dextran. In contrast, treatment of mo-DCs with pLAK-OM only induced moderate increases in CD86 and HLA-DR expression as well as a moderate reduction in FITC-dextran uptake. Production of cytokines and chemokines was also significantly higher in wild type OM-treated mo-DCs compared to pLAK-OM treated cells. Actions of LOS on phenotype of mo-DCs were similar to wild type OM. In addition, outer membranes from both strains of meningococci promoted the induction of mo-DC-driven T-cell proliferation.

5.6.2 Interactions of mo-DCs with viable *N. meningitidis*

5.6.2.1 Nitric oxide and bacterial killing

NO is an important effector molecule in the host innate defense against pathogens. IFN-γ alone or in conjunction with LPS is known to lead to the production of NO by murine DC or macrophages, respectively (MacMicking *et al.*, 1997). NO has been shown to be involved in the clearance of infections by *M. tuberculosis*, *S. typhimurium* and *S. aureus*, whereas it does not seem to be an essential part of the defense against *L. monocytogenes* (Leenen *et al.*, 1994; MacMicking *et al.*, 1997; Fehr *et al.*, 1997).

The present results demonstrated that in the presence of mo-DCs wild type meningococci were able to survive and proliferate, whereas LOS-deficient mutant bacteria failed to survive. The LOS-deficient mutant has been reported to grow more slowly *in vitro* compared with the wild type strain (Steeghs *et al.*, 1998). However, the fact that normal culture medium without mo-DC supported the growth of this bacterial strain (data not shown), excludes the possibility that the observed reduction in viability in the presence of mo-DC was due simply to poor growth. It was observed that mo-DCs produced the soluble mediator nitric oxide (NO) in increasing quantities over time. In the presence of LOS-deficient meningococci, NO production by mo-DCs was unaffected. The decreased ability of the LOS-deficient mutant to survive may be a consequence of mo-DC-derived NO production following exposure that in turn results in bacterial killing. In contrast, it was observed that nitric oxide production by mo-DC cultured with wild type bacteria was significantly reduced. This finding may suggest an important property of meningococcal LOS to facilitate evasion of bactericidal immune responses. Padrón and colleagues have reported that 24hr challenge of murine peritoneal macrophages with serogroup B wild type meningococci in the presence of the NO inhibitor L-NMMA, significantly increases the bacterial colony forming units (Padron *et al.*, 1999). Further studies are needed to determine whether the survival of LOS-deficient meningococci is restored following co-culture in the presence of inhibitors of NO synthase.

5.6.2.2 Production of cytokines and chemokines by mo-DCs

DCs have been reported to produce a range of soluble mediators following exposure to various bacterial stimuli (Wagner, 1999; Hessle *et al.*, 2000; Verhasselt *et al.*, 1997). In the present study, the effects of viable wild type *N. meningitidis* on the production of cytokines and chemokines by mo-DC were compared with those induced by LOS-deficient mutant meningococci. Levels of pro-inflammatory cytokines IL-1β, IL-6, IL-12p40, IL-12p70 and TNFα, and the anti-inflammatory cytokine IL-10 produced by mo-DCs challenged by the wild type bacteria were significantly higher than those produced by cells co-cultured with the LOS-deficient mutant. Only significant levels of IL-12p40 were produced by mo-DCs exposed to LOS-deficient mutant bacteria. The results presented in this study on the ability of wild type *N. meningitidis* to induce cytokine production by mo-DCs compared to the

LOS-deficient mutant strain are in agreement with the findings by Dixon and colleagues (Dixon *et al.*, 2001). However, those authors challenged mo-DCs with paraformaldehyde fixed wild type H44/76 or mutant LOS-deficient and detected increased intracellular production of TNF-α, IL-1α and IL-6 by flow cytometry (Dixon *et al.*, 2001). A separate study reported high levels of protein levels of TNF-α, IL-6, IL-β and IL-8 produced by mo-DCs exposed to *N. meningitidis* MC58 strain (Kolb *et al.*, 2001).

As observed with cytokine production, viable wild type bacteria induced mo-DCs to secrete higher levels of chemokines RANTES, MIP-1α and MIP-1β than cells stimulated with the LOS-deficient mutant. By contrast, there were no significant differences in the high levels of IL-8 secretion, over time, induced by both strains of bacteria. Thus, IL-8 appears to be induced by as yet uncharacterised but non-LOS component(s) of the meningococcus. The secretion of IL-8 by mo-DC observed here is consistent with its role in the *in vivo* chemotaxis of neutrophils, whereas MIP-1α and MIP-1β recruit natural killer cells, monocytes, macrophages as well as immature DCs to the sites of pathogen invasion (Rossi and Zlotnik, 2000; Sallusto and Lanzavecchia, 1999).

5.6.3 Activities of *N. meningitidis* outer membranes on mo-DCs

5.6.3.1 TLR expression on mo-DCs exposed to outer membranes

Initial events in the interactions of DCs with bacteria involve the recognition of pathogen-associated molecular patterns (PAMP) subsequently followed by cellular activation. Members of the TLR family recognise diverse PAMPs expressed on variety of microbial structures (Kopp and Medzhitov, 1999). Moreover, TLRs can discriminate between multitudes of microbial components. As described previously, TLR2 is preferentially involved in the inflammatory response to lipoteichoic acid, lipopeptides and glycans from a variety of gram-positive bacteria, whereas TLR4 is essential for responses to gram-negative LPS (Beutler, 2000; Lien *et al.*, 1999). In the current study, expression of TLRs appeared to be differentially regulated by the presence of meningococcal LOS in OM. Expression of TLR4 mRNA was up-regulated following exposure of mo-DCs to wild type OM and pure meningococcal

LOS, whereas expression of TLR2 mRNA was decreased. Conversely, mo-DCs incubated with LOS-deficient OM showed increased TLR2 mRNA expression, whilst expression of TLR4 mRNA remained unaffected. These findings are in accord with other reports demonstrating that activation of cellular responses in monocytes and macrophages by LOS-deficient meningococci was mediated through TLR2 (Ingalls *et al.*, 2000; Pridmore *et al.*, 2001).

These observations suggest that mo-DCs are capable of discriminating between different components of the meningococcal OM, which subsequently results in differential activation of TLRs. Moreover, expression of TLRs appears to be differentially regulated by the presence of meningococcal LOS in OM. *In vivo*, the presence of meningococcal OM within the environment may alert DCs through TLR signalling resulting in the appropriate induction of immune responses. Activation of human DCs through TLR2 and TLR4, by other pathogens, has been reported to induce cytokine and chemokine production (Means *et al.*, 1999; Re and Strominger, 2001; Kadowaki *et al.*, 2001b; Thoma *et al.*, 2000; Poltorak *et al.*, 2000). Thus, post-TLR signalling may dictate the nature of the immune response induced by the DC to initiate the appropriate immune response against meningococcal infection. Of note, it has been reported that susceptibility to meningococcal disease was not associated with a functional polymorphism of TLR4 in humans (Read *et al.*, 2001).

5.6.3.2 Mo-DC activation by meningococcal outer membranes

OM from wild type and LOS-deficient strains activated mo-DCs to different degrees of maturation. Incubation with wild type OM resulted in increases in MHC class II, DC maturation marker CD83 and co-stimulatory molecules CD80, CD86 and CD40, and was accompanied with a reduced capacity for antigen uptake. In contrast, LOS-deficient OM only partially altered mo-DC maturation with moderate increases in CD86 and MHC class II and a smaller reduction in antigen uptake. These data suggest that although LOS is likely to be the major determinant of mo-DC maturation, other components of the OM may also exert an effect. This potential stimulatory effect of bacterial components other than LOS has also been described by Dixon and colleagues (Dixon *et al.*, 2001), who reported increased expression of cell surface

CD86, CD40, CD83, CD25 and MHC class I and II molecules following exposure of mo-DCs to whole paraformaldehyde-fixed LOS-deficient meningococci.

Analysis of soluble mediators produced by mo-DCs following exposure to wild type OM or LOS-deficient-OM revealed that presence of LOS within the outer membrane plays a significant role in the induction of pro-inflammatory cytokines as well as IL-10. OM from the wild type strain induced the secretion of IL-8, RANTES, MIP-1α and MIP-1β; however, secretion of these chemokines was significantly lower from cells incubated with LOS-deficient OMs. The relative amounts of cytokines produced by mo-DCs were considerably lower than the observed chemokine levels produced following treatment of cells with outer membranes of either meningococcal strains. Nonetheless, the data presented here show that the presence of LOS within the OM plays a significant role in the production of pro-inflammatory, anti-inflammatory and chemoattractant cytokines by mo-DC. It is likely that the infiltration by immature DCs enables further antigen uptake, and the subsequent production of chemokines by activated DCs leads to movement of antigen-loaded DCs from infection sites to the secondary lymphoid organs where presentation of antigen to naïve T-cells occurs (Sallusto *et al.*, 1998). Thus, the production of cytokines and chemokines by mo-DC in response to meningococci is likely to be essential for inducing appropriate adaptive immune responses.

5.6.4 Induction of T-cell responses by mo-DCs activated by *N. meningitidis* outer membranes

The presentation by DCs of MHC class II/antigen peptide complexes to $CD4^+$ T-helper (Th) cells initiates the induction of acquired immunity to pathogens (Steinman, 1991; Inaba *et al.*, 1998). Interaction of immature DCs with many bacteria leads to their activation and acquisition of potent antigen presenting properties by virtue of increased expression of surface MHC-class II, co-stimulatory molecules and production of critical regulatory cytokines (Banchereau *et al.*, 2000). Overall, these factors influence the ability of DCs to drive naïve Th-cells to proliferate and differentiate into Th1 cells that produce IFNγ and IL-2 or Th2 cells that secrete IL-4, IL-5 and IL-13 (Mosmann and Coffman, 1989). Dendritic cells are known to produce biologically active IL-12p70 in response to a variety of bacterial components, including Gram-negative LPS (Langenkamp *et al.*, 2000; Hilkens *et al.*, 1997; Trinchieri, 1995). Bacteria-mediated IL-12 production by mo-DCs drives activated T-

helper cells and natural killer cells to produce IFNγ that in turn, enhances protective immunity against pathogens (Kalinski *et al.*, 1999b; Szabo *et al.*, 1997; Heufler *et al.*, 1996). Production of IFNγ together with DC-derived IL-12 influences the differentiation of naïve T-cells into Th1 effector cells that produce high levels of IFNγ and little or no IL-4 (Croft, 1994; Hilkens *et al.*, 1997).

In the current study, wild type OM were able to induce high levels of IL-12p70 by mo-DCs suggesting that these OMs may promote the induction of Th1 responses, largely mediated through the actions of LOS. Results from this investigation demonstrated that mo-DCs pre-exposed to OM from the parent strain were capable of inducing potent allogeneic $CD4^+$ T-cell proliferation as well as primary naïve T-cell responses. Interestingly, the relatively low level of mo-DC maturation induced by OM from the LOS-deficient mutant was sufficient to drive proliferation of both $CD4^+$ and naïve T-cells, albeit at levels lower than those observed by wild type OM-treated mo-DCs. Moreover, the capacity of LOS-deficient OM to induce T-cell proliferation suggests that LOS within OM is not solely responsible for T-cell activation.

In addition, mo-DCs pre-exposed to wild type OM acquired the capacity to prime naïve Th-cells into effector cells exhibiting a higher IFN-γ:IL-13 cytokine profile. In contrast, the reverse ratio of cytokine production (higher IL-13:IFN-γ) was observed by naïve T-cells primed with LOS-deficient OM treated-mo-DCs. These findings demonstrate the ability of meningococcal OM from either strain to modulate antigen-presenting capacity to initiate and promote differentiation of primary T-cell responses.

5.6.4.1 Polarization of T-cell responses by meningococcal outer membranes

In comparison to wild type OM, it would appear that the relatively small changes in mo-DC activation induced by LOS-deficient OM reflect a general state of unresponsiveness of mo-DCs to this stimulus. However, the release of significant levels of IL-6 and IL-10 and moderate increases in MHC class II molecules and CD86 co-stimulatory molecule expression, suggests a level of mo-DC activation/maturation induced by LOS-deficient OM. Indeed, the observed low levels of co-stimulatory and MHC class II molecules did not hamper proliferation of both allogeneic $CD4^+$ and naïve T-cells which was comparable with wild type OM-driven proliferation. APC-

derived IL-6 is reported to polarize naïve T-cells to effector Th2 cells (Rincon *et al.*, 1997). Anti-inflammatory IL-10 is believed to suppress IL-12 production and consequently IFN-γ production, thus inhibiting a Th1-response (De Smedt *et al.*, 1997). DCs that produce active 70kd IL-12 heterodimer secrete the isolated p40 chain, usually at levels higher than the heterodimer (Trinchieri, 1995). Only the IL-12p40 monomer was induced by mo-DCs following exposure to LOS-deficient OMs ($p>0.05$). The significance of this result is unclear at present. It has been proposed that excess p40 may down-regulate IL-12-mediated immune responses (Trinchieri, 1995; Gillessen *et al.*, 1995; Germann *et al.*, 1995).

There is increasing published evidence that distinct microbial products can modulate DC function to acquire the potential of directing the T-cell response toward either a Th1 or Th2 pattern (de Jong *et al.*, 2002). Exposure of immature murine DCs to *Shistosoma mansoni* egg antigen resulted in up-regulation of MHC class II molecules but had no effect on co-stimulatory expression and did not modify production of a range of Th1- or Th2-inducing cytokines (MacDonald *et al.*, 2001). Nevertheless, these authors reported that *S. mansoni* egg antigen pulsed-DCs were capable of strongly stimulating Th2 responses both *in vitro* and *in vivo* (MacDonald *et al.*, 2001). In a separate study, exposure of ES-62 antigen from the helminth *Acanthocheilonema vitteae* to murine DCs, promoted priming of $CD4^+$ T-cells expressing a Th2-phenotype despite the lack of DC maturation in terms of significant increases in MHC class II or co-stimulatory expression (Whelan *et al.*, 2000). Cholera toxin and hyphae of *Candida albicans* have also been reported to modulate DCs to promote Th2 polarization (Gagliardi *et al.*, 2000; d'Ostiani *et al.*, 2000). It is possible that OM from *N. meningitidis* deficient in LOS possesses similar attributes as these microbial agents. Unlike wild type OM, the inability of LOS-deficient OM to induce mo-DC-derived IL-12p70 together with the capacity of these activated mo-DCs to result in mixed Th1/Th2 differentiation with the predisposition to induce greater IL-13:IFN-γ production by naïve T-cells may be optimal for induction Th2-mediated responses. Further research is needed to determine whether LOS-deficient OM has the ability to induce these polarized Th2-cell responses *in vivo*.

In summary, the results presented in this chapter suggests that the recognition of outer membranes from wild type or LOS-deficient *N. meningitidis* through TLRs is likely to initiate signalling events that lead to mo-DCs interpreting the nature of the bacterial

stimulus. Additionally, outer membranes from both meningococcal strains are immunomodulatory and exert distinct effects on human mo-DCs resulting in the acquisition of potent T cell-activating properties.

CHAPTER 6

Effects of Class I Porin of *Neisseria meningitidis* on Human Monocyte-derived Dendritic Cells

6

6.1 INTRODUCTION

Class I (PorA) and class II/III (PorB) porins are abundantly present within the outer membrane of *N. meningitidis* (Nikaido, 1992). Neisserial porins are immunogenic in humans in the absence of exogenous adjuvants and thus, have been explored as candidates for anti-neisserial vaccine preparations (Claassen *et al.*, 1996; Christodoulides *et al.*, 1998; Haneberg *et al.*, 1998). In addition, neisserial porins have been used as adjuvants in vaccine preparations against *Haemophilus influenzae* type b (Donnelly *et al.*, 1990), malaria (Lowell *et al.*, 1988), anti-meningococcal polysaccharide conjugate vaccines (Fukasawa *et al.*, 1999) and melanoma (Livingston *et al.*, 1993).

The mechanisms of how porins exert their activity are not thoroughly understood. Porins act as pore-forming molecules in bacterial membranes (Nikaido, 1992). This characteristic was initially suggested as a possible mechanism by which porins could activate immune cells by forming ion channels in mammalian membranes (Mackinnon *et al.*, 1999). However, evidence on PorB neisserial porins have demonstrated their ability to act mitogenically on B-cells, stimulating proliferation and inducing IgM secretion (Snapper *et al.*, 1997; Wetzler *et al.*, 1996). In addition, PorB porins up-regulate the expression of CD86 co-stimulatory molecules on B-cells which may consequently have an effect on B-and T-cell interactions to direct an appropriate humoral response (Mackinnon *et al.*, 1999).

In Chapter 5, it was observed that outer membranes (OM) of wild type Serogroup B meningococci were able to modulate mo-DC function to drive T-cell proliferation. The enhanced functional ability of mo-DCs exposed to wild type OM may be attributed in part to the presence of endogenous LOS. However, despite the ability of LOS-deficient OM to induce a comparatively lower level of mo-DC maturation than the wild type OM, increased primary T-cell proliferation was observed. There are a multitude of as yet unidentified proteins present within the outer membrane that may be responsible for these observed effects on mo-DCs. It is possible that a number of individual proteins (including class I or II porins) in wild type or LOS-deficient OM possess immuno-stimulatory properties.

Following from published observations indicating the stimulatory potential of neiserrial porins, the objective of the studies presented in this chapter was to examine the effects of purified recombinant class I porin Serogroup B *N. meningitidis* on human mo-DCs. Changes in phenotype and functional ability of mo-DCs to drive allogeneic T-cell proliferation were investigated. Monitoring changes in CD83, HLA-DR and co-stimulatory molecules CD80, CD86 and CD40 assessed maturation of mo-DCs following incubation with purified class I porin. Production of a range of cytokines and chemokines by treated mo-DCs was also analysed. In addition, the capacity of porin-treated mo-DCs to drive proliferation of antigen-specific T-cells was also addressed.

6.2 MATERIALS AND METHODS

6.2.1 *N. meningitidis* class I porin

Preparation of purified recombinant meningococcal class I porin was carried out by Dr. M. Christodoulides (University of Southampton). In brief, the class 1 porin *porA* gene from strain H44/76 was cloned in the QIAGEN QIAexpressÔ type IV pQE vector plasmid. Cloning into the pQE-30 plasmid adds a His_6 affinity tag at the 5' end of the polylinker that functions as a high affinity nickel-binding domain in the translated protein. High level expression of the plasmid was achieved in *E. coli* and the recombinant protein was purified with nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography. Purified recombinant porin was solubilized in water containing 0.04% SDS (w/v).

6.2.2 Treatment of Mo-DCs with *N. meningitidis* class I porin

Monocyte-derived dendritic cells (mo-DC) were cultured as previously described for phenotypic analysis (2.2.2) and for use in functional assays with T-cells (2.2.2). Immature mo-DCs were exposed to purified recombinant class I porin (Por) for varying lengths of treatment. Contaminating endotoxin (*E. coli* LPS) within the porin preparation was measured at 0.2pg/ml (2.2.14). All studies were controlled either by treatment of mo-DCs with 0.04%(w/v) SDS vehicle alone (control) or 0.2pg/ml *E. coli* in 0.04%(w/v) SDS.

6.2.3 Analysis of phenotype and receptor-mediated endocytosis of mo-DCs

Class I porin-treated and control mo-DCs were harvested and analysed for surface expression of CD1a (FITC), CD14 (PE), CD83 (PE), CD80 (PE), CD86 (PE), CD40 (PE), HLA-DR (PE) and CD54 (FITC) by flow cytometry (2.1.5 and 2.2.7.1). Appropriate fluorochrome conjugated isotype control antibodies were also used in parallel (2.1.5). Changes in receptor-mediated endocytosis of FITC-dextran by treated mo-DCs were also assessed by flow cytometry (2.2.8.2). During flow cytometric analysis cells events were collected within a viable gate by the exclusion of dead cells using 7-AAD.

6.2.4 Co-culture of Mo-DCs: CD40Ligand-transfected CHO cells

Treated mo-DCs were harvested and washed twice by centrifuging for 10 minutes at 1500rpm (500 x *g*) in complete medium [10% (v/v) FCS phenol red-free RPMI 1640 medium supplemented with 100U/ml penicillin and 100μg/ml streptomycin]. For cytokine assays, $1x10^5$ mo-DCs were co-cultured with $2x10^5$ CD40Ligand-transfected or wild type CHO cells in 48-well plates in 500μl (final volume) complete medium. Supernatants were collected at increasing time points after initial co-culture to measure levels of IL-12p40 and IL-12p70 proteins by ELISA. For the detection of intracellular IL-12p40 and IL-12p70 expressed by mo-DCs by flow cytometry (2.2.7.4), 3 $x10^5$ mo-DCs were co-cultured with $6x10^5$ CD40Ligand or wild type CHO cells in 24-well plates in 1mL complete medium for 18hrs. Monensin (2.5μM), a protein transport inhibitor, was added for the last 12 hours of cultures.

6.2.5 Production of soluble mediators by mo-DCs

Supernatants obtained following direct 24hr treatment of mo-DCs with stimuli or after subsequent co-culture with CD40Ligand transfected cells were stored at –80°C pending analysis. Cytokine or chemokine secretion into supernatants was assessed with specific immunoassays using monoclonal matched pair antibodies (2.1.6). Where necessary, supernatants from co-culture experiments were diluted 1:5 - 1:200 in the appropriate assay buffer in order for cytokine/chemokines concentration to fall within the standard curve. Concentration of mediators produced by mo-DCs was calculated by relating the absorbance values of duplicate wells to the standard curve.

6.2.6 Measurement of TLR mRNA on mo-DCs

Total RNA from mo-DCs was extracted and treated with DNAse I as described (2.2.11 and 5.2.6). cDNA was prepared from 400ng of total RNA using the Omniscript reverse transcriptase preamplification system (Promega) with random hexamer primers (Promega). The cDNA levels of TLR2 and TLR4 were quantified by TaqMan PCR. The cDNA levels during the linear phase of amplification were normalized against ribosomal 18S rRNA. Relative RNA concentrations were extrapolated using a PBMC standard curve. Probes and primers used are listed in Table 2.8 and 2.9. Results are presented as fold increase in relative mRNA levels in treated cells compared to control.

6.2.7 Mo-DC driven allogeneic T-cell proliferation

$CD4^+$ or $CD4CD45RA^+$ were enriched using MACS beads and purity (>97 and >90, respectively) was assessed for every experiment by flow cytometry (representative flow cytometry profiles are shown in Figure 5.13A). Mo-DC driven allogeneic $CD4^+$ or naïve $CD4^+CD45RA^+$ T-cell proliferation was performed as previously described (2.2.4 and 5.2.8).

6.2.8 Mo-DC driven antigen-specific T-cell proliferation

Fourteen day cultured tetanus toxoid specific-$CD4^+$ T-cells lines were generated from PBMCs of healthy individuals recently vaccinated against tetanus (2.2.5). Autologous mo-DCs were treated for 24hrs with purified class I porin or appropriate controls, washed thoroughly (x4) by centrifugation at 1200rpm for 10minutes, and co-cultured with tetanus toxoid-specific $CD4^+$ short term lines. Five day mo-DC-driven T-cell proliferation assays were set up as described (5.2.8).

CHAPTER 6

Results

6.3 ACTIVATION OF Mo-DCs BY *N. meningitidis* CLASS I PORIN

6.3.1 Analysis of mo-DC phenotype

The effect of purified recombinant class I porin of *N. meningitidis* to induce phenotypic maturation of mo-DC was initially investigated. Incubation of mo-DCs for 24hrs with 1μg/ml class I porin resulted in morphological changes seen as cellular clumping and defined dendrite formation, viewed through an inverted microscope (Figure 6.1). Exposure of mo-DCs to purified class I porin for 24hrs induced marked elevation in surface expression of CD83 ($p=0.05$), CD80 ($p<0.05$), CD86 ($p<0.05$), CD40 ($p<0.05$), HLA-DR ($p<0.05$) and CD54 ($p<0.05$) as determined by flow cytometry (Figure 6.2 and Figure 6.3). Whereas, expression of all these molecules on mo-DCs treated with 0.2pg/ml *E. coli* LPS, to control for contaminating LPS present within the porin preparation, was comparable to those on mo-DCs treated with vehicle alone (Figure 6.2 and Figure 6.3). Summary of phenotypic changes following treatment with purified class I porin or contaminating levels of *E. coli* LPS is presented as median fluorescence intensity in Figure 6.3.

By comparison, the marked phenotypic changes exhibited by 1μg/ml porin-treated mo-DCs was less than that observed by mo-DCs pre-treated with 1μg/ml wild type meningococcal outer membranes (Figure 6.2 and Chapter 5 Figure 5.6). Preliminary dose-response studies showed increased expression of all surface molecules (except for CD83) on mo-DCs following treatment with purified class I porin at concentrations as low as 100ng/ml with maximal changes observed at 5μg/ml (data not shown). Induction of CD83 on mo-DCs by purified class I porin was noted at starting concentration of 500μg/ml (data not shown).

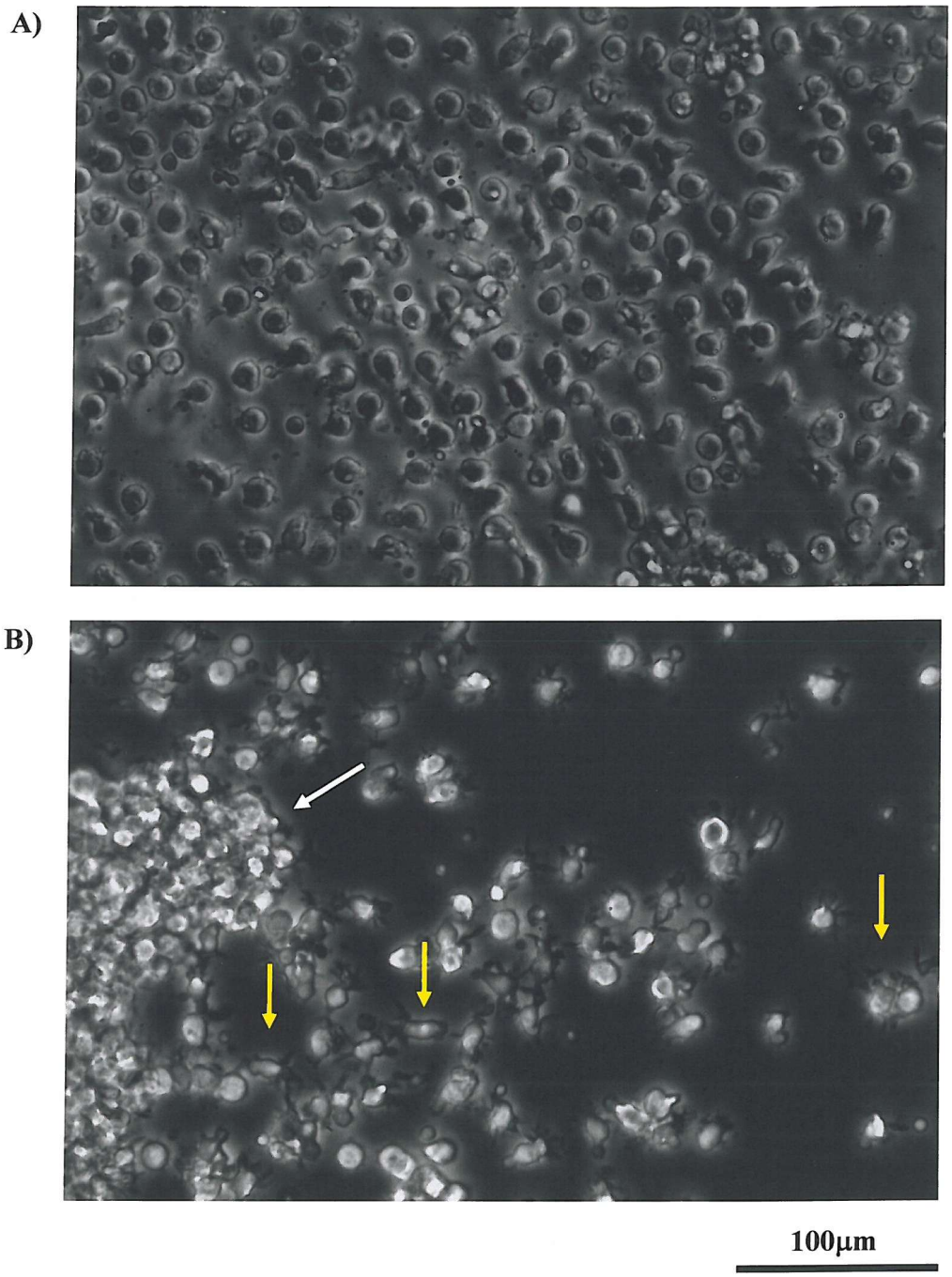

Figure 6.1: Morphology of mo-DCs treated with class I porin of *N. meningitidis*.

Five-day cultured mo-DCs were exposed for 24hrs to A) 0.04% SDS (w/v) vehicle control containing 0.2pg/mL *E. coli* LPS or B) 1µg/ml purified recombinant class I porin. Cells were viewed under phase contrast at x20 magnification. White arrows show mo-DC clustering and yellow arrows show veiled dendritic cells (compared to control treatment).

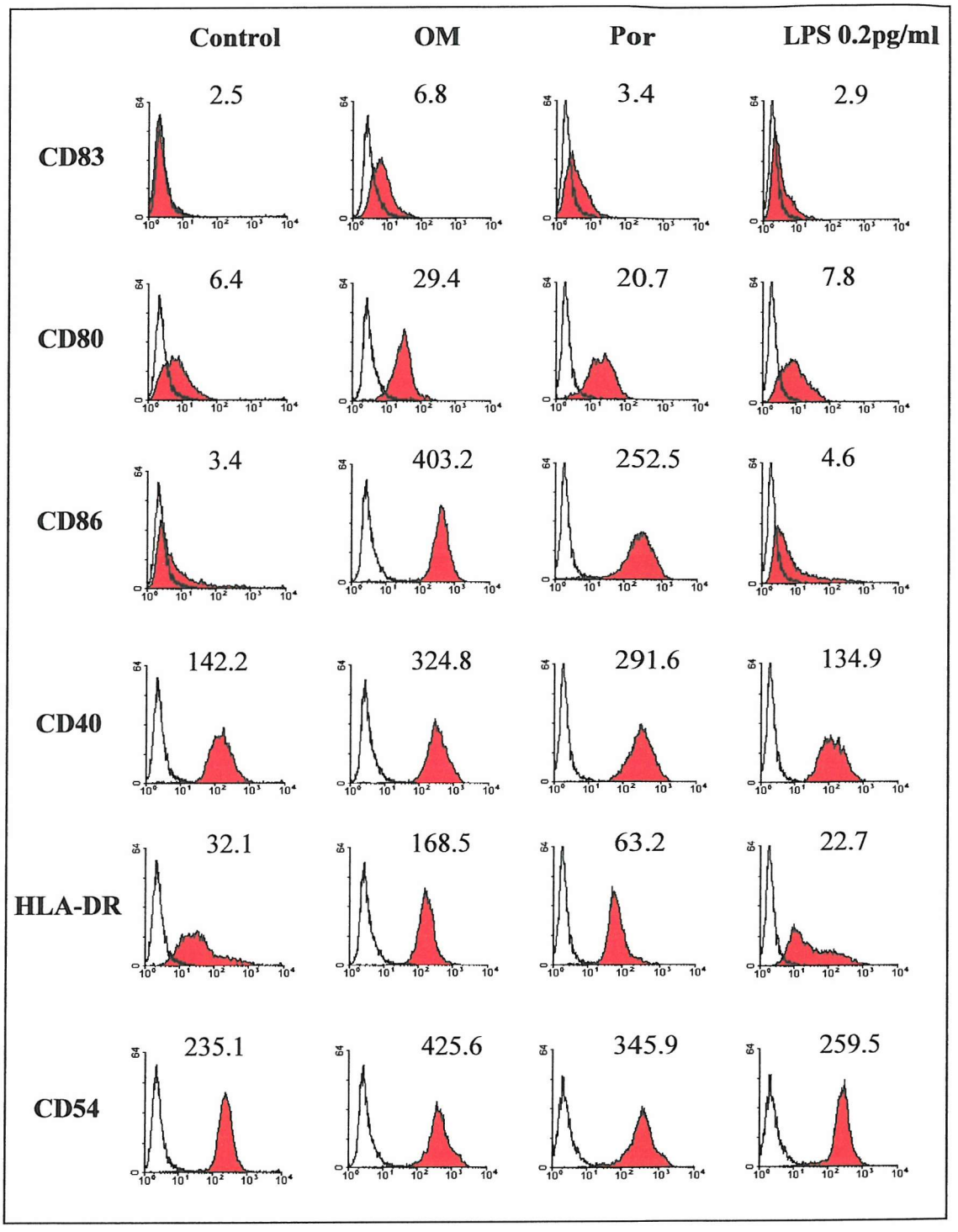

Figure 6.2: Phenotype of mo-DCs stimulated with meningococcal class I porin.

Cell surface expression of CD83, CD80, CD86, CD40, HLA-DR and CD54 on vehicle-treated (control) or cells stimulated for 24hr with 1μg/ml purified recombinant class I porin, 1μg/ml outer membranes from wild type H44/76 strain (OM) or 0.2pg/ml *E. coli* LPS. Expression of markers is shown by the solid red histograms. Cells stained with relevant isotype mAbs is shown as open clear histograms. Numbers on each histogram corresponds to the median fluorescence intensity of mAb staining. Data shown is a representative of eight experiments.

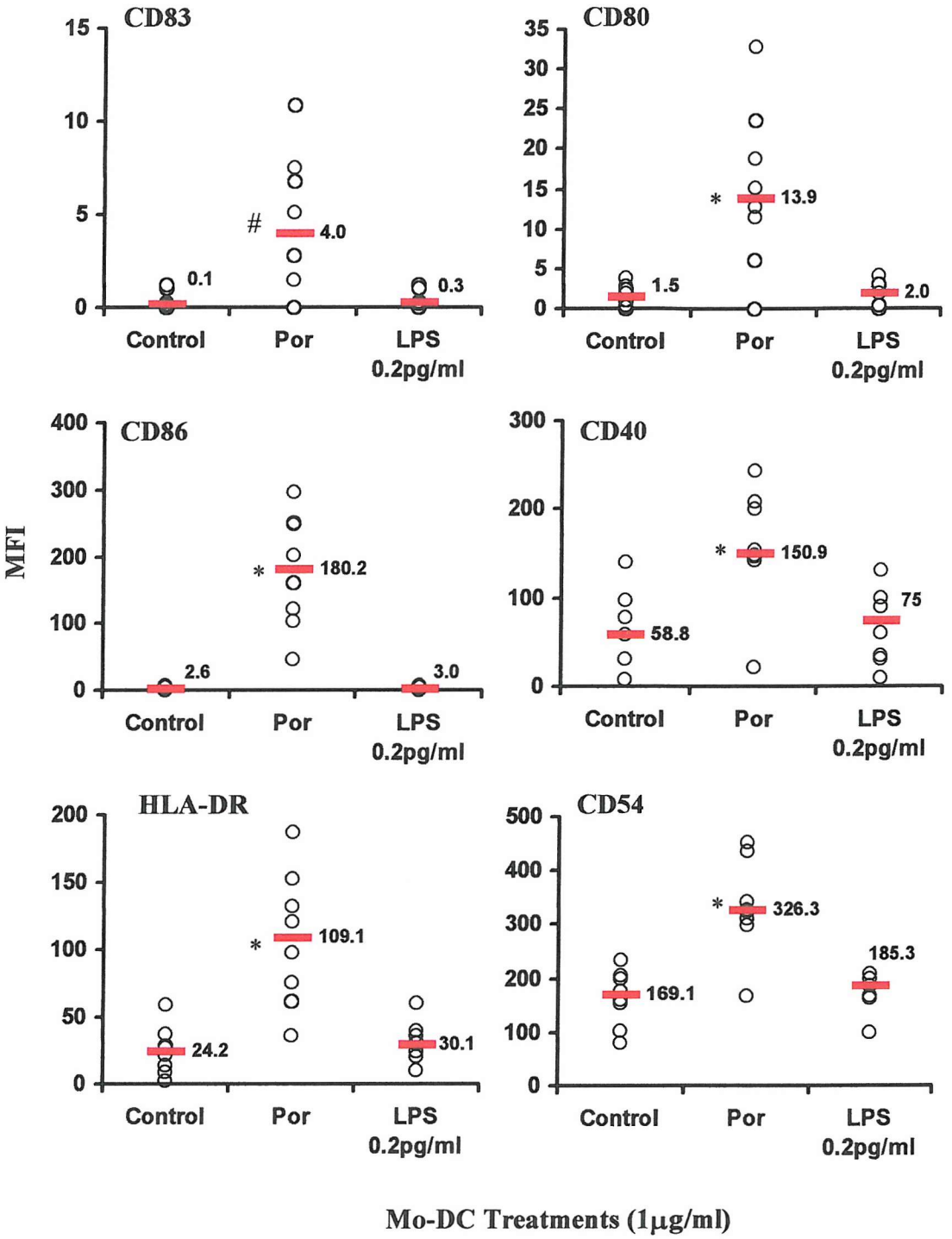

Figure 6.3: Summary data illustrating changes in mo-DC phenotype after treatment with *N. meningitidis* class I porin.

Mo-DCs were left untreated (control) or stimulated for 24hrs with 1μg/ml purified recombinant class I porin (Por) or 0.2pg/ml *E. coli* LPS. Expression of CD83, CD80, CD86, CD40, HLA-DR and CD54 was analysed by flow cytometry. Data shows all (n=8) the absolute individual median fluorescence intensity (MFI) after the respective treatments (○). Median for each treatment is shown as red bar with the value shown alongside. # denotes p=0.05; * denotes p>0.05 compared to control cells (Wilcoxon's test).

6.3.2 Receptor-mediated endocytosis

The ability of meningococcal class I porin to affect the uptake of FITC-labelled dextran by mo-DCs via receptor-mediated endocytosis was assessed (Figure 6.4). Representative flow cytometric profiles show FITC-dextran uptake by control and treated mo-DCs at 37°C (Figure 6.4, green histogram). Incubation of mo-DCs at 4°C determined the level of non-specific binding of FITC-dextran to these cells (Figure 6.4, clear histogram).

As observed in section 5.4.2, immature (vehicle control treated-) mo-DCs incubated at 37°C readily take up FITC-dextran (median fluorescence intensity 38.9.0 ± 52, n=6). However, pre-treatment of mo-DCs for 24hrs with purified recombinant class I porin significantly reduced its ability to subsequently take up FITC-dextran (median fluorescence intensity 13.7 ± 2.5, Wilcoxon's test $p<0.05$ n=6). In parallel, mo-DCs were stimulated with 0.2pg/ml *E. coli* LPS, to control for endotoxin within the porin preparation, or with 1μg/ml *E. coli* LPS. FITC-dextran uptake by mo-DCs pre-treated with 0.2pg/ml LPS (median fluorescence intensity 41.2 ± 7.0, n=6) was comparable with that observed by vehicle control-treated mo-DCs. However, the marked reduction in FITC-dextran observed by mo-DCs pre-exposed to 1μg/ml *E. coli* LPS (median fluorescence intensity 3.4 ± 0.9, Wilcoxon's test $p<0.05$ n=6), was comparable to that observed by meningococcal LOS (Chapter 5).

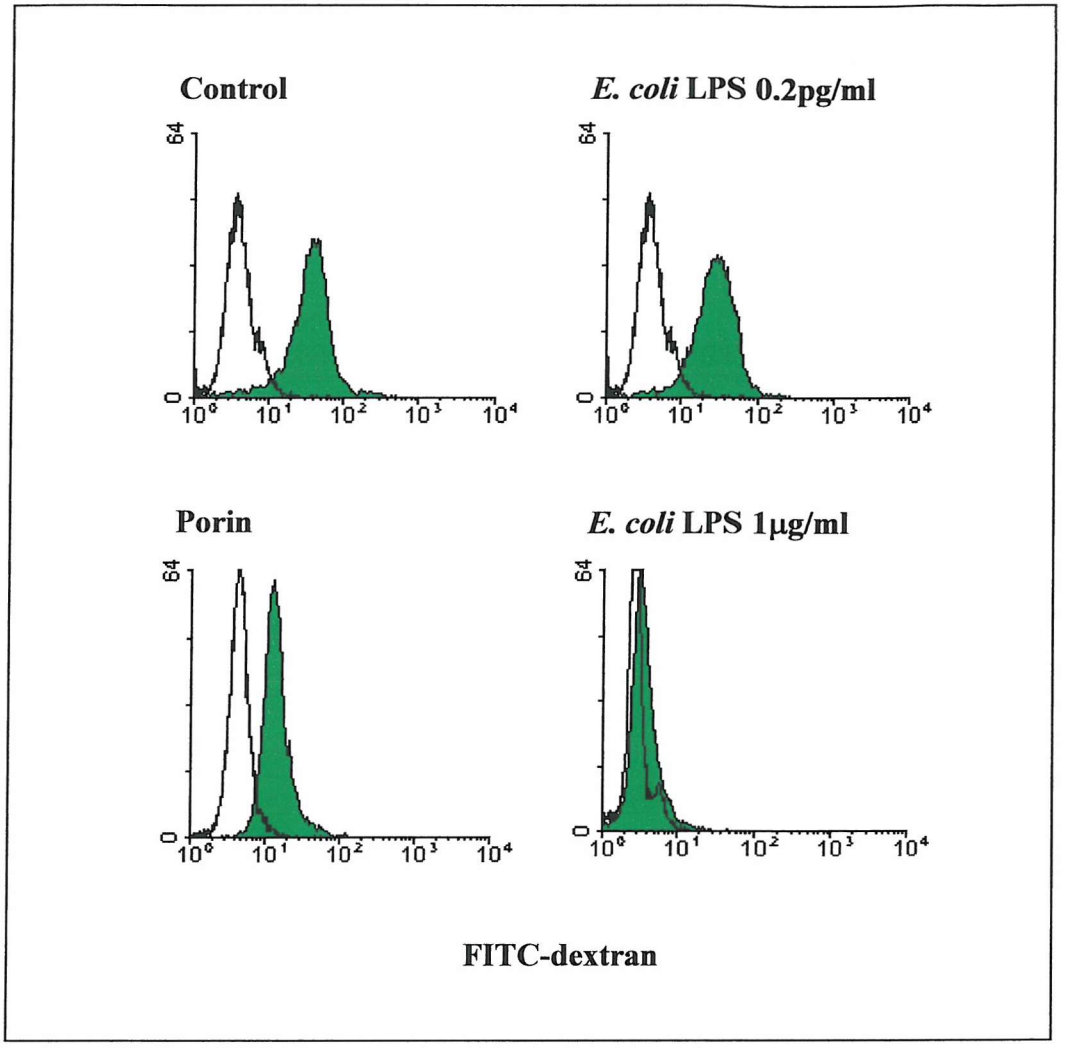

Figure 6.4: Activity of *N. meningitidis* class I porin on mo-DC receptor-mediated endocytosis.

Mo-DCs were treated with vehicle control or exposed for 24hrs to 1µg/ml purified recombinant class I porin, 1µg/ml *E. coli* LPS or 0.2pg/ml *E. coli* LPS. Following treatment, mo-DC were harvested and washed thoroughly prior to incubation with 250 mg/ml FITC-dextran for 1hr. Antigen uptake was assessed by flow cytometric analysis using a FACScan. Solid histograms represent cells incubated with FITC-dextran at 37°C, 5% CO_2. Clear histograms represent cells incubated with FITC-dextran at 4°C. Results are a representative of six separate experiments.

6.3.3 Effect of *N. meningitidis* class I porin on Mo-DC Toll-like receptor mRNA expression

Marked differences were observed in the abilities of wild type or LOS-deficient meningococcal outer membranes to affect TLR mRNA in mo-DCs (5.5.4). Therefore, the activity of meningococcal class I porin to induce changes in TLR mRNA expression was assessed. TaqMan RT-PCR was employed to measure TLR mRNA transcripts on mo-DCs treated with vehicle alone (control), purified class I porin or *E. coli* LPS (Figure 6.5). TLR2 mRNA levels were moderately reduced in mo-DCs stimulated for 24hrs with 1μg/ml porin (Wilcoxon's test $p>0.05$, $n=7$) and significantly reduced after 24hr treatment with 1μg/ml *E. coli* LPS (Wilcoxon's test $p<0.05$, $n=7$). In contrast, significantly increased TLR4 mRNA expression was observed after exposure to either treatment (Figure 6.5; Wilcoxon's test, $p<0.05$). TLR2 and TLR4 mRNA was unaffected following treatment of mo-DCs with 0.2pg/ml *E. coli* LPS. The observed TLR2 and TLR4 mRNA expression patterns following treatment of mo-DC with *E. coli* LPS or meningococcal LOS (Figure 6.5 and 5.10) were comparable.

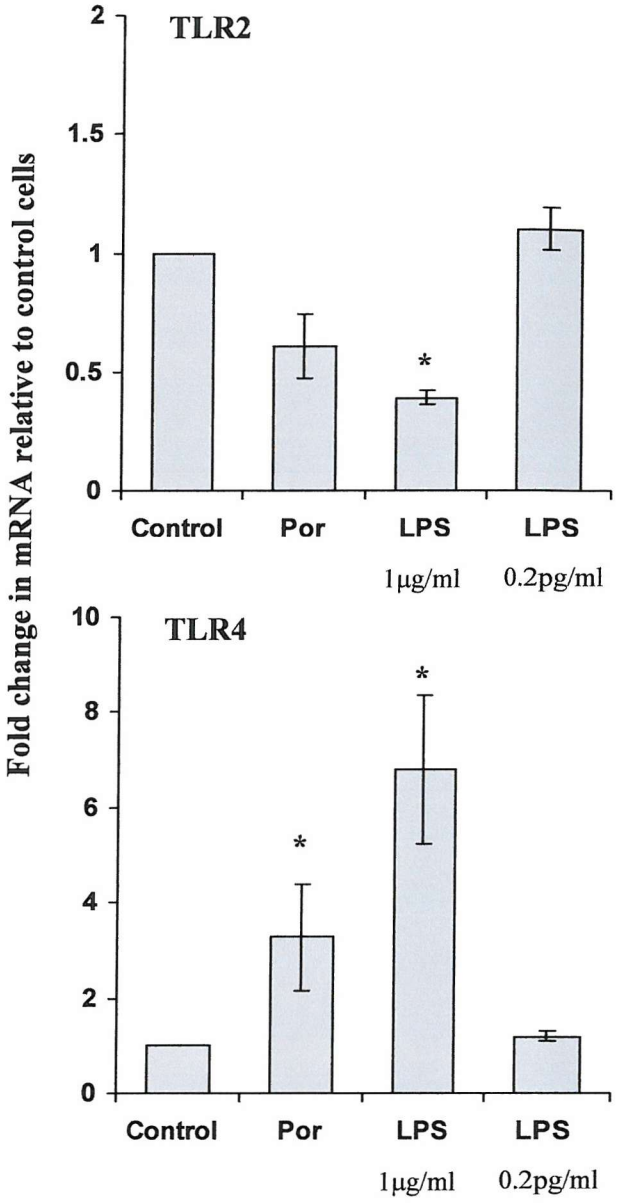

Figure 6.5: Expression of TLR2 and TLR4 mRNA on mo-DCs following treatment with *N. meningitidis* class I porin.

Cells were exposed to vehicle (control) or incubated for 24hrs to 1μg/ml purified recombinant class I porin, 1μg/ml *E. coli* LPS or 0.2pg/ml *E. coli* LPS. After treatment, extracted RNA was reverse transcribed into cDNA. Relative expression of TLR2 and TLR4 mRNA on mo-DCs were extrapolated from a PBMC standard curve following semi-quantitation by TaqMan PCR. TLR mRNA levels were normalized to the amount of endogenous ribosomal 18s RNA. Results are presented as average fold change in expression following treatment relative to control cells ± standard error (TLR2 n=7; TLR4 n=7). * $p<0.05$ compared to control cells.

6.3.4 Activity of class I porin of *N. meningitidis* on induction of soluble mediators by mo-DCs

Following incubation of mo-DC with purified class I porin, dose-dependent increases in IL-6, IL-10, TNFα and IL-12p70 production were observed (Figure 6.6). Although sample numbers are low, statistical significance was achieved at varying concentrations of treatment ($p<0.05$, Students t-test $n=4$). Moderately increased cytokine production was detected at concentrations of purified porin commencing at 0.01μg/ml ($p>0.05$).

Figure 6.7 and Figure 6.8 shows the activity of 1μg/ml porin on production of cytokines and chemokines by mo-DCs, respectively. Significant ($p<0.05$, Wilcoxon's test) production of IL-6, IL-10, TNFα and IL-12p40 were noted by 24hr porin-treated mo-DCs compared to that from cells stimulated with vehicle alone or 0.2pg/ml *E. coli* LPS (Figure 6.7). An increased pattern of IL-1β production was observed following treatment with purified porin, although this did not achieve statistical significance (Figure 6.7). Very low levels of biologically active IL-12p70 were produced by mo-DCs following treatment with purified porin (Figure 6.7). However, these increased levels did not achieve significance ($p>0.05$) over the amount of IL-12p70 produced by mo-DCs stimulated with 0.2pg/ml *E. coli* LPS. Of note, 1μg/ml *E. coli* LPS did not induce marked IL-12p70 production (32.6 pg/ml ± 5.1, $n=5$) by mo-DCs following 24hr exposure. This finding is in contrast to the effect of pure meningococcal LOS on IL-12p70 production by mo-DCs (Chapter 5: 349.8 pg/10^6 cells ± 50.3, $n=5$, $p<0.05$).

Induction of significant levels ($p<0.05$, Wilcoxon's test) of IL-8, RANTES, MIP-1α and MIP-1β by mo-DCs was also observed following treatment for 24hrs with purified class I porin (Figure 6.8). Whereas, production of chemokines by mo-DCs incubated with 0.2pg/ml *E. coli* LPS or vehicle alone was not altered.

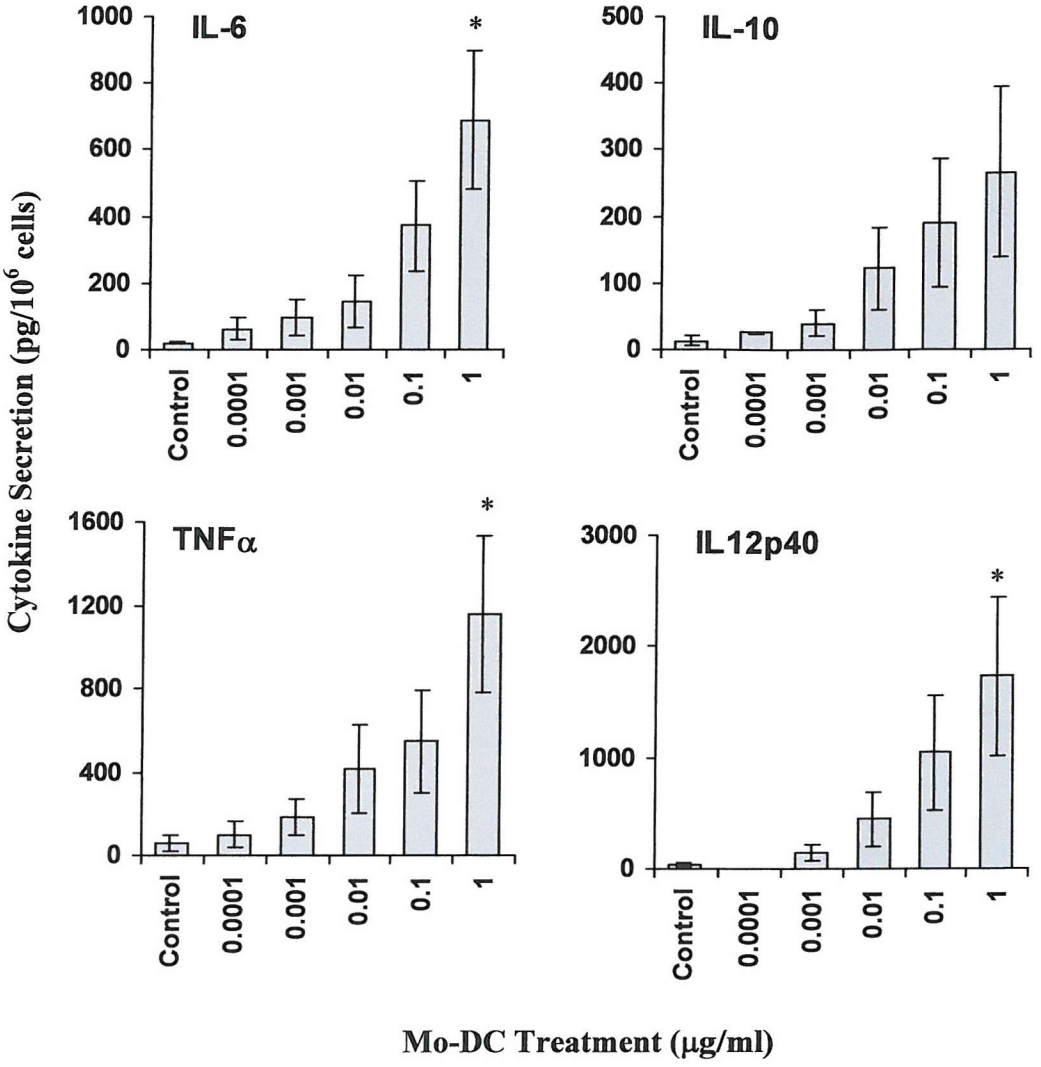

Figure 6.6: Dose dependent production of cytokine by mo-DCs following treatment with *N. meningitidis* class I porin

Cells were treated with vehicle alone (control) or stimulated for 24hrs with varying concentrations of purified recombinant class I porin (0.0001-1μg/ml). Supernatants were assayed for IL-6 (n=4), IL-10 (n=4), TNFα (n=4) and IL-12p40 (n=4). Results are presented as average cytokine production (pg/10^6 cells) with standard error bars. * = Significance observed ($p<0.05$, Student t-test) compared to control.

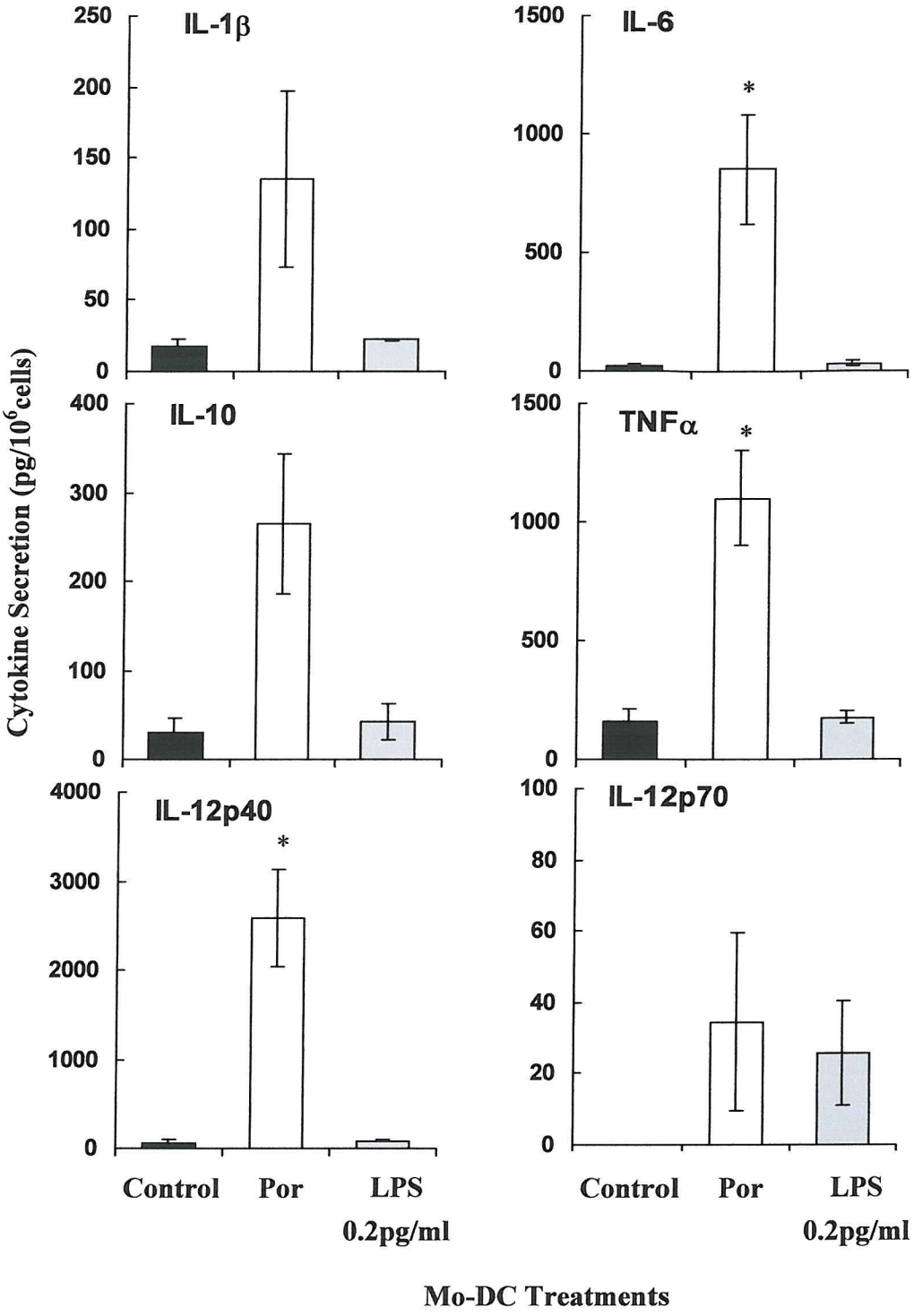

Figure 6.7: Effects of *N. meningitidis* class I porin on cytokine production by mo-DCs.

Cells were treated with vehicle alone (control, black bar) or stimulated for 24hrs with 1μg/ml purified recombinant class I porin (Por, white bar) or 0.2pg/ml *E. coli* LPS (grey bar). Supernatants were assayed for IL-1β (n=5), IL-6 (n=7), IL-10 (n=8), TNFα (n=7), IL-12p40 (n=9) and IL-12p70 (n=5). Results are presented as average cytokine production pg/10^6 cells with standard error bars. *=Significance observed ($p<0.05$) compared to control.

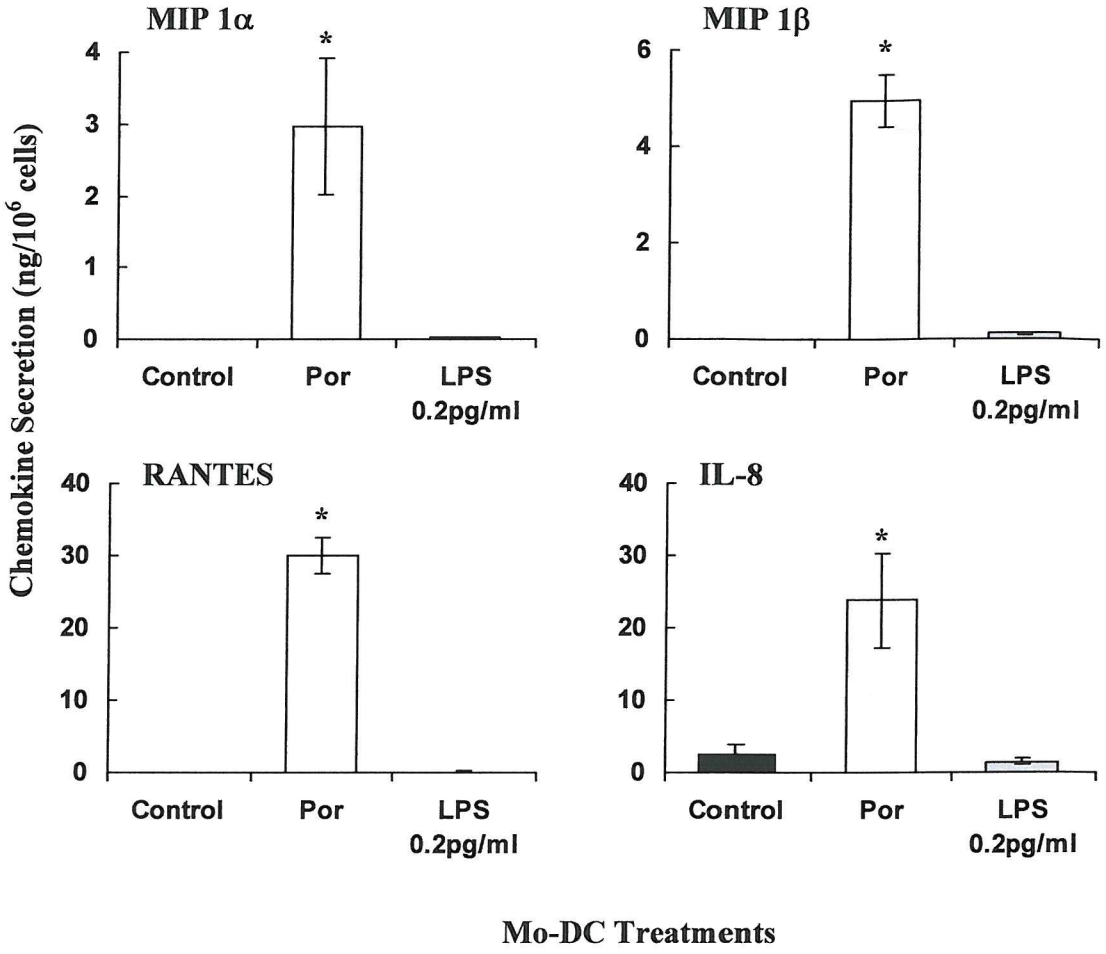

Figure 6.8: Effects of *N. meningitidis* class I porin on chemokine production by mo-DCs.

Cells were treated with vehicle alone (control, black bar) or stimulated for 24hrs with 1μg/ml purified recombinant class I porin (Por, white bar) or 0.2pg/ml *E. coli* LPS (grey bar). Supernatants were assayed for MIP-1α (n=5), MIP-1β (n=5), RANTES (n=5) and IL-8 (n=8) by sandwich immunoassay. Results are presented as average chemokine production ng/10^6 cells with standard error bars. *=Significance observed ($p<0.05$) compared to control.

6.3.5 Ligation of CD40 on mo-DCs

Biologically active IL-12p70 is an important cytokine that plays a role in regulating the balance of Th-cell immune responses (Heufler *et al.*, 1996; Hilkens *et al.*, 1997). Results presented so far in this chapter have demonstrated the potency of purified class I porin to induce mo-DC phenotypic maturation and induction of all soluble mediators tested, except for bioactive IL-12p70. Co-stimulation provided by the ligation of CD40 on DCs with CD40Ligand on T-cells is reported to induce high levels of IL-12 production (Cella *et al.*, 1996). Therefore, the results from studies presented in sections 6.3.5.1 and 6.3.5.2 addresses the ability of purified porin to affect the subsequent production of IL-12 by mo-DCs following CD40-ligation. In these experiments, CD40 cross-linking was brought about through co-culture of mo-DCs with CHO cells stably transfected with human CD40-ligand (CD154), acting as surrogate T-cells to provide co-stimulation.

6.3.5.1 Flow cytometric analysis of intracellular IL-12 expression in mo-DCs

Immature mo-DCs (treated with vehicle alone) or mo-DCs pre-exposed for 24hrs to purified recombinant class I porin were co-cultured with CD40Ligand-transfected or wild type CHO cells for 18hrs. Monensin was added to the cultures for the last 12hrs to facilitate detection of intracellular IL-12 by flow cytometry. Cells were subsequently harvested and doubly labelled for HLA-DR and intracellular IL-12p40 (Figure 6.9) or IL-12p70 (Figure 6.10). No intracellular IL-12p40 was detected in either control or porin-treated HLA-DR$^+$ mo-DCs following subsequent co-culture with wild-type CHO cells (Figure 6.9B). However, a high proportion of control immature (vehicle-treated) mo-DCs express IL-12p40 (53.7% ± 11.2, n=5) following co-culture with CD40Ligand-transfected CHO cells, indicating the ability of CD40-CD40L interactions to mediate IL-12 production (Figure 6.9B). Exposure of mo-DCs to 1μg/ml purified porin for 24hrs markedly decreased the percentage of cells expressing IL-12p40 following subsequent CD40-ligation (17.3% ± 4.4, $p<0.05$ Wilcoxon's test, n=5) (Figure 6.9B). This decrease in IL-12p40 was not observed on mo-DCs pre-treated with 0.2pg/ml *E. coli* LPS prior to CD40-ligation (data not shown).

In contrast to IL-12p40, no intracellular IL-12p70 was detected in control or porin-treated HLA-DR$^+$ mo-DCs co-cultured for a further 18hrs with wild-type or CD40Ligand-transfected CHO cells (Figure 6.10).

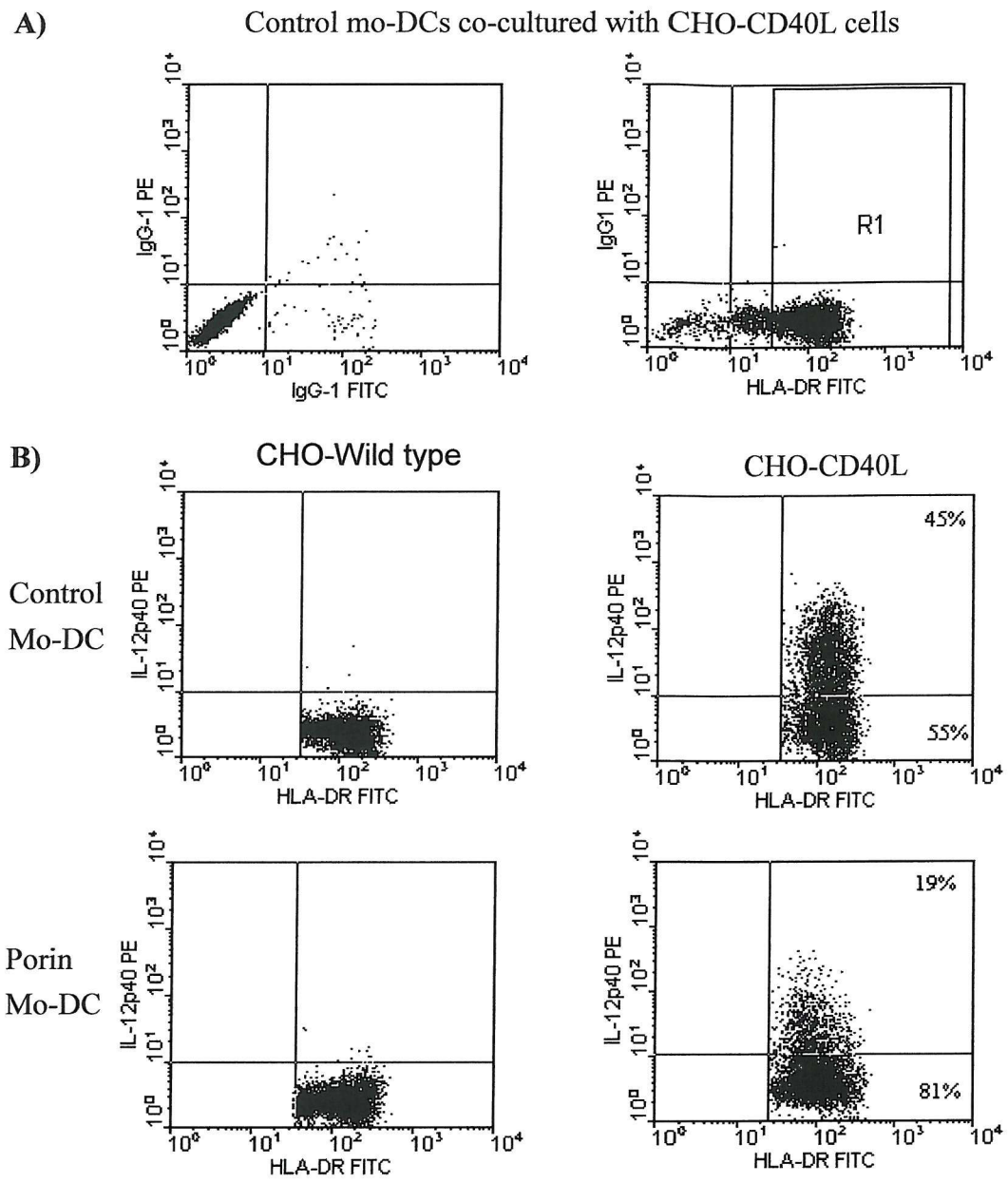

Figure 6.9: Production of intracellular IL-12p40 by CD40-ligated mo-DCs.

Mo-DCs were exposed to 1μg/ml purified class I porin or vehicle alone (control) for 24hr hours and subsequently co-cultured with wild type or CD40Ligand-transfected CHO cells for a further 18hrs. 2.5μM monensin was added to cultures for the last 12hrs of culture. Cells were harvested and doubly labelled for HLA-DR and intracellular IL-12p40. **A)** Profiles illustrate cell harvest (control mo-DCs co-cultured with CD40L transfectants) stained with isotype matched controls and HLA-DR. **B)** HLA-DR-positive gate (Region 1) showing percentage of mo-DCs expressing intracellular IL-12p40 following 12hr co-culture with wild type or CD40L-CHO cells. These dot plots are a representative from five experiments.

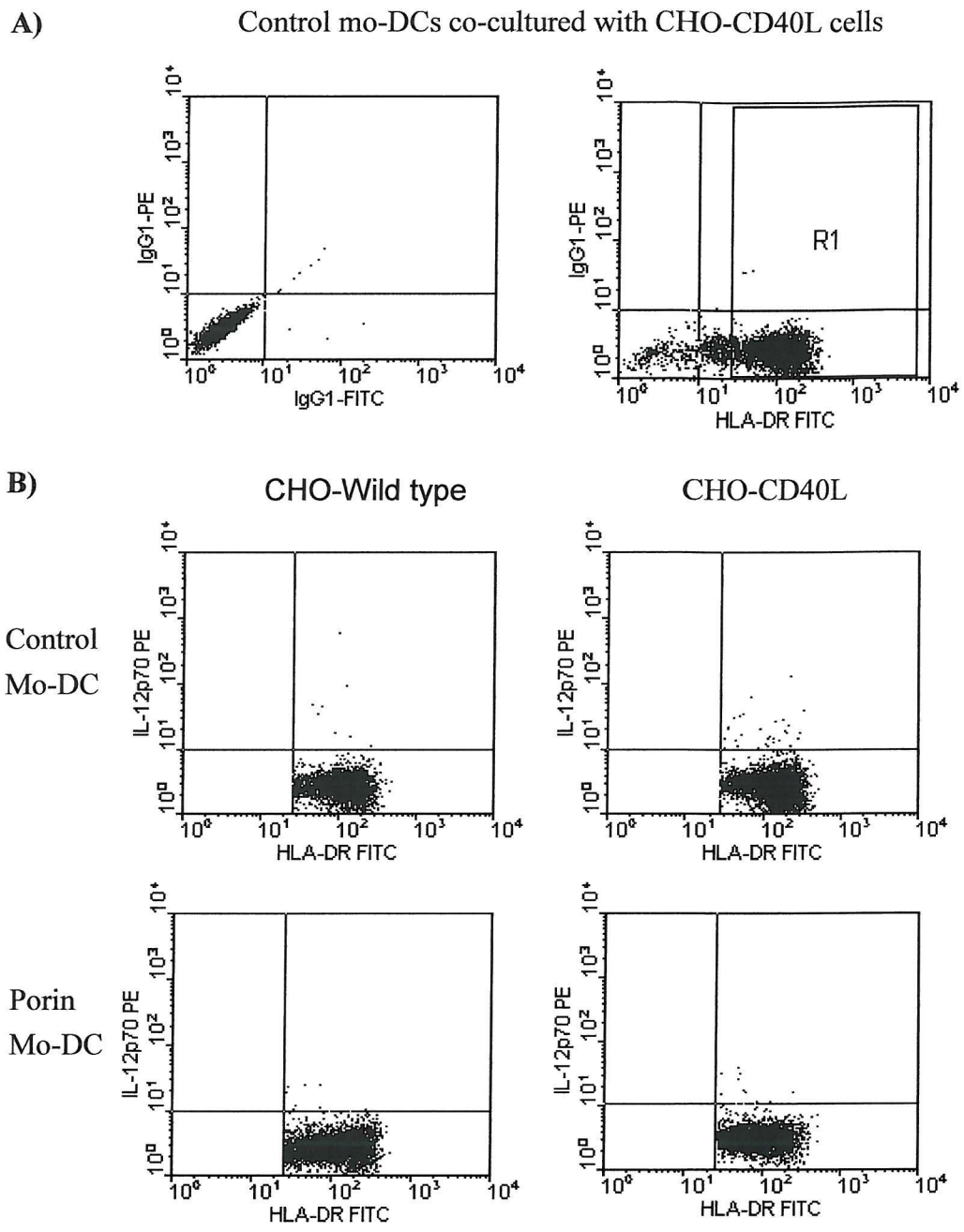

Figure 6.10: Production of intracellular IL-12p70 by CD40-ligated mo-DCs.

Mo-DCs were exposed to 1μg/ml class I Porin or vehicle alone (control) for 24hr hours and subsequently co-cultured with wild type or CD40Ligand-transfected CHO cells for a further 18hrs. 2.5μM monensin was added to cultures for the last 12hrs of culture. Cells were harvested and doubly labelled for HLA-DR and intracellular IL-12p70. **A)** Profiles illustrate harvested cells (control mo-DC treatment co-cultured with CD40L transfectants) stained with isotype matched controls and HLA-DR. **B)** Following 12hr co-culture with wild type or CD40L-CHO cells HLA-DR-positive gate (Region 1) was analysed for intracellular IL-12p70. These dot plots are a representative from five experiments.

6.3.5.2 Detection of IL-12 production by CD40-ligated mo-DCs by ELISA

Similar to the results obtained by flow cytometry (Figure 6.9) reduced secretion of IL-12p40 was detected in supernatants of mo-DCs pre-treated for 24hrs with 1μg/ml purified class I porin prior to subsequent 12hr co-culture with CD40Ligand-transfected CHO cells (Figure 6.11). Moreover, this decrease in IL-12p40 was dependent on the concentration of porin used to pre-treat mo-DCs (Figure 6.11A). In contrast to the flow cytometric data shown (Figure 6.10), high levels of IL-12p70 were produced by control mo-DCs following CD40-ligation (Figure 6.12). A dose-dependent decrease in IL-12p70 production by mo-DCs pre-treated with purified class I porin prior to CD40-ligation was observed (Figure 6.12A). Little or no production of IL-12p40 or IL-12p70 was detected by control or porin-treated mo-DCs following co-culture with wild-type CHO cells (Figures 6.11B and 6.12B). Pre-treatment of mo-DCs with 0.2pg/ml *E. coli* LPS had no effect on subsequent IL-12p40 (Figure 6.11C) or IL-12p70 (Figure 6.12C) after co-culture with CD40Ligand-CHO cells. Whereas, mo-DCs pre-treated with 1μg/ml *E. coli* LPS significantly increased IL-12p40 (Figure 6.11C) and IL-12p70 (Figure 6.12C) production upon subsequent CD40-ligation.

Ligation of CD40 in control (vehicle alone) mo-DCs induced significantly increased production of IL-12p40 (Figure 6.13A) and p70 (Figure 6.14A) that related to the duration of contact with CD40Ligand. This increase was apparent after 6hrs of CD40-ligation and had reached a maximum by 12hrs. Of note, the quantities of IL-12p40 were produced in ng, while p70 was produced in comparatively lower levels. The activities of mo-DCs pre-treated for 24hrs with 1μg/ml of purified class I porin or outer membranes from wild type (OM) or LOS-deficient mutant (pLAK-OM) of *N. meningitidis* on subsequent IL-12 production following CD40-ligation was compared. The trend observed for IL-12p40 production by mo-DCs pre-exposed to porin, OM or pLAK-OM was similar after CD40-ligation for 6, 12 or 24hrs. As illustrated in Figure 6.11, IL-12p40 released by porin-treated mo-DCs was reduced following CD40-ligation (Figure 6.13A). No significant difference in the production of IL-12p40 by CD40-ligated mo-DCs pre-treated with OM or pLAK-OM was observed compared to control mo-DCs (Figure 6.13A). IL-12p70 production by mo-DCs pre-treated with porin followed by CD40-ligation was also reduced (except for after 6hr

co-culture with CD40Ligand-CHO cells, where low levels were comparable with that of vehicle-treated mo-DCs, Figure 6.14A). After 12hr co-culture with CD40Ligand-CHO cells, OM activated mo-DCs exhibited a decreased ability to secrete IL-12p70 compared to control mo-DCs ($p>0.05$, $n=3$ Figure 6.14A). The reverse pattern was observed after 24hr CD40-ligation of OM-stimulated mo-DCs ($p>0.05$, $n=3$, Figure 6.13A). No change in IL-12p70 production by pLAK-OM treated mo-DCs was seen after CD40-ligation for 12 or 24hrs (Figure 6.14A). From these results IL-12p40 or p70 production by control mo-DCs was optimal after 12hr co-stimulation between CD40-CD40Ligand. In addition, pre-treatment with purified class I porin possesses a distinct ability, compared to OM or pLAK-OM, to affect the production of IL-12 by mo-DCs after co-culture with CD40-ligand transfected cells.

The effect of a shorter pre-stimulation of mo-DCs with purified class I porin was investigated on the subsequent ability to produce IL-12 following CD40-ligation. In these studies, mo-DCs were pre-treated for 8 or 24hrs with 1μg/ml purified class I porin, wild type OM or pLAK-OM and co-cultured with CD40Ligand-CHO cells for 12hrs. Pre-treatment of mo-DCs for 8hrs with purified porin did not significantly inhibit IL-12p40 (Figure 6.13B) or IL-12p70 (Figure 6.14B) production compared to vehicle-treated mo-DCs following CD40-ligation, whereas, a decreased production of p40 and p70 was noted in mo-DCs pre-treated for 24hrs with purified porin (Figure 6.13B IL-12p40: $p<0.01$ $n=7$; Figure 6.14B IL-12p70: $p<0.05$ $n=8$). Stimulation of mo-DC with wild type OM for 8hrs resulted in increased IL-12p40 (Figure 6.13B $p<0.01$ $n=7$) and IL-12p70 (Figure 6.14B $p<0.05$ $n=8$) production following CD40-ligation. In contrast, 24hr treatment of mo-DCs with OMs reduced the capacity of these cells to produce IL-12p40 ($p<0.05$ $n=7$) or p70 ($p<0.05$ $n=8$) after subsequent CD40 co-stimulation. However, this decrease in production was less than that observed by 24hr porin-treated mo-DCs. No differences in IL-12 production were observed by mo-CD40-ligated mo-DCs pre-treated with outer membranes from the LPS-deficient mutant (pLAK-OM) for 8 or 24hrs (Figure 6.13B and 6.14B).

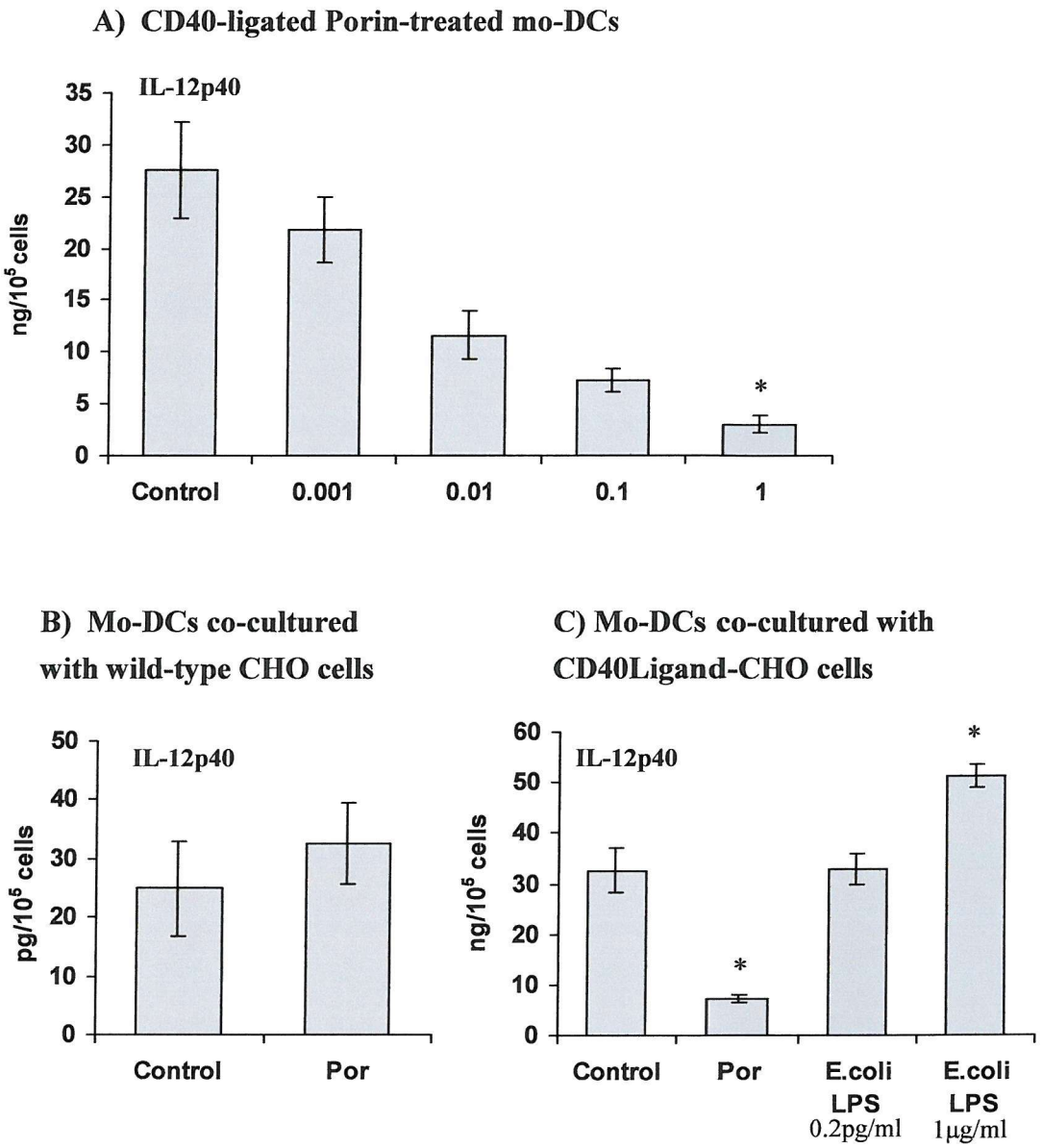

Figure 6.11: Influence of CD40-ligation and class I porin on production of IL-12p40 by mo-DCs.

Mo-DCs were stimulated for 24hrs with purified recombinant class I porin or vehicle control prior to 12hr co-culture with CD40Ligand-transfected CHO cells. **A)** Dose dependent effect on IL-12p40 production by mo-DCs stimulated with varying concentrations of porin (0.001-1µg/ml). **B)** IL-12p40 production by vehicle- or porin (1µg/ml)-treated mo-DCs co-cultured with wild-type CHO cells. **C)** Mo-DCs were pre-treated with 1µg/ml purified class I porin, 1µg/ml *E. coli* LPS or 0.2pg/ml *E. coli* LPS prior to co-culture with CD40Ligand-transfected CHO cells. Results are presented as mean production of IL-12p40 per 10^5 cells with standard error bars (A-C: n=5). *=Significance ($p<0.05$) observed compared to control cells..

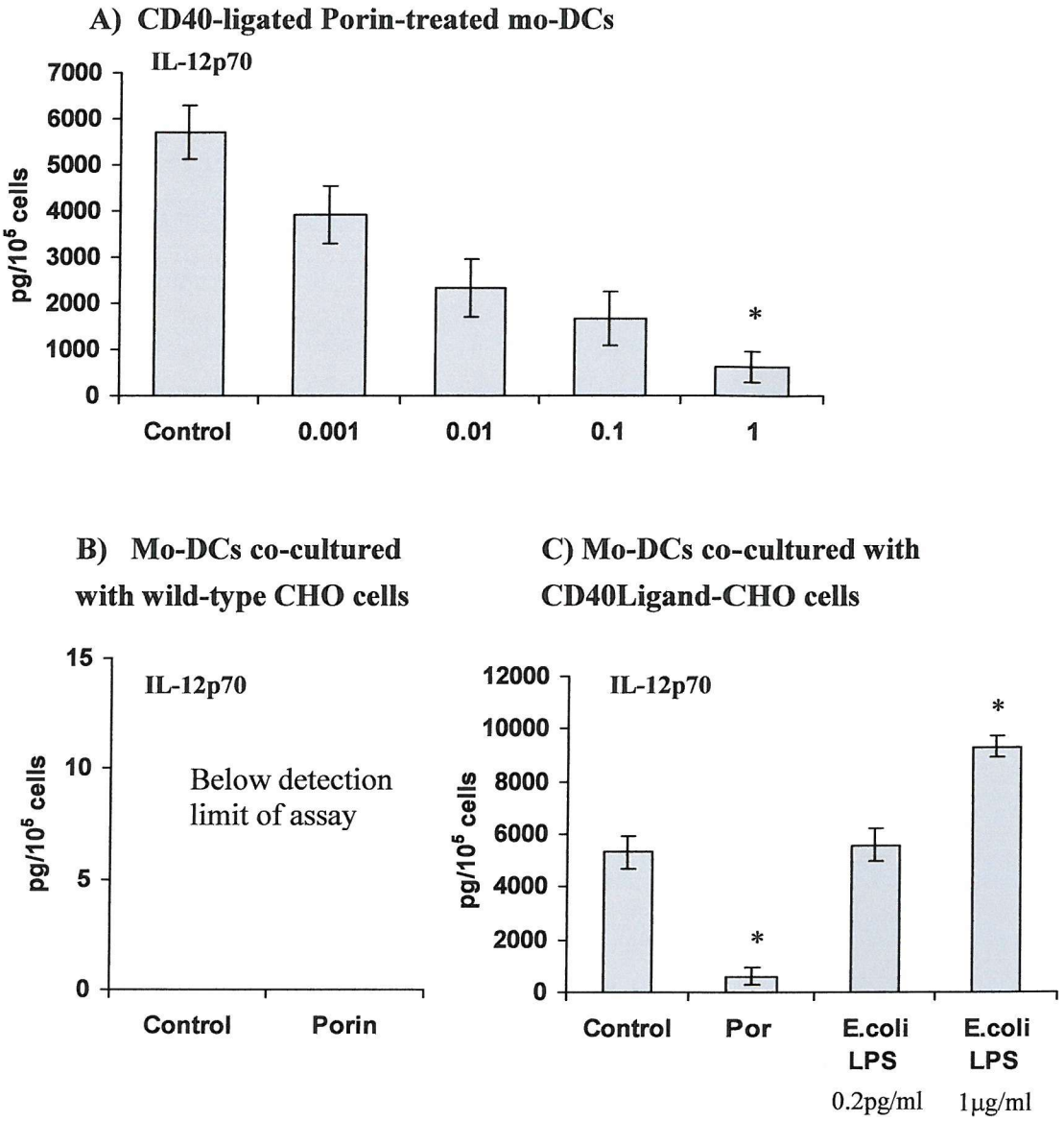

Figure 6.12: Influence of CD40-ligation and class I porin on production of IL-12p70 by mo-DCs

Mo-DCs were stimulated for 24hrs with purified recombinant class I porin or vehicle control prior to 12hr co-culture with CD40Ligand-transfected CHO cells. **A)** Dose dependent effect on IL-12p70 production by mo-DCs stimulated with varying concentrations of porin (0.001-1µg/ml). **B)** IL-12p70 production by vehicle- or porin (1µg/ml)-treated mo-DCs co-cultured with wild-type CHO cells. **C)** Mo-DCs were pre-treated with 1µg/ml purified class I porin, 1µg/ml *E. coli* LPS or 0.2pg/ml *E. coli* LPS prior to co-culture with CD40Ligand-transfected CHO cells. Results are presented as mean production of IL-12p70 per 10^5 cells with standard error bars (A: n=4, B: n=8, C: n=8). *=Significance ($p<0.05$) observed compared to control cells.

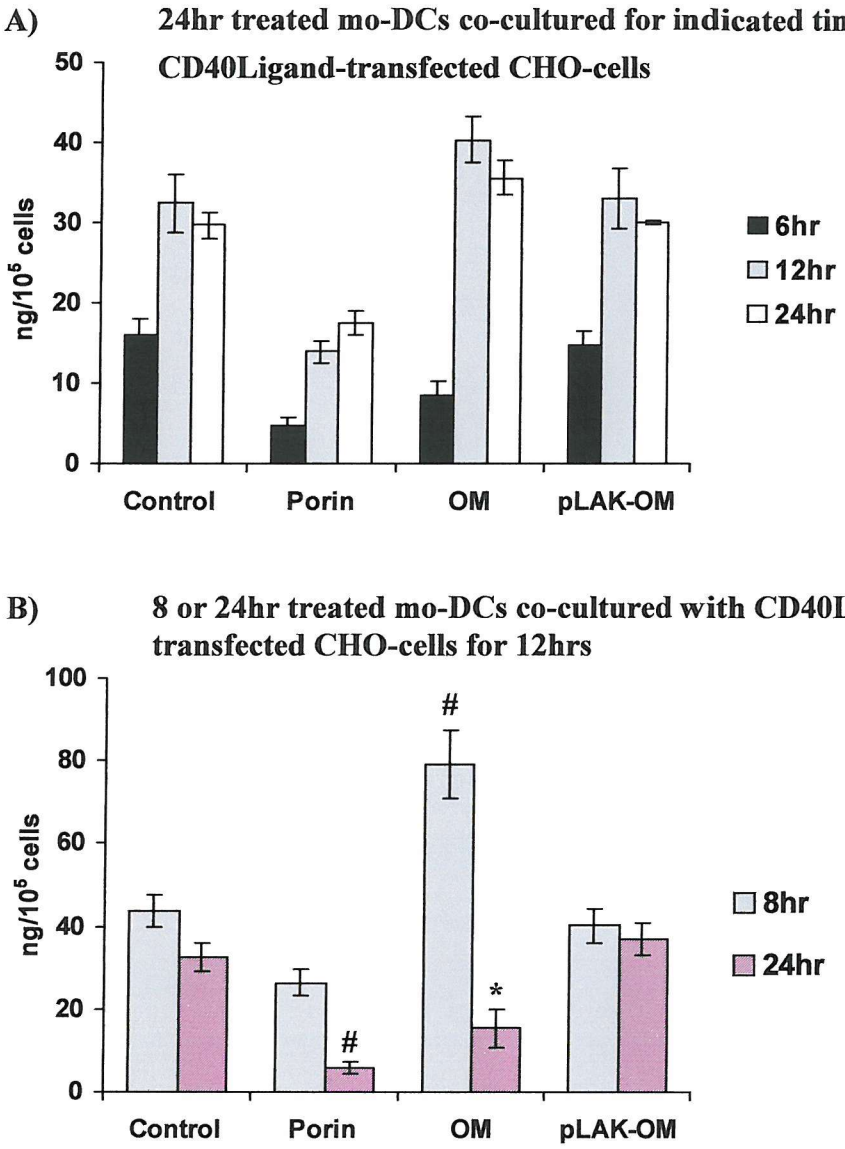

Figure 6.13: Production of IL-12p40 by CD40-ligated mo-DCs pre-treated with purified class I porin or outer membranes of *N. meningitidis*.

A) Mo-DCs were stimulated for 24hrs with 1μg/ml of purified recombinant class I porin, wild type outer membranes (OM) or LOS-deficient outer membranes (pLAK-OM). Control cells were treated with vehicle alone. Mo-DCs were subsequently co-cultured for 6, 12 or 24hrs with CD40Ligand-transfected CHO cells.

B) Mo-DCs were stimulated as described above for 8 or 24hrs and subsequently co-cultured with CD40Ligand-transfected cells for 12hrs.

Results are presented as mean production of IL-12p40 per 10^5 cells with standard error bars (A: n=3, B: n=7). *=Significance ($p<0.05$) observed. # = Significance ($p<0.01$) observed.

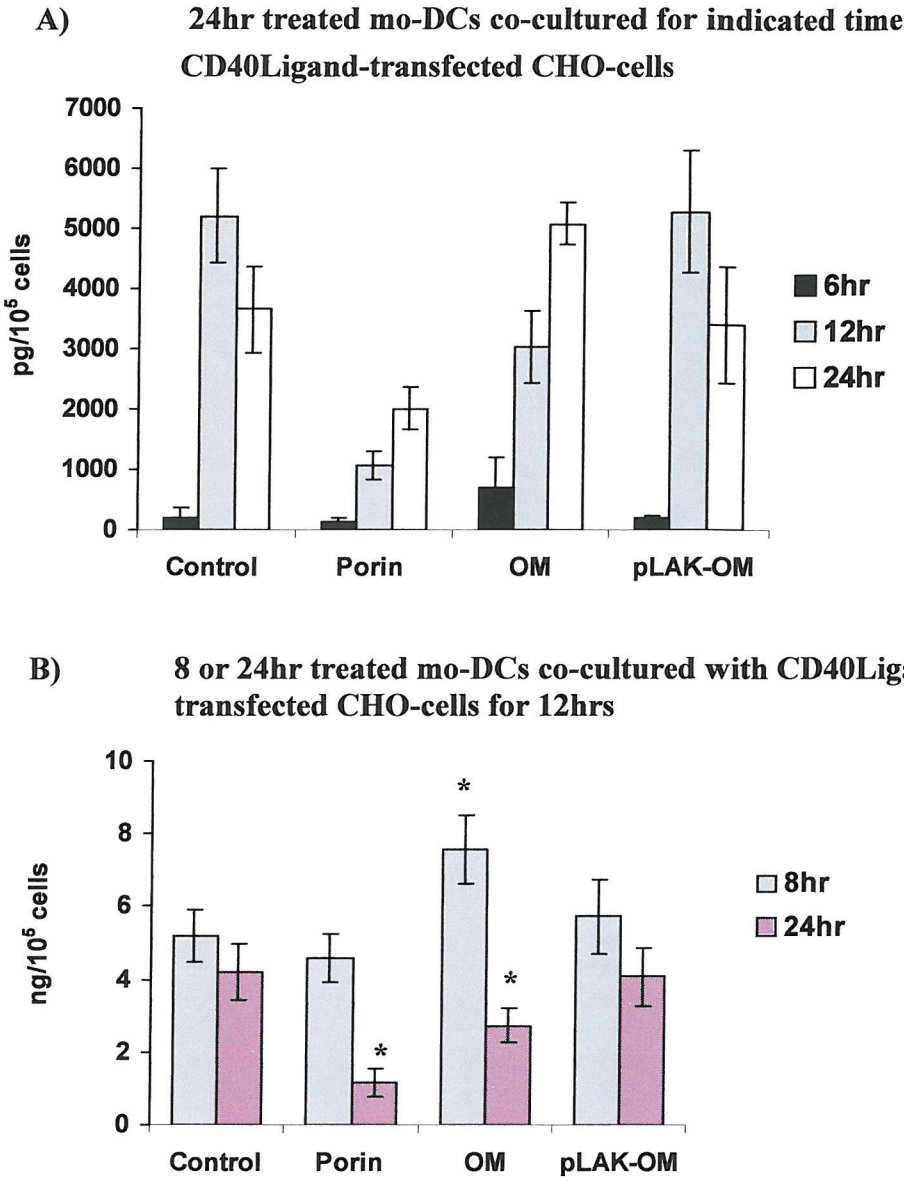

Figure 6.14: Production of IL-12p70 by CD40-ligated mo-DCs pre-treated with purified class I porin or outer membranes of *N. meningitidis*.

A) Mo-DCs were stimulated for 24hrs with 1μg/ml of purified recombinant class I porin, wild type outer membranes (OM) or LOS-deficient outer membranes (pLAK-OM). Control cells were treated with vehicle alone. Mo-DCs were subsequently co-cultured for 6, 12 or 24hrs with CD40Ligand-transfected CHO cells.

B) Mo-DCs were stimulated as described above for 8 or 24hrs and subsequently co-cultured with CD40Ligand-transfected cells for 12hrs.

Results are presented as mean production of IL-12p70 per 10^5 cells with standard error bars (A: n=4, B: n=8). *=Significance ($p<0.05$) observed.

6.4 EFFECT OF *N. meningitidis* CLASS I PORIN ON Mo-DC DRIVEN T-CELL PROLIFERATION

6.4.1 Mo-DC driven proliferation of allogeneic T-cells

The ability of DCs, matured by a range of microbial stimuli, to present antigen to T-cells is facilitated in part by increased expression of co-stimulatory molecules on DCs following bacterial encounter (Banchereau and Steinman, 1998). Thus, the effect of purified class I porin pre-treatment on the capacity of mo-DCs to induce allogeneic T-cell proliferation was assessed.

Incubation of 10^5 allogeneic purified $CD4^+$ (Figure 6.15A) or $CD4^+CD45RA^+$ naïve (Figure 6.16A) T-cells with vehicle-treated mo-DCs induced low T-cell proliferation, in wells containing higher numbers of mo-DCs. The T-cell proliferative response driven by porin-treated mo-DCs was comparable with that observed by mo-DCs pulsed with meningococcal wild-type outer membranes (OM) (Figure 6.15A and 6.16A). Stimulation indices for porin-treated mo-DC (1000 cells / well only) driven proliferation of $CD4^+$ and naïve T-cells is presented in Figures 6.15C and 6.16C, respectively. Mo-DCs incubated with 0.2pg/ml *E. coli* LPS did not show allostimulatory capacity to induce proliferation of purified $CD4^+$ or naïve T-cells.

Polarization of allogeneic naïve T-cells by mo-DCs, pre-treated with 1μg/ml purified class I porin or vehicle alone, was investigated by co-culturing cells for four-days followed by 24hr re-stimulation of T-cells on plate-bound anti-CD3. However, analysis of T-cell derived IFN-γ or IL-13 production revealed inconclusive pattern of results (ELISA data not shown, n=3).

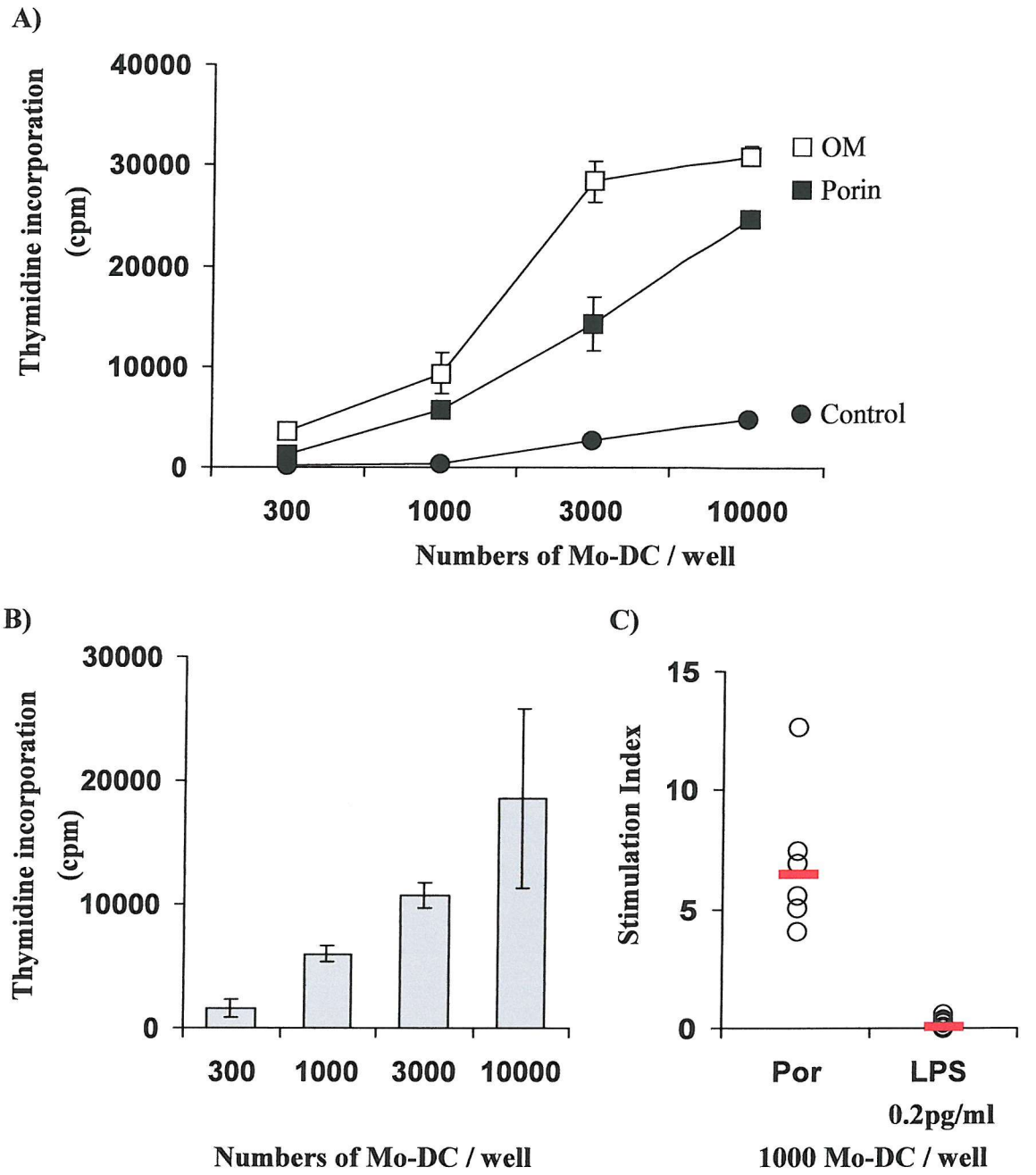

Figure 6.15: Effect of *N. meningitidis* class I porin on mo-DC driven allogeneic CD4$^+$ T-cell proliferation.

Graded numbers of mo-DCs were co-cultured with 1x10^5 purified allogeneic CD4$^+$ T-cells. After 5 days, T-cell proliferation was assessed by H^3 thymidine incorporation over the last 18hrs. **A)** Mo-DCs were treated for 24hrs with vehicle alone or exposed to 1μg/ml wild type outer membranes (OM) or 1μg/ml purified recombinant class I porin. Data is representative of one donor ± standard error of triplicate cultures for each treatment. **B)** Data is presented as average of CPM for each treatment minus CPM for respective control cells with standard error bars (n=6). **C)** Data is presented as stimulation index (SI) for treatment with class I porin (Por) or 0.2pg/ml *E. coli* LPS at the 1000 mo-DC/well concentration only (mo-DC: T cell, 1:100). Individual SI is presented as open circles (n=6) and the median SI is shown as a red bar.

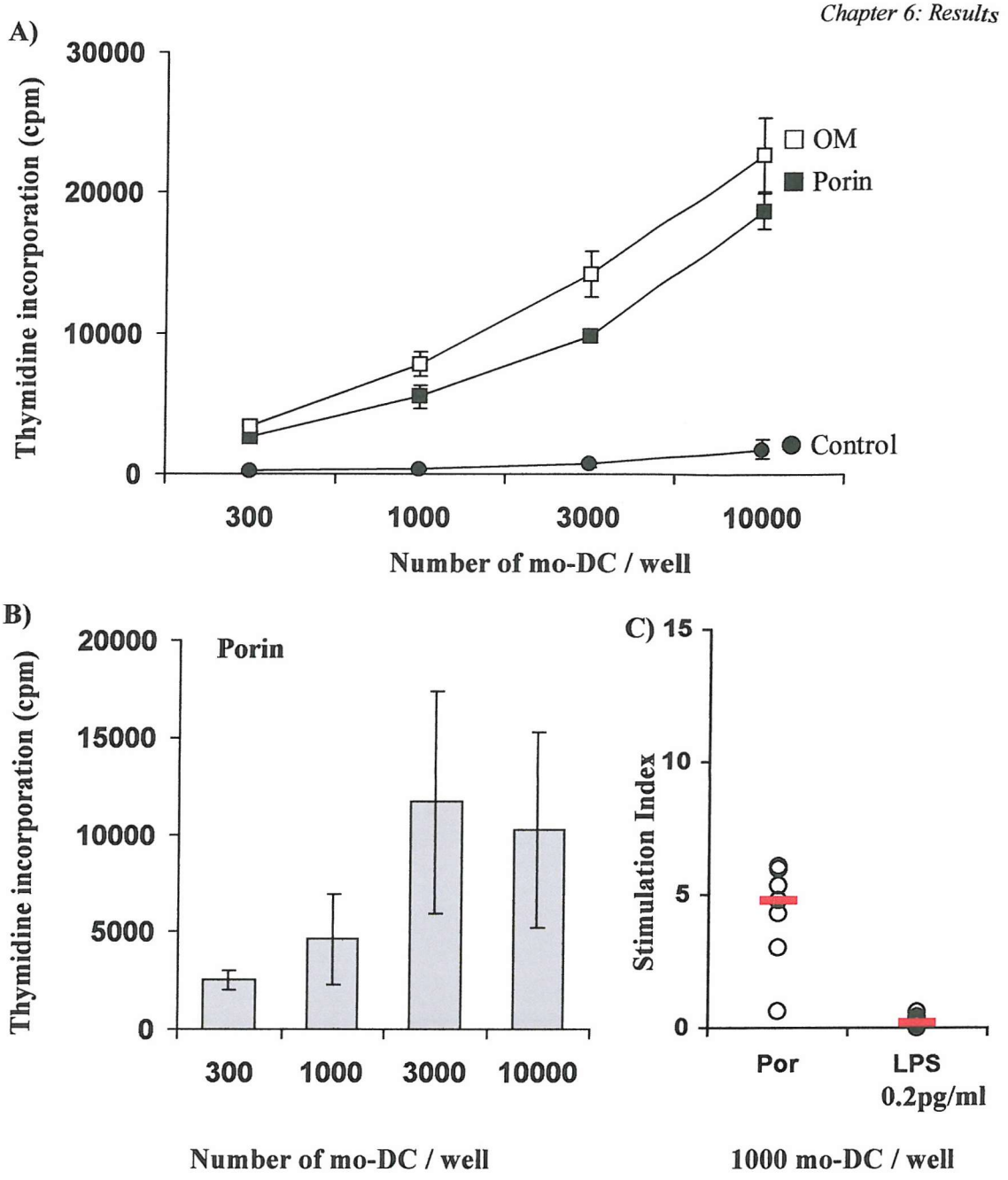

Figure 6.16: Effect of *N. meningitidis* class I porin on mo-DC driven allogeneic naïve T-cell proliferation.

Graded numbers of mo-DCs were co-cultured with $1x10^5$ purified allogeneic $CD4^+CD45RA^+$ T-cells. After 5 days, T-cell proliferation was assessed by H^3 thymidine incorporation over the last 18hrs. **A)** Mo-DCs were treated for 24hrs with vehicle alone or exposed to 1μg/ml wild type outer membranes (OM) or 1μg/ml purified recombinant class I porin. Data is representative of one donor ± standard error of triplicate cultures for each treatment. **B)** Data is presented as average of CPM for porin treatment minus CPM for control treatment with standard error bars (n=7). **C)** Data is presented as stimulation index (SI) for treatment with class I porin (Por) or 0.2pg/ml *E. coli* LPS at the 1000 mo-DC/well concentration only (mo-DC: T cell, 1:100). Individual SI is presented as open circles (n=7) and the median SI is shown as a red bar.

6.4.2 Mo-DC driven proliferation of antigen-specific T-cells

Neisserial porins have been described to possess adjuvant properties (Mackinnon *et al.*, 1999). Therefore, activities of purified class I porin of *N. meningitidis* to impact on the ability of DCs to augment activation of antigen-specific T-cells was investigated. Short-term tetanus toxoid $CD4^+$ lines were generated from PBMCs of donors recently vaccinated against tetanus. Mo-DCs stimulated for 24hrs with tetanus toxoid did not alter expression of co-stimulatory molecules or HLA-DR compared to vehicle-treated mo-DCs (Figure 6.17), whereas, maturation of mo-DC phenotype was observed following incubation with purified class I porin (Figure 6.17). Changes in phenotype by mo-DCs treated with purified porin and tetanus toxoid were comparable to mo-DCs treated with porin alone (Figure 6.17).

Autologous mo-DCs, pre-pulsed with tetanus toxoid for 24hrs, induced proliferation of tetanus toxoid-specific T-cells lines (Figure 6.18). Enhanced ability to drive proliferation of tetanus toxoid-T-cells was observed by the pre-treatment of mo-DCs for 24hrs with purified class I porin plus tetanus toxoid (Figure 6.18A and 6.19). This enhanced effect was comparable to that seen by mo-DCs pre-treated with wild type OM (Figure 6.18B). No effect was seen with mo-DCs stimulated with 0.2pg/ml *E. coli* LPS (data not shown). Stimulation indices for porin-treated mo-DC-driven proliferation of tetanus toxoid specific T-cell lines are presented in Figure 6.19.

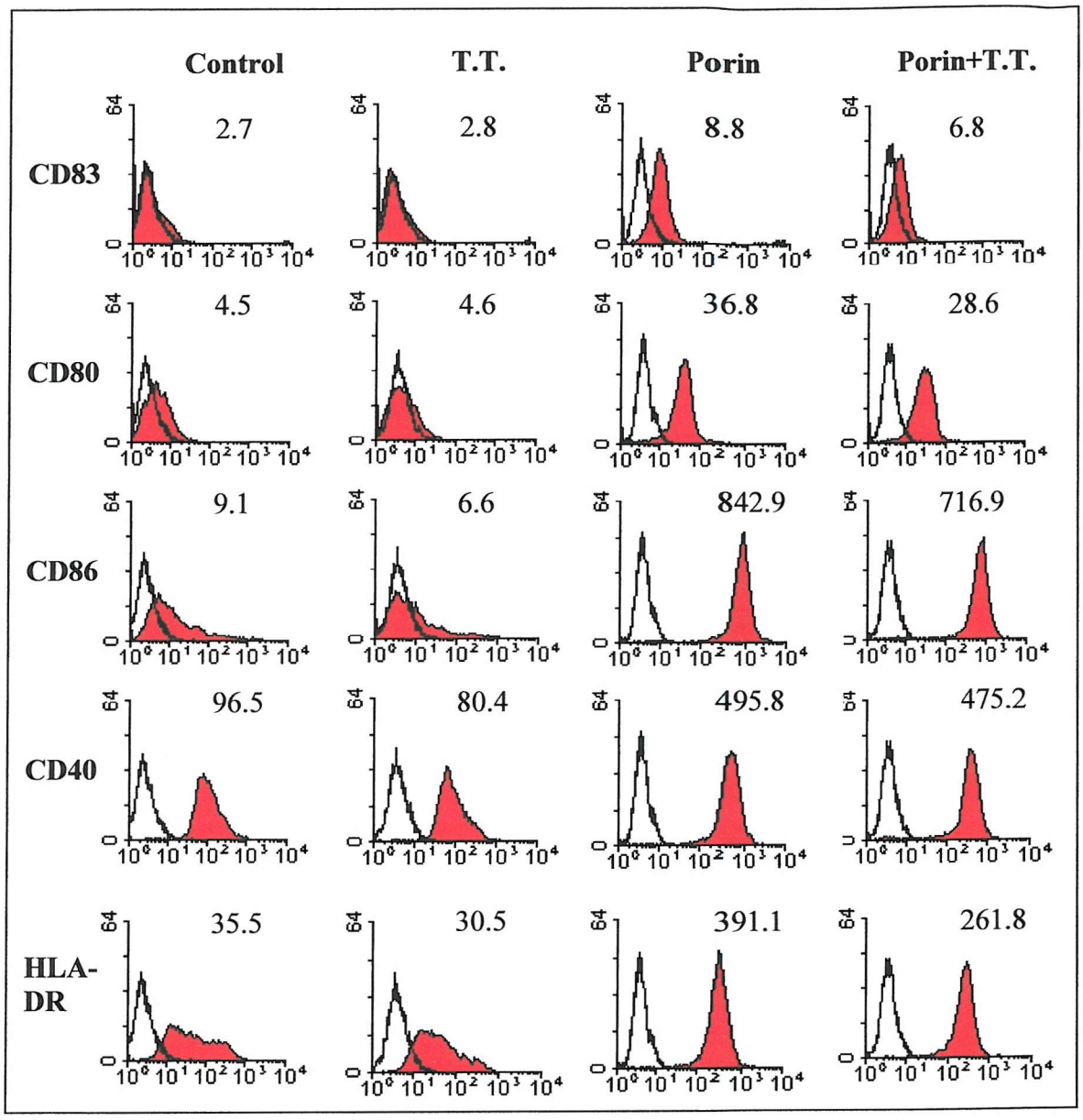

Figure 6.17: Effect of tetanus toxoid and/or neisserial class I porin on mo-DC phenotype.

5-day mo-DCs were exposed for 24hrs to 1μg/ml tetanus toxoid (T.T), 1μg/ml purified recombinant class I porin (Por) or both (T.T+Por). Control mo-DCs were stimulated with vehicle alone. Surface expression of CD83, CD80, CD86, CD40 and HLA-DR was determined by mAb staining (red histograms) and analysed by flow cytometry. Open clear histograms represents cells stained with appropriate isotype controls. Numbers on each histogram corresponds to the median fluorescence intensity of mAb staining. Profiles are representative of four donors.

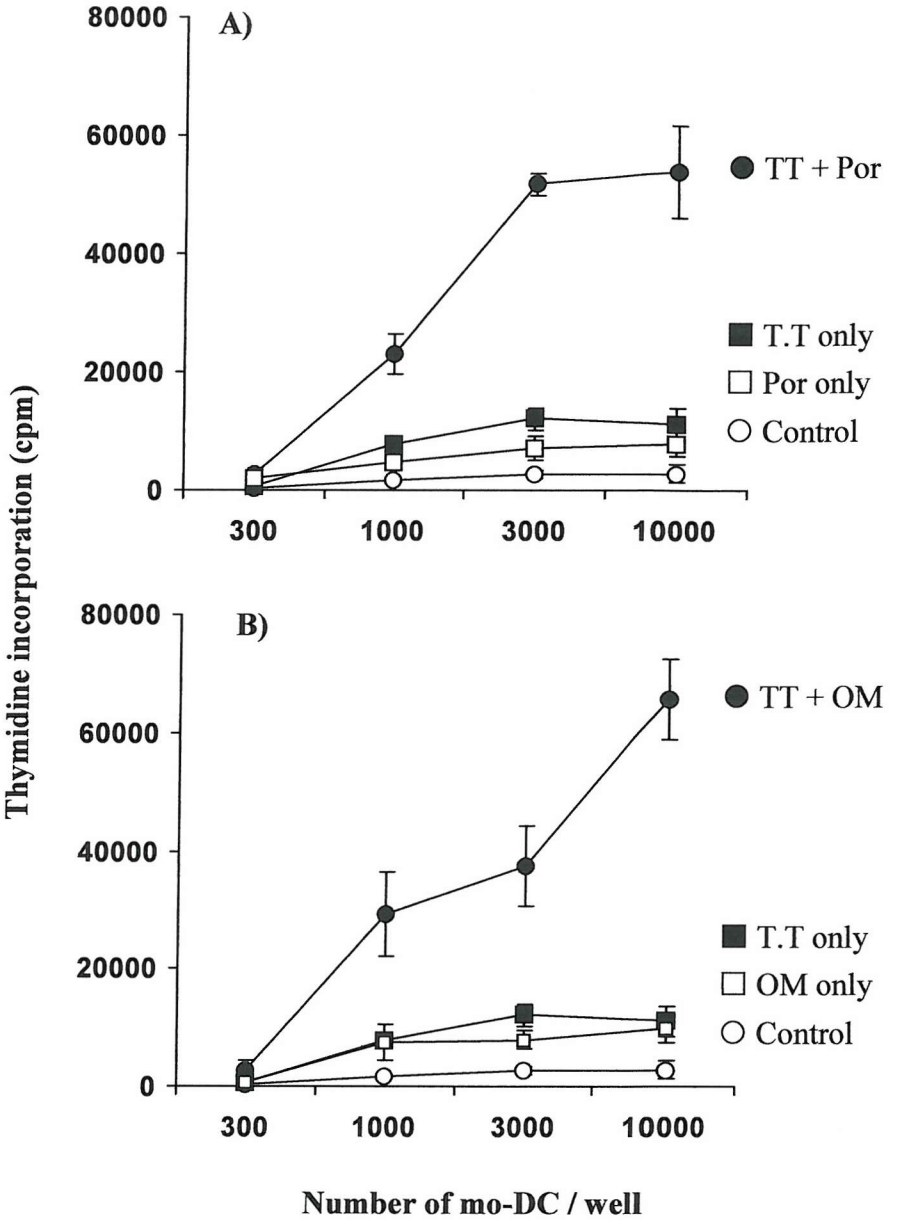

Figure 6.18: Effect of class I porin of *N. meningitidis* on mo-DC driven proliferation of tetanus toxoid-specific T-cells.

Graded numbers of mo-DCs were co-cultured with $1x10^5$ autologous short-term generated tetanus toxoid specific $CD4^+$T-cells. After 6 days, T-cell proliferation was assessed by H^3 thymidine incorporation over the last 18hrs. **A**) Mo-DCs were treated for 24hrs with vehicle control or exposed to 1μg/ml purified recombinant class I porin (Por), 10μg/ml tetanus toxoid (T.T) or both (T.T+Por). **B**) Mo-DCs were treated for 24hrs with vehicle control or exposed to 1μg/ml wild type outer membranes (OM), 10μg/ml tetanus toxoid (T.T) or both (T.T+OM). Proliferation data is representative of one donor ± standard error of triplicate cultures for each treatment (A, n=4; B, n=3).

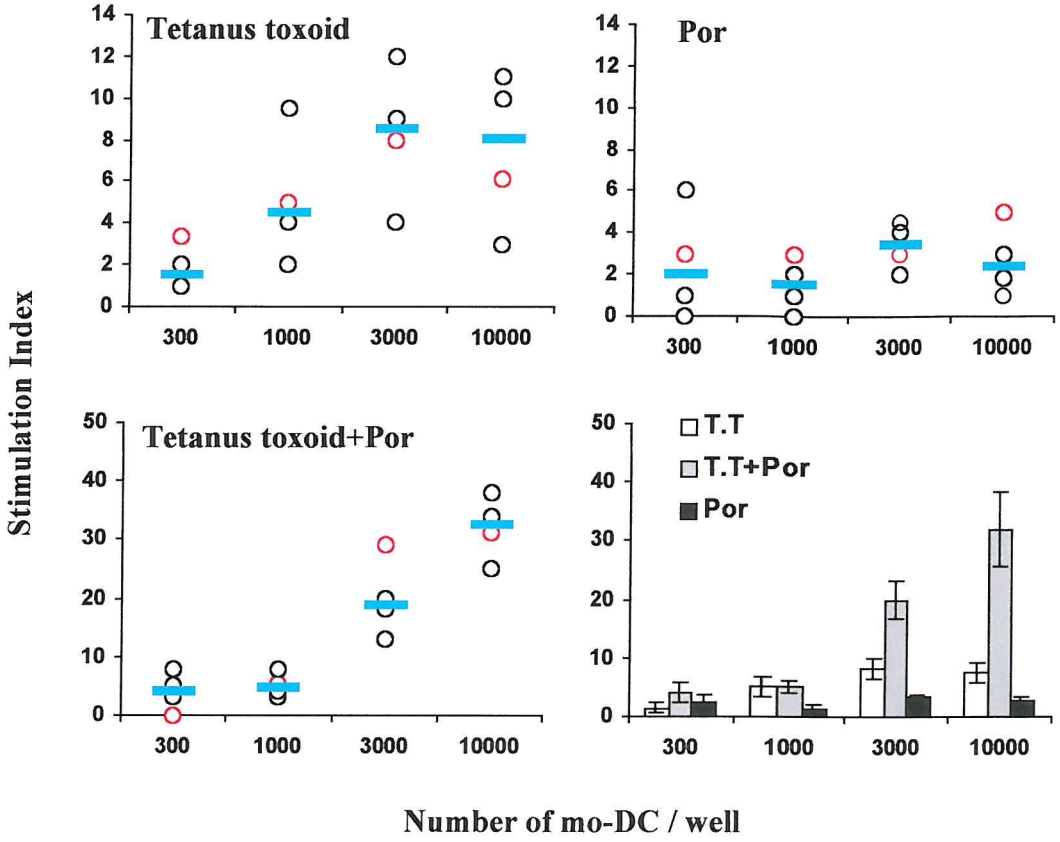

Figure 6.19: Summary of the effect of meningococcal class I porin on mo-DC driven proliferation of tetanus toxoid-specific T-cells.

Mo-DCs were treated for 24hrs with vehicle control or exposed to 1μg/ml purified recombinant class I porin (Por), 10μg/ml tetanus toxoid (T.T) or both (T.T+Por). Graded numbers of mo-DCs were co-cultured with $1x10^5$ autologous short-term generated tetanus toxoid specific $CD4^+$T-cells. After 6 days, T-cell proliferation was assessed by H^3 thymidine incorporation over the last 18hrs. Data is presented as stimulation index (SI) following treatment of mo-DCs as indicated. Individual SI is presented as open circles and the median is shown as a blue bar (n=4). Red open circles represent an experiment in which the mo-DCs used were differentiated from cryopreserved monocytes.

CHAPTER 6

Discussion of Results

6.5 DISCUSSION

6.5.1 Summary of results

Purified recombinant class I porin of *N. meningitidis* induced marked changes in mo-DC phenotype and function. Induction of mo-DC maturation was observed by increased expression of CD83 together with the reduced capacity to take up FITC-dextran via receptor-mediated endocytosis. Maturation of mo-DCs was also paralleled with increased expression of MHC class II and co-stimulatory molecules CD80, CD86 and CD40. Moreover, productions of significant levels of IL-6, IL-10, TNFα, IL-12p40, IL-8, MIP-1α, MIP-1β and RANTES by mo-DCs stimulated with purified porin were observed. In contrast, bioactive IL-12p70 was not induced by class I porin-treated mo-DCs. High levels of IL-12p70 were secreted by mo-DCs following a second stimulus provided by CD40-ligation. However, pre-treatment of mo-DCs with purified porin inhibited this production. This inhibitory effect appeared to depend on the duration of mo-DC exposure to porin. CD40-ligated mo-DCs pre-treated with class I porin for 8hrs produced comparable levels of IL-12p70 to control cells, whereas pre-treatment for 24hrs significantly decreased IL-12p70 secretion compared to control cells.

Mo-DCs pre-treated with purified class I porin demonstrated enhanced ability to drive allogeneic $CD4^+$ and naïve T-cell proliferation. However, examination of naïve T-cell polarization into either Th1 or Th2 effector cells was inconclusive. Purified class I porin was also observed to augment the ability of mo-DCs to stimulate tetanus toxoid-specific T-cell proliferation. In all studies, the effect of treating mo-DCs with concentrations of *E. coli* LPS (0.2pg/ml) equivalent to LPS levels that might contaminate the purified meningococcal porin produced effects comparable to those observed by treatment with vehicle alone. This suggests that it is indeed the purified class I porin that is responsible for the findings presented.

6.5.2 Comparative effects of meningococcal class I porin and outer membranes

The effect of purified recombinant class I porin on inducing the activation and maturation of mo-DCs was comparable with that of equal amounts of whole meningococcal outer membrane. The relative amounts of class I porin in meningococcal outer membranes is approximately 40% (Professor J. Heckels, personal communication). Thus, 1μg/ml purified recombinant porin is approximately twice the amount of porin present in 1μg/ml outer membranes. However, the levels of surface molecule up-regulation and production of soluble mediators was generally greater in mo-DCs treated with 1μg/ml whole outer membranes. Responses of mo-DCs to wild type outer membranes are likely to result from the recognition of various components within the preparation including endogenous LOS as well as porins (class I and class II/III). Meningococcal LOS exhibited mark potency in inducing mo-DC maturation (Chapter 5). Indeed, the relatively low maturation levels displayed by mo-DCs stimulated with LOS-deficient mutant outer membranes does suggest that LOS is primarily responsible for the results observed with the wild type outer membranes. *N. meningitidis* mutant lacking LOS expresses the full complement of proteins within its outer membrane (Steeghs *et al.*, 1998). Thus, taking into account the maturation induced by purified class I porin, it was surprising that greater differences were not observed after treatment of mo-DCs with the LOS-deficient outer membranes. A point to consider is the possibility that lack of LOS within outer membrane may affect the stabilization and arrangement of other proteins, such as porins, in the membrane. In this case, recognition of class I porin within LOS-deficient outer membranes by mo-DCs may be moderately impaired. It is also conceivable that other, as yet unidentified proteins within the outer membrane may possess activities to counteract the effects of porins on mo-DCs. Therefore, the actions of wild type or LOS-deficient mutant outer membranes may reside in the cumulative activities of individual proteins within the outer membrane.

6.5.3 Class I porin induced changes on TLR expression by mo-DCs

Moderate reductions in TLR2 mRNA transcripts were measured in mo-DCs following exposure to purified recombinant class I porin. Whereas, TLR4 mRNA expression was significantly enhanced after treatment, which was not the result of contaminating endotoxin within the preparation. These data suggest the ability of purified class I

porin to modulate TLR expression on mo-DCs. Recently, it has been shown that the ability of neisserial purified class II porin (PorB) to stimulate murine B-cells and induce CD86 up-regulation is dependent on their expression of MyD88 and TLR2 (Massari *et al.*, 2002). Further assessment of the findings presented in this thesis will be important to determine whether class I and class II porins exert distinct effects on mo-DCs.

6.5.4 Effect of class I porin on mo-DC-derived IL-12 production

It was observed that purified class I porin generally exhibited similar actions compared to whole wild-type outer membranes on mo-DC maturation, in terms of increasing surface molecule expression and production of soluble mediators. However the magnitude of cell activation was generally higher in cells exposed to wild type outer membranes. Data presented in Chapter 5 demonstrate the ability of wild type outer membranes to induce IL-12p70 production by mo-DCs. In contrast to expectation, class I porins failed to induce mo-DCs to produce significantly increased levels of IL-12p70 although all other soluble mediators analysed were induced.

Since the Th1-promoting capacity of DCs correlates with their ability to produce IL-12p70 (Heufler *et al.*, 1996; Hilkens *et al.*, 1997), the inability of class I porin-treated mo-DCs to produce IL-12p70 initially suggested the modulation of these cells to drive Th2-mediated responses. As observed following the direct stimulation of mo-DCs with LOS-deficient outer membranes (Chapter 5), purified class I porin induced significant levels of the IL-12p40 monomer despite the lack in production of p70 heterodimer. However, differences in the overall activation of mo-DCs by purified porin or LOS-deficient outer membranes were observed, suggesting that these meningococcal components exhibit distinct properties.

Results presented in this chapter on the potency of ligation of CD40 on mo-DCs for secretion of high levels of IL-12p70 concur with published reports (Langenkamp *et al.*, 2000; de Jong *et al.*, 2002). Moreover, co-stimulation provided through CD40 is reported to induce higher levels of IL-12 than direct stimulation of mo-DCs with bacterial products, such as LPS (Langenkamp *et al.*, 2000). In the case of standard *E. coli* LPS, it has been reported that biologically significant levels of IL-12 are produced by mo-DCs following additional stimulation with IFN-γ (Hilkens *et al.*,

1997). Of note, it was observed in this study that meningococcal LOS was more potent than *E. coli* LPS at inducing mo-DC to secrete IL-12p70.

In this chapter, experiments were devised to assess the ability of purified porin to affect subsequent production of IL-12 by mo-DCs following CD40-ligation. Comparison of the relative effects of wild type outer membranes with those of purified class I porin also demonstrated differences in production patterns for IL-12 that depended on the duration of pre-treatment prior to CD40-ligation. Mo-DCs pre-treated for 8hr with OM released significant levels of IL-12p40 or p70, whereas, no difference was observed after 8hr pre-treatment of mo-DCs with purified class I porin. In contrast, 24hr matured class I porin or OM-treated mo-DCs, produced significantly less IL-12p40 and p70 after CD40-ligation compared to control cells. This decrease in IL-12 production was greater in porin- rather than OM-treated mo-DCs.

Mo-DCs matured in the presence of *Staphylococcus aureus* Cowan I strain, are less capable of producing IL-12 upon further co-culture with CD40-Ligand expressing cells compared to immature mo-DCs (Ebner *et al.*, 2001). Moreover, there is evidence suggesting that the duration of DC maturation by bacterial stimuli, affects DC-derived IL-12 production, subsequently influencing the type of T-cell response generated (Langenkamp *et al.*, 2000). In the present study, the altered ability of wild type OMs or purified class I porin to induce CD40-ligated mo-DC to produce IL-12 may relate in part to a high level of mo-DC maturation induced by either stimulus during the 24hr pre-treatment compared to 8hr. However, an inconsistency in this observation is the ability of whole wild type OMs to induce IL-12p70 production following direct stimulation of mo-DCs for 24hrs, which was not observed by porin-treated mo-DCs. Following 8hr pre-treatment with whole wild type OM, mo-DCs retain the capacity to induce high levels of IL-12 production upon CD40-ligation; whereas, no difference was observed for 8hr porin-treated mo-DCs subsequently CD40-ligated compared to control cells. Interestingly, IL-12 production by CD40-ligated mo-DCs pre-treated with OMs from the LOS-deficient mutant for 8 or 24hrs was similar to that of control mo-DCs. Therefore, it is likely that in addition to the duration of maturation, the nature of the meningococcal stimulus is critical for determining mo-DC-derived IL-12 production.

6.5.5 Effect of class I porin on mo-DC-driven T-cell proliferation

Data presented in this chapter demonstrate the capacity of purified meningococcal class I porin to modulate mo-DC function, increasing their capacity to stimulate T-cell proliferation. Mo-DCs exposed to purified class I porin induced high levels of allogeneic $CD4^+$ and naïve T-cell proliferation. Unfortunately, in the time available it was not possible to determine whether purified class I porin-treated mo-DCs had the ability to differentiate naïve T-cells into T-cells displaying Th1 or Th2 cytokine profiles. Further investigation on the nature of this naïve T-cell polarization is needed to facilitate the understanding of the decreased IL-12 production observed in the CD40-ligation experiments.

It was also observed that purified class I porin augmented the capacity of mo-DCs to drive proliferation of tetanus-toxoid specific $CD4^+$ cells. Mo-DCs pulsed with tetanus toxoid induced proliferation of antigen-specific $CD4^+$ cells despite the comparable levels of surface co-stimulatory molecules on these cells to those on control mo-DCs. Porin-treated mo-DCs exhibited significantly augmented expression of co-stimulatory molecules suggesting the ability of these cells to provide adequate co-stimulation following presentation of antigen. Moreover, tetanus toxoid specific-$CD4^+$ proliferation was much greater when stimulated by mo-DCs pre-exposed to purified porin plus tetanus toxoid, than proliferation induced by tetanus toxoid or porin-pulsed mo-DCs alone. Thus, using this *in vitro* model, purified class I porin was shown to possess potent immunostimulatory properties by modulating mo-DC function, in part by increasing co-stimulatory molecule expression, to promote antigen-specific $CD4^+$ proliferation.

CHAPTER 7

General Discussion

7

The aim of the work presented in this thesis was to investigate the ability of certain bacterial components to behave as adjuvants and enhance antigen presenting cell function. Two distinct types of bacterial modulins, CpG ODN and outer membrane components of *N. meningitidis,* were chosen for this study due to their inherent ability to provoke potent immune responses *in vivo.* Human mo-DCs were generally used throughout this study as *in vitro* model APCs, however, the responsiveness of monocytes to ODN was also investigated.

The ability of monocytes/mo-DCs to recognise the various microbial stimuli that resulted in subsequent cellular activation was demonstrated in this report by measuring changes in MHC class II and co-stimulatory molecule expression as well as production of soluble mediators. Modulation of mo-DC function following exposure to CpG ODN or *N. meningitidis* outer membrane proteins was assessed in order to determine the consequences of these changes on induction of $CD4^+$ T-cell responses.

7.1 CpG ODN – DISTINCT RESPONSES BETWEEN HUMAN MONOCYTES AND Mo-DCs

7.1.1 Summary of results

Unmethylated CpG-motifs present in bacterial DNA or synthetic ODN have been described to act as microbial molecular patterns that alert the immune system upon its recognition (Krug *et al.*, 2001a; Hemmi *et al.*, 2000). CpG '1668' and CpG '2006' significantly increased the expression of CD86 on monocytes, whilst having little or no effects on the other co-stimulatory molecules investigated. A number of additional ODN sequences were found to affect monocyte phenotype. Examination of non-CpG'1745' and '1720' on monocytes also revealed stimulatory potential resulting in CD86 up-regulation suggesting the presence of motifs other than 'CpG' in the ODN that might contribute to the maturation of human monocytes. No effects were seen following treatment of monocytes with non-CpG '100', which was also devoid of 'TpG' motifs in the sequence. Although not investigated in great detail, clear

differences in phenotypic expression of surface markers were noted between monocytes treated with CpG or *E. coli* LPS. Similar patterns of changes were demonstrated between cells treated with CpG ODN or plasmid DNA, suggesting differences in signalling events induced by CpG/plasmid DNA and LPS. Stimulatory activity of CpG '1668' was also shown by its ability to induce cytokine secretion by monocytes.

In contrast to monocytes, mo-DC/LC exposed to CpG ODN did not show altered expression of any of the surface molecules analysed nor induce cytokine production. Furthermore, mo-DC/LCs exposed to CpG ODN were not able to induce allogeneic $CD4^+$ proliferation. Collectively, the findings presented here show that the responsiveness to CpG ODN is altered during the *in vitro* differentiation of monocytes to mo-DC/LC. This decrease in responsiveness to CpG ODN correlates with the loss of TLR9 expression during culture of monocytes to mo-DCs or mo-LCs, as demonstrated by TaqMan RT-PCR. As a result of these observations it was not possible to fulfil an objective of this thesis, which was to use these mo-DC/LC as model APCs to examine CpG actions on the *in vitro* differentiation of primary T-cells into Th1 or Th2 effector cells. Of the other DC subsets investigated, surface TLR9 expression was not detected on $CD34^+$-derived DCs or LCs. However, preliminary findings demonstrated surface TLR9 on human skin epidermal Langerhans' cells.

7.1.2 DC activation by CpG ODN requires TLR9

A feature of CpG ODN is their ability to stimulate a variety of immune cells to induce the production of pro-inflammatory and Th1-like cytokines (Heeg and Zimmermann, 2000). The adjuvanticity of CpG ODN has largely been elucidated in murine models; however, there are growing numbers of reports on the actions of CpG ODN on a variety of human systems. Murine bone marrow-derived DCs are strongly activated by CpG ODN resulting in the up-regulation of MHC class II and co-stimulatory molecules together with production of Th1-skewing cytokines, such as IL-12 (Sparwasser *et al.*, 1998). The activities of CpG ODN/DNA on human DCs appear to be confined to plasmacytoid DCs subset only, with some resultant effects similar to and some distinct from those observed by murine cells (Sparwasser and Lipford, 2000; Krug *et al.*, 2001a; Bauer *et al.*, 2001b). Collective evidence suggests that differences observed between murine and human models may generally be attributed to 1) species-specific sequences present in the ODN as well as the presence of

additional stimulatory or inhibitory motifs and 2) the origin of the DC, whether myeloid or lymphoid.

DCs express a wide range of the TLR pattern recognition receptors, that serve to recognize PAMPs and signal the presence of invading pathogens (Kopp and Medzhitov, 1999). The suggestion that the receptor for ODN belonged to the TLR family stemmed from observations that CpG-induced maturation and cytokine production by APCs was dependent on the adaptor proteins, MyD88 and TRAF6, known to be involved in TLR signalling (Hacker *et al.*, 2000). TLR9 has been reported to act as the signal transducer for CpG ODN. TLR9 knock-out mice were found to be unresponsive to CpG ODN (Hemmi *et al.*, 2000). Moreover, DCs from TLR4 or TLR9-deficient mice retained their ability to respond to CpG ODN or LPS, respectively. These findings indicated that CpG ODN and LPS preferentially mediate signalling through distinct receptors (Hemmi *et al.*, 2000; Poltorak *et al.*, 2000). However, it has not yet been demonstrated whether CpG ODN/DNA directly binds to TLR9.

During infection, the presence of a number of pathogenic products will generate signals that may potentially alter the maturation status of resident peripheral monocytes/macrophages and DC. Bacterial DNA, that may be present *in vivo* following the death of pathogens, may strongly activate human monocytes/macrophages (Wagner, 1999; Sparwasser *et al.*, 1997). Additionally, following uptake and degradation of bacteria in phagosomes, receptors that recognize DNA, such as TLR9, may sample the contents of phagosomes ultimately leading to cellular activation (Hemmi *et al.*, 2000; Underhill *et al.*, 1999). Randolph and colleagues have shown using an endothelial reverse-transmigration model, that monocytes crossing an endothelial monolayer and contacting particulate collagen matrix can acquire characteristics of DCs in as few as two days (Randolph *et al.*, 1998). It was proposed that this system may model monocyte extravasation, entry into peripheral tissues, and phagocytosis of particulate matter that forms part of the normal DC maturation process (Randolph *et al.*, 1998). Thus, monocytes can serve as a reservoir for human DCs. Findings presented in this thesis demonstrate that human monocytes express TLR9, although this observation has been contested in a separate study (Kadowaki *et al.*, 2001b). Monocyte-related cells, such as microglial cells and astrocytes, are also reported to be activated by CpG ODN (Schluesener *et al.*, 2001;

Deng *et al.*, 2001). Monocytes are also able to differentiate into macrophages, *in vitro* (Chomarat *et al.*, 2000). CpG ODN can stimulate human macrophages (Stacey *et al.*, 2000). Thus, it is interesting that depending on environmental instruction monocytes may be differentiated into CpG responsive (macrophages) or non-CpG responsive (mo-DCs) cells. It may also be conceivable that mo-DCs are not required to respond to CpG ODN/DNA, and that CpG-mediated responses are brought about by the presence of other responsive cells at the time and site of infection.

7.1.3 Langerhans' cells

LCs reside in the epidermis where they are able to sample the external environment for foreign agents. The preliminary findings presented in this study that human epidermal LC express moderate levels of surface TLR9 suggests that CpG motifs may trigger signals resulting in the activation of these cells. In contrast to primary human LCs, data presented here showed $CD34^{+}$-derived LCs and mo-LC to be devoid of TLR9 expression. Further preliminary data showing that non-CD1a expressing cells from human epidermal skin express surface TLR9 warrants further characterization of these cells, such as keratinocytes, that may also be participate in CpG ODN/DNA recognition. It is unlikely that CD1a dermal DCs are contaminating the human LC preparation since the blistering protocols used only remove the epidermis (Friedmann, 1981; Friedmann *et al.*, 1987). Moreover, intracellular mAb staining of Langerin, expressed specifically in Birbeck granules of LC (Valladeau *et al.*, 2000), was demonstrated in the CD1a enriched fraction of the dissociated epidermal skin.

The most common route of CpG DNA administration employed in vaccination studies is parenterally, although non-invasive application onto skin may confer protection (Shi *et al.*, 1999; Yu *et al.*, 1999). Whilst DNA immunization is largely associated with Th1 responses that prime specific CTLs, the induction of Th2 responses is favoured using the gene gun or topical methods of DNA administration (Yu *et al.*, 1999; Pertmer *et al.*, 1996). Plasmid DNA bound to gold beads and bombarded into the skin have been shown to elicit humoral responses (IgG1> IgG2a). A suggestion to explain the generation of predominantly Th2-responses induced by gene gun methods, is that plasmid vectors are delivered directly into the APCs bypassing surface interaction of CpG motifs in the vector (Krieg, 2002). Delivery of plasmid vectors intradermally and via a gene gun targets epidermal LCs and interstitial DCs (Banchereau and Steinman, 1998). After injection into the skin, keratinocytes and

LCs are reported to become transfected with plasmid DNA (Yang *et al.*, 1990; Raz *et al.*, 1994). Moreover, following intradermal injection of CpG DNA murine LCs have been shown to rapidly migrate out of the skin to the draining lymph node (Ban *et al.*, 2000).

Future studies understanding the mechanisms underlying the activation of human LC by CpG ODN/DNA will be desirable for the development of immunization strategies against pathogens that primarily infect the skin. It may be feasible to trigger a desired Th-response by specifically targeting these LCs to enhancing their antigen presenting capacity and induce their maturation and migration.

7.1.4 Response of APCs from atopic and non-atopic to bacterial stimuli

Atopic diseases such as allergy and asthma are characterized by increases in Th2 cells and serum IgE antibodies (Comoy *et al.*, 1998; Gereda *et al.*, 2000; Sutton and Gould, 1993). In this thesis, a comparison of the effects of CpG ODN on monocytes obtained from non-atopic and atopic subjects was initially made to determine whether these cells from atopic individuals possess altered responsiveness to this stimulus. However, no differences in phenotypic change were noted between these two subject groups. Moreover, no difference was observed by these monocytes in response to *E. coli* LPS. In addition it was observed that IL-1β, IL-6, IL-10 and IL-12p40 production was enhanced in both subjects groups following CpG ODN '1668' treatment compared to non-CpG '100' indicating the immunostimulatory activity of CpG '1668'. However, differential patterns of cytokine production by CpG-treated atopic or non-atopic monocytes were observed. A more thorough investigation is warranted to confirm altered responsiveness of atopic monocytes to ODN. Collective studies demonstrating the effects of CpG ODN/DNA to induce Th1-like responses both *in vitro* and *in vitro* present an attractive approach to developing therapies for allergic diseases. Administration of nuclease-digested DNA orally into mice resulted in approximately 50% decrease in serum IgE, IgG1 and IgM levels, suggesting the induction of a Th1 favouring response (Krieg, 2002). It is feasible that the activity of CpG DNA in murine models of asthma to prevent disease results from the induction of Th1-inducing cytokines such as IL-12 and IFN-γ (Kline *et al.*, 1999). However, mice genetically deficient in either or both of these cytokines do not develop allergic disease following administration of CpG DNA indicating that IL-12 and IFN-γ may

be involved in the mechanisms of CpG action but are not required (Kline *et al.*, 1999). In a separate study using a similar murine model of asthma, IFN-γ knockout mice were not protected against eosinophilic inflammation in the airways (Sur *et al.*, 1999). Other Th1-cytokines, IL-18 and IFN-α, are also produced in response to CpG DNA treatment that may play important roles (Bohle *et al.*, 1999; Van Uden *et al.*, 2001). There is also evidence of CpG ODN inhibiting Th2-responses to cedar pollen and birch pollen in mice (Kohama *et al.*, 1999; Hartl *et al.*, 1999).

The involvement of DCs in atopic individuals to participate in skewing immune responses towards a Th2 response has been suggested. However, the mechanisms by which this occurs remain controversial (Kapsenberg *et al.*, 2000). The dose of antigen, route of exposure, genetic background of the host, type of DCs interacting with naïve T-cells, co-stimulatory molecules expressed by DCs and the polarizing cytokine microenvironment during antigen presentation are some critical factors that determine the differentiation of Th1 or Th2 cells (Hosken *et al.*, 1995; Hsieh *et al.*, 1995; Romagnani, 1997). CD80 and CD86 molecules have been implicated in directing Th differentiation (Keane-Myers *et al.*, 1998a; Nakada *et al.*, 1999). Increased CD86 expression on murine DCs in the absence of other factors that promote Th1 responses may skew towards Th2 responses in rodents, inducing allergen-induced asthma (Tsuyuki *et al.*, 1997). CD86 expression on LCs has been suggested to play a role in the pathogenesis of atopic dermatitis from findings demonstrating anti-CD86 antibodies inhibiting T-cell proliferation against extracts of *D. pteronyssinus* (Ohki *et al.*, 1997). Expression of CD86 on B-cells from patients with atopic dermatitis correlates with the total serum IgE level (Jirapongsananuruk *et al.*, 1998). Mo-DCs from house dust mite allergic individuals express a higher CD86:CD80 ratio than mo-DCs from healthy donors after stimulation with Der p 1 (Hammad *et al.*, 2001), whereas, mo-DCs from healthy individuals preferentially showed an up-regulation of CD80 expression (Hammad *et al.*, 2001). Exposure of mo-DCs from allergic and healthy individuals to LPS is reported to result in comparable increases in CD80/CD86 expression (Hammad *et al.*, 2001).

In some allergen-induced asthma models, the ability of LPS to induce IL-12 and IFN-γ results in the switching of Th2 responses to favour Th1 responses (Keane-Myers *et al.*, 1998b; Gereda *et al.*, 2000). However, in some human studies, LPS exposure has been associated with increased risk of inducing asthma (Park *et al.*, 2001). LPS is a

ligand for TLR4 and polymorphisms in human TLR4 have been identified where amino acid alterations result in decreased LPS responsiveness (Arbour *et al.*, 2000). Recently, Dabbagh and colleagues reported that TLR4-mediated signals are necessary for the development of Th2 responses to nonmicrobial allergens (Dabbagh *et al.*, 2002). TLR4-defective mice challenged with protein allergen had reduced airway inflammation with eosinophils, allergen-specific IgE levels and Th2 cytokine production compared to wild type mice. The authors also demonstrated lower expression of CD86 on DCs from TLR4-deficient mice, and a reduced capacity of these DCs to produce IL-12p40 that could contribute to the reduced Th2 responses (Dabbagh *et al.*, 2002).

Further research into the regulation of TLR expression on monocytes or DCs from atopic individuals in comparison to non-atopics may reveal differences in the functional ability of these cells to respond to bacterial stimuli.

7.2 *N. meningitidis* OMs – DIFFERENTIAL PROPERTIES OF OM PROTEINS

7.2.1 Summary of results

OMs of wild type or LOS-deficient mutant variants of serogroup B *N. meningitidis* H44/76 exerted different properties on mo-DCs resulting in differential levels of cellular activation and maturation. These differences were primarily due to the presence of endogenous LOS within the native OMs. Induction of mature mo-DCs was observed by wild type OMs, reflected by marked increased expression CD83, CD80, CD86, CD40, HLA-DR and CD54, reduced capacity to take up antigen and increased production of various soluble mediators. By contrast, LOS-deficient OM only induced minor phenotypic changes and was selective at inducing cytokine secretion. Although the presence of LOS within the OM appeared to be essential for enhancing mo-DC function, LOS-replete or LOS-deficient OMs had the capacity to modulate mo-DCs to drive primary T-cell responses but had distinct effects on the T-cell cytokine profiles.

Purified recombinant class I porin was observed to possess the intrinsic capacity to mature mo-DC in a similar fashion to that observed by wild type OMs. However, an

important difference in the activity of this porin was its inability to induce IL-12p70 production by mo-DCs. Nonetheless, porin-stimulated mo-DCs were able to promote naïve T-cell proliferation and augment tetanus toxoid specific-T-cell responses demonstrating adjuvant properties.

TLR2 and TLR4 mRNA expression also revealed major differences in the regulation of these receptors in mo-DCs in response to OMs from either variant or purified class I porin.

7.2.2 Meningococcal OMs

The actions of wild type OM on mo-DCs may be attributed to the presence of membrane-associated LOS, as phenotypic maturation of mo-DCs is reproduced by purified meningococcal LOS. However, potent $CD4^+$ and $CD4CD45RA^+$ T-cell stimulatory capacity was observed by wild type OM-treated mo-DCs that were not observed by LOS-treated mo-DCs. These findings suggest that factors other than LOS within the OM are responsible for these effects. The natures of the bacterial components within LOS-deficient OM that modulate mo-DC function are not known. However, it is possible that a number of as yet uncharacterised individual OM proteins possess immuno-stimulatory properties through their actions on mo-DCs (Hertz *et al.*, 2001; Sester *et al.*, 1999). Results presented in this thesis demonstrate the preferential upregulation of TLR2 and TLR4 mRNA expression in mo-DCs following exposure to LOS-deficient OM and wild type OM, respectively. Recognition of the separate components within the different OM variants is likely to initiate signalling events that lead to mo-DCs interpreting the nature of the bacterial stimulus. In turn, mo-DCs become equipped by exhibiting changes in surface molecule expression and production of soluble mediators that may instruct distinct patterns of adaptive immunity. Therefore, the response induced by multiple components within the meningococcal OM may be the overall net effect of all signals transduced to the DC to mount the necessary immune response.

The proliferative responses of human PBMCs to OMs following meningococcal infection are described to be independent of bacterial strain and the subtype of class I or II/III porins (Pollard *et al.*, 1999b). However, the authors reported that cytokine production was age-dependent with a higher IL-10:IFN-γ (Th2) ratio produced by PBMCs from older children and more IFN-γ:IL-10 (Th1) ratio produced by infants

(Pollard *et al.*, 1999b). These results suggest that the reduced IL-10 production in infants may provide less help to naïve B-cells for class switching antibody production from IgD to IgG1 and IgG3, which are important for providing protection against infection (Briere *et al.*, 1994). It should be taken into account that the authors do not specify which cells within the PBMCs secrete IL-10, since this cytokine can also be released by monocytes; other Th2 cytokines (IL-4, IL-5 or IL-13) may have been better indicatives of Th2 activity (Pollard *et al.*, 1999b).

7.2.3 Meningococcal class I porin

The observation that purified recombinant class I porin potently activates mo-DCs also suggests that the presence of native class I porin in both wild type OM and LOS-deficient OM, contributes in part to the activation of mo-DCs. However, the reason for the observed greater ability of purified class I porin to induce mo-DC activation/maturation compared to LOS-deficient OM is presently unclear. These observations may depend on a number of factors including the relative concentrations of purified porin used to treat mo-DCs compared to that found in LOS-deficient mutant as well as the presence of a number of uncharacterised inhibitory proteins within the LOS-deficient OM that may counteract the stimulatory properties of the class I porin.

Isolated porins from a number of bacteria, such as *Salmonella typhimurium*, *Yersinia enterocolitica*, *Haemophilus influenzae* and *Helicobacter pylori*, have been shown to stimulate monocytes and lymphocytes to release a range of proinflammatory and immunomodulatory cytokines including IL-1, IL-4, IL-6, IL-8, TNFα, GM-CSF and IFN-γ (Galdiero *et al.*, 2001a; Galdiero *et al.*, 1998a; Galdiero *et al.*, 2001b; Tufano *et al.*, 1994a; Tufano *et al.*, 1994b). These published observations can be extended with findings from this thesis to demonstrate the ability meningococcal class I porins to stimulate the production of a variety of cytokines and chemokines by mo-DCs. It is reported that actions of bacterial porins are not mediated solely by binding to specific receptors but a consequence of perturbation of the cell membrane lipoproteic phase induced during absorption or porin penetration (Nikaido, 1992). *S. typhimurium* porins are reported to be internalised by human monocytes and lymphocytes (Galdiero *et al.*, 1998b). CD11a/CD18 integrins are reported to be involved in mediating signal transduction of purified porins of *S. typhimurium* in THP-1 cells

(Galdiero *et al.*, 2001b). However, blocking these receptors only partially decreased porin-induced cytokine secretion by THP-1 cells, therefore, suggesting the involvement of additional receptors (Galdiero *et al.*, 2001b). Recently neisserial class II porins have been described to mediate signals through TLR2, resulting in the up-regulation of CD86 and MHC class II molecules on murine B-cells (Massari *et al.*, 2002). The present findings from this thesis that LOS-deficient OMs induced increased TLR2 mRNA expression in mo-DCs are in accord with published reports on the involvement of TLR2 in response to *N. meningitidis* LOS-deficient mutant (Pridmore *et al.*, 2001; Ingalls *et al.*, 2001). Therefore, it is feasible that porins within the LOS-deficient OM are being recognized by and signal through TLR2. TLR4 mRNA expression in mo-DCs was significantly enhanced following treatment with purified class I porin, whilst there was a trend towards decreased TLR2 mRNA expression in mo-DCs although this did not reach significance. Although the findings from this study can not be compared with those published by Massari and colleagues due to 1) different cells (mo-DC vs. B-cells) and models (human vs. murine) and 2) different classes of porins investigated (class I porin solubilized in SDS vs. class II porin in proteosomes) used in both studies, it would be interesting to determine whether different classes of porins signal through different TLRs and whether different cells expressing these TLRs respond in a similar fashion to the neiserrial porins. Future analysis of TLR2 and TLR4 receptor expression by immunostaining different DC subsets following exposure to neisserial porins, as well as blocking TLR activity using antagonists, may increase understanding of the signalling mechanisms involved in porin activation of human DCs.

Protein conformation is essential for gram-negative membrane-spanning outer membrane porins to exhibit their functional properties (Barlow *et al.*, 1989; Minetti *et al.*, 1997). Immunologically relevant epitopes involved in bactericidal activity have been attributed to meningococcal porin conformation. The antigenic diversity of the class I protein is confined to two variable regions VR1 and VR2 (Peeters *et al.*, 1996; Mandrell and Zollinger, 1989; Idanpaan-Heikkila *et al.*, 1995). Monoclonal antibodies binding to these regions have been shown to be bactericidal *in vitro* and protective in *in vivo* animal models (Sacchi *et al.*, 2000; Peeters *et al.*, 1996). Immunization of mice with denatured porin, solubilized by denaturing agents such as SDS, fails to elicit protective antibodies (Muttilainen *et al.*, 1995a). Refolding of the

protein to exhibit native-like epitopes is necessary for the formation of bactericidal and protective epitopes. Typically, refolding of class I porin into phospholipid vesicles (such as liposomes) gives it its β-sheet conformation (Muttilainen *et al.*, 1995b; Christodoulides *et al.*, 1998). Preliminary studies were conducted early during this study to determine the activities of meningococcal class I porin in liposomes. Unlike the observations for recombinant porin solubilized in SDS (Chapter 6), porin in liposomes had little effect on mo-DC activation and maturation (data not shown). Moreover, exposure to liposomes alone increased mo-DC death in cultures as determined by 7-AAD staining using flow cytometry (data not shown). Dissolving in SDS is reported to generate α-helical instead of β-sheet conformation of porins (Muttilainen *et al.*, 1995a). Nonetheless, results presented here suggest that the native conformation of class I porin is not necessary to activate mo-DCs and subsequently stimulate allogeneic $CD4^+$ and primary T-cell proliferation. However, reconstitution of porins is essential to attain their appropriate structure for the synthesis of anti-bactericidal antibodies to react to porin epitopes on meningococcal surfaces (Zhang *et al.*, 1999; Jansen *et al.*, 2000). Other agents, including proteosomes, zwitterions and Triton-X are reported to be efficient at re-folding porin proteins (Idanpaan-Heikkila *et al.*, 1996; Minetti *et al.*, 1997). Future investigation of these agents to re-fold class I porins may be advantageous in attempting to modulate DC function in addition to enabling antibody formation.

Adult volunteers have lymphoproliferative responses to purified class I porins as well as to OMs (Wiertz *et al.*, 1996; Naess *et al.*, 1998). The T-cell epitopes of PorA on adults have been identified in conserved regions of the protein (Wiertz *et al.*, 1992). MHC restriction of these epitopes has also been documented. Human T cell responses to purified meningococcal OMs were higher to class 5 OMs (Opa and Opc) than class I porins with some epitopes more widely recognized by different HLA types and some showing greater HLA restriction (Wiertz *et al.*, 1996). Wiertz and colleagues have also demonstrated that class I porin T-cell epitopes are in regions of OMs which are not only conserved between strains but also highly conserved amongst neisserial porin proteins, increasing the chances for T-cell help in producing antibodies (Wiertz *et al.*, 1992; Wiertz *et al.*, 1991). Unlike bactericidal antibody specificities, PBMC responses to meningococci are not strain specific, and epitope-

specific T-cells are likely to response to a wide range of porins (Wiertz *et al.*, 1991; Pollard *et al.*, 1999b).

The adjuvant properties of neisserial porins at the cellular level are described to involve the up-regulation of CD86 and MHC class II molecules on B-cells, that may in turn influence antibody production (Wetzler *et al.*, 1996; Snapper *et al.*, 1997). To date the effects of isolated neiserrial porins on human DCs have not been documented. Thus, the results presented in this thesis, demonstrate that purified class I porins are able to act directly on immature mo-DCs increasing co-stimulatory expression of CD40, CD80 and CD86 as well as MHC class II molecules. This subsequently enhances allo-stimulatory activity of $CD4^+$ or $CD4^+CD45RA^+$ T-cells. Moreover, the adjuvanticity of class I porin is demonstrated by its ability to synergise with tetanus toxoid antigen and augment the development of mo-DC-driven $CD4^+$ T proliferative responses.

7.3 ACTIVATION OF DCs BY PATHOGENS

Different pathogens affect DC function in various ways resulting in differential levels of cellular activation and maturation. *Trypanosoma cruzi* is reported to induce the release of soluble factors that prevent DC maturation (Van Overtvelt *et al.*, 1999). *Plasmodium falciparum* increases surface expression of PfEMP-1 Ag on infected erythrocytes that subsequently bind to immature DCs and indirectly inhibits their maturation and function upon exposure to LPS (Urban *et al.*, 1999). Vaccinia virus is also reported to inhibit DC maturation (Larsson *et al.*, 2001). By inhibiting the maturation pathway of DCs and possibly inducing their death, these pathogens can subvert the development of efficient T-cell immunity. In contrast, pathogens such as influenza virus induce DC maturation following uptake (Larsson *et al.*, 2000).

The observation from this thesis that *N. meningitidis* activates human mo-DCs to secrete a wide range of cytokines has recently been documented (Kolb *et al.*, 2001; Dixon *et al.*, 2001). Markedly weaker levels of intracellular IL-6, IL-α and TNF-α were induced in mo-DCs exposed to the LOS-deficient H44/76 strain compared to the wild type organism (Dixon *et al.*, 2001). Moreover, these authors reported the production of intracellular IL-12 (p70/p40-specific mAb) only in mo-DCs exposed to the wild type bacteria (Dixon *et al.*, 2001), whereas in this thesis, IL-12p40 secretion

by mo-DCs was detected following challenge with LOS-deficient mutant bacteria. Although, there are a number of differences in the methodologies used in the current study compared with that of Dixon and colleagues, results from both studies demonstrate the importance of meningococcal LOS in wild type meningococci for induction of cytokine production, in particular IL-12, compared to the LOS-deficient mutant bacteria. It is noteworthy that Dixon and colleagues treated mo-DCs with paraformaldehyde-fixed bacteria and then measured cytokine release (Dixon *et al.*, 2001). Paraformaldehyde treatment is likely to significantly alter the composition of bacterial surface antigens. Thus, the biological consequences of the interactions of mo-DC with meningococci are only relevant with live bacteria (Dr. Christodoulides, personal communication).

In the present work the cytokine inducing properties of *N. meningitidis* were shown to apply to chemokines with LOS within the wild type bacteria being the major inducing factor. This suggests the participation of DCs in the innate immunity against meningococcal infection. Also, this data suggests that increased cytokine and chemokine production by DCs may indeed be associated with the pathology seen during meningococcal infection (van Deuren *et al.*, 1995). Apart from being directly involved in instigating high levels of soluble mediators by mo-DCs, it was also observed here that an additional role for LOS, present in wild-type *Neisseria,* may be to inhibit nitric oxide production by mo-DCs, that in turn, results in inadequate killing of the bacteria.

7.3.1 Nature of pathogenic stimuli directs immune responses

The pivotal role of DC activation is believed to be influenced by a number of factors including 1) the nature of the microbial/environmental stimuli, 2) the DC subset involved/expression of TLR receptors, 3) co-stimulatory interactions and 4) DC-derived cytokines (Langenkamp *et al.*, 2000; Vieira *et al.*, 2000; Kadowaki *et al.*, 2001a). DCs exposed to Th1-promoting bacterial components, such as bacterial LPS, display elevated levels of CD40 expression and increased ability to produce IL-12 in response to CD40 engagement, these interactions being important for DC activation (Caux *et al.*, 1994; Buelens *et al.*, 1997; Cella *et al.*, 1996). Moreover, DC-derived IL-12 contributes to the development of Th1 responses (Macatonia *et al.*, 1995). Indeed, the high levels of biologically active IL-12 produced by mo-DCs, as well as

CD40 up-regulation, in response to viable wild type meningococci or their isolated OMs, suggests that this bacteria or it's components induce Th1 responses.

The mechanisms that govern how DCs 'interpret' pathogen-associated signals to induce Th2 responses are not thoroughly understood. The developmental origins of DCs, whether myeloid or lymphoid, is thought to favour polarization of T-cells into Th1 or Th2 cells, respectively (Rissoan *et al.*, 1999). However, DCs also display functional plasticity and can be instructed to polarize T-cells depending on the foreign agents/inflammatory mediators that are present in the peripheral microenvironment (de Jong *et al.*, 2002; Cella *et al.*, 2000). A number of stimuli are known to induce DCs to produce high levels of IL-12 and promote Th1 differentiation (see Introduction). By contrast, agents such as PGE_2, calcium ionophore, ATP and histamine can favour mo-DCs priming Th2 cells by inhibiting DC-derived IL-12 production (La Sala *et al.*, 2001; Kalinski *et al.*, 1997; Mazzoni *et al.*, 2001; Faries *et al.*, 2001). Human plasmacytoid DCs mature and secrete high doses of IL-12 and IFNγ upon viral infection and, thus, can prime Th1 cells (Cella *et al.*, 2000; Dalod *et al.*, 2002). Mo-DCs and plasmacytoid DCs are reported to regulate each other; IFNα favours DC2 differentiation and inhibits IL-12 production, thus producing Th2 responses, whereas IL-4 induces apoptosis of preDC2 thus favouring DC1 differentiation and IL-12 production (Liu *et al.*, 2001a). Pathogen-induced changes in surface molecule expression on DCs may affect subsequent interactions with T-cells. Co-stimulation through molecules such as OX40L can deliver Th2 polarizing signals to $CD4^+$ T-cells (Ohshima *et al.*, 1998). Mo-DCs cultured in PGE_2 (DC2 favouring conditions) express surface OX40L only following CD40-ligation (de Jong *et al.*, 2002). Priming of mo-DCs with *Schistosoma mansoni* soluble egg antigens (SEA) in the presence of anti-OX40L mAb reduced the development of Th2 cells from naïve cells, suggesting the involvement of OX40L to promote Th2 differentiation. However, this was not observed by DCs primed with other DC2 agents such as PGE_2 or cholera toxin clearly indicating that polarization of Th cells is determined by a number of factors (de Jong *et al.*, 2002). Poly (I:C), a synthetic dsRNA molecule, is a potent stimulus for inducing Th1 response. Cella and colleagues have reported the ability of poly (I:C) to stimulate IL-12p70 as well as IFN-α production by mo-DCs that may participate in the development of Th1 responses (Cella *et al.*, 1999b). In contrast, Smits and colleagues reported poly (I:C) to be a poor stimulator of IL12p70

by CD40-ligated mo-DCs (Smits *et al.*, 2002). However, it is noteworthy that Smits and colleagues detected approximately 200-300pg/ml of IL-12p70 from poly (I:C)-treated mo-DCs, and much lower concentrations of this potent cytokine may be sufficient to induce Th1 responses. Nonetheless, these authors propose that ICAM-1/LFA-1 interactions contribute to the Th1-driving capacity of DCs when factors such as IL-12 are limiting or absent, as in the case of poly (I:C)-stimulated mo-DCs (Smits *et al.*, 2002).

It is probable that the ability of OMs from the LOS-deficient mutant to promote the differentiation of Th-cells displaying a higher IL-13:IFN-γ profile is collectively brought about through the inability of this stimulus to induce IL-12p70 and/or induce marked up-regulation of numerous co-stimulatory molecules as compared with the wild type OM (described in Chapter 5). The inability of purified class I porin to stimulate IL-12p70 by mo-DCs also suggests the modulation of these cells to drive Th2 responses. However, the marked up-regulation of co-stimulatory and MHC class II molecules as well as CD54 (ICAM-1) may suggest that these DCs would favour Th1 responses. Further studies will be necessary to determine the Th-cell polarizing capacity of neisserial porins.

7.4 MUCOSAL IMMUNIZATION

DCs present within the mucosal immune system have been described to be strongly associated with the potentiation of immune responses and development of active immunity against foreign agents (Weiner, 2001; Stumbles *et al.*, 1998; Stagg *et al.*, 2002; McWilliam *et al.*, 1996). Therefore, attempts at immunizing with vaccines that may specifically target these DCs to improve their antigen presenting properties may be advantageous. There are increasing reports demonstrating that administration of DNA vaccines, which encode specific antigens, through mucosal routes may be advantageous at generating protective immune responses against diseases that predominate at those specific sites. Administration of oral DNA vaccines containing the gene for the main peanut allergen (Arah2) protected mice against peanut-induced anaphylaxis, a response correlating with a reduction of IgE production (Roy *et al.*, 1999). Intranasal and intratracheal administration of DNA vaccines have also demonstrated high efficacy at inducing protection in animal models against infectious

agents (Sasaki *et al.*, 1998; McCluskie *et al.*, 2001; Meyer *et al.*, 1995). Thus, it would be interesting to determine whether DNA vaccines, empty vectors or vectors encoding meningococcal antigens, administered through such mucosal routes would confer protection against *N. meningitidis,* which colonizes the upper respiratory tract.

Phosphorothioate CpG ODN induces potent antibody and CTL responses, as opposed to phosphodiester CpG ODN which give weaker antibody responses but may give stronger CTL responses in the absence of T cell help. However, in humans, DNA vaccines are reported not to be very efficient at inducing humoral immunity (Wang *et al.*, 1998; Calarota *et al.*, 1998). Since the production of bactericidal antibodies is needed for both natural and vaccine-induced protection against meningococci, since disease occurs in individuals who lack serum bactericidal antibodies or complement, the development and delivery of DNA vaccines that induce both arms of the immune responses will need to be optimised. An advantage of mucosally delivered vaccines over conventional parenteral immunization is that it may stimulate both mucosal and systemic immune responses (Yao *et al.*, 2002; Debin *et al.*, 2002). The immunogenicity of DNA vaccines will be provided in part by the presence of numerous CpG sequences that possess adjuvant properties that act on specific APCs. However, from the results presented in this thesis and by others, it will be important to identify the underlying mechanisms of CpG ODN/DNA actions in human APCs that reside in the areas of vaccine administration in order to optimise the process. Knowing which cells are affected by the DNA will enable further development of how to increase the efficiency of its uptake into the cell and introduction into the nucleus where it undergoes transcription. The mechanisms of how CpG ODN is taken into cells and whether this is facilitated by human TLR9 has not been determined to date.

Improved vaccination strategies against meningococci are needed in infants. The immune system of newborns/infants is functionally different from that of adults, due to general immaturity. This results in poor responses to vaccine antigens (Barrios *et al.*, 1996; Cadoz, 1998). DNA immunizations are showing potential at inducing immune responses in infant animals. Vaccinal responses of newborn mice are skewed towards Th2 responses under conditions that normally induce Th1 responses in adult animals (Barrios *et al.*, 1996). Newborns also display Th2-type responses upon in utero contact with environmental antigens (Prescott *et al.*, 1998). However, induction

of IFN-γ secreting cells is found in children vaccinated at birth with bacillus Calmette-Guérin (Marchant *et al.*, 1999).

The failure of neonatal T-cells to induce Th1 responses for efficient elimination of a number of pathogens is suggested as a factor contributory to the increased susceptibility to intracellular infections (Dadaglio *et al.*, 2002). This reduced T lymphocyte function is manifested as a low lympho-proliferation rate in response to anti-CD3 stimulation (Adkins *et al.*, 1994), predisposition towards Th2 responses (Adkins and Du, 1998) or weak expression of CD40 ligand upon activation (Durandy *et al.*, 1995). Moreover, it has been proposed that low yields of neonatal DCs and their impaired function in antigen presentation could contribute to the dysfunction of T-cells and in turn susceptibility to infection (Liu *et al.*, 2001b). Neonatal DCs derived from human cord blood monocytes have lower levels of MHC class II and co-stimulatory molecule expression compared to adult mo-DCs (Goriely *et al.*, 2001). In addition, synthesis of IL-12p70 in response to LPS, poly (I:C) or CD40 ligation was impaired in neonatal mo-DCs (Goriely *et al.*, 2001). However, murine neonatal $CD11c^+$ DCs are comparable to adult DCs in maturing following LPS activation and have been shown to efficiently induce specific CTL responses, *in vivo* (Dadaglio *et al.*, 2002). Although the mechanisms involved in neonatal immune responses to pathogens are not fully elucidated, it is possible that altered TLR expression patterns on newborn APCs may play a role in the response to environmental antigens and invading pathogens. Since numerous microbial components are reported to initiate cell activation through TLRs, it is feasible that bacterial adjuvants exert their actions through TLRs. Thus, by using specific adjuvants in vaccine preparations it could be possible to evoke a desired immune responses, whether Th1 or Th2, through targeting specific TLRs. Adult intestinal epithelial cells are reported to be deficient of TLR4 by immunohistochemistry (Cario and Podolsky, 2000; Asai *et al.*, 2001). Gingival epithelial are also reported to lack TLR4 expression and these cells respond to Gram-negative bacteria via TLR2 ligands (Asai *et al.*, 2001). Thus, it will be important to elucidate the TLR signalling mechanisms in different cells at various mucosal sites. Studies on the responses of human neonatal/infant DCs to meningococcal outer membranes or individual proteins, such as the porins, will be useful to qualitatively compare their responses to the responses by adult DCs. In addition, these studies may

be useful for evaluating new methods to induce optimal humoral and cell-mediated responses in young individuals against infection.

7.5 FINAL SUMMARY

Work presented in this thesis provides evidence that human APCs are differentially activated by distinct bacteria or their separate components to exhibit mature phenotypes, and secrete soluble mediators. Interpretation of bacterial signals depends on the presence of particular TLRs on different APCs that transduce signals provided by specific bacterial ligands upon its recognition. These processes eventually determine the fate of APCs as to whether they will become activated, enhance their antigen presenting function and induce primary immune responses.

In this study, differences in patterns of expression of TLR 2, 4 and 9 by human monocytes, mo-DC/LC, $CD34^+$ derived-DC/LC and epidermal LCs were observed. The lack of responsiveness of mo-DC/LC to CpG ODN was shown to result from the failure of these cells to express TLR9 receptors, compared to their monocyte precursors. Thus, the presence or absence of TLRs on these cells may indicate whether they are directly involved in the activation of their respective TLR-ligands. The mechanisms involved in this decreased TLR9 expression throughout the *in vitro* culture of mo-DCs have yet to be elucidated and may facilitate greater understanding on the regulation of TLR9 *in vivo*. Overall, the ability of distinct subsets of APCs to recognize distinct pathogenic components through different TLRs may contribute to the strength and duration of the immune response elicited.

OMs of wild type or LOS-deficient *N. meningitidis* were shown here to modulate mo-DC function, and the level of maturation is dependent on the nature of the OM. Moreover, purified meningococcal class I porin exhibits potent adjuvant properties on mo-DCs to potentiate their ability to stimulate $CD4^+$ proliferation to tetanus toxoid antigen. The study of the effects of LOS-deficient mutant meningococci on DC is not biologically relevant, since individuals are only infected with wild type bacteria. Nonetheless, comparative studies using these variants will facilitate greater understanding of the actions of meningococcal LOS as well as individual proteins during the pathogenesis of disease. Importantly, the availability of LOS-deficient OMs will be beneficial for designing future vaccines against meningococci.

The findings of this report have added to the limited knowledge of the effects of CpG ODN and *N. meningitidis* on human mo-DCs. It is only by increasing scientific understanding with studies such as these, that it will be possible to evaluate therapeutic strategies.

CHAPTER 8

References

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