Studies Into The Characteristics And Basic Biology Of Dendritic Cells And Their Use In The Immunotherapy Of Malignancy.

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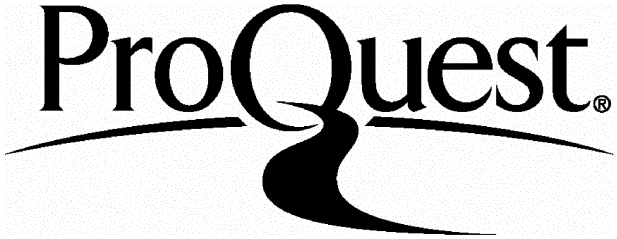

Abstract

Dendritic cells (DCs) are the most potent of all antigen presenting cells, playing a central role in initiating and orchestrating immune responses. It has recently become possible to generate large numbers of DCs from either CD34+ haemopoietic progenitor cells or peripheral blood monocytes under the influence of various cytokines. With this advance there has been a great explosion of interest in using *ex vivo* generated DCs as part of immunotherapy programmes directed towards the treatment of malignancy, chronic infections and autoimmune diseases, as well as in the context of inducing tolerance following allogeneic transplantation. In all these settings the DCs need to be loaded with the appropriate antigens. This can be achieved by several methods, each of which has its advantages and disadvantages.

In this thesis a comparison is made between the methods used to generate DCs from either CD34+ progenitor cells or CD14+ monocytes. The morphology, phenotype and functional characteristics of the resulting DCs are reported.

Results are presented which demonstrate that the differentiation of monocytes into DCs under the influence of GM-CSF and IL-4 is a non-proliferative process and therefore does not lend itself to retroviral transduction, rendering this method of loading DCs with antigen inapplicable. The signal transduction pathways which are involved in the lipopolysaccharide (LPS)-induced maturation of monocyte derived DCs are also explored. The p38SAPK pathway and NF-κB pathways are shown to be important in regulating some but not all of the phenotypic changes which occur with LPS-induced maturation of DCs. The PI3 kinase pathway is shown to be important in maintaining the viability of LPS stimulated DCs.

Finally, a clinically applicable method for generating DCs from CD34+ progenitor cells is described, and preliminary results are presented on two patients with non-Hodgkins Lymphoma treated with DCs generated in this manner and loaded with tumour antigens in the form of an autologous tumour cell lysate.

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List of Abbreviations

APC	Antigen Presenting Cell
ATF	Activating Transcription Factor
CCR	Chemokine Receptor
CD40L	CD40 Ligand
CDR	Complementarity Determining Region
CLIP	Class-II-Associated Ii-Chain Peptide
CREB	cAMP Response Element Binding Protein
CTL	Cytotoxic Lymphocyte
CTLA	Cytotoxic T Lymphocyte Antigen
DAB	Diamino Benzidine
DC	Dendritic Cell
DIFP	Diisopropyl fluorophosphate
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethyl Sulphoxide
DTH	Delayed Type Hypersensitivity
EDTA	Disodium Ethylenediamine Tetra-Acetic acid
EPO	Erythropoietin
ERK	Extracellular signal related protein kinase
FITC	Fluorescein isothiocyanate
FLT3	Fms-like tyrosine kinase
G-CSF	Granulocyte -Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HLA	Human Lymphocyte Antigen
HPC	Haemopoietic Progenitor Cell
HRP	Horseradish Peroxidase
HSA	Human Serum Albumin
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
Ii	Invariant Chain
IL	Interleukin
JNK	c-Jun N-terminal kinase
KLH	Keyhole Limpet Haemocyanin
LFA	Leukocyte Function Associated Antigen
LNGFR	Low Affinity Nerve Growth Factor Receptor
LPS	Lipopolysaccharide
MIIC	Major Histocompatability Class II Compartment
MAP(K)	Mitogen Activated Protein (Kinase)
MCF	Mean Cell Fluorescence
MEF	Myogenic Enhancer Factor
MEK	MAP Kinase Kinase (MEK=MKK1)
MGG	May Grunwald Giemsa
MHC	Major Histocompatibility Complex
MIP	Macrophage/Monocyte Inhibitory Protein
MKK	MAP Kinase Kinase (MKK1=MEK)
MLR	Mixed Lymphocyte Response
MNC	Mononuclear Cell
Mnk	MAP Kinase Interacting Protein Kinase
MoMLV	Murine Moloney Leukaemia Virus

Msk	Mitogen and Stress Activated Protein Kinase
NF-κB	Nuclear Factor-kappa B
NHL	non-Hodgkins Lymphoma
NK	Natural Killer
p38SAPK	p38 Stress Activated Protein Kinase
PBS	Phosphate Buffered Saline
PBSC	Peripheral Blood Stem Cell
PCR	Polymerase Chain Reaction
PI3	Phosphoinositide-3-OH
PPD	Purified Protein Derivative
pRb	Retinoblastoma Protein
RANTES	Regulated on Activation of Normal T cell Expressed and Secreted
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCF	Stem Cell Factor
SD	Standard Deviation
SEM	Standard Error of the Mean
SLC	Secondary Lymphoid Chemokine
TAP	Transporter Associated with Antigen Processing
TCR	T Cell Receptor
TNF	Tumour Necrosis Factor

Work Accepted for Publication During the Course of this Thesis

MONOCYTE-DERIVED DENDRITIC CELLS DO NOT PROLIFERATE AND ARE NOT SUSCEPTIBLE TO RETROVIRAL TRANSDUCTION
K.M.Ardeshna, A.R. Pizzey, N.S.B.Thomas, S.Orr, D.C.Linch and S.Devereux.
Brit J Haem **108** (4) 2000 p817-24

THE PI3-KINASE, p38 SAP KINASE AND NF-κB SIGNAL TRANSDUCTION PATHWAYS ARE INVOLVED IN THE SURVIVAL AND MATURATION OF LIPOPOLYSACCHARIDE-STIMULATED HUMAN MONOCYTE-DERIVED DENDRITIC CELLS.
K.M.Ardeshna, A.R.Pizzey, S.Devereux and A Khwaja.
Blood In Press – Provisionally due to be published in Vol **96** (3)

A CLINICALLY APPLICABLE METHOD FOR THE EX-VIVO GENERATION OF ANTIGEN PRESENTING CELLS FROM CD34+ PROGENITORS.
KM Ardeshna, CP Corney, SJ Ings, MJ Watts, DC Linch and S Devereux.
Vox Sanguinis In Press – Provisionally due to be published in Vol **79/1**

Chapter 1

Introduction

1.0.1 Summary.

Dendritic cells were first visualised as Langerhans cells in the suprabasal layer of the epidermis in 1868 by the medical student Paul Langerhans (Langerhans, 1868). It was not until the 1970s however, that these cells were characterised further (Steinman & Cohn, 1973). Dendritic cells (DCs) are now known to be central to the orchestration of an immune response, being important in initiating and modulating immune responses (Banchereau & Steinman, 1998). DCs are 'professional' antigen presenting cells being unique in their ability to stimulate naïve T cells and are therefore responsible for initiating primary immune responses. In their immature form DCs are proficient at antigen uptake, whilst in their mature form they are specialised in antigen presentation. These phases of the DCs' life cycle are not only separated in time but also in space, the immature form residing in the peripheral parts of the body and the mature form in the lymph nodes and spleen through which T cells relentlessly traffic. With the discovery of methods by which DCs can be generated in large numbers *in vitro*, the interest in using these sentinels of the immune system in vaccination strategies against cancer and infectious agents, together with their potential use in the treatment of autoimmune disorders, has grown. In the remainder of this section a more detailed account of the biology of the DC is given together with the aims that underlie the work which is presented in the following chapters of this thesis.

1.0.2 Morphology and distribution.

Dendritic cells are large cells (the body of the cell being ~20μm in diameter) which have numerous, long, delicate processes. When the cells are in culture and viewed by phase-contrast microscopy, these arbourising processes can be seen to extend and retract in a non-polarised fashion. In situ, in tissue sections, DCs can be seen to have a stellate appearance, whilst on cytospin preparations fine dendrites can be seen. Under the scanning electron microscope, thin spiny or sheet like processes are visible. These dendrites provide a large surface area over which antigens in the environs of the DC can be initially sampled and then later presented to lymphocytes in order to generate an immune response (Banchereau & Steinman, 1998).

DCs are found in the epidermis of the skin, lung and gastrointestinal tract where, after antigen uptake, they migrate to the regional lymph nodes through the afferent

lymphatics where they appear as veiled cells. In humans, DCs are said to be located in the interstitium of all organs excluding the brain, parts of the eye and testis (Steinman, 1991), though the absence of DCs in these organs is now controversial (Lotze & Thomson, 1999). These interstitial DCs are in close proximity to the vasculature and gain access to the blood stream after encounter with antigen (Avigan, 1999). DCs constitute between 0.1-1% of peripheral blood mononuclear cells and are at different stages of maturation (Reid, 1997).

1.0.3 Methods of antigen uptake.

In their immature state, DCs are proficient at taking up antigen. This occurs by several mechanisms, all of which are downregulated as the DC matures.

DCs exhibit constitutive macropinocytosis during which actin-dependent membrane ruffling occurs, and large vesicles up to 5μm in diameter are formed as the ruffles fold back against each other or the cell membrane itself (Sallusto & Lanzavecchia, 1994; Swanson, 1989). The soluble antigens, which are sampled in this way, are concentrated within the macropinosome and the vesicle is recycled back to the plasma membrane. This is an efficient method by which fluid phase or non-binding antigens can be sampled (Steinman & Swanson, 1995; Swanson & Watts, 1995).

DCs also use mannose receptors to bind antigens which have exposed mannose or fucose residues. Having bound their ligands, these receptors are internalised in clathrin coated vesicles of < 0.1 μm diameter (Stahl, 1992). After release from the receptor, the antigen is delivered to an endocytic compartment and the receptor recycled to the cell surface. Uptake by this mechanism has been shown to be 100 times more efficient than bulk macropinocytosis, and mannosylation of antigenic proteins or peptides has been shown to increase the potency of the T cell responses to the antigen by 200-10000 fold (Tan *et al.*, 1997). The efficiency of macropinocytosis and mannose receptor-mediated uptake of antigen enables picomolar concentrations of antigen sufficient to allow adequate antigen presentation by DCs. Other antigen presenting cells (APCs) require micromolar quantities of antigen (Sallusto *et al.*, 1995).

DCs also use the Fcγ receptor (FcγIIR) and the Fcε receptor (FcεR) to bind and internalise immune complexes. In these cases, the receptors are degraded along with their ligands (Sallusto & Lanzavecchia, 1994).

DCs demonstrate only a modest degree of phagocytosis when compared with macrophages (Steinman & Swanson, 1995). This is thought to reflect the difference in function of the two cells; macrophages clear debris and scavenge whereas DCs only need to sample the environment.

DCs are now also known to internalise apoptotic cells by means of the integrins $\alpha_v\beta_5$ and $\alpha_v\beta_3$ together with the CD36 adhesion molecule (Albert *et al.*, 1998a;1998b). This is thought to account for the cross priming phenomenon whereby, *in vivo*, major histocompatibility complex (MHC) class I restricted tumour antigens are almost exclusively presented by DCs, which take up dying tumour cells, rather than by the tumour cells themselves (Huang *et al.*, 1994). macrophages do not cross present antigens in this way.

1.0.4 Antigen processing.

Association of exogenous antigen with MHC class II molecules.

MHC class II molecules are dimeric structures consisting of one α and one β chain. These are synthesised in the endoplasmic reticulum and assembled together there in association with the invariant chain (Ii). The Ii chain is a non-MHC product which binds three MHC II dimers and allows transport to the *trans*-Golgi network , and then directs the complex to the endosomal compartment. Here, the Ii chain is degraded, leaving behind an internal Ii peptide named CLIP (class-II-associated Ii-chain peptide). CLIP occupies the groove of all MHC II molecules, preventing premature antigen loading. CLIP is removed under conditions of low pH, as found in the endosomes, with or without the assistance of the peptide editing molecules human lymphocyte antigen (HLA) DM and HLA DO. Exogenous antigen which has entered the cellular endocytic compartment by the various mechanisms outlined above, is degraded to peptides composed of 9-16 amino acids and these then associate with the empty peptide binding groove, which lies between the α and β chains of the MHC class II molecules. (Fig1.1a) (Mellman *et al.*, 1998).

In the immature DC, the MHC II-peptide complex reaches the cell surface but is then removed and degraded relatively quickly, the complex having a half life of approximately 10 hours (Cella *et al.*, 1997). The MHC II molecules removed from the cell surface in this way are held in peripherally located endosomes before being recycled .

Thus, there are two distinct MHC class II compartments. The major compartment is the MHC class II-rich compartment (MIIC) which contain newly synthesised MHC II

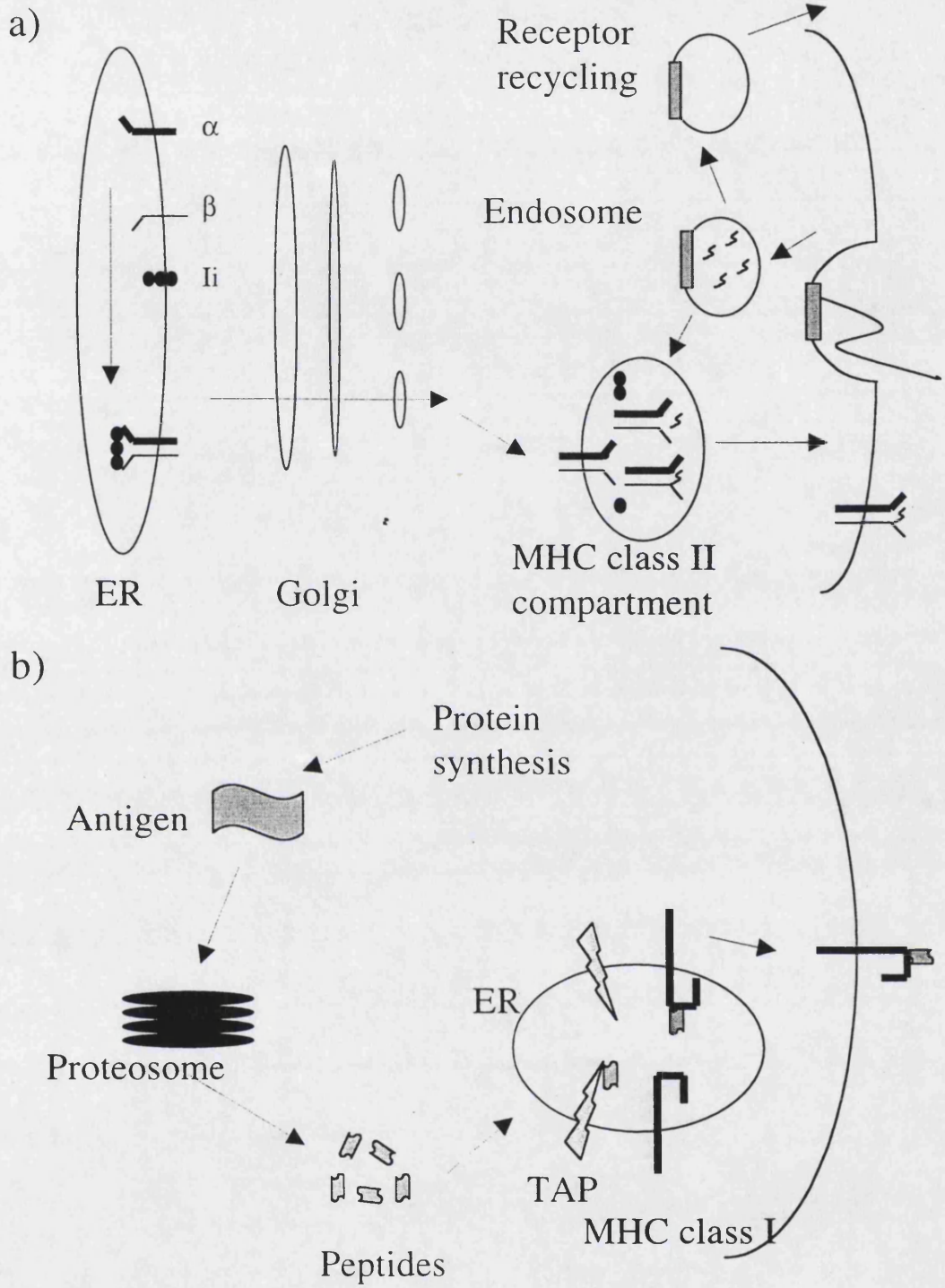


Figure 1.1 Pathways of antigen processing.
a) The α and β chains of the MHC class II complex are synthesised and assembled together with the invariant chain in the endoplasmic reticulum (ER). This is then transported through the Golgi network before entering the MHC class II compartment. Antigen which has been endocytosed is degraded to peptides of 9-13 αα in length in endosomes. These peptides are then loaded onto the MHC class II complexes after the invariant chain has been degraded. The MHC II-peptide complex is then transported to the cell surface. b) Protein which has been synthesised in the cytoplasmic compartment is degraded to peptides in the proteasome. Some of these peptides are transported by transporter associated with antigen processing (TAP) to the ER. These peptides are then loaded onto the MHC I molecules which have been synthesised in the ER before being displayed on the cell surface. It is now known that exogenous antigen can also enter this pathway .

molecules in association with the Ii chain. These are abundant in immature DCs, appearing as multilaminar or multivesicular inclusions (Peters *et al.*, 1995). The minor compartment consists of recycled MHC II complexes, which are not associated with Ii.

Immature DCs have high total levels of class II molecules, but only a small fraction are on the cell surface and they are rapidly internalised. 24 hours after receiving a maturation stimulus there is a large increase in both total and surface MHC class II molecules, indicating both a redistribution and increased biosynthesis of MHC class II molecules. Indeed, MHC class II synthesis has been shown to increase by 2-3 fold soon after a maturation stimulus is received and is sustained for 10-16 hours before synthesis stops. The recycling pool of MHC II gradually disappears by 40 hours and the half life of the MHC II-peptide complexes is increased to over 100 hours (Cella *et al.*, 1997).

The antigen processing machinery of an immature DC has been compared to an idling engine, that is running, but not in the right gear to allow effective antigen presentation. Upon encounter with a maturation stimulus however, the DC engages the correct gear to allow effective antigen presentation (Watts, 1997).

Association of endogenous antigen with MHC class I molecules.

Endogenously generated antigens, such as those produced by virally infected cells and tumour cells, are hydrolysed by proteasomes in the cytoplasm to form oligopeptides. A small fraction of these are transported by a carrier, known as transporter associated with antigen processing (TAP), to the endoplasmic reticulum where they associate with newly synthesised MHC class I molecules. These MHC I-peptide complexes are transported to the cell surface for display to CD8+ cytotoxic lymphocytes (Fig 1.1b)(Raychaudhuri & Rock, 1998).

Association of exogenous antigen with MHC class I molecules.

Although most cells cannot present exogenously derived antigens on MHC class I molecules, DCs are able to transfer internalised antigens from endosomal compartments into the cytoplasm by an unknown mechanism. This enables DCs to present exogenous antigens, such as those found on tumour cells but not synthesised by the DCs themselves, on to MHC class I molecules, thereby allowing a cytotoxic immune response against tumour antigens to be primed (Rock *et al.*, 1993; Rodriguez *et al.*, 1999). This process of presenting exogenous soluble antigen in association with MHC I is very inefficient however, requiring high concentrations of exogenous protein (Raychaudhuri & Rock, 1998).

As the generation of a cytotoxic T cell response against tumour cells is a key component of anti-tumour immunity, strategies to enhance this process are being actively investigated. One method which would encourage the presentation of tumour antigens on MHC class I molecules would be to retrovirally transduce the DC with a gene encoding a tumour-associated antigen. This would allow endogenous expression of the tumour antigens and thus enhance the formation of MHC class I-tumour peptide complexes, which would, in turn, promote the generation of anti-tumour cytotoxic T cells.
The possibility of retrovirally transducing different types of DCs is explored in chapter 4 of this thesis.

1.0.5 Antigen presentation.

Having taken up and processed antigen, the DC migrates to the T cell dependent areas of the regional lymph nodes or spleen to present antigen to the T cells constantly passing through these organs. This maximises the chance of the DC encountering the potentially very rare T cell whose receptor recognises the MHC-peptide complex presented by the DC. DCs present antigenic peptides in the context of MHC class I molecules to the T cell receptors (TCRs) of CD8+ cytotoxic T cells. Peptides in association with class II molecules are in turn presented to the TCR of CD4+ helper T cells. Engagement of the TCR delivers the first signal to naïve T cells. A second signal is required for the T cell to exceed the activation threshold and allow T cell proliferation (Lanzavecchia, 1997). This second signal is provided by the interaction of the CD28 receptor on the T cell with the co-stimulatory molecules B7.1/CD80 and B7.2/CD86, which are expressed to a high degree on mature dendritic cells (Fig 1.2). If no second signal is received, anergy ensues (Banchereau & Steinman, 1998). Cytotoxic T lymphocyte Antigen (CTLA) 4 is another receptor found on T cells for the B7 molecules. Upon engagement of CTLA-4 with B7 signals are transduced that shut off T cell activation. CTLA-4 blocks signals transduced by CD28 suggesting that these 2 molecules function as mutual antagonists (Van Parijs & Abbas, 1998).

If one of the signals delivered by the DC to the T cell is selectively interrupted, it would be possible to convert a DC from one which activates a T cell to one which tolerises a T cell. This would, of course, be valuable in the context of treating autoimmune disorders and in the introduction of post-transplant tolerance. In chapter 5 of this thesis the signal transduction pathways involved in DC maturation and hence MHC and co-stimulatory molecule expression are investigated with this possibility in mind.

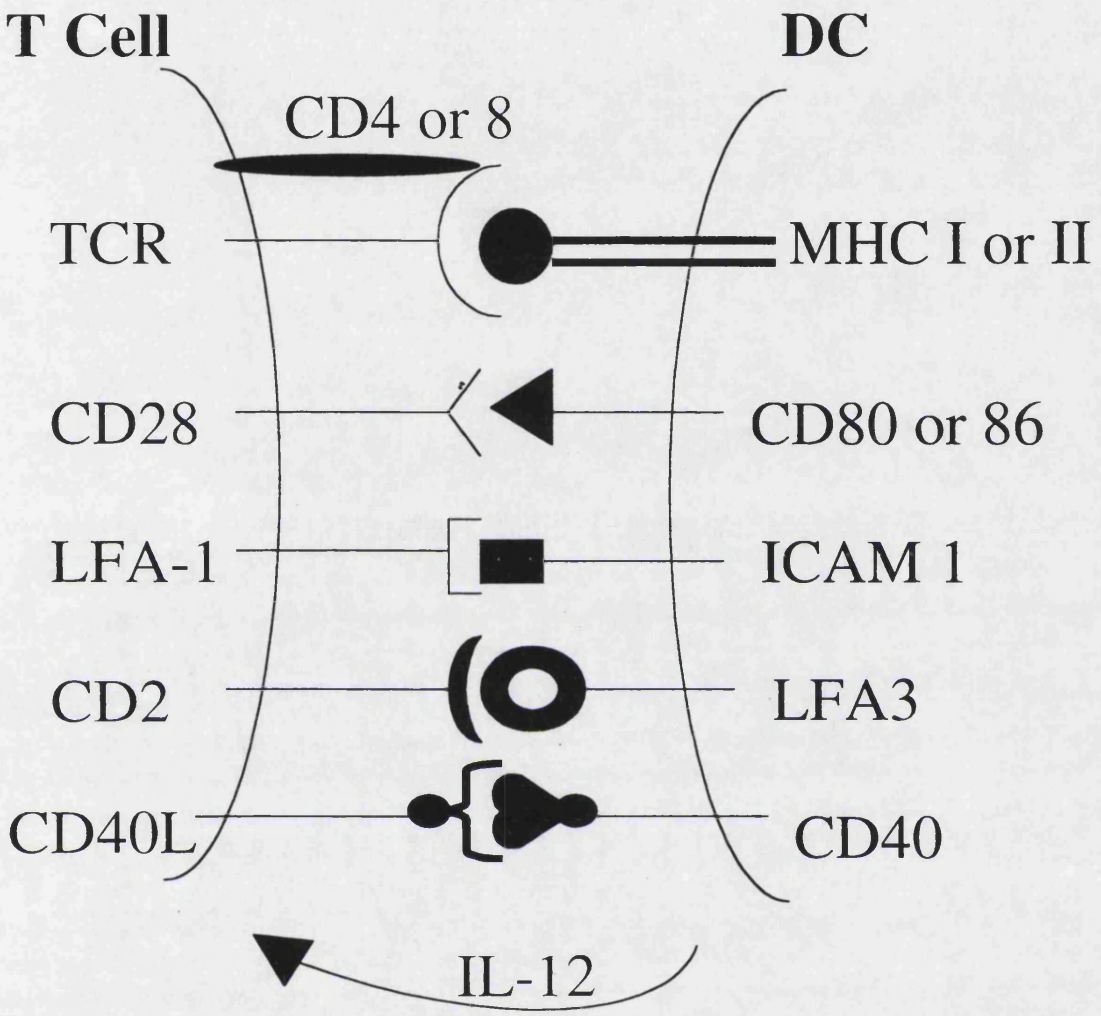


Figure 1.2. Dendritic cell: T cell interactions.
This diagram displays some of the many ligands and their receptors which interact when a mature DC activates a T cell.

CD40 is another co-stimulatory molecule expressed by DCs. Its expression is upregulated upon DC maturation. Its ligand (CD40L) is expressed on T cells. Engagement of CD40 with CD40L results in a two-way interaction. This interaction acts as a co-stimulus for the T cell (McLellan *et al.*, 1996) but also results in DC activation, as seen by the increased expression of MHC II, CD80, CD86 and leukocyte function associated antigen (LFA) 3 by the DC and increased DC survival (Caux *et al.*, 1994). CD40 ligation also results in the production of interleukin (IL)-12 by the DC, which skews the T helper response to a γ-interferon (IFN) producing, T_{H1} type response, which promotes the differentiation of T cells into killer cells and activates macrophages (Fig 1.2) (Cella *et al.*, 1996).

Recently it has been shown that DCs initially activate T helper cells, which then stimulate the DC via CD40-CD40L interactions. This 'conditioned' DC is then able to prime antigen-specific CD8+ cytotoxic T cells. It is in this way that T cell help is delivered to cytotoxic T cells. Thus, the DC acts as a temporal bridge between helper and cytotoxic T cells (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998).

Dendritic cell-T cell clustering is a feature of mature DCs. It is enhanced by the low level of DC surface sialic acid and further encouraged by the engagement of TCRs with MHC-peptide complexes, co-stimulatory molecules with their ligands and the adhesion molecules (intercellular adhesion molecule (ICAM) 1, ICAM 3 & LFA 3) with their respective ligands. (Fig 1.2)

DCs are also essential for the initiation of primary B cell immunity. This occurs in both a T_H dependent manner as wellas through the direct interaction of B cells with DCs. DCs activate T_H cells and these subsequently promote B cell survival and proliferation through the release of cytokines and via CD40-CD40L interactions. T_H cells also promote the IL-2 dependent differentiation of B cells into IgM producing plasma cells. However, DCs are required to deliver the appropriate signals to the B cells in order to initiate isotype switching and thus enable IgG secretion. This occurs in a T-independent fashion. DCs are also responsible for capturing and retaining unprocessed antigen *in vitro* and *in vivo*. This antigen is subsequently transfered to naive B cells resulting in the initiation of antigen specific antibody responses (MacPherson *et al.*, 1999).

1.0.6 Dendritic cell maturation.

Initial work showed that when DCs were isolated from skin, a terminally differentiated population was obtained. These cells had high expression of MHC molecules but low

new MHC biosynthesis. These cells were not good at capturing new antigens (Schuler & Steinman, 1985). It is now known that DCs reside in the tissues in an immature form, where they sample the antigens in their environment. Upon encounter with antigen, they undergo a maturation process which can be induced by numerous stimuli.

Numerous changes occur as part of this maturation process including the alteration in the chemokine receptors expressed by the DC. Thus an immature DC expresses the chemokine receptors CCR1 and CCR5. These respond to the inflammatory chemokines macrophage inflammatory protein (MIP) 1α, MIP 3α and RANTES (regulated on activation of normal T cell expressed and secreted) which are produced at sites of inflammation. Within 1 hour after exposure to a maturation stimulus, these receptors are downregulated and there is a slower upregulation of the CCR7 receptor. The ligands for CCR7 are MIP 3β and secondary lymphoid tissue chemokine (SLC). MIP 3β is produced in lymphoid organs and SLC is produced by the endothelial cells of the lymphatics and the T cell zones of the lymph nodes (Dieu *et al.*, 1998; Kellermann *et al.*, 1999). These changes together with the downregulation of the skin homing molecule E-cadherin, result in the migration of the maturing DCs to the T cell areas of the regional lymph nodes (Sallusto *et al.*, 1998; Sozzani *et al.*, 1999). Here, the DCs in turn produce chemokines, which preferentially attract naïve T cells and thus facilitate the generation of a primary immune response (Adema *et al.*, 1997).

As mentioned earlier, upon DC maturation there is an initial increase in MHC synthesis, together with a redistribution of MHC molecules to the cell surface where they remain for a longer period of time. Cell surface expression of the co-stimulatory molecules CD80, CD86 and CD40 is also increased, as is the expression of the marker of mature DCs, CD83. The expression of the CD1a molecule found in immature DCs is down-regulated, as are the various mechanisms for antigen uptake.

It is in this way that the DC becomes specialised in presenting the antigens sampled in its previous environment to the circulating T cells in the lymphoid organs.

There are many stimuli which can initiate the maturation process *in vitro*. They include the proinflammatory cytokines tumour necrosis factor-α (TNF-α) and IL-1β, and bacterial products such as lipopolysaccharide (LPS) (Sallusto *et al.*, 1995; Sallusto & Lanzavecchia, 1994). Ligation of CD40 by CD40L and the engagement of Fcγ receptor by immune complexes have also been shown to stimulate maturation as have CpG DNA motifs found in prokaryotic DNA and viral double-stranded RNA (Caux *et al.*, 1994; Cella *et al.*, 1999; Hacker *et al.*, 1998; Regnault *et al.*, 1999). Monocyte conditioned

medium is a potent maturation stimulus but the causative factor remains unknown (Reddy *et al.*, 1997). LPS has also been shown to lead to the maturation of DCs *in vivo* (De Smedt *et al.*, 1996).

1.0.7 Definition of a dendritic cell

It is clear that the phenotype of a DC changes depending on its maturation state, this together with the finding that there is no single immunophenotypic marker that identifies either an immature or a mature DC, makes the definition as to what constitutes a DC difficult. This is further complicated by the finding that there are several different types of DCs. It is therefore necessary when examining whether a cell is a DC or not, to evaluate the cell on the basis of its morphology and broad cell surface immunophenotype, together with some assessment of its functional capabilities.

The morphology of the DC has been described earlier. Functional tests could include a) the ability of the cell to stimulate a mixed lymphocyte response (MLR), some would even insist on a strong MLR indicated by a stimulation index of 20 or more at DC:T cell ratios of 1:1000 (Schuler & Steinman, 1997), b) the ability to present primary and recall antigens to autologous CD4+ T cells and c) the ability of the cell to take up antigen by macropinocytosis or via the mannose receptor.

DCs are CD45+ leukocytes, which express high levels of MHC II molecules but with the absence of the following lineage specific molecules: CD3, CD15, CD16, CD56, CD19, CD20 and CD14. As mentioned earlier, DCs express the co-stimulatory molecules CD40, CD80 and CD86 to varying degrees. The antibody CMRF44 recognises a molecule of unknown function which is expressed by early activated DCs. Expression of this molecule has been proposed by some to identify DCs, though it has not gained widespread acceptance (Hock *et al.*, 1994). CD83, which is a member of the immunoglobulin superfamily, is another molecule of unknown function which is almost exclusively expressed on mature DCs. Expression of this molecule is now widely accepted as an indication of a mature DC, though not all mature DCs express CD83 (Zhou & Tedder, 1995). The molecule CD1a is expressed on Langerhans cells and other immature DCs. It is downregulated upon DC maturation. It is of interest as it is a member of the CD1 family of molecules, which appear to have an ancestral link with the MHC molecules in terms of their genetic evolution. Like the MHC molecules, the CD1 molecules appear to be involved in antigen presentation in that the CD1b and CD1c molecules have been shown to present glycolipid and lipid antigens to T cells

(Brenner & Porcelli, 1997). Unlike the MHC molecules however, the CD1 molecules are non-polymorphic.

1.0.8 The in vitro generation of dendritic cells.

DCs are present in the peripheral blood at very low levels (0.1-1% of mononuclear cells) and are a trace cell type in most lymphoid and non-lymphoid tissues. The isolation of DCs in substantial numbers has therefore been both laborious and difficult, and this largely accounts for the initial slow progress in unravelling DC biology.
In 1990 Reid *et al* demonstrated that colonies of cells with dendritic morphology and dendritic cell function could be generated *in vitro,* in semi solid cultures, from both bone marrow and peripheral blood MNC (Reid *et al.*, 1990). In 1992 Reid *et al* andCaux *et al* demonstrated that cord blood CD34+ progenitor cells can develop into DCs under the influence of granulocyte-macrophage colony stimulating factor (GM-CSF) and TNF-α. (Caux *et al.*, 1992; Reid *et al.*, 1992). Others soon demonstrated that bone marrow, peripheral blood and mobilised peripheral blood CD34+ cells can also act as a source of dendritic cells using the same or different (and often numerous) cytokines (Herbst *et al.*, 1996; Mackensen *et al.*, 1995; Siena *et al.*, 1995; Strunk *et al.*, 1996; Szabolcs *et al.*, 1995; Young *et al.*, 1995). In 1994 it was discovered that CD14+ monocytes could be differentiated into DCs under the influence of GM-CSF and IL-4 (Romani *et al.*, 1994; Sallusto & Lanzavecchia, 1994) though it remains unclear whether the DCs generated *in vitro* in this way have their equivalent *in vivo*. The ability to generate such large numbers of DCs *in vitro* has given the DC research field a great boost, allowing not only the more detailed study of the function and molecular biology of DCs, but also enabling sufficient DCs to be generated to be used *in vivo*, with the aim of inducing immunity either in the context of infection or malignancy. The relative merits of the different methods of generating DCs *ex vivo* are further discussed in chapters 3 and 4 of this thesis.

More recently it has been shown that the immediate precursors of endstage neutrophils can be differentiated into DCs under the influence of GM-CSF, IL-4 and TNF-α (Oehler *et al.*, 1998), though once again whether these cells have counterparts *in vivo* is unclear. This curious finding emphasises the extraordinary plasticity of what were previously thought to be committed cells and highlights our limited understanding of DC ontogeny.

1.0.9 Dendritic cell ontogeny.

Using bone marrow chimera studies, Katz *et al* have shown that epidermal dendritic cells are of haemopoietic origin (Katz *et al.*, 1979). Other groups have made similar observations regarding DCs in other tissues. Caux *et al* have shown that CD34+ cells give rise to two discrete DC populations; the epidermal type Langerhans cells (LC) and the dermal or interstitial type dendritic cell (Caux *et al.*, 1996). The LC progenitor is CD1a+CD14- and can only differentiate into DCs, whereas the dermal DC progenitor is CD1a-CD14+ and is capable of differentiating into either macrophage/monocytes or DCs depending on the cytokine stimulus. Different DC populations have also been described depending on the expression of CD11c (O'Doherty *et al.*, 1994), CD33 (Thomas & Lipsky, 1994) and CD13 (Rosenzwajg *et al.*, 1996).

In addition to the myeloid type of DC described above, there is evidence for a lymphoid-derived DC. The precursors to these cells have the appearance of plasmacytoid lymphocytes, express CD4 but lack CD11c and develop into DCs under the influence of IL-3 and CD40L (Grouard *et al.*, 1997). These DCs appear to be involved in tolerance induction.

To further confuse the picture, it appears that lymph node germinal centres contain two types of DC. Follicular DCs, which appear not to have a bone marrow origin, are important in sustaining the viability, growth and differentiation of B cells. CD11c+ DCs are also found in the germinal centres and may be the DC that brings antigens to the germinal centre and displays antigens to the memory T cells. Both types of germinal centre DCs carry antigen in the form of immune complexes (Banchereau & Steinman, 1998).

1.1.0 The use of dendritic cells in tumour vaccines.

Conventional therapies directed against malignancies are often inadequate, and alternative less toxic treatments are currently being sought. There is a large body of evidence that amongst the lymphocytes of tumour-bearing hosts are cells whose receptors recognise tumour-associated antigens (Boon & Old, 1997; Vanderbruggen *et al.*, 1991). However, these cells have not been activated and expanded, and are therefore unable to kill the tumour cells.

Recently it has also become clear that most tumours express antigens, which, are also expressed by the normal cell counterpart at the same stage of differentiation. These

antigens are normal self-antigens and not truly tumour-specific antigens. Some tumours express mutant forms of normal cellular proteins i.e altered self antigens e.g mutant forms of Ras, whilst others express viral proteins e.g in Epstein Barr Virus associated lymphomas. Only in a few tumour types is a true tumour-specific antigen known to be present (e.g. the *BCR-ABL* fusion protein found in chronic myeloid leukaemia). It is however possible to generate an effective immune response against cells bearing these self/altered-self antigens as well as against truly foreign antigens.

The possibility of vaccinating patients with malignancies with these antigens in order to generate an appropriate and effective anti-tumour immune response has therefore become more of a reality. This is especially so as our knowledge of the exact nature of some of these 'tumour-specific antigens' has grown. In particular, it is now known, for some 'tumour-specific antigens', which peptide fragments are most immunogenic in the context of the appropriate HLA molecule (Rosenberg *et al.*, 1996; Schuler & Steinman, 1997).

Immunisation with antigen alone results in a weak immune response at best. Conventional adjuvants such as alum appear to be ineffective and pre-clinical studies have shown that although some types of immune responses are elicited, conventional adjuvants fail to generate an effective cytotoxic T cell response, which is crucial if cancer cells are to be killed (Raychaudhuri & Rock, 1998).

It should be clear from the preceding account that T cells are activated only when they are presented with antigen in conjunction with the correct MHC molecule and together with the appropriate co-stimulatory signals. DCs are in the unique position of being able to provide all of these appropriate signals. It is for this reason that DCs have been called 'nature's adjuvant'(Steinman, 1996).

Indeed, a substantial number of pre-clinical studies have demonstrated the effectiveness of using DCs to generate effective immune responses, and the ability of DCs to stimulate the production of antigen-specific cytotoxic lymphocytes (CTLs) in various settings has been clearly shown.

Initial *in vitro* studies using DCs loaded with peptides produced from the HIV gag or envelope proteins, have shown that antigen specific CTLs which lyse peptide-pulsed or virally-infected cells can be generated (Mehta *et al.*, 1994). Further studies have demonstrated the ability of DCs pulsed with OVA peptide to stimulate CTL *in vivo* which are able to lyse syngeneic tumour cells pulsed with OVA peptide or transfected

with the OVA gene (Porgador & Gilboa, 1995). Bhardwaj has been able to show that DCs infected with the influenza virus can generate CTLs, which are able to lyse influenza-infected targets (Bhardwaj *et al.*, 1994). Other groups have shown that DCs generated from normal human volunteers can be pulsed with antigens found in human tumours. DCs treated in this way are able to induce the generation of CTLs, which in turn, are able to lyse HLA-matched tumour cell lines (Alters *et al.*, 1998; Bakker *et al.*, 1995; Mannering *et al.*, 1997).

In vivo studies in mice have shown that OVA peptide-pulsed DCs can confer protection to subsequent challenge with a tumour cell line transfected with the OVA gene (Celluzzi *et al.*, 1996). Peptide-pulsed DCs have also been shown to result in therapeutic benefit in tumour bearing mice as well as providing protection against subsequent tumour challenge (Mayordomo *et al.*, 1995). Protein-pulsed DCs have also been shown to induce CTLs *in vivo* (Paglia *et al.*, 1996). This method allows the DC to select the optimal peptide for presentation.

Adenoviruses and retroviruses have been used to insert genes encoding tumour-associated antigens into DCs. These methods of loading DCs with antigen have also been shown to result in protective and therapeutic immunity in animal models (Song *et al.*, 1997; Specht *et al.*, 1997).

Several methods of antigen loading of DCs are suitable when relevant tumour antigens are not known. These include DCs pulsed with peptides eluted from tumour cells, DCs pulsed with tumour lysates, DCs pulsed with whole tumour RNA and DCs fused with tumour cells to generate DC-tumour hybridomas. All these methods of antigen loading have been shown in animal models to provide protective and therapeutic benefit (Ashley *et al.*, 1997; Fields *et al.*, 1998; Gong *et al.*, 1997; Zitvogel *et al.*, 1996).

The positive results of these pre-clinical studies has opened the door to the therapeutic use of DCs in humans. Several phase I clinical trials have begun and the results of some of these have been very encouraging. These will be reviewed in chapter 6.

The use of DCs as part of a vaccine has been hampered by the difficulty in isolating sufficient numbers of DCs. With the discovery of methods to generate large numbers of dendritic cells *ex vivo* this is no longer an obstacle. However, methods of generating DCs in a laboratory are not necessarily appropriate for generating DCs which will be returned to patients. Of particular concern has been the presence of foreign proteins in culture medium containing fetal calf serum (FCS), as the patient may subsequently

become sensitised to these proteins. Also of concern is the possibility of prion transmission, especially if bovine products are used.

A further limitation to the use of DC vaccines has been the limited number of tumours for which tumour-specific antigens are known. In the case of the non-Hodgkin's lymphomas (NHL), each tumour cell expresses a tumour-specific antigen in the form of its idiotypic determinant. However, this determinant will be unique to each individual patient's tumour, and therefore the characterisation of this tumour-specific antigen will be relevant only to that one patient. In one of the first clinical studies to have used DC based vaccines for the treatment of NHL, the idiotypic determinant for each individual patient was characterised in order to generate a vaccine (Hsu *et al.*, 1996). However, generating a patient-specific DC vaccine by this method will necessarily require much time and labour. This in turn will be associated with an increase in cost, and therefore ultimately reduced availability, of any DC-based vaccine for lymphoma, which necessitates the knowledge of the exact nature of the idiotypic determinant. In view of this, and because tumour-specific antigens have not been identified for most tumour types, alternative, more broadly applicable methods for antigen loading of DCs will be required, such as those used in the animal studies outlined above.

In chapter 3 of this thesis a clinically applicable method for the generation of DCs is described. In chapter 6, the initial results of the first two patients with NHL treated with DCs generated in this manner and loaded with antigen by means of a tumour lysate are presented.

Chapter 2

General Materials and Methods

2.0.1 Cytokines.

Recombinant human GM-CSF (Hoechst, Marburg, Germany) and IL-4 (Schering Plough, England) were used at a concentration of 100ng/ml and recombinant TNF-α (R+D systems, Minneapolis MN) was used at 2.5ng/ml

2.0.2 Cell counts.

Cell counts were performed using a Neubauer counting chamber (Hawksley, England) with at least 200 cells being counted per sample. Cell viability was assessed using trypan blue exclusion (Sigma Chemical Co.).

2.0.3 Source of CD34+ cells.

Mobilised peripheral blood mononuclear cells (MNCs) were collected by apheresis from patients with multiple myeloma, non-Hodgkin's and Hodgkin's lymphoma who were about to undergo peripheral blood stem cell (PBSC) transplantation for their haematological malignancy as well as from healthy donors. Patients received either cyclophosphamide 1.5g/m^2 followed by a vial of granulocyte-colony stimulating factor (G-CSF) subcutaneously daily (lenograstim™ 263μg/vial or Filgrastim™ 300μg/vial) or ESHAP chemotherapy (etoposide 40mg/m^2 for 4 days, *cis*-platin 25mg/m^2 over 4 days, cytarabine 2g/m^2 on day 1 only, methylprednisolone 500mg for 5 days), followed by a single vial (263μg) of lenograstim™ subcutaneously daily. Mobilised peripheral blood MNCs were harvested by leukapheresis 10-11 days after the end of the chemotherapy. The normal donor received 5 days of G-CSF alone (Filgrastim™ 10mg/kg/d) prior to harvesting. Local ethical approval was obtained to use some of the cells obtained in this manner for experimental purposes (UCL 97/0282).

2.0.4 Source of monocytes, CD4+ T cells, and MNCs.

Monocytes which were destined to be differentiated into DCs were obtained from a MNC preparation from the resting peripheral blood of normal volunteers. Monocytes were also required for use in autologous T cell proliferation assays and MLRs as control stimulator cells. These were purified from 60ml of peripheral blood obtained from the original donor of the cells used to make DCs. In the case of CD34+ DCs, the blood was obtained from the patient 12-14 days after leukapheresis. In the case of monocyte derived DCs, the blood was obtained from the donor 7 days after the first

venesection. In both cases, collecting these cells on the day they were required obviated the need to freeze and thaw them.
CD4+ T cells were required for use in the autologous T cell proliferation assays as responder cells. These were purified from the same sample of blood used to collect the monocytes required for these assays.
MNCs were used as responder cells in the allogeneic MLR. These were obtained on the day required from the peripheral blood of one of several normal volunteers.

2.0.5 Preparation of MNCs.

Peripheral blood was collected in disodium ethylenediaminetetra-acetic acid (EDTA) from normal volunteers. Red cells were removed by dextran sedimentation using 1% w/v dextran (Pharmacia Biotech, Uppsala Sweden), the supernatant was then layered on Ficoll-Paque (Pharmacia Biotech) and centrifuged at 800g for 15 min at room temperature. Cells at the interface were harvested, diluted with 30ml phosphate buffered saline (PBS) (Gibco BRL, Scotland) and centrifuged at 725g for 5 minutes at room temperature.

2.0.6 Separation of CD34+ cells

CD34+ cells were isolated from the leukapheresis product in one of 2 ways. In some patients the CD34+ cells from the entire harvest product were positively selected using either the Ceprate™ stem cell concentration system as described by Watts (Watts *et al.*, 1997) or the Clinimacs device™ (Miltenyi Biotec, Bergish Gladbach, Germany)(Schumm *et al.*, 1999). An aliquot of $1x10^6$ cells were then removed and expanded (Fig 2.1).
In the remaining cases an aliquot (2ml) of leukapheresis product was removed and the CD34+ cells were positively selected using the Variomacs™ device (Miltenyi Biotec) as per manufacturer's instructions (Fig 2.1). In brief, the cells were processed using Ficoll-Hypaque density centrifugation (Pharmacia Biotech AB, Uppsala Sweden). Cells obtained from the interface were washed and the pellet incubated with 100µl of CD34+ multisort magnetic beads (Miltenyi Biotec) for 30 minutes on ice, prior to washing and selection on a positive selection VS+ column (Miltenyi Biotec).

2.0.7 Separation of CD14+ cells.

Monocytes were selected from MNCs using an anti-CD14 antibody (Dako A/S, Denmark). The MNCs from 50ml of blood were incubated with 100µl of antibody for 30 minutes at 4°C, after this 25ml of PBS was added and the cells centrifuged (800g,

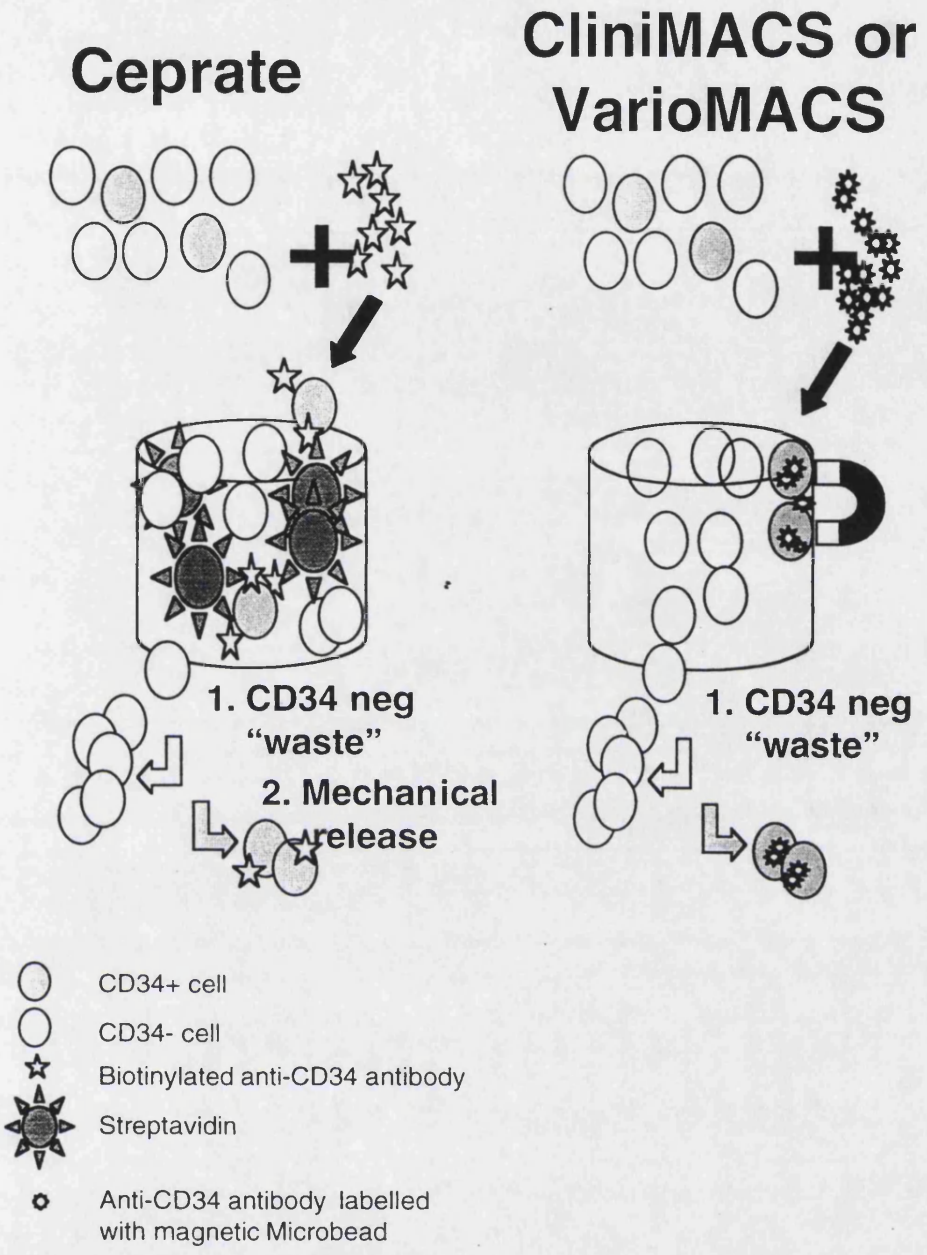


Figure 2.1 Methods of selecting CD34+ progenitor cells.
Schematic representation of the methods used to select CD34+ cells from the leukapheresis product collected from donors. With the Ceprate system the cells are incubated with biotinylated anti-CD34 antibodies and then passed through a column of avidin-coated polystyrene beads. The biotin moiety forms a complex with the avidin-coated polystyrene beads, retaining the CD34+ cells in the column. These are then released mechanically. In the MACS system the cells are incubated with an anti-CD34 antibody which is labelled with a magnetic bead. These cells are then passed through a column to which a stong magnetic force is applied which retains the CD34+ cells within the column. When the magnetic force is removed the CD34+ cells are flushed out of the column. The Clinimacs device is used to select cells on a clinical scale whilst the VarioMacs device is used to select cells on a laboratory scale.

5mins, 4°C), resuspended in 200µl PBS and further incubated with 50µl goat anti-mouse microbeads (Miltenyi Biotec) for 30 min at 4°C. These cells were then washed with PBS once more and the monocytes were then selected using a VS+ column. The resulting samples were greater than 95% pure monocytes by morphology. It has been shown by Miltenyi Biotec (Germany) that cells which have been selected using their antibodies have shed 25% of their microbeads (50nm diameter) after 4 hours in culture at 37°C, and that by 24 hours almost no microbeads remains on the cells (personal communication E. Schultz).

2.0.8 Separation of CD4+ T cells for T cell proliferation assays and monocytes for MLR and T cell proliferation assays.

Monocytes and CD4+ T cells were selected sequentially from MNCs prepared from peripheral blood using the Variomacs™ device as follows; MNCs were pelleted, incubated with 100µl of anti-CD14 fluorescein isothiocyanate (FITC)-conjugated antibody (Dako A/S, Denmark) for 30 minutes on ice, washed, incubated with 100µl of anti-FITC multisort beads (Miltenyi Biotec) then positively selected using a VS+ column. The unadsorbed cells were pelleted, incubated with 100µl of anti-CD4 antibody (Dako A/S, Denmark) for 30 minutes on ice, washed, incubated with 100µl of goat anti-mouse multisort beads (Miltenyi Biotec) for 30 minutes followed by positive selection using a further VS+ column. The purity of the cells collected by all of these methods was 55-90% as determined by morphology in the case of the monocytes, or alkaline phosphatase anti-alkaline phosphatase immunoenzymatic staining of CD4 in the case of CD4+ T cells.

2.0.9 Culture conditions for the generation of DCs from CD34+ cells.

In these experiments $1x10^6$ purified CD34+ cells were cultured for 14 days in medium consisting of X-VIVO 10 (supplied without phenol red or gentamicin by Biowhittaker, Walkersville Maryland), 10% citrated autologous plasma (collected at the time of leukapheresis), GM-CSF 100ng/ml (Novartis, Basel Switzerland), recombinant TNF-α 2.5ng/ml (R+D systems, Minneapolis) and heparin 2U/ml henceforth referred to as complete medium (CM). From day 7 IL-4 100ng/ml was added to the CM. The initial cell concentration was $1x10^5$/ml. On days 5 and 10 the volume of medium with added growth factors was doubled to allow for cell proliferation.

2.1.0 Culture conditions for the generation of DCs from CD14+ monocytes.

CD14+ monocytes were cultured at a starting concentration of $5x10^5$ cells/ml in RPMI 1640 (Gibco BRL) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco BRL, Scotland) and GM-CSF and IL-4 for 7 days in 6 well plates (Costar, Cambridge, MA). On days 3 and 5 half the original medium was removed and replaced by fresh medium containing growth factors. All cells were cultured at 37°C in 100% humidified 5% CO_2 in air.

2.1.1 Flow cytometric analysis.

$5x10^5$ cells were pelleted and resuspended in 100µl of 50:50 PBS and AB serum in 5ml (12 x 75mm) polystyrene round bottomed tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA). These cells were stained using either a direct or indirect method. In the direct method the cells were stained was for 60 minutes at 4°C using an antibody to which a fluorochrome was directly conjugated. In the indirect method, cells were first incubated with an unconjugated primary antibody for 60 minutes, this then detected by staining for 45 minutes at 4°C with a FITC-conjugated anti-immunoglobulin antibody. Cells were washed with 4 mls of ice cold PBS for 5 minutes at 320g after each incubation. Appropriate isotype controls were used at the same protein concentration as the test antibody. The subtype of isotype controls and test antibody were identical and both test and control antibodies were purchased from the same company. Samples were analysed using the Coulter EPICS Elite™ flow cytometer (Fig 2.2). Fluorochrome-conjugated murine antibodies directed against the following antigens were used: CD1a (IgG2a), CD80 (IgM), CD86 (IgG1), CD40 (IgG1) (Serotec, UK): CD14 (IgG2a) (Sigma Chemical Co, St Louis), HLA-DR (IgG1) (Dako A/S, Denmark), and CD83 (IgG2a) (Immunotech, France). Murine anti-DQ (IgG 1) was kindly provided by Prof. B. Chain, Dept of Immunology, UCL. This was detected using an FITC-conjugated rabbit anti-mouse antibody (Sigma Chemical Co, St Louis), diluted 1 in 20, and as a control only the second layer was applied. IgM antibody to CMRF 44 was kindly provided by Dr G Risden, Cellpro Inc, Seattle. The second layer antibody used to detect this antibody was FITC-conjugated $F(ab)^2$ goat anti-mouse IgM (Jackson Immunoresearch), diluted 1 in 50.

2.1.2 Allogeneic mixed lymphocyte reaction (MLR).

$1x10^5$ allogeneic peripheral blood MNCs were cultured in CM for 5 days in 96 well U bottom microtitre plates (Nunc, Denmark) with up to $3x10^4$ γ-irradiated (25Gy) stimulator cells. Stimulator cells were either those removed from the DC cultures on the final day of culture or fresh monocytes obtained from the same patient. 1µCi of ^3H-thymidine

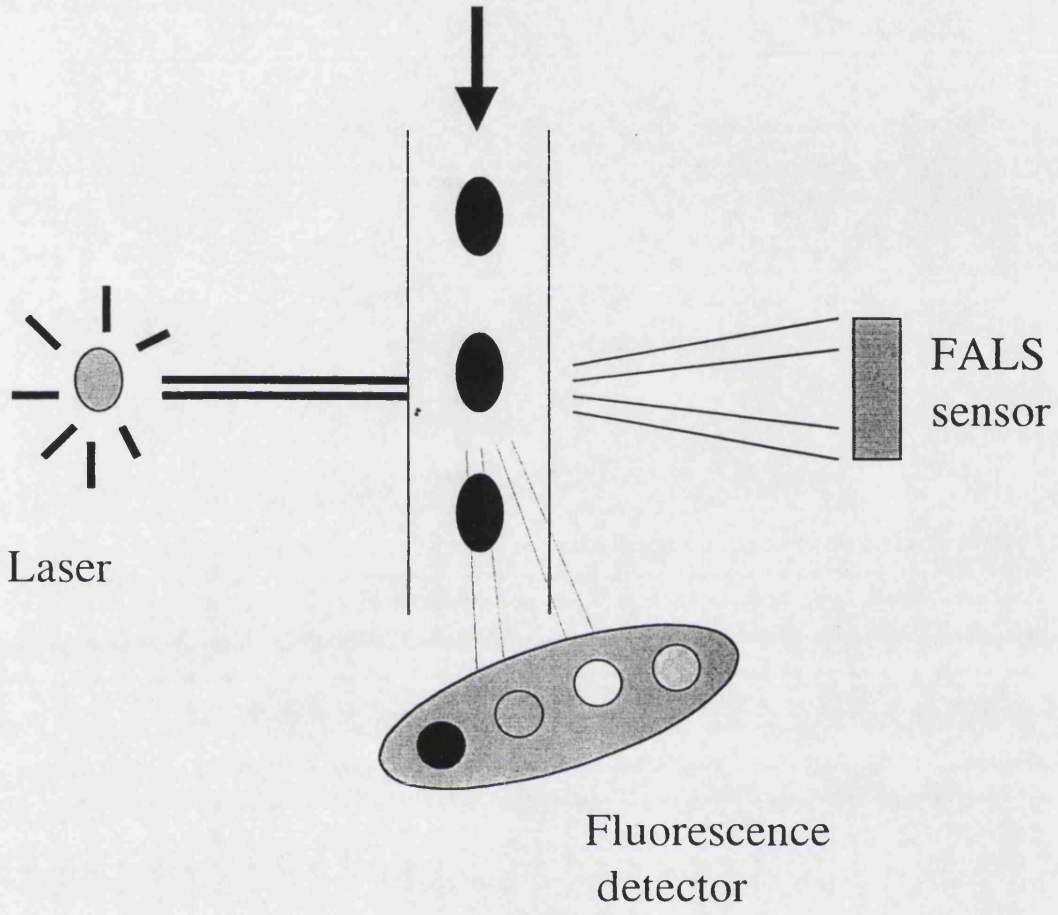


Figure 2.2 The principle of the flow cytometer.
Cells which have been incubated with fluorochrome-conjugated antibodies are injected into a sheath fluid as it passes through a small orifice (100μm). This causes the cells to run in single file past a laser beam. Each cell is sequentially interrogated by the laser beam and light is scattered both in the forward direction and at 90° to this.This scattered light is detected by light sensors placed in the forward direction (forward angle light scatter (FALS) sensor) and at 90° to this (not shown).
Forward scatter tends to be more sensitive to the surface properties of a cell whereas side scatter tends to be more sensitive to inclusions within cells.The fluorescence detected by each fluorochrome-conjugated antibody is detected in a specific fluorescence channel. All this information is collected, converted to digital values and displayed on a computer screen.

(Amersham, UK) was added to triplicate wells for 16-18 hours before the cells were harvested on fibreglass filters and counted.

2.1.3 Endocytosis assay with FITC-dextran.

The method described by Sallusto *et al* was used (Sallusto *et al.*, 1995). In brief, FITC-dextran (Molecular Probes, Oregon) was added to DCs or MNCs resuspended in X-VIVO 10, at a final concentration of 1mg/ml. After incubation for varying time intervals up to one hour at 37°C, cells were removed and washed 4 times with ice cold PBS and then analysed on the Coulter EPICS Elite™ flow cytometer. Dead cells were gated out on the basis of propridium iodide staining. In some experiments the cells were pre-incubated with mannan (Sigma Chemical Co, St Louis.), 0.3mg/ml for 15 minutes to inhibit uptake via the mannose receptor.

Chapter 3

The Generation of Dendritic Cells from CD34+ Progenitor Cells and CD14+ Monocytes

3.0 Introduction

Dendritic cells are present in the peripheral blood at very low levels (0.1-1% of mononuclear cells) and are a trace cell type in most lymphoid and non-lymphoid tissues. Thus the isolation of DCs in substantial numbers has been both laborious and difficult. The discovery of methods to generate large numbers of DCs *in vitro* from either CD34+ progenitor cells or CD14+ monocytes has given the DC research field a great boost. This has allowed not only the more detailed study of the function and molecular biology of the DC, but has also enabled sufficient DCs to be generated *ex vivo* with the ultimate intention of returning these to the host in an attempt to induce immunity, either in the context of infection or malignancy.

In order for DCs to be suitable for clinical use they must be generated in an appropriate culture system. This not only means that they should be generated under sterile conditions but also that the culture medium should be free of foreign proteins, in particular the bovine proteins found in FCS. Failure to do so may render the recipient of these potent APCs sensitised to bovine proteins. For similar reasons the culture medium should be free of antibiotics. A further reason to avoid exposure of DCs destined for clinical use to FCS is the possibility of contamination with prion proteins.

In this chapter, a clinically applicable method for the generation of DCs from mobilised peripheral blood CD34+ progenitor cells is described. These cells are shown to be DCs on the basis of their appearance, cell surface immunophenotype and, most importantly, on the basis of their functional properties. These cells are compared with DCs generated from CD14+ monocytes under standard conditions which are used to explore the biology of DCs. The relative merits of each cell type are then discussed.

3.1 Materials and Methods

3.1.1 Primary T cell proliferation assays.

The method of Mehta was followed (Mehta *et al.*, 1995). In brief, $1x10^5$ autologous CD4+ T cells were cultured in CM in 96 well U bottom microtitre plates (Nunc, Denmark) with up to $3x10^4$ γ-irradiated (25Gy) stimulator cells (DCs or monocytes).

Keyhole limpet haemocyanin (KLH)(Sigma Chemical Co, St Louis.) was added at a final concentration of 25μg/ml, purified protein derivative (PPD)(Evans Medical Ltd, Leatherhead, UK) was used at 10μg/ml and tetanus toxoid (Pasteur Merieux, France) was used at 5 level of flocculation units (Lfu) per ml. The proliferative response of the cells was measured by the uptake of ^{3}H thymidine (1μCi/well) added 16-18 hours prior to the harvesting of cells on the 7th day of culture. Background proliferation was determined by culturing responders and irradiated stimulators without added antigen. Further controls consisted of each cell type cultured alone.

3.2 Results

3.2.1 Generation of DCs from mobilised peripheral blood CD34+ progenitor cells is a proliferative process.

Mobilised peripheral blood CD34+ cells were cultured for 14 days in medium which consisted of X-VIVO 10 and 10% citrated autologous plasma collected at the time of leukapheresis. GM-CSF and TNF-α were present throughout the culture period. IL-4 was added on day 7. Over the 14 day period there was a median 38 fold expansion in cell number (range 18-72, n=12). The characteristics of the patients from whom the CD34+ cells were derived, as well as the fold expansion which occurred over the 14 day period is shown in table 3.1

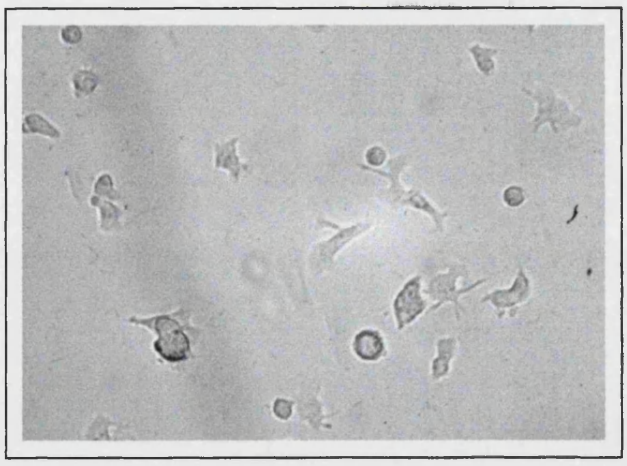
3.2.2 A minor proportion of cells generated from CD34+ progenitor cells have the morphological appearance of DCs.

Cells generated under the culture conditions described were examined by phase contrast microscopy. A small but variable proportion of cells had the characteristic appearance of DCs. In their adherent form these cells were seen to be large with numerous long irregular cytoplasmic processes. Most of the cells were loosely adherent as judged by the ease with which they were dislodged by gentle repeated pipetting of the culture medium. In their non-adherent form the cells had the appearance as shown in Fig 3.1a. Cytospin preparations of cultured cells were stained with May Grunwald Giemsa (MGG) stain to enable more detailed examination of the morphology (Fig 3.1b). Between 5 and 16% (n=6) of the cells had a typical dendritic morphology, being large cells with a single round nucleus and abundant slightly basophilic cytoplasm. Numerous fine cytoplasmic projections were present. On one occasion electron microscopy was performed and the characteristic multilamellar MHC class II compartments were seen (Kleijmeer *et al.*, 1996). However no Birbeck granules were noted (Fig 3.1c).

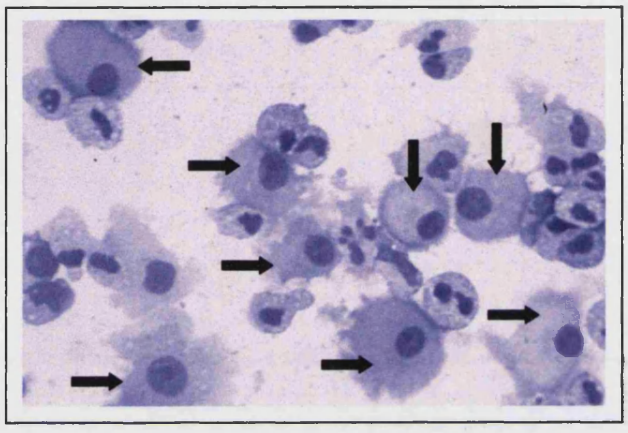
Age	Sex	Disease	Mobilisation Regimen	Separation	Purity	Previous Treatment	Fold Exp^n
52	M	MM	Cyclophosphamide Filgrastim	Variomacs	---	CVAMPx5 IFN	36
50	M	MM	Cyclophosphamide Filgrastim	Ceprate	83%	CVAMPx5 spinal R/T	48
35	M	Normal Donor	Filgrastim	Ceprate	62%	nil	60
54	M	HGNHL	ESHAP Lenograstim	Variomacs	---	CHOPx6 epirubicin dexaBEAM	29
48	M	MM	Cyclophosphamide Lenograstim	Ceprate	64%	CVAMPx4	18
36	M	HGNHL	ESHAP Lenograstim	Variomacs	96%	CHOPx4	72
45	M	MM	Cyclophosphamide Lenograstim	Variomacs	98%	CVAMPx4 oral melphalan DCEx2	30
49	F	HD	ESHAP Lenograstim	Variomacs	---	PABLOEx6 mediasinal R/T	40
51	M	HGNHL	ESHAP Lenograstim	Ceprate	87%	CHOPx6 ESHAP	28
56	F	MM	Cyclophosphamide Filgrastim	Ceprate	55%	CVAMPx4	48
44	F	MM	Cyclophosphamide Filgrastim	Ceprate	73%	CVAMPx3	52
54	M	HGNHL	ESHAP Lenograstim	Variomacs	---	CHOPx3	32

Table 3.1 Characteristics of the donors of the CD34+ cells used to generate CD34+ derived DCs.
The age, sex and haematological disease of the patients from who CD34+ cells were obtained is shown together with their prior treatment and the mobilising regimen used. The mode of CD34+ selection, the purity of the selection and the final total cell expansion is also shown.
HGNHL=high grade non-Hodgkins lymphoma, MM=multiple myeloma, HD= Hodgkins disease,
CVAMP=cyclophosphamide,vincristine.adriamycin.methylprednisolone
CHOP= cyclophosphamide, daunorubicin,vincristine. prednisolone
DCE=dexamethasone, cyclophosphamide, etoposide
DexaBEAM=dexamethasone, BCNU, etoposide, cytarabine. melphalan
ESHAP=etoposide,methylprednisolone,cytarabine.*cis*-platin
PABLOE=procarbazine,adriamycin,bleomycin.vincristine.etoposide
R/T=radiotherapy

a)



b)



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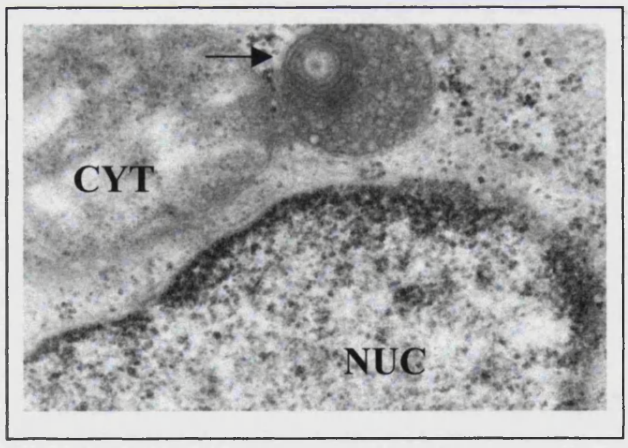


Figure 3.1 Photographs of CD34+ derived DCs. a) DCs as seen by phase contrast microscopy. b) May Grunwald Giemsa stain of a cytospin preparation of the output cells from the CD34+ cultures. DCs are marked with an arrow. The remaining cells are a mixture of other myeloid cells. c) An electron micrograph of a CD34+ DC. A characteristic multilamellar inclusion can be seen (arrow).

3.2.3 The cell surface immunophenotype of CD34+ derived DCs.

The cells generated after 14 days of culture were stained with fluorochrome-conjugated antibodies, either directly or indirectly, and analysed by flow cytometry. 12.6% of cells were CD1a+CD14- (range 6.4-24%, n=13) on day14. Representative dual parameter histograms displaying the relative number of cells which were CD1a+CD14- on day 0 and day 14 of culture are shown in Figs 3.2a & b. The percentage of cells expressing the MHC class II molecules HLA DR and DQ, the co-stimulatory molecules CD80, CD86 & CD40, and the cell surface antigens to which the antibodies CMRF44 and anti-CD83 are directed is displayed in figure 3.2c, figure 3.3 and table 3.2.

3.2.4 CD34+ derived DCs are potent stimulators in a MLR.

The population of cells generated from CD34+ haemopoiesis progenitor cells (HPCs) after 14 days of culture, which contained DCs, were tested for their ability to induce proliferation of allogeneic MNCs. As shown in figure 3.4, DC cultures induced marked proliferation of responder cells over 5 days when compared with autologous monocytes, as determined by ^{3}H-thymidine incorporation. The level of proliferation of MNCs correlated with the number of DCs per well.

3.2.5 CD34+ derived DCs are able to stimulate both naïve and memory T cells.

The ability of CD34+ derived DCs generated *ex vivo* to present primary and recall antigens to autologous CD4+ cells was tested. Fresh autologous CD4+ T cells were purified from peripheral blood by positive selection 14 days after CD34+ cells had been collected. These cells acted as responders and a fixed number were added to variable numbers of irradiated APCs, which were either DCs or autologous monocytes, the latter also having been positively selected from fresh peripheral blood. Soluble protein antigens were added to these cultures. KLH was chosen as a primary antigen and PPD was used as a recall antigen. The cultures were maintained for 7 days and the proliferative response of the CD4+ cells was assessed by ^{3}H-thymidine incorporation in the last 16-18 hrs of culture. As can be seen in figure 3.5, DCs pulsed with primary or recall antigens were able to induce a greater proliferation of autologous CD4+ cells than monocytes or DCs not exposed to exogenous antigen. This indicates that the *in vitro* generated DCs are able to stimulate both naive and memory T-cells.

3.2.6 A population of cells generated from CD34+ progenitors are able to take up FITC-dextran efficiently.

Mannose receptor-mediated uptake and fluid phase endocytosis of FITC-dextran by the DC cultures was measured by flow cytometry. A population of cells stained brightly after incubation with FITC-dextran after one hour. The intensity of staining was

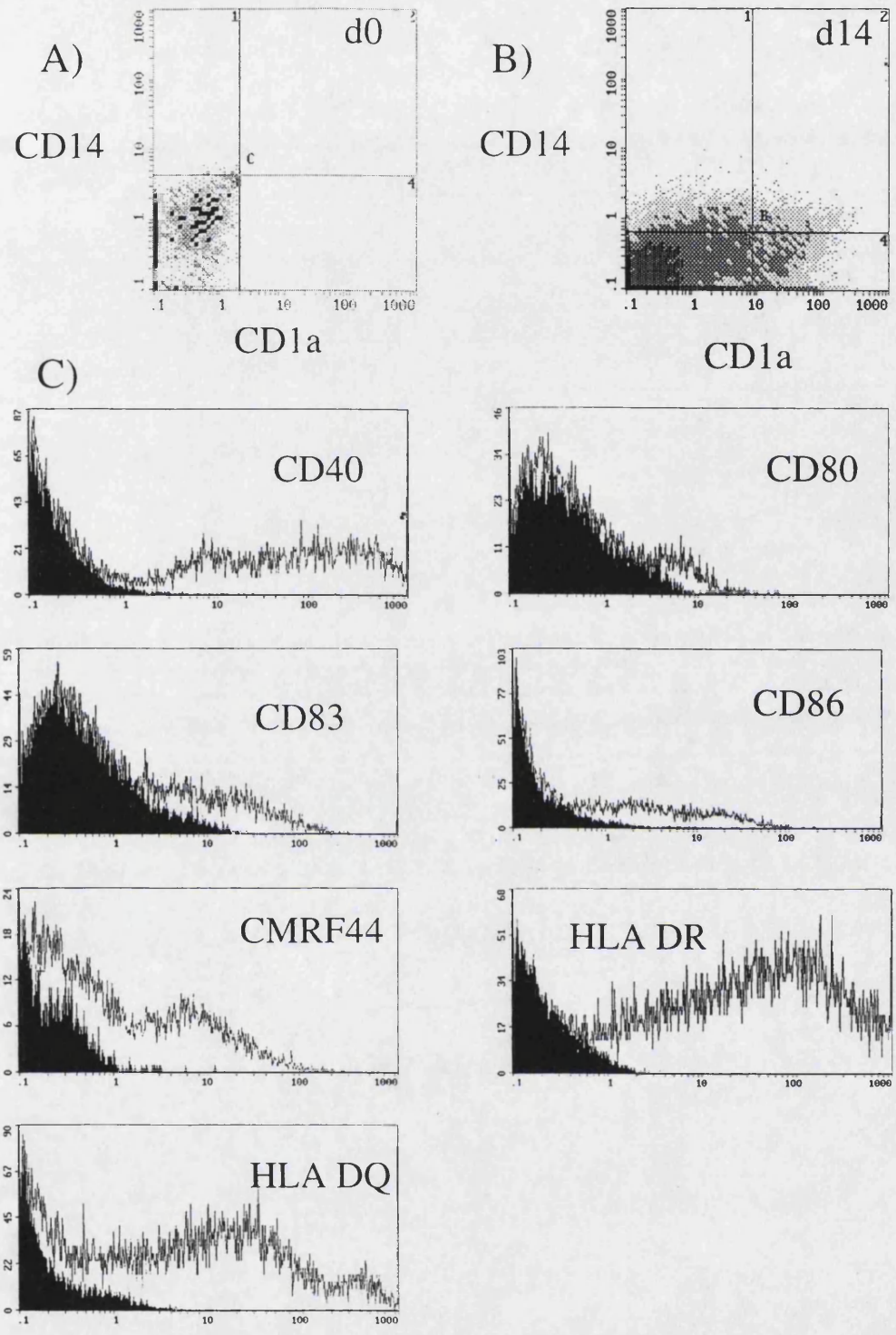


Figure 3.2. Cell surface antigen expression on day 0 and day14 CD34+ derived DCs.
Flow cytometric data from one experiment is shown. (A & B) Dual parameter histograms displaying the relative numbers of cells which were CD1a+ CD14- (A) d0, (B) d14.
(C) Single parameter histograms showing the binding of a variety of fluorochrome-conjugated monoclonal antibodies. Shaded areas represent binding by isotype matched control antibodies.

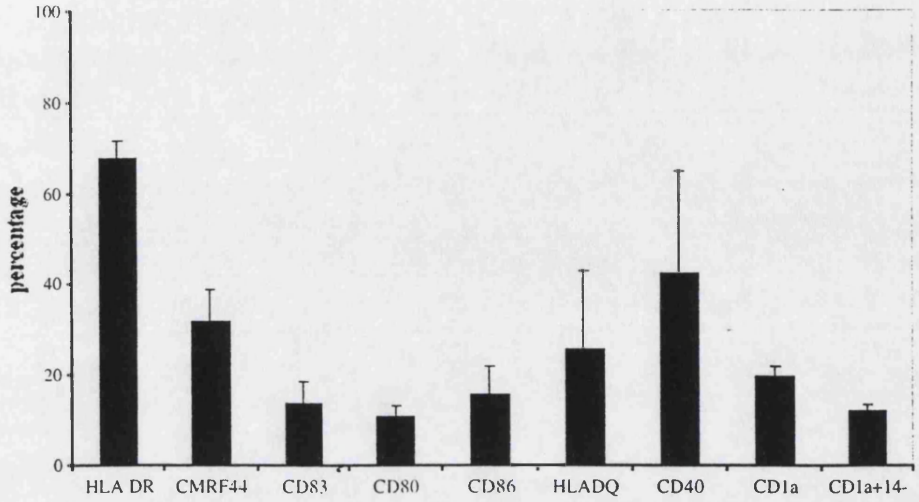


Figure 3.3 Histogram of the cell surface antigen expression on CD34+ derived DCs.
The mean percentage of output cells from the CD34+ DC cultures expressing various cell surface antigens is shown. Error bars represent the SEM. n values are the same as in the table below

CELL SURFACE ANTIGEN	MEDIAN PERCENTAGE (RANGE)	No OF EXPTS
HLA DR	70.5 (58.2-72.3)	n=4
CMRF 44	31.1 (18.9-47.2)	n=4
CD83	11.1 (9.1-21.5)	n=3
CD80	9.0 (8.6-15.8)	n=3
CD86	15.7 (6.9-24.7)	n=3
HLA DQ	19.9 (4.8-52.5)	n=3
CD40	42.4 (26.4-58.4)	n=2
CD1a	17.8 (12.2-32.9)	n=10
CD1a+14-	12.6 (6.4-24)	n=12

Table 3.2 Cell surface antigen expression on CD34+ derived DCs.
Table displaying the cell surface expression of various antigens on the d14 output cells from the CD34+ DC cultures. A median value is shown together with the range.

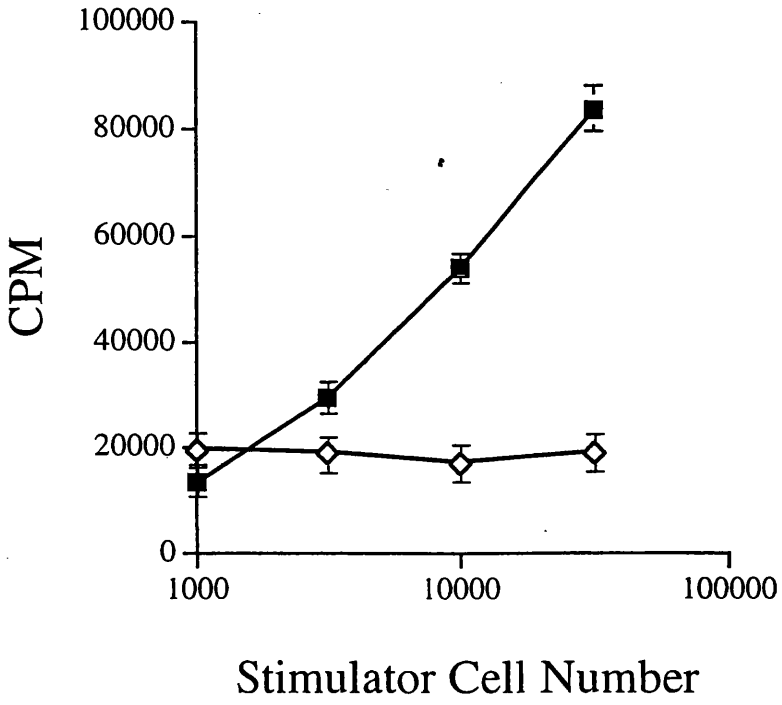


Figure 3.4 CD34+ derived DCs MLR.
DCs (■) or monocytes (◇) were used as stimulators and allogeneic MNCs were used as responders in an MLR. ^{3}H-thymidine incorporation, which quantifies DNA synthesis and thus cell proliferation, was measured. DCs can be seen to induce a greater degree of proliferation of MNCs than monocytes. This experiment is representative of 4 experiments. Each data point is the mean of 3 replicates. Error bars represent SEM.

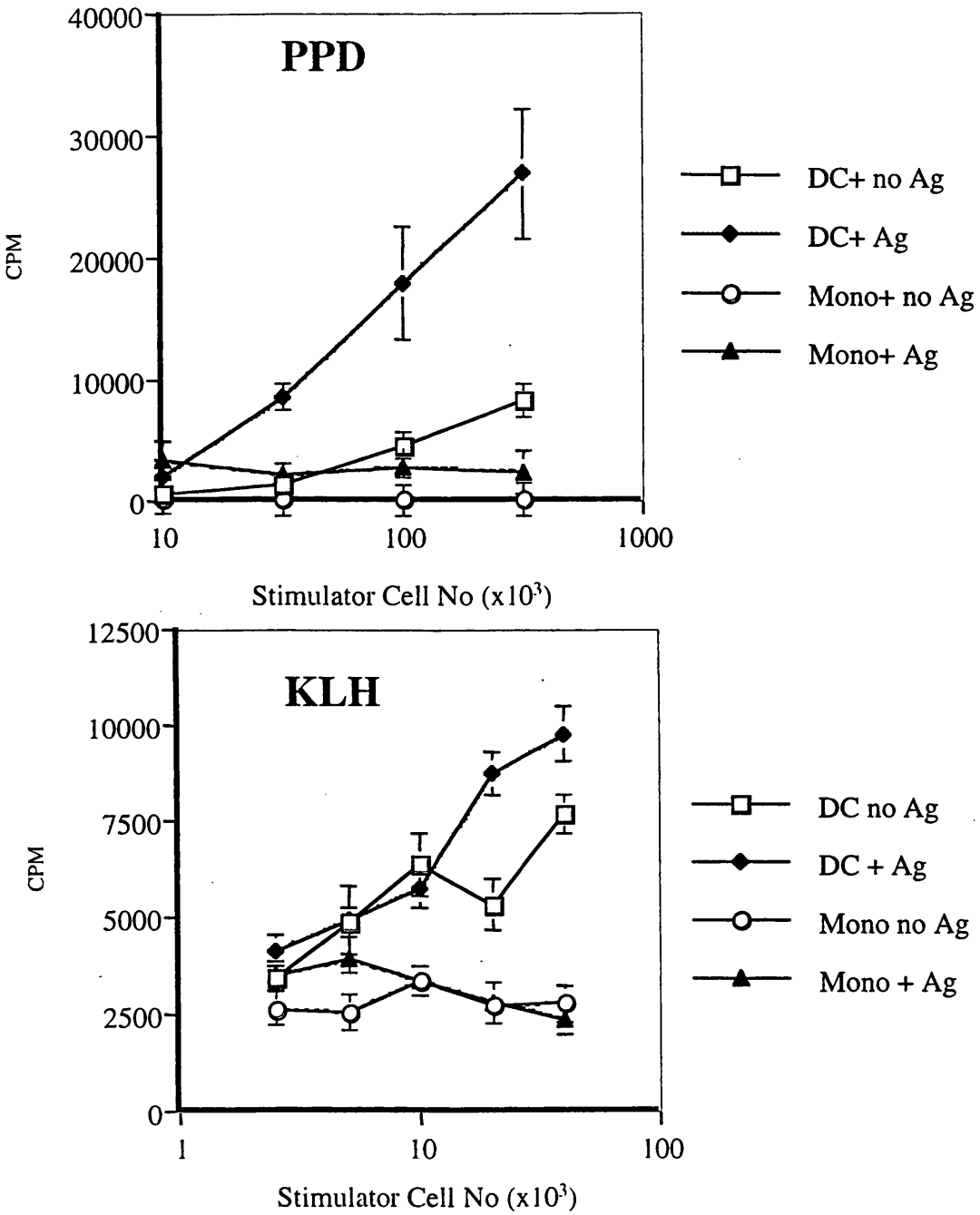


Figure 3.5 CD34+ derived DCs. CD4+ T cell proliferative response to recall and primary antigens. Graphs showing the CD4+ T cell proliferative response, as measured by ^{3}H-thymidine uptake, in response to antigens presented either by autologous CD34+ derived DCs (♦) or monocytes (▲). KLH was used as a primary antigen and PPD as a recall antigen. DCs without antigen (□) and monocytes without antigen (o) were also incubated with CD4+ cells as controls. DCs pulsed with primary or recall antigens can be seen to induce greater proliferation of autologous CD4 T cells than compared with monocytes or DCs not exposed to exogenous antigen, indicating that the *in vitro* generated DCs are able to stimulate both naive and memory T-cells. Each data point is the mean of 3 replicates. Error bars represent SEM. n=2

noticeably diminished if the cells were pre-incubated with mannan, which inhibits uptake via the mannose receptor (Fig 3.6). MNCs were used as controls.

3.2.7 Generation of DCs from peripheral blood CD14+ monocytes is accompanied by a reduction in cell number.

Peripheral blood monocytes were positively selected from the blood of normal donors on the basis of their expression of CD14+. Monocytes selected in this way were at least 95% pure by morphology. These cells were cultured in RPMI/10% FCS supplemented with GM-CSF and IL-4, for 7 days. At the end of the culture period there was a reduction in cell number with only 56.5%±6.3% of the original number of cells remaining (mean±SD; n=10). (Table 3.3).

3.2.8 The majority of cells generated from CD14+ monocytes have the appearance of DCs.

Monocytes which had been cultured for 7 days in the presence of GM-CSF and IL-4 were examined under phase contrast. The cells were non-adherent or loosely adherent, being dislodged by gentle repeated pipetting. The vast majority of cells were large and had numerous cytoplasmic projections characteristic of DCs. Cytospin preparations were made and stained using MGG stain. Again the vast majority of cells had the appearance of DCs, being large cells with a single central nucleus and ample basophilic cytoplasm with numerous fine cytoplasmic projections (Fig 3.7).

3.2.9 The cell surface immunophenotype of monocyte derived DCs

The DCs generated after 7 days of culture were stained with fluorochrome-conjugated antibodies, either directly or indirectly, and analysed by flow cytometry. Over 95% of cells expressed HLA DR and the co-stimulatory molecule CD40. The majority of cells expressed CD1a and HLA DQ. The co-stimulatory molecules CD80 and CD86, and the antigens detected by the antibodies CMRF44 and anti-CD83 which are considered to be relatively specific for DCs, were expressed at low to moderate levels. By day 7, less than 10% of cells still expressed CD14 (Fig 3.8 and Table 3.4).

3.2.10 Monocyte derived DCs are potent stimulators in a MLR.

The alloreactivity of the monocyte derived DCs were compared to that of monocytes from the same donor in a one way MLR. Allogeneic MNCs were used as responder cells. As shown in figure 3.9, DC cultures induced marked proliferation of responder cells over 5 days when compared with autologous monocytes, as determined by ^{3}H-thymidine incorporation. The level of proliferation of MNCs increased as the number of DCs per well increased.

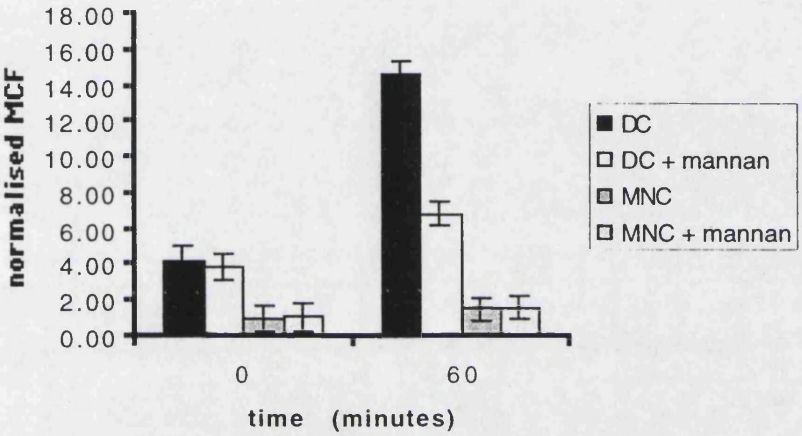


Figure 3.6 Uptake of FITC-Dextran by CD34+ DCs and MNCs.
Mean cell fluorescence (mcf) of cells at 0 and 60 minutes after incubation with FITC-dextran in the presence and absence of mannan is shown. DCs can be seen to have a greater capacity to take up FITC-dextran. This is inhibited to some extent by preincubation with mannan, Indicating that some of the uptake of FITC-dextran occurs via the mannose receptor. This data is the mean of 3 experiments. Error bars indicate the SD.

EXPT	INPUT NO OF CELLS x10^6	OUTPUT NO OF CELLS x10^6	% O F CELLS SURVIVING
1	31.0	18.0	58.1
2	15.0	9.0	60.0
3	14.0	6.0	42.9
4	25.0	16.3	65.2
5	45.6	25.0	54.8
6	25.0	14.0	56.0
7	25.0	14.0	56.0
8	28.0	17.0	60.7
9	13.5	6.8	50.4
10	18.0	11.0	61.1

Table 3.3 The generation of DCs from monocytes results in a reduction in total cell number. Table showing the number of monocytes put into culture with GM-CSF and IL-4 and the resulting number of monocyte-derived DCs generated at the end of the 7 day culture period. The remaining number of cells is expressed as a percentage in the final column.

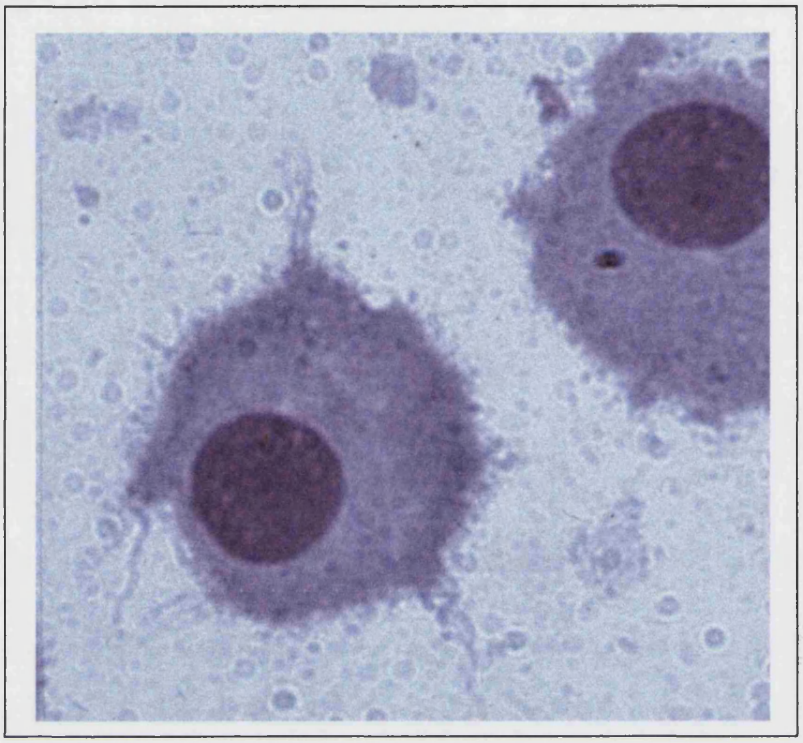


Figure 3.7 Photograph of a cytospin preparation of monocyte derived DCs stained with a MGG stain.

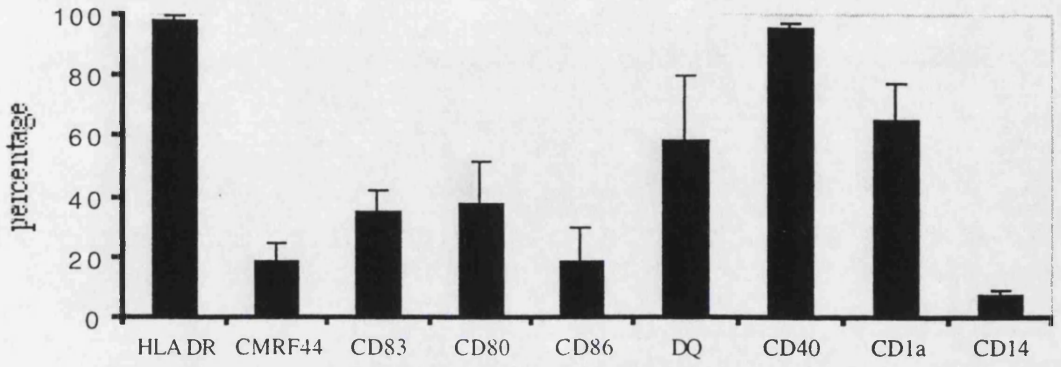


Figure 3.8 Histogram of the cell surface antigen expression on monocyte derived DCs. The mean percentage of output cells from the monocyte derived DC cultures expressing various cell surface antigens is shown. Error bars represent the SEM.
n values are the same as in the table below.

CELL SURFACE ANTIGEN	MEDIAN PERCENTAGE (RANGE)	No OF EXPTS
HLA DR	99.7 (95.4-99.9)	n=5
CMRF 44	13.4 (12.3-31.3)	n=3
CD83	30.9 (18.6-73.7)	n=8
CD80	34.7 (12.3-69.3)	n=4
CD86	15 (0.7-43.5)	n=4
HLA DQ	48.5 (27.8-99.7)	n=3
CD40	96.5 (92.2-98.5)	n=4
CD1a	66.8 (23.8-93.2)	n=6
CD14	7.2 (5.4-10.5)	n=3

Table 3.4 Cell surface antigen expression on monocyte derived DCs.
Table displaying the cell surface expression of various antigens on the d7 output cells from the monocyte derived DC cultures. A median value is shown together with the range.

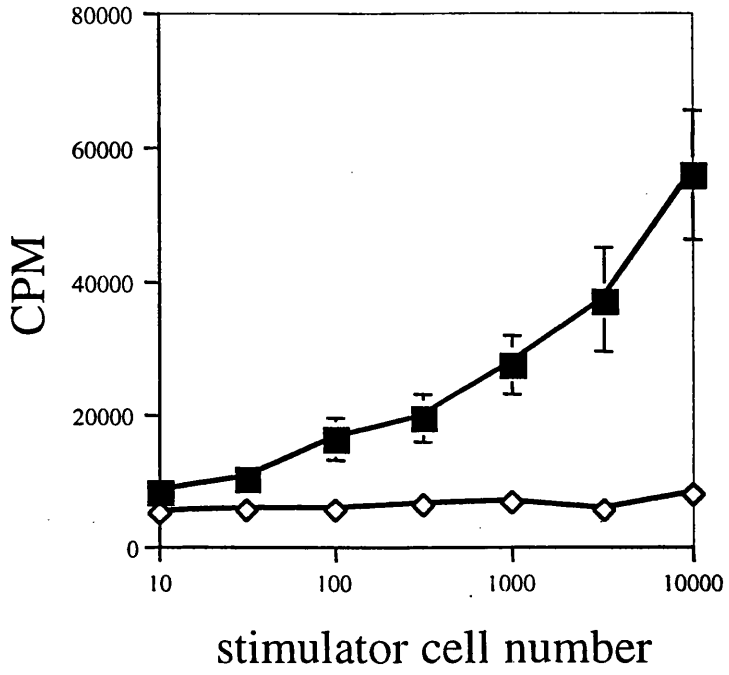


Figure 3.9 Monocyte derived DCs: MLR
Monocyte derived DC (■) or monocytes (◇) were used as stimulators and allogeneic MNCs were used as responders in this MLR. ^{3}H-thymidine incorporation, which is a measure of DNA synthesis and thus cell proliferation, was quantified. DCs can be seen to induce a greater degree of proliferation of MNCs than monocytes. This experiment is representative of 4 experiments. Each data point is the mean of 3 replicates. Error bars represent SEM.

3.2.11 Monocyte derived DCs are able to stimulate both naïve and memory T cells.

Monocyte derived DCs were able to induce the proliferation of autologous CD4+ T cells in response to both primary and recall antigens over a 7 day period. The proliferative response of the CD4+ cells was assessed by ^{3}H-thymidine incorporation in the last 16-18 hrs of culture. DCs pulsed with primary or recall antigens were able to induce greater proliferation of autologous CD4+ T cells than monocytes or DCs not exposed to exogenous antigen, thus indicating their ability to stimulate both naïve and memory T cells. (Fig 3.10)

3.2.12 Monocyte derived DCs are efficient at FITC-dextran uptake.

The whole population of cells generated after culturing monocytes with GM-CSF and IL-4 for 7 days were efficient at taking up FITC-dextran as compared with MNCs (Fig 3.11a). This uptake was prevented to a large extent by pre-incubating the cells with mannan, indicating that in part uptake is occurring by the mannose receptor (Fig 3.11b).

3.3 Discussion

DCs, as defined by appearance, cell surface immunophenotype and functional properties, have been generated here from two distinct cell populations, namely mobilised peripheral blood CD34+ progenitor cells and resting blood CD14+ monocytes. The former have been generated in a novel serum free culture system with the ultimate aim of using them in clinical immunotherapy programmes, whilst the latter have been cultured under standard conditions with the express aim of studying various aspects of DC biology. Although a direct comparison between the two types of DC has not been carried, out certain similarities and differences are noted in the discussion that follows.

3.3.1 Procurement of CD34+ progenitor cells and CD14+ monocytes.

Obtaining mobilised peripheral blood CD34+ progenitor cells in order to expand them into DCs for use in immunotherapy protocols is a lengthy process involving mobilisation, leukapheresis and selection of CD34+ cells. These procedures require specialist staff and are not without their own risks. The majority of donors of CD34+ cells used in this small scale study were patients with haematological malignancies (multiple myeloma, high grade NHL, Hodgkin's disease) and mobilisation was performed using a combination of disease-orientated chemotherapy and G-CSF with a view to stem cell collection for future autologous stem cell transplantation. It is known

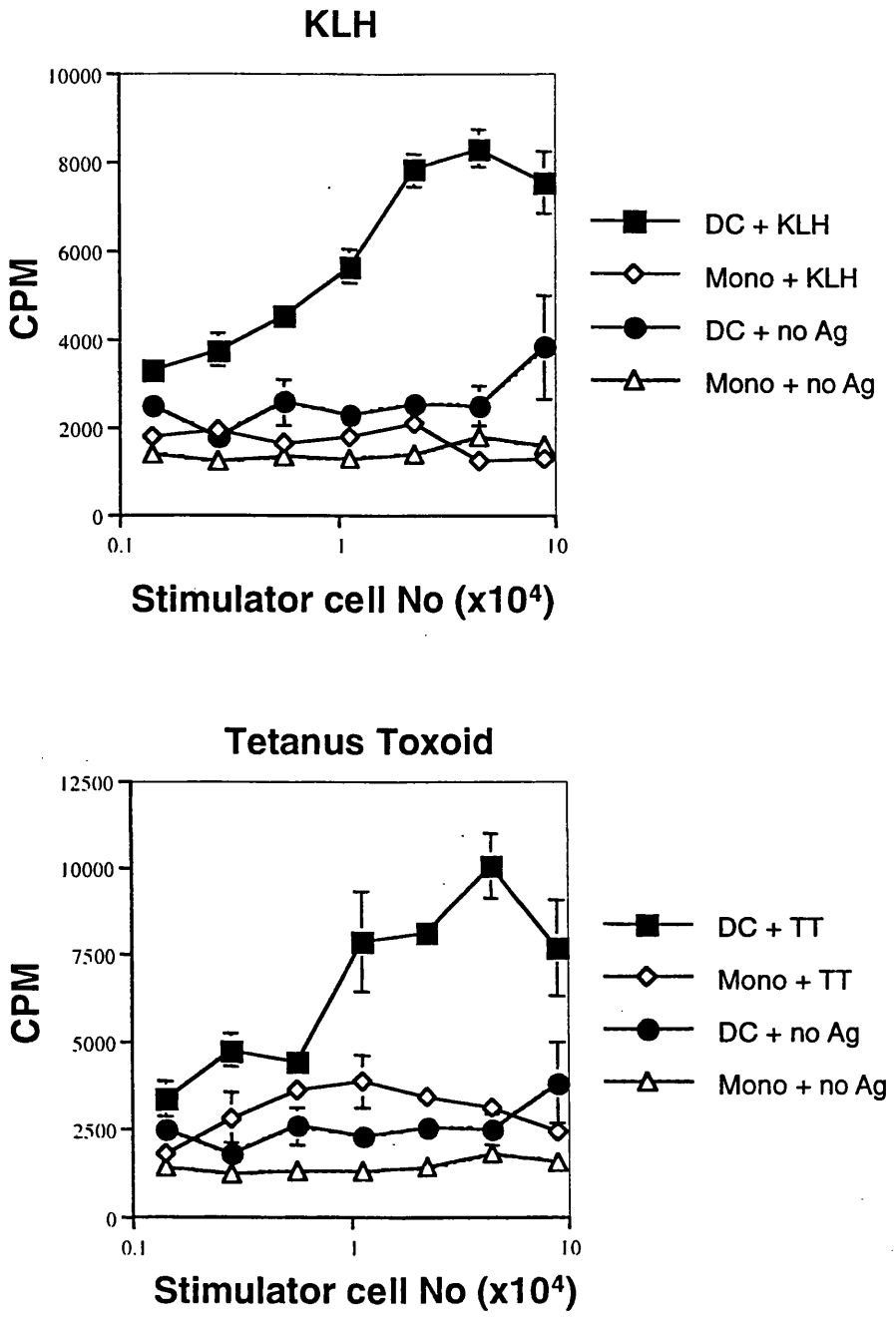


Figure 3.10 Monocyte derived DCs: CD4+ T cell proliferative responseto primary and recall antigens.
Graphs showing the CD4+ T cell proliferative response, as measured by ^{3}H-thymidine uptake, in response to primary and secondary antigens presented either by autologous monocyte derived DCs or monocytes. DCs without antigen and monocytes without antigen were also incubated with CD4+ cells as controls. Keyhole limpet haemocyanin (KLH) was used as a primary antigen and tetanus toxoid (TT) as a recall antigen. Each data point is the mean of 3 replicates. Error bars represent SEM. n=3

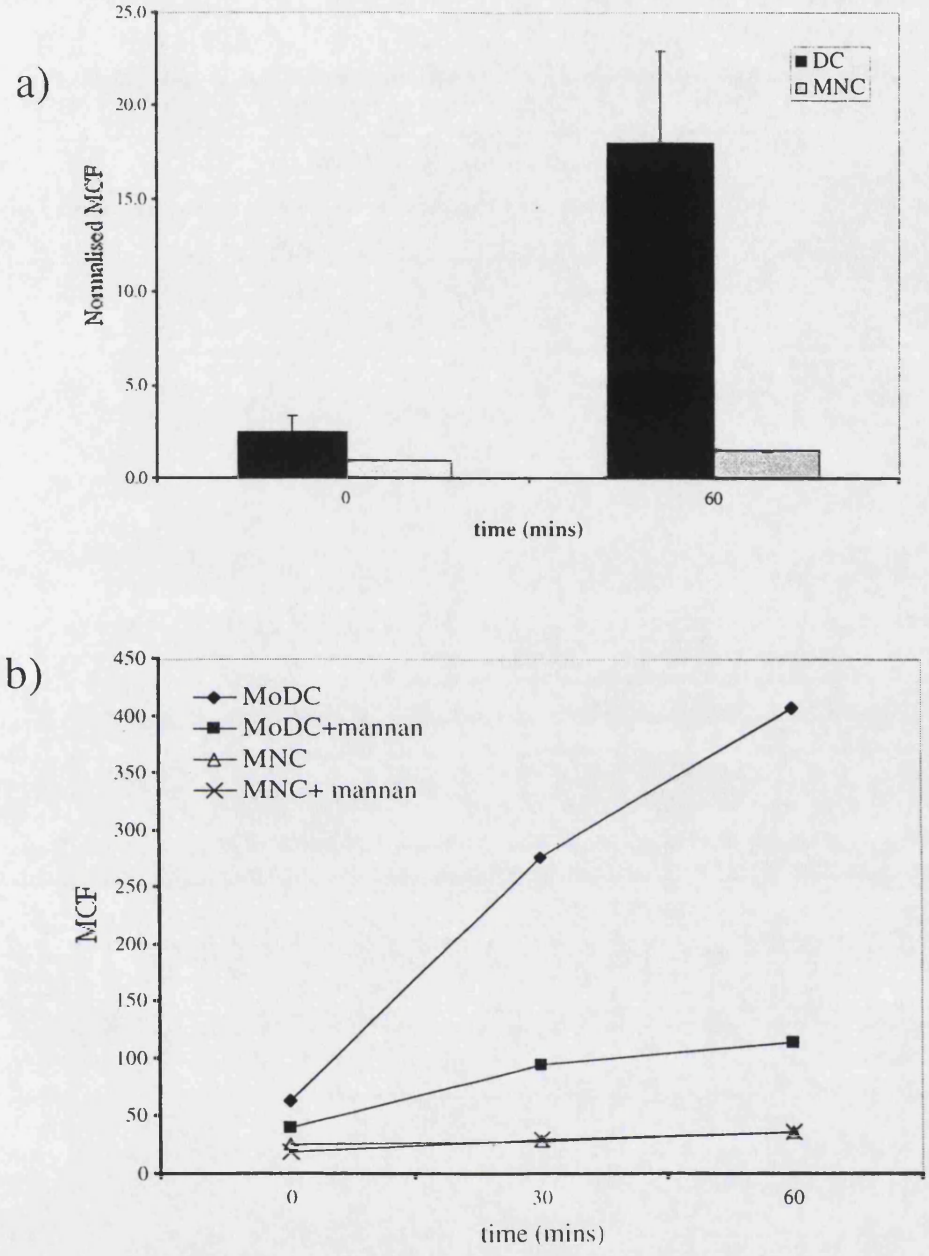


Figure 3.11 Uptake of FITC-Dextran by monocyte derived DCs and MNCs.
a) Mean cell fluorescence of cells immediately and 60 minutes after incubation with FITC-dextran is shown. DCs can be seen to have a greater capacity to take up FITC-dextran. (n=4)
b) The uptake of FITC-dextran by monocyte derived DCs is to a large extent inhibited by mannan. (n=1)

that 5-10% of patients mobilised with chemotherapy and G-CSF become neutropenic and septic, requiring hospitalisation and treatment with intravenous antibiotics (Watts *et al.*, 2000). However, this risk is thought to be outweighed by the benefit of an autologous transplant in the vast majority of cases. If CD34+ mobilisation and collection was performed with the sole aim of generating DCs, then this same risk applies. However, using DCs as part of immunotherapy protocols is likely to be used initially in the context of minimal residual disease. Thus, in many circumstances patients may have already undergone a peripheral blood stem cell transplant and an aliquot of CD34+ cells would have been cryopreserved at the time of the initial collection for use in the future. Generating DCs from these CD34+ cells need not involve any additional risk to the patient.

Leukapheresis has its own inherent untoward effects, ranging from patient discomfort, hypocalcaemia and the symptoms thereof, to the rare occurrence of circulatory collapse. However, leukapheresis, though not pleasant, is usually well tolerated and provides an opportunity to obtain large amounts of citrated autologous plasma, which is a constituent of the culture medium used here for the generation of CD34+ derived DCs. Clearly there will be a minimum time delay of 10-15 days between the decision to mobilise CD34+ progenitor cells in a patient and the point at which CD34+ cells are collected, depending on which mobilisation regimen is used (10 days for cyclophosphamide/G-CSF, 15 days for ESHAP/G-CSF).

In this research study, CD34+ cells were selected from the leukapheresis product in one of two ways: 1) if the patient was to receive a CD34+ selected transplant, a clinical scale selection was performed using the now obsolete Ceprate device and $1x10^6$ CD34+ cells were reserved for this research project. 2) If, however, the patient was to receive an unselected transplant, a small aliquot of the leukapheresis product was removed and CD34+ cells were selected on a small scale using MACS technology (Fig 2.1). For clinical immunotherapy programmes, CD34+ selection would need to be performed on a large scale using the currently available selection devices such as the CliniMacs (Miltenyi Biotec, Bergish Gladbach, Germany) or Isolex 300i (Nexell, Irvine, California) devices. Selection on this scale takes 2.5 hours and 4.5 hours respectively, and requires skilled, trained staff in addition to the specialised equipment.

In addition to generating DCs from CD34+ progenitor cells, CD14+ monocytes were also used as a starting cell population for generating DCs. In contrast to the collection of CD34+ cells, monocytes were collected from the resting peripheral blood of normal volunteers, there being no requirement for mobilisation or leukapheresis and thus the

inherent time delay which ensues is avoided. MNCs were separated from the peripheral blood and monocytes were positively selected on the basis of their expression of CD14. This was done on a small scale by immunomagnetic means, 50mls of blood yielding on average $14x10^6$ monocytes. This procedure could be completed in 2.5 hours. The equipment used would, however, need to be adapted if the resulting cells were to be used clinically and this may alter the length of the process. Monocytes can be enriched from MNCs by many other means such as simple adherence to plastic, adherence followed by metrizamide gradient centrifugation, T cell depletion by E rosetting, T+B cell ± natural killer (NK) cell depletion using immunomagnetic beads or by elutriation. However, the CD14+ selection method used here yielded a very pure population of monocytes which was advantageous as these cells were intended for the study of DC biology.

3.3.2 Culture conditions.

The mobilised peripheral blood CD34+ cells were cultured in medium which would allow the development of DCs over a 14 day period. As the ultimate aim was to use CD34+ derived DCs in clinical immunotherapy protocols, the medium needed to be compatible with clinical usage of the resultant cells. This meant avoiding the presence of foreign antigens in the culture medium in view of the possibility of rendering the recipient of these potent APCs sensitive to certain foreign antigens. In particular this meant avoiding the bovine proteins found in FCS, and antibiotics which are often used in culture medium to reduce the risk of infection. Bovine products were also avoided in view of the possibility of transmissible prion disease. The medium used consisted of X-VIVO 10 and 10% citrated autologous plasma supplemented with GM-CSF and TNF-α. IL-4 was added during the second week of culture. Unlike many other studies the cytokine cocktail used was kept to a near minimum, thus making the used of this culture system in a clinical setting more feasible.

X-VIVO 10 is a serum-free medium manufactured in a manner acceptable to regulatory agencies for use in the processing of tissue-based products which has previously been used in clinical studies (Williams *et al.*, 1996). X-VIVO 10 without phenol red or gentamicin was specifically used to prevent the theoretical problems of potential recipients of DCs becoming sensitised to these agents. However, use of X-VIVO 10 alone without any supplementation with serum or plasma was found in preliminary studies to result in poor cell expansion (S.Devereux, personal communication). It was therefore decided to supplement X-VIVO 10 with citrated autologous plasma which was collected from mobilised peripheral blood at the time of leukapheresis. Substantial volumes of plasma can be collected at this time and frozen until required.

The CD14+ monocytes were cultured for 7 days in RPMI/10%FCS supplemented with GM-CSF and IL-4. These are the standard culture conditions used to generate monocyte derived DCs (Romani *et al.*, 1994; Sallusto & Lanzavecchia, 1994) and it is noteworthy that the culture period is half that required for the generation of DCs from CD34+ cells. These monocyte derived DCs were intended to be used to study the biology of DCs and therefore no attempt was made to culture these cells in medium compatible with clinical use.

3.3.3 Changes in cell number.

Culturing CD34+ cells in the manner described is a proliferative process. By the end of the 14 day culture period there was a median 38 fold expansion in cell number, though the range was quite large (18-72, n=12). There was no significant association between the degree of cell expansion obtained and the mobilisation regimen used, underlying haematological disease, prior treatment or patient age. However, the statistical power was limited by the small sample size. This degree of expansion is comparable with results of other groups, though those who included the early acting cytokines (stem cell factor or fms-like tyrosine kinase (flt) 3 ligand) in their culture system tended to have a greater degree of proliferation (Bernhard *et al.*, 1995; Fisch *et al.*, 1996; Mackensen *et al.*, 1995; Siena *et al.*, 1995) (Table 3.5). Most other groups have used FCS in their culture medium, with the notable exception of Siena *et al.* (1995) who, as here, used autologous plasma collected at the time of mobilisation of haemopoietic progenitors into the peripheral blood following chemotherapy plus added growth factors. In earlier pilot studies the use of X-VIVO 10 with10% autologous plasma was found to be associated with less cell proliferation compared to the use of X-VIVO 10 with 10%FCS (mean fold expansion ± SEM; 7.7± 2.7 vs 15.5±7.7: n=3) but this was not significantly different using a two tailed paired t-test.

In contrast, the generation of DCs from monocytes was accompanied by a consistent reduction in cell number, with only 56.5% ± 6.3% of the original number of cells remaining after 7 days in culture (mean ± SD, n=10). This, of course, does not rule out an underlying proliferative process which is overshadowed by cell death (see chapter 4).

3.3.4 Dendritic cell morphology.

It is clear that morphologically the output cells from the CD34+ cultures were a heterogenous population of cells with only 5 to16% of cells having typical DC morphology, the remaining cells being mainly early and late granulocytes. This does not preclude their use in immunotherapy trials but limits their usefulness in studying DC biology in this unselected state. In contrast the output cells from the monocyte cultures were much more homogenous and the occasional granulocyte which is seen was

Investigator	CD34 Purity	Culture length	Cytokines used	FCS	Total cell expansion	% DC	Criteria for DC
Bernhard 1995	30-80%	12-16d	GM-CSF TNF-α	Y	x40 (20-80)	30-60%	CD1a+
Fisch 1996	>87%	40d	SCF EPO IL-1β IL-3 IL-6	Y	x190 (inferred)	<5%	CD1a+14-DR+
Mackensen 1995	55-60%	27d	SCF IL-4 IL-1β EPO IL-3 GM-CSF IL-6	Y	x37	up to 45%	CD1a+
Siena 1995	81-99%	up to 21d	GM-CSF TNF-α SCF Flt3	Y/N	x30	25% (estimate)	CD1a+

Table3.5 Table summarising the studies in which DCs have been generated from mobilised peripheral blood CD34+ cells. SCF=stem cell factor, EPO=erythropoietin, GM-CSF=granulocyte macrophage-colony stimulating factor, TNF-α=tumour necrosis factor-α, IL=Interleukin, Flt3=fms like tyrosine kinase 3

probably contamination from the input population of monocytes. The morphological appearance of the two types of DC themselves was, however, very similar.

3.3.5 Immunophenotypes.

As mentioned earlier, there is no cell surface immunophenotype unique to DCs. This situation is further confused by the fact that different subsets of DCs have a different combination of markers and that the immunophenotype of a DC changes as it matures. The CD1a+CD14- immunophenotype has often been used to define DCs and this combination of markers was used to determine the percentage of DCs generated in the CD34+ cultures. A mean of 12.6% of cells were found to be CD1a+CD14-, though the range was fairly broad (range 6.4-24%, n=13). Direct comparison of this result with other published data is confounded by the fact that often only the CD1a positivity is reported. If, however, only the CD1a status is considered wherever possible in the present study, a mean of 19.7% (range 12.2-32.9%, n=10) of the output cells could be considered to be DCs. This figure is compatible with the results of other published work (Table 3.5). In view of the fact that not all DCs will be CD1a+, the cells were analysed for the expression of other markers often found on, but not exclusive to, DCs. This flow cytometric data is difficult to interpret in isolation, not only because many of the markers are found on other cell types, but also because single antibody staining has been performed. Over two thirds of the output cells in these cultures expressed HLA DR (70.5%), though a smaller fraction expressed the other MHC class II molecule HLA DQ (19.9%). Cells bearing the relatively specific DC markers recognised by the antibodies CMRF44 and anti-CD83 were present at low to moderate levels (31.1% and 11.1% respectively), as were cells bearing the co-stimulatory molecules CD80, CD86 and CD40 (9.0%, 15.7% and 42.4%).

The interpretation of the cell surface immunophenotype of monocyte derived DCs is easier due to the homogeneous cell population on day 7 of culture. Nearly all of the cells expressed HLA DR (99.7%) and CD40 (96.5%) and almost half expressed HLA DQ (48.5%). It is of note that only 66.8% of these cells expressed CD1a. There was only moderate expression of CD83 (30.9%), the antigen detected by the CMRF44 antibody (13.4%) and the co-stimulatory molecules CD80 & CD86 (34.7% and 15% respectively), as might be expected in these immature DCs. The broad range of expression of some of these markers e.g. CD86, may reflect biological variability between individuals or it may reflect that in some of the cultures some degree of maturation had occurred inadvertently. In general, a higher percentage of monocyte derived DCs expressed the aforementioned markers when compared with CD34+ derived DCs. This, to a large extent, is due to the difference in purity of the two

populations of cells, and statements about the quality of the two types of DCs cannot be made from the data shown here, though comparisons have been made by others. In one study no significant difference in expression of cell surface markers was noted (Ferlazzo *et al.*, 1999), whereas in the other study monocyte derived DCs were shown to have on average a slightly higher expression of co-stimulatory molecules (Meierhoff *et al.*, 1998).

3.3.6 Functional studies.

Ultimately, as there is no cell surface marker unique to DCs, and because the surface phenotype of the cells varies as the cell matures, the definition of a DC must be based on its functional properties. DCs generated from either CD34+ progenitor cells or CD14+ monocytes as described above have potent allostimulatory capacity when compared with monocytes, as demonstrated in the one way MLRs. In addition, these cells were able to present both primary and recall antigens to autologous CD4+ T cells and induce their proliferation. This indicated the ability of both types of DCs to stimulate naïve as well as memory T cells, which is the hallmark of a DC.

In these experiments there was a significant degree of proliferation in cultures of irradiated DCs and CD4+ cells without added antigen. In the case of the monocyte derived DCs, this could be attributed to the presentation of foreign bovine proteins present in the culture medium by the DCs. However, this could not be the cause in the case of the CD34+ derived DCs where no bovine proteins were present. This autoreactivity has been noted by others (Bernhard *et al.*, 1995; Mehta *et al.*, 1995) and described as an autologous MLR (Bernhard *et al.*, 1995).

It was not possible to directly compare the allostimulatory capacity or antigen presenting capabilities of the two types of DC as, firstly, the culture medium used to generate each type of DC was different and secondly, in no case were both type of dendritic cell generated from the same donor. This has, however,been investigated by other groups. Meierhoff *et al* found that CD34+ derived DCs were more potent stimulators in an allogenic MLR than monocyte derived DCs, Troizzi and Aldrich found the opposite and Ferlazzo *et al* concluded that the two DC types were equivalent in an allogeneic MLR (Ferlazzo *et al.*, 1999; Meierhoff *et al.*, 1998; Triozzi & Aldrich, 1997).

Antigen uptake by both types of DCs was demonstrated by measuring FITC-dextran uptake. In the case of the CD34+ derived cells this was limited to a small proportion of the output cells, as might be expected. FITC-dextan uptake is known to occur by a

combination of macropinocytosis and mannose receptor-mediated uptake (Sallusto *et al.*, 1995). In keeping with this, uptake was prevented to a large degree by pre-incubating the cells with mannan, indicating that, in part, uptake is occurring by the mannose receptor.

3.3.7 Conclusions.

Generating DCs from mobilised peripheral blood CD34+ progenitors requires that the cells are mobilised and that the patient is leukapheresed. This is a lengthy, labour intensive and therefore expensive process which is not without its own risks. The time to culture CD34+ derived DCs is also protracted, and although there was a considerable expansion in cell number, only a minority of these cells were DCs. In contrast, monocytes can easily be obtained from resting blood without any need for mobilisation or leukapheresis. The culture time is shorter and although there was almost a 50% reduction in cell number, the vast majority of these cells were DCs. These features of monocyte derived DCs promoted their use in experiments studying aspects of DC biology presented in this thesis. However, it was unclear whether the differentiation of DCs from monocytes was to any degree a proliferative process or not, whereas, it was without doubt that the generation of DCs from CD34+ progenitors was a proliferative process. As one long term aim was to be able to retrovirally transduce DCs with genes encoding tumour antigens for use clinically, and, as retroviral transduction requires a cell to be undergoing cell division, CD34+ derived DCs were the favoured cell type to use in clinical trials. This necessitated the development of a clinically suitable culture system. Such a culture system has been described here for the generation of DCs from CD34+ cells. These cells have been proven to have the appearance, immunophenotype and functional characteristics of DCs. Clearly, the true merits of each type of DC will only become apparent after their efficacy in a clinical situation has been tested.

Chapter 4

Cell Cycle Kinetics and Retroviral Transduction of CD34+ and Monocyte Derived DCs

4.0 Introduction

When devising immunotherapy protocols it is important to consider the method by which DCs will be loaded with the relevant antigens. Retroviral transduction of DCs with the gene encoding the protein of interest is a method which has several theoretical advantages. A prerequisite for retroviral tranduction is that the target cell is actively dividing. While it was clear that proliferation accompanies the generation of DCs from CD34+ cells, it was less clear whether proliferation accompanies the differentiation of monocytes into DCs under the influence of GM-CSF and IL-4 (Chapuis *et al.*, 1997; Romani *et al.*, 1994; Sallusto & Lanzavecchia, 1994). This ambiguity heavily biased the decision to use CD34+ derived DCs for clinical studies, despite the fact that they are more difficult to generate and are a more heterogenous population of cells. In 1997 and 1998, reports of highly efficient retroviral transduction of monocyte derived DCs (Aicher *et al.*, 1997; Russo *et al.*, 1997; Westermann *et al.*, 1998) prompted the experiments presented in this chapter. These were designed to determine whether proliferation accompanies the differentiation of monocytes into DCs, and whether they are indeed amenable to retroviral transduction.

4.1 Methods

4.1.1 Cell selection.

Monocytes were purified from MNCs from normal donors either by positive or negative selection techniques. In the experiments to determine whether monocytes proliferate as they differentiate, the MNCs were enriched for monocytes employing one of two negative selection techniques. Initial experiments used aminoethylisothiouronium bromide hydrobromide (AET)(Sigma Chemical Co, St Louis, MO, USA) treated sheep erythrocytes to deplete the MNCs of T cells (Chanarin, 1989). Subsequently immunomagnetic depletion was used. MNCs were incubated with anti-CD3 and anti-CD19 mouse anti-human monoclonal antibodies (0.25μg/1x10^6 cells and 1μg/1x10^6 cells respectively) (Sigma Chemical Co) for 30 min at 4°C, followed by incubation with 50μl goat anti-mouse microbeads (Miltenyi Biotec, Germany) for a further 30 min, with a washing step using 20ml PBS after each incubation. The T and B cells were removed

immunomagnetically using a VS+ column (Miltenyi Biotec) as per manufacturers instructions. Purity was assessed by Giemsa staining of cytospin preparations and were greater than 70% monocytes (n=3). Negative selection techniques were used to ensure analysis of the entire non-lymphoid mononuclear compartment and to avoid exclusion of a subpopulation of proliferating cells.
The monocytes used for retroviral transduction experiments were positively selected from MNCs using a CD14 antibody (Dako A/S, Denmark) as described in chapter 2 section 2.0.7.
Non-dividing T cells constituted the vast majority of cells remaining after monocytes have been positively selected from peripheral blood MNCs. These cells were used in the spiking experiments together with T cells which were stimulated to proliferate. In order to obtain proliferating T cells, non-dividing T cells were exposed to phytohaemagglutinin (1μg/ml) for 3 days, thoroughly washed and then stimulated with IL-2 (20ng/ml) for 24 hours.

4.1.2 Tritiated Thymidine Uptake.

Monocytes, irradiated monocytes (25Gy γ-particles) and U937 cells were each cultured in triplicate in round bottomed 96 well microtitre plates (Nunc, Denmark). The starting cell concentration of monocytes and irradiated monocytes was $5x10^4$/ml and 200μl was aliquoted into each well ($1x10^4$/well). These cells were cultured in RPMI/10%FCS supplemented with GM-CSF and IL-4 (100ng/ml). U937 cells (the monocytic cell line) were used as controls in these experiments and seeded at a five fold lower concentration in medium without growth factors. Seven plates were prepared in this way per experiment. At daily intervals 1μCi of ^{3}H-thymidine (Amersham Pharmacia Biotech, England) was added to each well of one plate and left for 6 hours. After this time cells were harvested using an Automash (Dynatech Laboratories Ltd., Billinghurst., Sussex.). The glass fibre filters were dried and placed in scintillation fluid and counted using a scintillation counter.

4.1.3 Cell cycle analysis.

At daily intervals, $5x10^5$ cells were removed from culture, pelleted, resuspended and fixed in 70% (v/v) ethanol at -20°C. At the time of analysis the samples were again pelleted and the ethanol removed. The cells were resuspended in PBS containing 20μg/ml propidium iodide (Sigma Chemical Co) together with 50μg/ml ribonuclease A (Sigma Chemical Co) and incubated for 20 min at 37°C. Propidium iodide is a planar molecule which is non-fluorescent in its native state, however, when it intercalates with DNA it forms a dimer which is strongly fluorescent, emitting light at the red end of the

spectrum. The amount of fluorescence is proportional to the amount of DNA in a cell. RNAse is added to destroy single stranded RNA which can interfere with the analysis by taking up a secondary structure that also interacts with propidium iodide. The DNA content of the cells was then analysed by flow cytometry (EPICS Elite, Coulter Electronics). A Forward Angle Light Scatter (FALS) pulse height/integral comparison and a FALS red fluorescence/integral comparison was utilised to eliminate cell doublets from the analysis as far as possible (Shapiro, 1995).

4.1.4 Western Blotting.

0.5-1x10^6 cells were centrifuged at 400g for 5 min, resuspended in PBS containing 1mM diisopropyl fluorophosphate (DIFP) on ice for 15 min, then pelleted again at 400g for 5 min. The supernatant was completely removed and the cells were boiled for 7 min in 2 x sodium dodecyl sulphate (SDS) sample buffer containing protease and phosphatase inhibitors (sodium fluoride (NaF), sodium orthovanadate ($NaVO_4.14H_2O$), phenylmethylsulfonyl fluoride, DIFP, aprotinin, pepstatin A, leupeptin) (40µl/1 x 10^6 cells) as decribed by Thomas (Thomas, 1989). Proteins were separated by electrophoresis at 150V through 6% polyacrylamide/SDS 10cm minigels (Hoeffer Mighty Small II, San Fransisco, USA), according to the method of Laemmli (Laemmli, 1970). The proteins were blotted onto Immobilon P membrane (Millipore Corp, Bedford MA), blocked with 10% (w/v) nonfat-dried milk (Marvel)/ 0.1% (v/v) Tween 20 in PBS and incubated with 500ng/ml anti-pRb (PMG 3-245, Pharmingen) or anti-p130 (C-20) (Santa Cruz Biotech, Inc.) antibodies for an hour. The membranes were then washed thoroughly with 0.1% (v/v) Tween 20 in PBS before incubation for 1 hour with a goat anti-mouse antibody to which horseradish peroxidase (HRP) was conjugated. The membranes were washed thoroughly once more. Detection was by ECL Plus (Amersham Pharmacia Biotech).

4.1.5 Retroviral transduction of monocyte derived DCs and CD34+ derived DCs.

The PG13 packaging cell line containing a retrovirus encoding the low affinity nerve growth factor receptor (LNGFR) gene (kindly provided by Dr A Thrasher, London) was grown to confluency in a T175 flask in Dulbeccos Modified Eagles Medium (DMEM), supplemented with 10% (v/v) FCS together with penicillin and streptomycin 50IU/ml (Gibco BRL). The medium was then removed and replaced with a further 10ml of the same medium and the cells were cultured for a further 6 hours at 32°C or 37°C. The supernatant was then harvested and filtered through a 0.45µm filter (Millipore, Bedford MA). The viral titre was 1.1 x 10^5 infectious vector particles per ml of supernatant as determined by titration on HeLa cells.

Positively selected monocytes were cultured with GM-CSF and IL-4 for 7 days as described above. After 24 hours (day1) $1x10^6$ cells were removed, pelleted and resuspended in 100µl of medium and placed in a well of a 6 well plate (Costar). To this 1.5ml of viral supernatant was added together with protamine sulphate (5µg/ml) for 6 hours. The protamine sulphate is positively charged and helps to overcome the negative charges expressed by the viral particles and cell surface thereby bringing the virus and cell together and hence aiding retroviral binding. After 6 hours the potentially toxic protamine sulphate was diluted out by the addition of a further 7.5 ml of medium containing growth factors. The following day the cells were pelleted and exposed to fresh viral supernatant. On day 5, half the medium was removed and replaced with fresh medium containing growth factors. As a positive control Jurkat cells (a T cell line) were transduced, as above, but only on day 1. In view of their rapid growth the Jurkat cells were split twice during the 7 days of culture. Monocytes which had been treated in the same way as above, except that the viral supernatant was substituted by culture medium alone, were used as a negative control (mock-transduced monocytes). A similar protocol was used to transduce CD34+ derived DCs. The CD34+ cells were cultured in X-VIVO 10 (Gibco BRL) supplemented with 10% (v/v) autologous plasma together with TNF-α and GM-CSF. They were transduced on days 3 ± 4 ± 6. On day 7 IL-4 was added to the cultures. The cells were counted daily and kept between $2x10^5$/ml and $1x10^6$/ml. The growth factors were replenished on days 5 and 10, and the cells were collected for analysis on day 14. As a positive control HeLa cells (cervical carcinoma cell line) were transduced as above but only on day 3. In view of their rapid growth, these cells were split as necessary during the 14 days of culture. Mock transduced CD34+ cells were used as a negative control.

The level of transduction (as assessed by LNGFR expression) was measured by flow cytometry on day 14. $2.5x10^5$ cells were pelleted and resuspended in 100µl of 50:50 PBS and human AB serum. These cells were stained for 60 min at 4°C using a FITC-conjugated murine anti-human CD1a antibody (isotype IgG2a) and an unconjugated murine anti-human LNGFR antibody (isotype IgG1). Appropriate isotype controls were used at the same immunoglobulin concentration as these first layer antibodies. Cells were washed twice in ice cold PBS and then incubated for a further 60 mins at 4°C with a second layer goat anti-mouse IgG1 antibody which was conjugated to phycoerythrin (Serotec). After a further wash the cells were then analysed using a Coulter EPICS Elite flow cytometer.

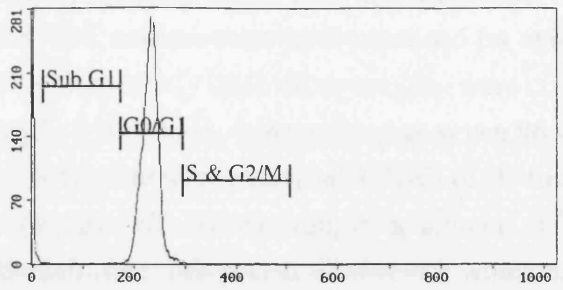
4.1.6 Staining of cytospin preparations of monocyte derived DCs with Ki-67 antibody.

Cytospin preparations of monocyte derived DCs were fixed in acetone at room temperature for 10 minutes. The slides were allowed to air dry and then a 1 in 10 dilution of normal goat serum (Dako) was applied to the slide for 10 minutes to prevent non-specific Fc receptor binding by the test antibodies.This was then removed and a 1 in 50 dilution of the murine antibody against Ki-67 derived from the MIB-1 clone (Immunotech, France) was applied to the slide for 60 minutes. This was washed off with tris buffered saline (TBS) pH 7.4, after which a 1 in 100 dilution of a biotinylated goat anti-mouse antibody (Dako) was applied for 35 minutes. Following a TBS wash the slide was incubated with an avidin-biotin complex to which horseradish peroxidase (HRP) was conjugated (Dako) for 35 minutes. This was washed off with TBS before diaminobenzidine hydrochloride (DAB)(500μg/ml) (Kem-en-Tec, Denmark) and hydrogen peroxide (H_2O_2)(final conc 0.03%) was added. The HRP releases oxygen radicals from the H_2O_2 which in turn react with the DAB to form a brown compound. The slides were then counterstained with haematoxylin.

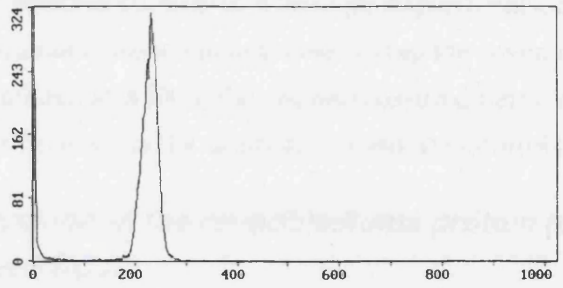
4.2 Results - Cell cycle kinetics

4.2.1 Flow cytometric analysis of cell cycle status of monocyte derived DCs.

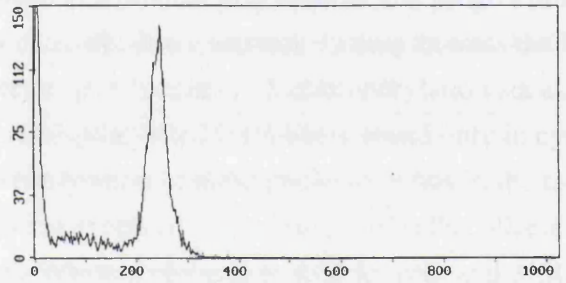
Peripheral blood MNCs which had been obtained from normal volunteers and enriched for monocytes by negative selection techniques (purity >70%) were set up in culture in RPMI/10%FCS supplemented with GM-CSF and IL-4. At almost daily intervals a sample of cells was removed then fixed and permeablised with 70% v/v ethanol at –20°C. The DNA content of these cells was later analysed by flow cytometry using propidium iodide. Less than 2% of the cells were determined to be in S, G_2 and M phases of the cell cycle on each day of culture. It was also noted that from early on in the culture period, a proportion of cells had a sub-diploid DNA content. This is indicative of apoptosis, which occurred throughout the period of culture but was most marked on days 4 and 5 and indeed at the end of the culture (day 7) 12.3% (range 8.1-17.1, n=3) of cells were undergoing apoptosis. Fig 4.1 displays the cell cycle profiles of cells taken from the monocyte derived DCs cultures on days 1, 3 & 6. In comparison the cell cycle profile of continuously proliferating U937 cells is shown.



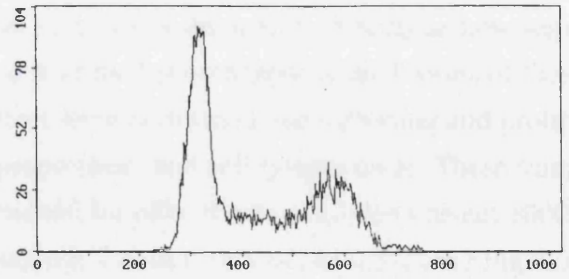
Day 1 MoDCs



Day 3 MoDCs



Day 6 MoDCs



Day 4 U937

Figure 4.1 Flow cytometric analysis of DNA content of MoDCs. Representative cell cycle profiles of monocytes cultured in GM-CSF and IL4 over a 7 day period. U937 cells were used as a positive control. The gates set to determine the proportion of cells in G_0/G_1, S & G_2/M phases of the cell cycle, as well as those with a sub-G_0/G_1 DNA content, are shown in the first panel. Y axis =cell number, X axis = DNA content.

4.2.2 ^{3}H-thymidine uptake by monocyte derived DCs

The monocyte derived DC cultures were next examined for evidence of DNA synthesis on each day of culture. Negatively selected monocytes were cultured in RPMI/10% FCS supplemented with GM-CSF and IL-4 in triplicate in seven 96 well plates. At daily intervals the wells of one plate were pulsed with 1μCi of ^{3}H-thymidine and left for 6 hours before harvesting the cells and measuring the amount of ^{3}H-thymidine incorporated into the cells over this period. Monocytes which had been irradiated (25Gy) prior to culture were used as a negative control and proliferating U937 cells were included as a positive control. In 3 separate experiments, no significant ^{3}H-thymidine incorporation occurred at any time during the seven days of culture as monocytes differentiated into DCs, the counts measured being equivalent to the background counts detected in the irradiated monocyte controls (Fig 4.2).

4.2.3 Phosphorylation of the retinoblastoma protein (pRb) and p130 in monocyte derived DCs.

pRb and p130 are members of the pocket protein family of cell cycle regulators. The phosphorylation status of p130 and pRb regulate the progression of cells through the cell cycle and only cells which are actively cycling express the hyperphosphorylated forms of these proteins. p130 occurs in 3 phosphorylation states (forms 1, 2 and 3). Form 3 is the hyperphosphorylated form being found only in cycling cells. In order to determine the phosphorylation of these pocket proteins in the monocyte derived DC cultures, protein lysates prepared from samples of cells collected throughout the culture period were run on a Western blot and probed for pRb and p130. pRb was found to be present only in its hypophosphorylated form, and p130 was present in forms 1 and 2 (Fig 4.3a). The hyperphosphorylated forms of pRb and p130 were not detected, even after long exposures of the blots. In order to determine how sensitive this method is at detecting the presence of the hyperphosphorylated forms of these proteins, parallel "spiking" experiments were performed. Non-dividing and proliferating T cells were mixed in varying proportions and cell lysates made. These samples were run on a Western blot and probed for pRb. It was possible to detect 1000-5000 cycling T cells amongst $2x10^{5}$ quiescent T cells (ie a level of 0.5-2.5%)(Fig 4.3b). These results provided further evidence that no more than 2.5% of cells in the monocyte derived cultures were in cycle at any time during the culture period.

4.2.4 Ki-67 staining of monocyte derived DCs

One cytospin preparation of monocyte derived DCs prepared on day 3 was stained with the Ki-67 antibody (derived from the MIB-1 clone) which detects an antigen found in cycling cells. No cells out of 500 cells examined were found to stain positively with this antibody.

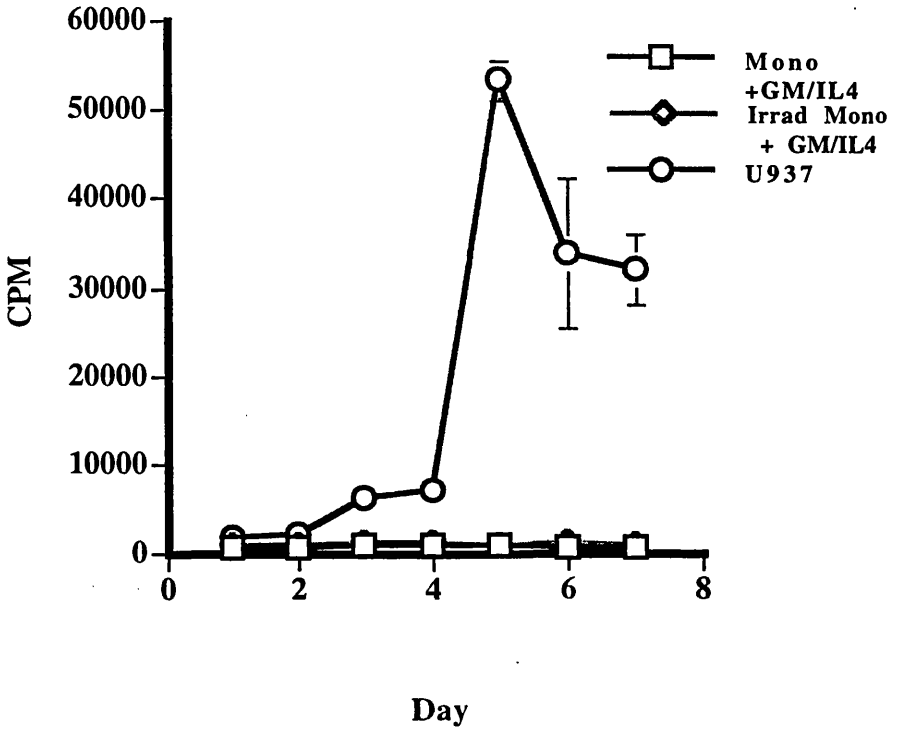


Figure 4.2 Measurement of DNA synthesis in cultures of monocyte derived DCs by ^{3}H-thymidine incorporation.
Monocytes were cultured in GM-CSF and IL-4 over a 7 day period. Irradiated monocytes and U937 cells were used as controls.

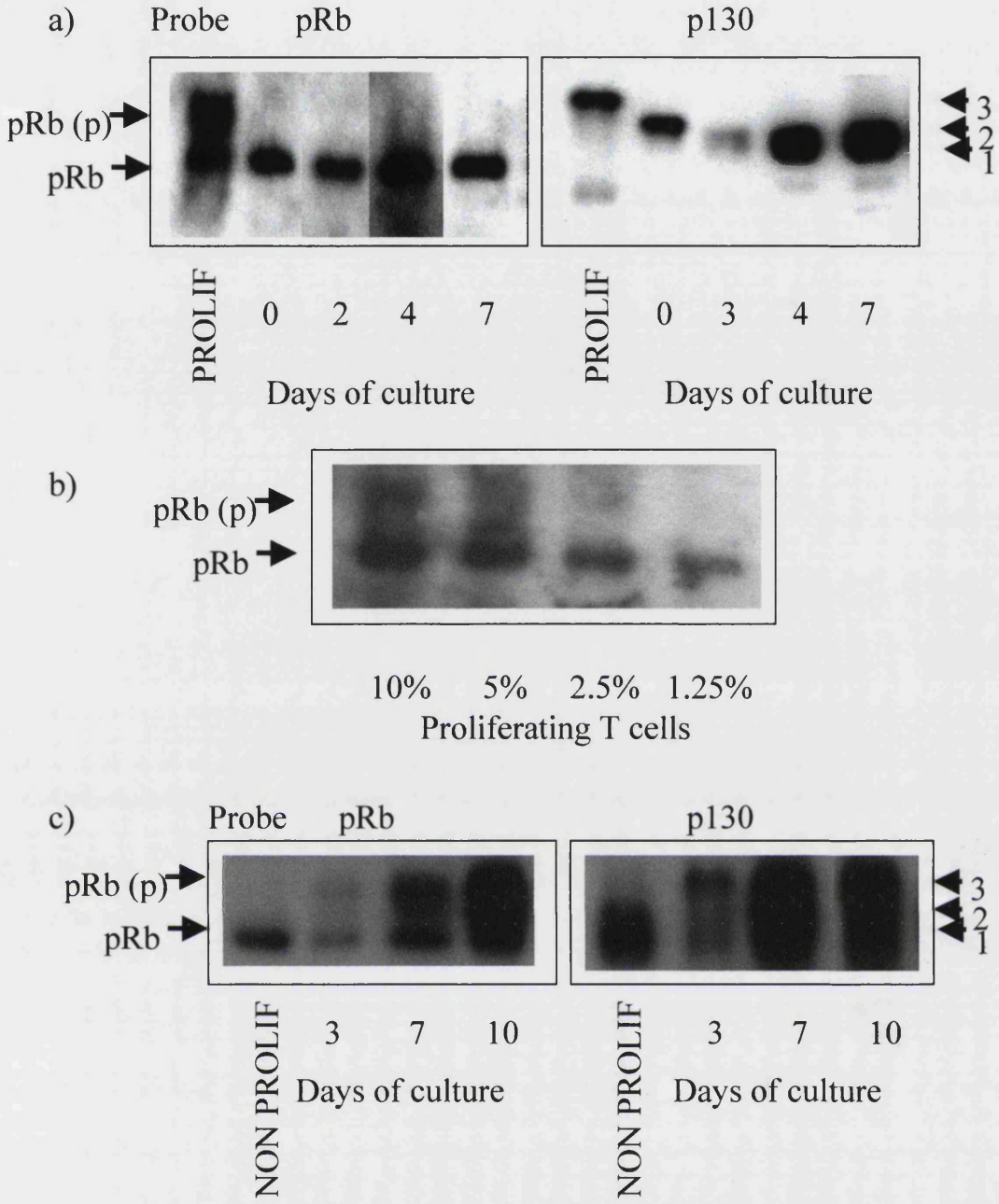


Figure 4.3 pRb and p130 Western Blotting of cell lysates from cultures of monocyte and CD34+ derived DCs.
a) Cell lysates from proliferating Daudi cells (Prolif), monocyte derived DCs removed from culture on the days indicated. Rb remains in its hypophosphorylated form (pRb) throughout the culture period. p130 remains in forms 1 & 2. b) Cell lysates made from mixtures of proliferating and non-proliferating T cells. The percentages of proliferating cells in the samples are given. c) Cell lysates from non-proliferating T cells (non prolif) and CD34+ derived DCs removed from culture on the days indicated. Rb can seen to be present in its phosphorylated (pRb(p)) form throughout the CD34+ DC culture period. p130 can be seen to be in forms 1, 2 & 3.

4.2.5 Flow cytometric analysis of cell cycle status of CD34+ derived DCs

CD34+ cells which were cultured in X-VIVO10/10% citrated autologous plasma supplemented with TNF-α and GM-CSF from the beginning of culture and IL-4 from day 7 of the 14 day culture period, were analysed with respect to their DNA content. By day 3 of culture, greater than 15% of cells were in S and G_2/M phases as analysed by flow cytometry, and this persisted throughout the remaining culture period (n=2). Apoptosis was prominent early in culture but occurred at a lower level throughout the remainder of the 14 day period. Figure 4.4 shows data from a representative experiment.

4.2.6 Phosphorylation of the retinoblastoma protein (pRb) and p130 in CD34+ derived DCs.

Cells were taken from cultures of CD34+ derived DCs and lysates made. These were then run on Western blots and probed for pRb and p130. These blots revealed pRb to be present in its hypo- and hyperphosphorylated forms. p130 was present in form 3 in addition to forms 1 and 2. These findings are compatible with the presence of proliferating cells in the CD34+ cultures (Fig 4.3c).

4.3 Results - Retroviral transduction

4.3.1 Monocyte derived DCs are not susceptible to retroviral transduction.

Monocytes which had been positively selected were cultured in medium containing GM-CSF and IL-4 for 7 days. On days 1 and 2 the cells were retrovirally transduced using a PG13 pseudotyped retroviral vector encoding the LNGFR. As a positive control, proliferating Jurkat cells were also transduced but only on day 1. On day 7, the cells were harvested and the proportion of cells expressing the LNGFR was measured by flow cytometry using an antibody directed against this antigen. Although between 12% and 32% (n=3) of Jurkat cells expressed the LNGFR, none of the monocyte derived DCs expressed this antigen (n=3). Fig 4.5 shows a representative result.

4.3.2 CD34+ derived DCs are susceptible to retroviral transduction.

DCs were generated from CD34+ progenitor cells by culturing them for 14 days in medium containing GM-CSF and TNF-α with the addition of IL-4 on day 7. Using the same retroviral vector as above expressing the LNGFR, these cells were transduced either once, twice or three times on days 3 ± 4 ± 6. As a positive control, HeLa cells were transduced with the same vector but only on day 3. On day 14, the percentage of cells expressing the LNGFR was analysed by flow cytometry.

In general, the percentage of cells transduced increased with increasing rounds of transduction, although this was not always the case. The percentage of cells which were transduced varied with a median of 7.7% (range 3-19.3%, n=8) being transduced after

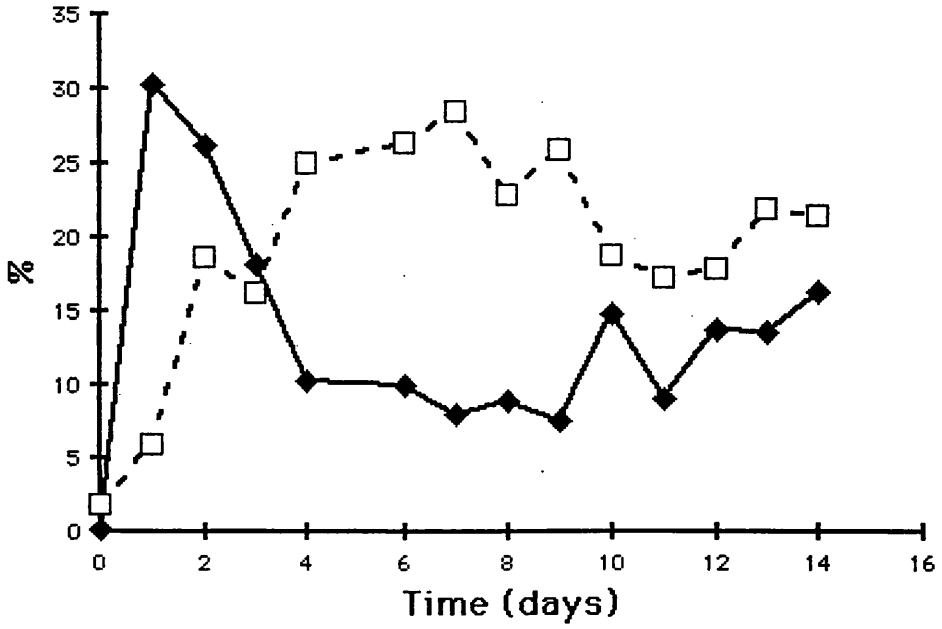


Figure 4.4 Cell cycle analysis of CD34+ cells during differentiation into DCs
Graph showing the proportion of cells in S and G_2/M cell cycle phase (□) and sub-G_0/G_1 (♦) on each day of culture.

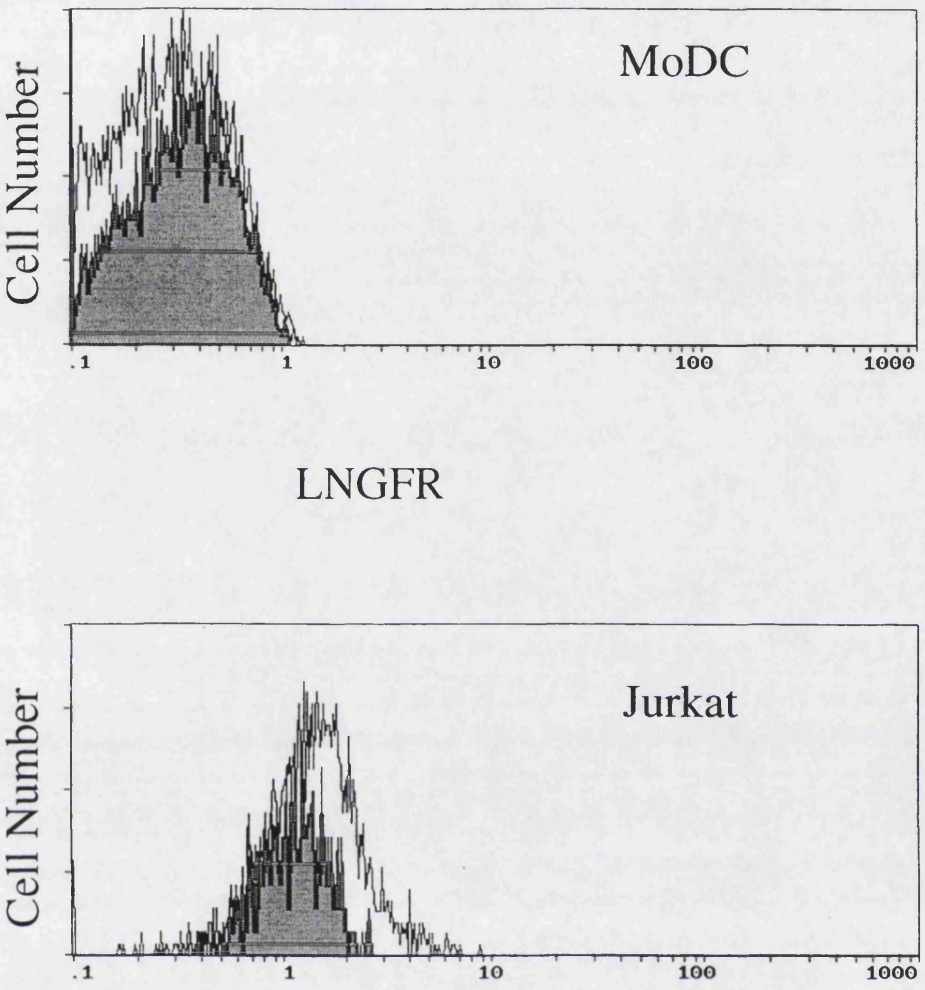


Figure 4.5 Monocyte derived dendritic cells are not susceptible to retroviral transduction.

Fluorescence histograms demonstrating the proportion of MoDCs and Jurkat cells expressing LNGFR after retroviral transduction with an LNGFR containing vector (open areas). Shaded areas represent binding by isotype matched controls.

one round of transduction, 12.5% (range 3.4-26.2%, n=8) after 2 rounds and 13.2% (range 4-28.3%; n=7) after 3 rounds.
In six of these experiments the percentage of cells expressing CD1a was also measured. A median of 0.8% of the output cells from the various transductions were CD1a+ (range 0-9.8%, n=17). The untransduced control cells had a median CD1a expression of 2.8% (range 0.2-6.9%, n=6). Between 0 and 30.8% of CD1a+ cells were found to be transduced with the LNGFR, although these comprised only between 0 and 1.4% of the output cells from these cultures. (Tables 4.1 & 4.2 and Fig 4.6).

4.4 Discussion

DCs retrovirally transduced with genes encoding tumour-associated antigens may have advantages over DCs loaded with antigens by other methods. A prerequisite to retroviral transduction is that the target cells need to undergo cell division to allow integration into genomic DNA. The findings in chapter 3 showed that generating DCs from monocytes is easier than from CD34+ progenitors and that the cells produced are more homogeneous. However, despite this, and because it is known that the generation of DCs from CD34+ progenitors is a proliferative process, the decision was made to use CD34+ derived DC as the source of DCs in clinical studies. This process would ultimately lend itself to retroviral transduction, which could potentially be an important component of future clinical studies.

It was less clear whether the *in vitro* GM-CSF/IL-4-induced differentiation of DCs from monocytes involved proliferation. Initial reports suggested that monocyte derived DCs were derived from proliferating precursors as they appeared to develop as enlarging aggregates of cells. The process was shown to be radiosensitive and 10% of cells stained positively with the Ki-67 monoclonal antibody that identifies an antigen present in cycling cells (Romani *et al.*, 1994). Others also noted an increase in cell number in these cultures (Sallusto & Lanzavecchia, 1994). However, subsequent studies showed that this process was associated with a decrease in cell number and was radioresistant. In addition, there appeared to be a lack of uptake of ^{3}H-thymidine in the first two days of culture, although the later 5 days of culture were not investigated in this way (Chapuis *et al.*, 1997). Somewhat surprisingly, in 1997 and 1998 several groups reported the susceptibility of these monocyte derived DCs to transduction with retroviral vectors (Aicher *et al.*, 1997; Russo *et al.*, 1997; Westermann *et al.*, 1998). These groups reported transduction rates of up to 95% (Russo *et al.*, 1997). The essential features of peer reviewed published experiments are displayed in table 4.3.

	CD34+ selection Device	% HeLa Cells Trans-duced	No of transduc-tions	LNGFR+ cells (%)	CD1a+ cells (%)	CD1a+ cells which are LNGFR+ (%)	% of total output cells which are CD1a+ & LNGFR+
1	Clini-Macs	48.4	0	-	4.9	-	-
			1	6.9	0.1	30.8	0.03
			2	3.6	0	0	0.00
			3	4	0	0	0.00
2	Vario-Macs	48.4	0	-	0.2	-	-
			1	4.9	0	0	0.00
			2	6.3	1.4	4.2	0.06
			3	13.2	2.7	15.1	0.41
3	Vario-Macs	17.5	0	-	6.9	-	-
			1	3	6.4	1.4	0.09
			2	3.4	9.5	0	0.00
			3	4.4	9.8	3.9	0.38
4	Clini-Macs	49.8	0	-	1	-	-
			1	11.5	0	0	0.00
			2	17.2	0.8	14.2	0.11
			3	18.7	0	0	0.00
5	Clini-Macs	47.0	0	-	0.4	-	-
			1	15.3	0.5	0	0.00
			2	19.2	0.7	0	0.00
6	Clini-Macs	38.5	0	-	4.6	-	-
			1	7	3.8	23.7	0.90
			2	9.7	4.5	24.4	1.10
			3	10.4	7.0	20	1.40
7	Clini-Macs	36.7	0	-	-	-	-
			1	8.4	nd	-	-
			2	15.3	nd	-	-
			3	28.3	nd	-	-
8	Clini-Macs	22.0	0	-	-	-	-
			1	19.3	nd	-	-
			2	26.2	nd	-	-
			3	25.7	nd	-	-

Table 4.1 Retroviral transduction of CD34+ DCs (i)
Data from 8 experiments in which the retroviral transduction of CD34+ DCs was attempted using a PG13 pseudotyped retroviral vector encoding the low affinity nerve growth factor receptor (LNGFR).

Median Percentage CD1a expression in output cells. (Categorised by number of rounds of transduction.)			Median percentage of output cells transduced (Categorised by number of rounds of transduction.)			Median Percentage of output cells which were both transduced and CD1a positive
0+1+2+3	1+2+3	0	1	2	3	
1% (0-9.8) n=23	0.8% (0-9.8) n=17	2.8% (0.4-6.9) n=6	7.7% (3-19.3) n=8	12.5% (3.4-26.2) n=8	13.2% (4-28.3) n=7	0.02% (0-1.4) n=18

Table 4.2 Retroviral transduction of CD34+ DCs (ii)

Data from 8 individual experiments where the retroviral transduction of CD34+ DCs was attempted using a PG13 pseudotyped retroviral vector encoding low affinity nerve growth factor receptor(LNGFR). The median is followed by the range in brackets.

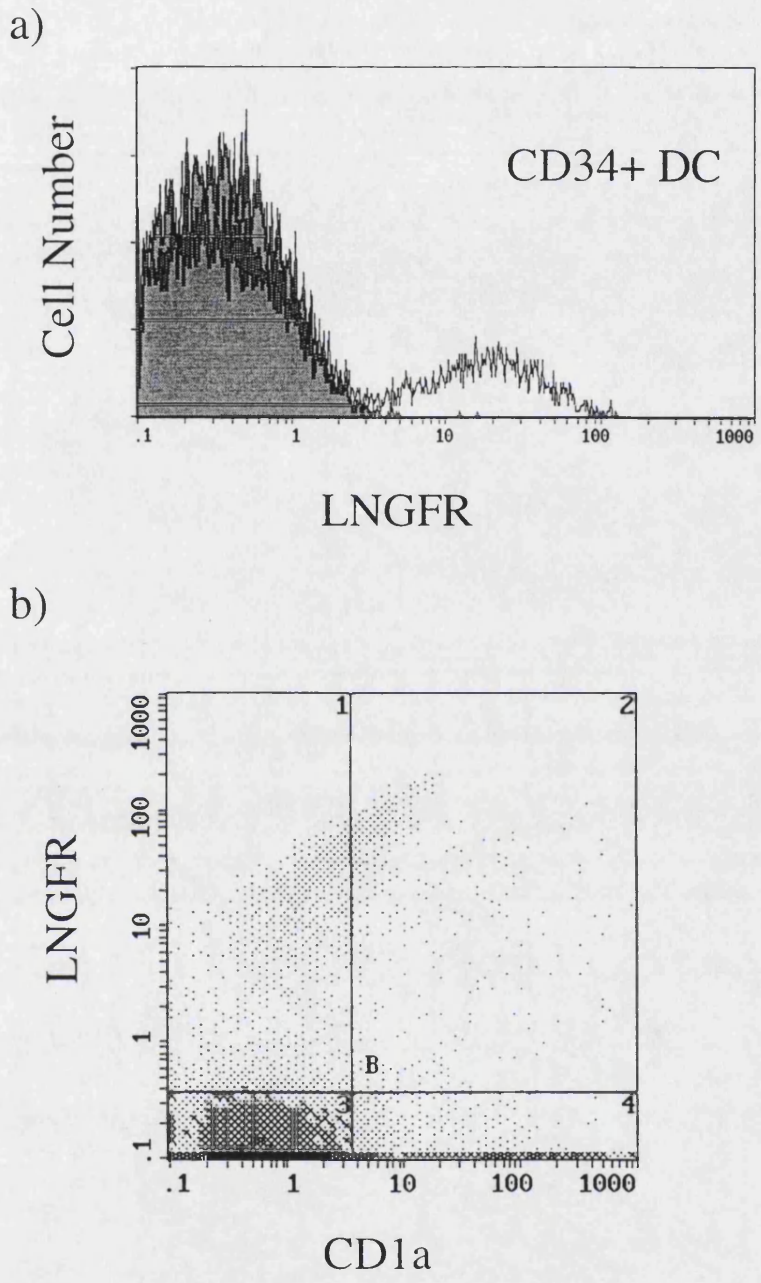


Figure 4.6 Retroviral transduction of DCs derived from CD34+ cells a) Single parameter histogram displaying the expression of the LNGFR on CD34+ derived DCs after transduction. Shaded areas represent nonspecific binding as determined using an irrelevant isotype control antibody. b) Dual parameter histograms displaying expression of the LNGFR and CD1a on the total output of cells derived from the CD34+ cultures.

Clearly, if retroviral transduction of monocyte derived DCs was a viable option this would have a significant impact on the choice of the type of DC used in clinical studies. In view of this, experiments were undertaken to investigate more fully whether monocytes undergo proliferation at any point over the seven day period of culture during which they differentiate into DCs.

Results presented in the previous chapter showed that there was a consistent reduction in cell number as monocytes differentiated into DCs under the influence of GM-CSF and IL-4. However, this finding does not exclude the possibility that proliferation may be occurring in a small subpopulation of cells whilst the majority undergo cell death. Therefore experiments were designed to detect proliferating cells during the differentiation of monocytes into DCs. In these experiments, negative selection techniques were used to enrich for monocytes prior to culture, to ensure analysis of the entire non-lymphoid mononuclear compartment, and to avoid exclusion of a subpopulation of proliferating cells. Initial experiments were performed analysing the DNA content of the cells on each day of the culture period by flow cytometry. These demonstrated that greater than 98% of all cells were in G_0/G_1. The remaining 2% or less of cells were scored in the G_2/M compartment. The lack of any cells in S-phase strongly suggests that these presumptive G_2/M cells are artefactual, as cells may not enter G_2/M without passing through S-phase, and they are probably composed of cell doublets and coincident events. The significant amount of apoptosis, which was also noted to occur during the culture period, was consistent with the reduction in cell number previously noted. Measurement of DNA synthesis on each day of culture by ^{3}H-thymidine uptake further confirmed the lack of proliferation. It is of note, however, that others have detected proliferation in these ^{3}H-thymidine assays if greater than $1x10^5$ cells are pulsed with ^{3}H-thymidine. This was found to be due to contaminating CD34+ progenitor cells (Cavanagh *et al.*, 1998). In the experiments described here, $1 x10^4$ cells were pulsed with ^{3}H-thymidine, rendering any contaminating CD34+ cells too insignificant in number to contribute to the incorporation of ^{3}H-thymidine.

Further evidence for the lack of proliferation was provided by the phosphorylation status of the cell cycle regulatory proteins pRb and p130. Throughout the 7 day period, pRb was present only in its hypophosphorylated form and p130 was only present in forms 1 and 2. In these forms, the proteins bind E2F transcription factors and thus repress E2F-responsive genes which encode proteins required for the progression through S phase (Thomas, 1999). In addition, it was shown that as few as 1000-5000 cycling T cells could be detected amongst $2x10^5$ quiescent T cells when probing a Western blot for Rb using the same conditions as those for analysing the monocyte

Investigators	Method of monocyte enrichment of PBMNCs	Retroviral vector	Titre	Days transduced	Cells transduced on day analysed (%)	Evidence for DC transduction	Additional evidence cited for successful retroviral transduction
Aicher et al 1997	Immuno-magnetic depletion of T and B cells	MFG-nlslacZ	1×10^5 cfu/ml on NIH 3T3 cells	days 1 + 2 + 4	d7: mean 54.2% n=10 d20: mean 38% n=2	42-60% of CD1a positive cells transduced	PCR of genomic DNA at d7 and d20 = positive for LacZ
Westermann et al 1998	Immuno-magnetic depletion of T and B cells	LXSN-hIL7	2×10^6 cfu/ml on NIH 3T3 cells	days 1 or 2 + 3 + 5	Day not specified 28±5%	RT-PCR for mRNA expression of neomycin phosphotransferase gene in CD1a + cells	IL7 production by cells on d7. RT-PCR mRNA expression of IL7 on d7

Table 4.3 Retroviral transduction of MoDCs: published data
RT-PCR = reverse transcriptase - polymerase chain reaction

derived DCs. This implies the monocyte derived DC cultures contain no more than 0.5-2.5% proliferating cells at any time, though, in fact, far fewer proliferating cells may be present, as evidenced by the striking lack of staining with the Ki 67 antibody on cytospin preparations of cultured monocyte derived DCs.

The results obtained in these studies conclusively demonstrate that monocyte derived DC generation can occur without cellular proliferation. In accordance with these findings, monocyte derived DCs were found to be resistant to transduction using a MoMLV based retroviral vector. This is in contrast to the findings of others (Aicher *et al.*, 1997; Russo *et al.*, 1997; Westermann *et al.*, 1998). It may be that, although proliferation was not observed in these studies, and is clearly not a prerequisite for differentiation, it may occur under some culture conditions, thus explaining the ability of other groups to retrovirally transduce these cells. Alternatively, artefacts such as passive uptake by target cells of reporter proteins present in retroviral supernatants, or PCR amplification of DNA derived from producer cells, could explain some of the reported instances of monocyte derived DC transduction. It is of note that definitive evidence of retroviral integration by Southern blotting was not demonstrated in any of the reports of high efficiency monocyte derived DC transduction.

The generation of DCs from CD34+ progenitors is known to be a proliferative process. The increase in cell number in the cultures was observed in the present experiments and has been noted previously (Caux *et al.*, 1992). This was further confirmed when the DNA content and phosphorylation status of pRb and p130 in these cells were analysed.

Retroviral transduction of CD34+ derived DCs on days 3 ± 4 ± 6 of culture did indeed result in a proportion of the output cells being transduced. However, in this series of experiments only a very small proportion of cells were CD1a+ (0-9.8%) which was much less than would be expected on the basis of previous experiments. The percentage of the output cells which were transduced CD1a+ cells was therefore even less (0-1.4%).

CD1a is not a universal marker of immature DCs, being only expressed by a proportion of immature DCs. It is downregulated upon DC maturation. The low expression of CD1a in these cultures may have been due to the maturation of DCs *in vitro*, especially as the double-stranded RNA found in the retroviral vectors is known to be a stimulus for DC maturation (Cella *et al.*, 1999). An alternative explanation is that the transduction process itself may have altered the phenotype of the cells produced in this culture system, or even resulted in selective death of the very cells which were the desired

target of the transduction procedure. However, other groups have reported retroviral transduction of human CD34+ cells which are being driven to differentiate into DCs under the influence of a cocktail of cytokines, have normal growth characteristics, express a normal phenotype and retain their potent T cell stimulatory capacity, making the latter explanation less likely (Szabolcs *et al.*, 1997). In both of these scenarios, the CD1a expression in the mock transduced cells should have been significantly higher, which was only the case in 1 out of 6 experiments. In this context it is noteworthy that in 4 out of 6 experiments the CD34+ cells were selected using the CliniMacs device. This was not the case in the experiments described in the previous chapter in which the median number of cells expressing CD1a was 17.8% (12.2-32.9%). This raises the possibility that a different population of CD34+ cells are being selected by the CliniMacs. Despite these findings, there may have been a significant transduction of cells which were functionally DCs but CD1a negative. Broader immunophenotyping to characterise the cells more fully would clarify this issue. Further investigation into these findings is clearly warranted.

In conclusion, these results show that monocytes do not proliferate as they differentiate into monocyte derived DCs under the influence of GM-CSF and IL-4. The demonstration that monocyte derived DCs are not susceptible to retroviral transduction is consistent with this finding and alternative methods of gene delivery will be required for these non-dividing cells. Evidence has also been presented which further confirms that the generation of CD34+ cells is a proliferative process. In keeping with this, significant retroviral transduction of the output cells from these cultures was also demonstrated, though further investigation is warranted to explain the low expression of CD1a in these cultures.

Chapter 5

Signal Transduction Pathways Involved in the Lipopolysaccharide-Induced Maturation and Survival of Monocyte Derived Dendritic Cells.

5.0 Introduction

In their immature state, DCs, take up antigens from their environment with high efficiency. Upon encounter with foreign antigen, DCs undergo a complex maturation process and become specialised in antigen presentation. This is achieved by upregulation of cell surface MHC class I and II molecules and the co-stimulatory molecules CD80, CD86 and CD40. Concomitantly, the DC downregulates its antigen capture mechanisms (Banchereau & Steinman, 1998). Many stimuli are known to induce DC maturation including bacterial LPS. This maturation process has a pivotal role in DC function enabling a single cell to sequentially perform different, highly specialised functions.

Understanding the molecular events involved in DC maturation may lead to the ability to manipulate the immune response with greater precision. This would naturally be of value when devising immunotherapy protocols and in the context of inducing tolerance post-transplant. However, to date, little is known concerning the signal transduction pathways involved in this complex process.

Signal transduction via mitogen-activated protein (MAP) kinases plays an important role in cellular responses including growth factor-induced cell proliferation, differentiation and survival. Three groups of MAP kinases have been identified in mammals: the extracellular signal-regulated protein kinases (ERKs) (Boulton *et al.*, 1991; Boulton *et al.*, 1990), the c-Jun N-terminal kinases (JNKs) (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994) and the p38 stress activated protein kinases (p38SAPKs) (Han *et al.*, 1994; Lee *et al.*, 1994). The ERK pathway appears mainly to respond to mitogens and growth factors, which regulate cell proliferation and differentiation. The JNK and p38SAPK pathways are predominantly activated by stress, such as osmotic changes and heat shock, but also by inflammatory cytokines such as IL-1β and TNF-α.

In addition to MAP kinases, other signal transduction pathways may mediate cellular responses to external stimuli. These include the phosphoinositide-3-OH kinase (PI3

kinase) pathway in which Akt kinase is a downstream target which is known to be important in cell survival, (Downward, 1998) and the activation of the transcription factor NF-κB, which is stimulated by pro-inflammatory cytokines and growth factors (May & Ghosh, 1998). It is becoming increasingly clear that there is cooperation between different signaling pathways and, with the development of specific inhibitors, it has become possible to dissect out further the roles of each component in important cellular processes (Fig 5.1).

In this chapter the signal transduction pathways important in the survival and LPS-induced maturation of monocyte derived DCs are investigated.

5.1 Materials and Methods

5.1.1 Inhibitors.

SB 203580, the p38SAPK inhibitor, (Calbiochem-Novabiochem UK Ltd, Nottingham, UK) was used at a final concentration of 40μM. PD 98059, the MAP kinase kinase 1 (MKK1/MEK) inhibitor, (Biomol Research Labs Inc., Plymouth Meeting, PA, USA) was used at a final concentration of 50μM. LY294002, the PI3 kinase inhibitor, (Biomol Research Labs Inc.) was used at a final concentration of 25μM. SN50 peptide and a control peptide (Calbiochem-Novabiochem UK Ltd) were used at a final concentration of 50μg/ml. SB203580, PD 98059 and LY294002 were all dissolved in dimethyl sulphoxide (DMSO), whereas SN50 peptide was made up in aqueous solution.

5.1.2 Cell selection.

Monocytes were selected from the MNCs of normal donors using the murine anti human CD14 antibody (Dako A/S, Denmark) as described in the main materials and methods chapter.

5.1.3 Cell culture and flow cytometric analysis of cell surface antigens.

Monocyte derived DCs were generated as previously described. On day 7 of culture, the resulting immature DCs were split as appropriate and LPS (100ng/ml)(Sigma Chemical Co, St Louis) and/or inhibitors added and the cultures continued for 2 more days. The resulting cells were analysed afterwards by flow cytometry.
In preliminary studies it was shown that DMSO (at the same concentration as diluent for SB203580 i.e 1μl/ml) did not inhibit the LPS-induced changes in expression of the any of the cell surface antigens under scrutiny (ratio of $LPS_{(\%\ positive\ x\ MCF)}$:$LPS/DMSO_{(\%\ positive\ x\ MCF)}$=1.00:1.05 (n=7)).

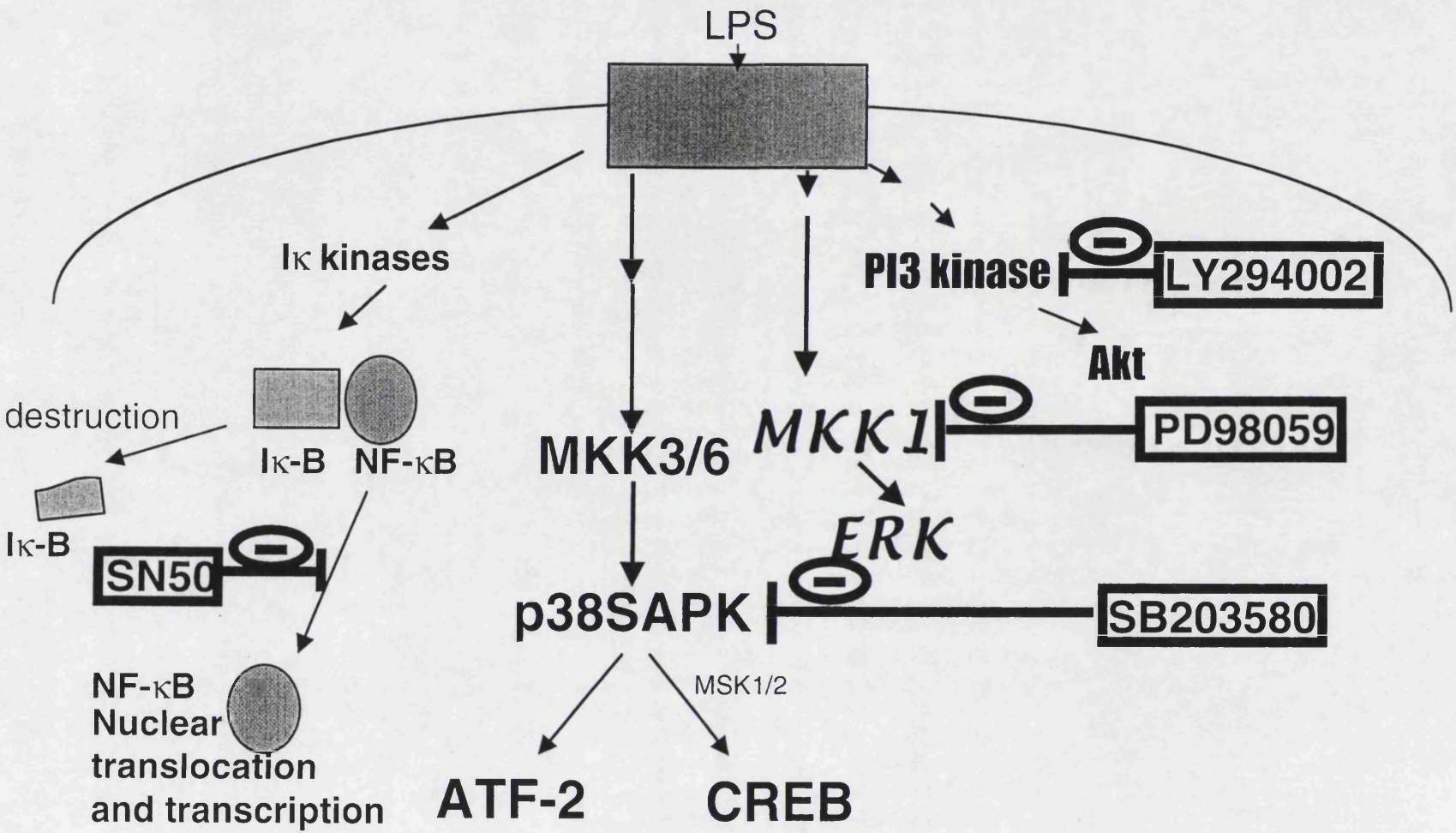


Figure 5.1 Schematic representation of some of the signal transduction pathways activated by LPS. The site of action of the inhibitors PD98059, LY294002, SB203580 and SN50 are shown

5.1.4 Quantification of cell survival.

At the indicated time points, cells were washed in annexin V binding buffer (140mM NaCl, 5mM $CaCl_2$, 10mM HEPES, pH7.4) and resuspended in buffer containing annexin V-FITC (1µl per $1x10^6$ cells according to the manufacturer's instructions)(Boehringer Mannheim) and propidium iodide (5µg/ml). After 10 minutes incubation at room temperature, samples were placed on ice and directly analysed by flow cytometry. Cells negative for annexin V and propidium iodide staining were considered viable.

5.1.5 SDS/PAGE and Western Blotting.

Immature monocyte derived DCs were washed twice and incubated in RPMI alone for 2 hours at 37°C, at a density of 0.5-1 x 10^6/ml. Cells were stimulated with LPS (100ng/ml) and at the indicated time points $1x10^6$ cells were removed, washed once with cold PBS and the pellet resuspended in 2xSDS sample buffer and boiled for 5 minutes. Where inhibitors were used, cells were incubated with the inhibitor for 30 minutes prior to the addition of LPS. Proteins were separated by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels (SDS/PAGE) and blotted onto nitrocellulose membranes (Hybond C-Extra, Amersham). Membranes were blocked with 5% (w/v) nonfat-dry milk (Marvel)/ 0.1% (v/v) Tween 20 in PBS for one hour at room temperature and incubated overnight with primary antibody at 4°C. Antibodies to the phosphorylated and total forms of p38SAPK, Akt, ERK, activating transcription factor (ATF) 2, and cAMP response element binding protein (CREB) were all from New England Biolabs. Anti-IκBα was from Santa Cruz and anti-tubulin from Boehringer Mannheim. Detection was by ECL or ECL Plus (Pharmacia Biotech, Amersham).

5.1.6 Nuclear NF-κB pull-down assay.

Day 7 monocyte derived DCs ($5x10^6$ per point) were stimulated with LPS (100ng/ml) after preincubation for 2 hours with SN50 peptide (concentration 50µg/ml) and nuclear extracts prepared as follows. Cells were pelleted and resuspended in 0.4ml hypotonic lysis buffer (20mM HEPES pH7.9, 10mM KCl, 1mM EDTA, 0.2% Triton X-100, 1mM sodium orthovanadate plus protease inhibitors) on ice for 20 minutes. After centrifugation at 14000g for 5 minutes at 4°C, the nuclear pellet was extracted with 0.1ml hypertonic lysis buffer (20mM HEPES pH7.9, NaCl 0.4M, 1mM EDTA, 1mM sodium orthovanadate plus protease inhibitors) on ice for a further 20 minutes. After centrifugation at 14000g for 5 minutes at 4°C, the supernatant was diluted to 100mM NaCl with distilled water and incubated with 25µl of agarose beads conjugated to a consensus NF-κB binding oligonucleotide (Santa Cruz) for 1 hour at 4°C. After 3 washes, 25µl of 2 x sample buffer was added and boiled for 5 minutes. The result was

analysed by SDS-PAGE and immunoblotting using a polyclonal anti-p65 NF-κB antibody (Santa Cruz).

5.2 Results

5.2.1 LPS induces phenotypic maturation of monocyte derived DCs.

Immature DCs were generated by culturing peripheral blood monocytes with GM-CSF and IL-4 for 7 days. Incubation of these cells with LPS at a concentration of 100ng/ml for a further 48 hours led to a significant upregulation of cell surface CD80, CD86, HLA-DR, CD83 and CD40 (Table 5.1 & Fig 5.2). Others have shown that incubation of immature monocyte derived DCs with LPS results in the downregulation of CD1a (Sallusto & Lanzavecchia, 1994). In this series of experiments, however, although there was a decrease in CD1a expression, this was not statistically significant (Table 5.1 & Fig 5.2). The uptake of FITC-dextran by immature and LPS-matured monocyte derived DCs over a one hour period was also measured. This is known to be maximal in the immature monocyte derived DC (Sallusto *et al.*, 1995), and consistent with this, a reduction in FITC-dextran uptake by 70%±10% (n=4) was demonstrated when the monocyte derived DCs were matured with LPS (Fig 5.2).

5.2.2 LPS activates p38SAPK, ERK and Akt in immature monocyte derived DCs.

The classical MAP kinase pathway (MEK/ERK), the PI3 kinase/Akt and the p38SAPK pathways are known to be important in many cell types in the regulation of cell survival, proliferation and differentiation. In view of this, and the fact that LPS has been shown to activate multiple signaling pathways in macrophages including ERK, JNK and p38SAPK (Hambleton *et al.*, 1996; Han *et al.*, 1994; Weinstein *et al.*, 1992), experiments were performed to determine if these pathways were activated upon stimulation of monocyte derived DCs with LPS. Monocyte derived DCs were incubated with LPS ± inhibitors and cell lysates prepared at varying time points afterwards up to 120 minutes. Activation of ERK, Akt and p38SAPK results in their phosphorylation and this was assessed by Western blotting using phosphorylation specific antibodies. As can be seen in figure 5.3, p38SAPK, Akt and ERK were activated within 15-30 minutes of the addition of LPS to immature monocyte derived DCs.

5.2.3 Inhibition of PI3-kinase leads to decreased survival of LPS stimulated monocyte derived DC.

In order to evaluate the role of these pathways in monocyte derived DC survival and maturation, specific inhibitors of the pathways were used (Fig 5.1). PD 98059

CELL SURFACE MARKER	Immature DCs			LPS Matured DCs		
	% positive	MCF	% positive x MCF	% age positive	MCF	% positive x MCF
CD83 n=7	34.8±10.8	0.63±0.26	29.6±21.3	76.1±8.7	1.73±0.59	150.2±58.5
CD80 n=5	26.9±10.7	1.12±0.54	25.3±15.6	60.3±16.2	2.23±0.70	161.1±58.7
CD86 n=5	11.4±4.2	0.94±0.46	9.6±4.5	66.1±8.5	3.25±1.10	220.8±83.6
CD40 n=4	90.9±9.5	8.44±3.35	768.4±307.6	90.7±9.5	20.03±8.66	1784.9±751
HLA DR n=5	96.4±1.1	47.56±18.79	4625.9±1868	95.9±1.1	159.5±54.13	15313.5±5176.5
CD1a n=5	71.9±9.9	11.70±7.29	1055.1±695.2	70.9±10.6	9.66±5.89	858.2±561.9

Table 5.1 Flow cytometric analysis of immature and LPS-matured populations of monocyte derivedDCs.
Peripheral blood monocytes were cultured with GM-CSF and IL4 (both 100ng/ml) for 7 days. The cells were then divided into 2 aliquots. To one half LPS (100ng/ml) was added, to the other half no addition was made. The cells were then cultured for a further 48 hours before being analysed by flow cytometry. The percentage of cells expressing various cell surface antigens (±SEM) together with the mean cell fluorescence (MCF) of the whole population of cells (±SEM) is shown together with the product of these 2 variables.

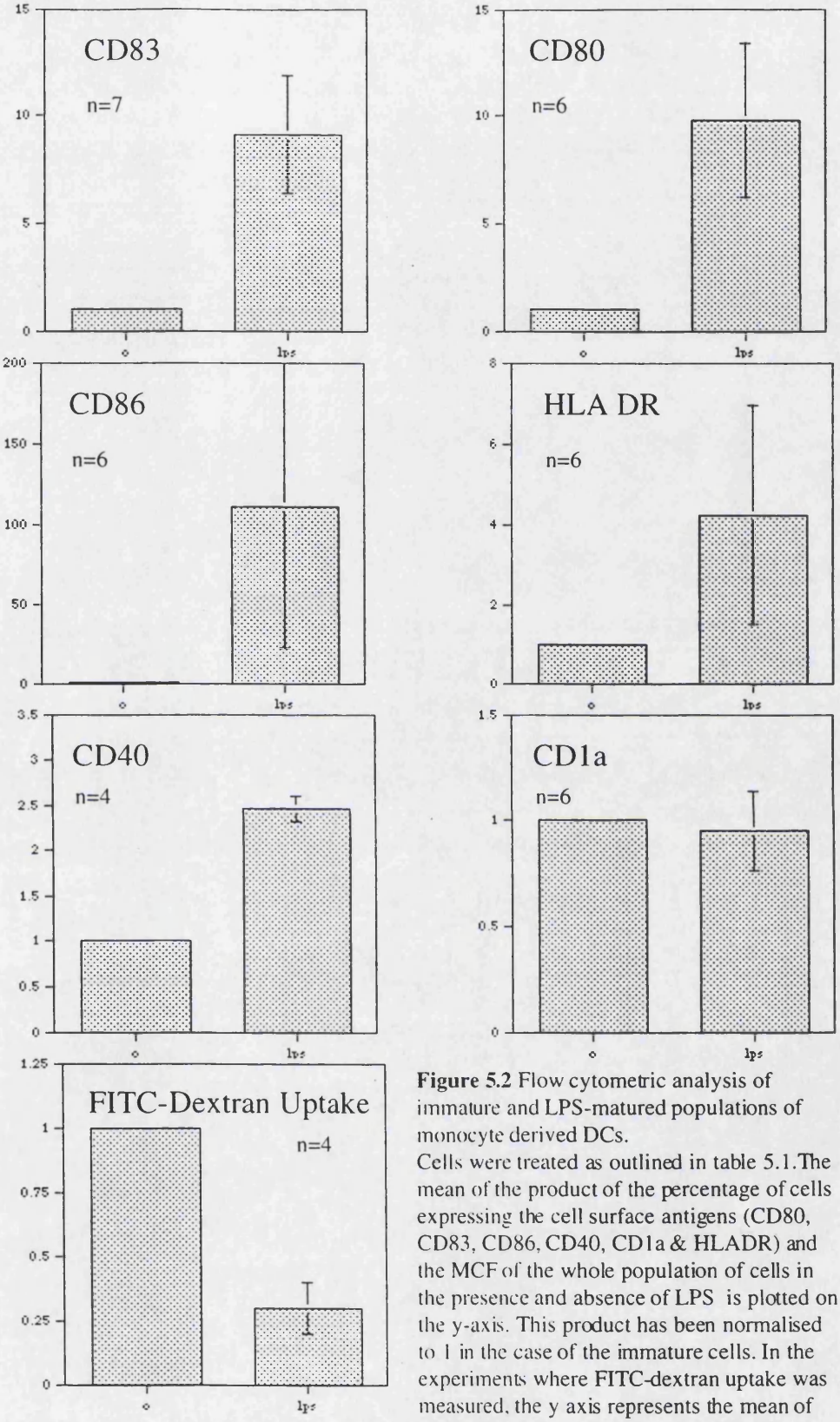


Figure 5.2 Flow cytometric analysis of immature and LPS-matured populations of monocyte derived DCs.
Cells were treated as outlined in table 5.1.The mean of the product of the percentage of cells expressing the cell surface antigens (CD80, CD83, CD86, CD40, CD1a & HLADR) and the MCF of the whole population of cells in the presence and absence of LPS is plotted on the y-axis. This product has been normalised to 1 in the case of the immature cells. In the experiments where FITC-dextran uptake was measured, the y axis represents the mean of the MCF of the whole population of cells. Again this value has been normalised to 1 in the case of the immature cells. Error bars represent theSEM.

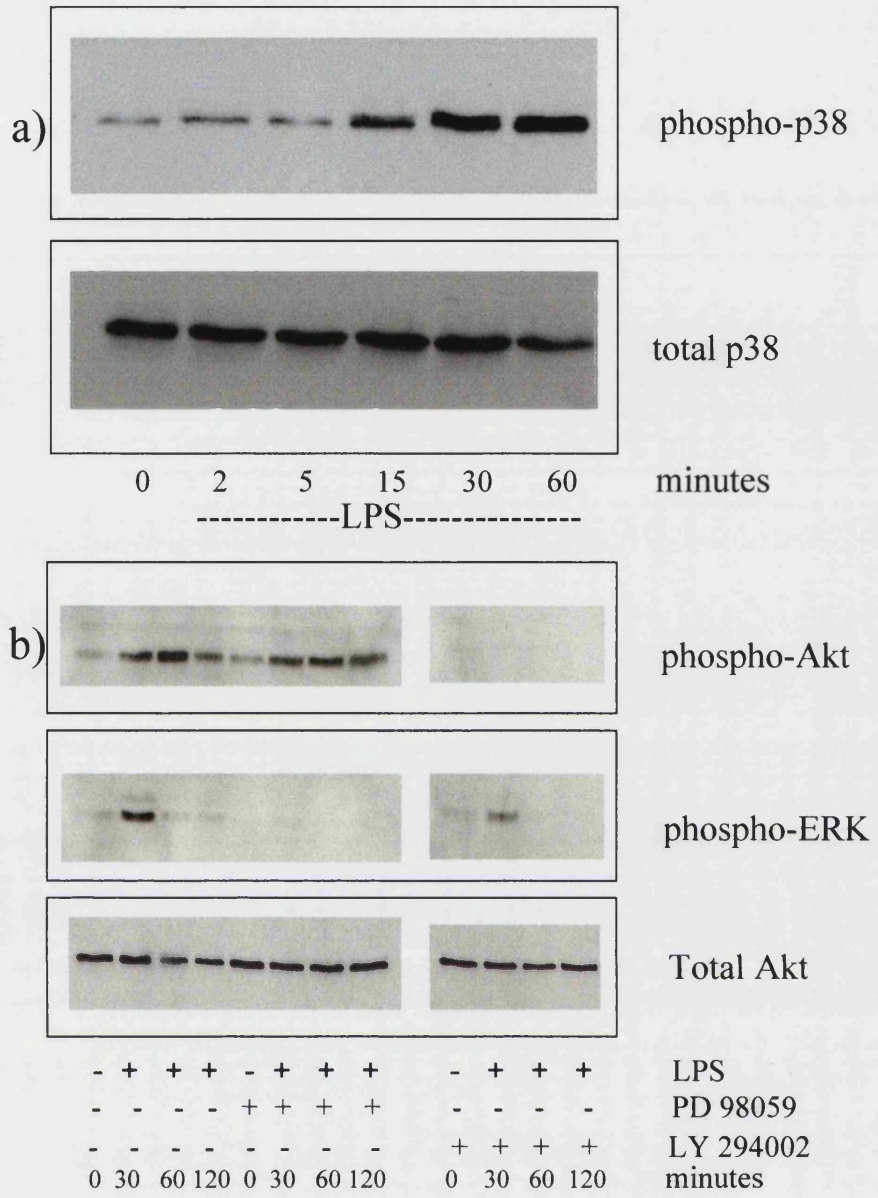


Figure 5.3 LPS induces the phosphorylation of p38SAPK, ERK and Akt kinase Peripheral blood monocytes cultured with GM-CSF and IL-4 for 7 days (immature monocyte derived DCs) were exposed to LPS (100ng/ml) and after variable lengths of time, samples were removed and analysed by probing western blots with phosphorylation specific antibodies. In some cases the d7 monocyte derived DCs were preincubated with signal transduction pathway inhibitors for 30 minutes prior to exposure to LPS. Panel (a) shows that the phosphorylation (and hence activation) of p38 SAPK induced by LPS occurs within 15 minutes and persists for at least 60 minutes. The lower part shows the same blot probed for total p38 SAPK to demonstrate equal loading of samples. Panel (b) shows that the phosphorylation (and hence activation) of Akt and ERK induced by LPS occurs within 30 minutes. The phosphorylation of Akt is inhibited if MoDCs are preincubated with the PI3 kinase inhibitor, LY294002. Similarly the phosphorylation of ERK is inhibited if MoDCs are preincubated with the MKK1/MEK inhibitor PD98059. PD 98059 and LY 294002 are both dissolved in DMSO therefore monocyte derived DCs which had been incubated with DMSO alone were used as controls. The lower part shows the same blot probed for total Akt to demonstrate equal loading of samples. Similar results were obtained in 4 separate experiments.

suppresses the activation of MAP kinase/ERK by inhibiting the upstream MAP kinase kinase-1 (MKK1/MEK) (Alessi *et al.*, 1995). LY294002 is a specific inhibitor of PI3-kinase and prevents activation of Akt kinase and other targets of PI3-kinase (Vlahos *et al.*, 1994). SB203580 binds to the ATP binding pocket of p38SAPK, inhibiting its activity but not its phosphorylation (Tong *et al.*, 1997). Immature monocyte derived DCs were incubated with the inhibitors for 30 minutes on ice, then stimulated with LPS and aliquots removed at varying time points. Fig 5.3b shows that incubation of monocyte derived DC with LY294002 or PD98059 effectively blocked the LPS-induced activation of the PI3K/Akt and MEK/ERK pathways respectively. Inhibition of PI3-kinase with LY294002 led to reduced viability as a result of increased apoptosis (Fig 5.4), with only a quarter of monocyte derived DCs remaining viable at 48 hours. Viability was assessed by their ability to exclude propidium iodide ± their lack of binding of FITC-conjugated annexin V. Inhibition of either MEK/ERK pathway did not affect monocyte derived DC survival (Fig 5.4).

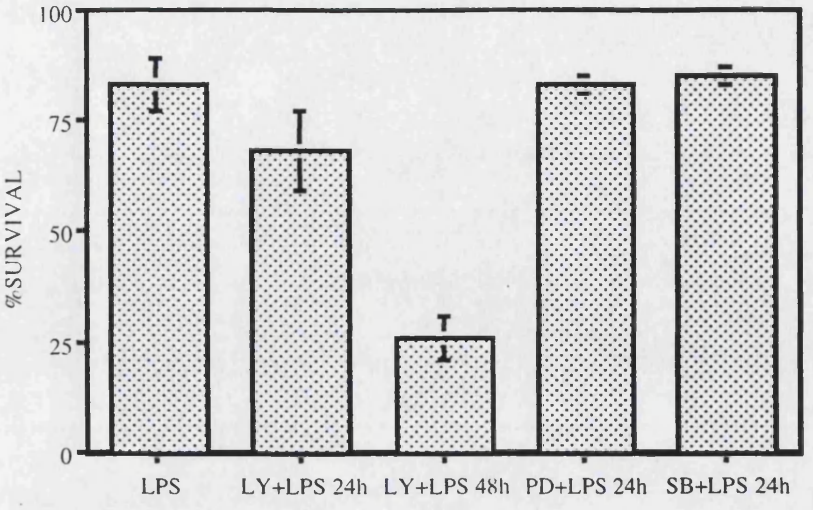
5.2.4 Inhibition of p38SAPK prevents some but not all of the maturation changes induced by LPS.

As inhibition of PI3-kinase resulted in apoptosis, the effect of blocking this pathway on monocyte derived DC maturation could not be reliably assessed. Inhibition of MEK with PD98059 had no effect on the LPS-induced upregulation of CD83 and HLA DR in monocyte derived DCs (Table 5.2). Blocking the p38SAPK pathway with SB203580 significantly inhibited the LPS-induced upregulation of CD83, CD86 and, to a lesser extent, CD80 (Fig 5.5 & Table 5.3). Inhibition of p38SAPK did not, however, affect the LPS-induced upregulation of CD40 and HLA-DR. The reduced uptake of FITC-dextran seen in LPS-matured monocyte derived DC was also unaffected. The effects of SB203580 were not likely to be due to non-specific toxicity as there was no increase in apoptosis (Fig 5.4). These results show that certain features of monocyte derived DC maturation are regulated by signaling via p38SAPK and imply that different aspects of the maturation process induced by LPS may be regulated by distinct signal transduction pathways.

5.2.5 The addition of LPS results in the phosphorylation of CREB and ATF-2 transcription factors in a p38SAPK-dependent manner.

These finding suggest that the activation of p38SAPK resulted in the transcription of various genes involved in the maturation of monocyte derived DCs. CREB and ATF-2 are two transcription factors which are known to lie downstream of p38SAPK. They become active upon phosphorylation. Changes in the phosphorylation status of CREB and ATF-2 were therefore studied. Figure 5.6 shows that both transcription factors were

a)



b)

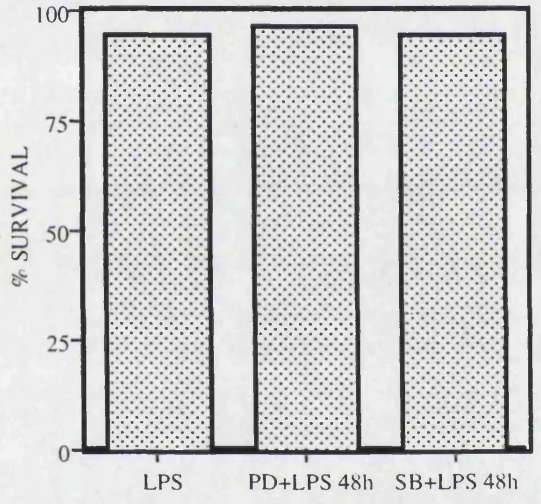


Figure 5.4 Viability of LPS-stimulated monocyte derived DCs exposed to inhibitors. Peripheral blood monocytes cultured with GM-CSF and IL-4 for 7 days were incubated with LPS with or without inhibitors for 24 or 48 hours. The percentage of cells surviving at the end of the incubation was measured flow cytometrically. a) Only cells which did not bind FITC-conjugated annexin V and take up propidium iodide were classified as viable. Mean ± SEM of 4 experiments. b) Only cells which did not take up propidium iodide were classified as viable (n=1). PD=PD98059 (MKK1/MEK inhibitor). LY=LY294002 (PI3 kinase inhibitor). SB= SB203580 (p38 SAPK inhibitor). Blocking the PI3 kinase pathway has a marked effect on MoDC survival whereas blocking the MAPK or p38 SAPK pathways does not effect MoDC survival even after 48 hours.

Variable		% Positive	MCF	% Positive x MCF
DR	0	98	4.1	402
	lps	98	15.7	1539
	pd/lps	98	14.0	1372.
CD83	0	32	1.5	48
	lps	91	3.3	300
	pd/lps	87	3.2	278
	(sb/lps)	(58)	(1.8)	(104)

Table 5.2 The LPS-induced upregulation of HLA DR and CD83 in monocyte derived DCs is not inhibited by PD98059. Results of one experiment are shown. PD98059 did not significantly inhibit the LPS induced upregulation of the 2 cell surface antigens shown. For comparison the inhibitory effect of SB203580 on the LPS-induced upregulation of CD83 is shown in brackets.

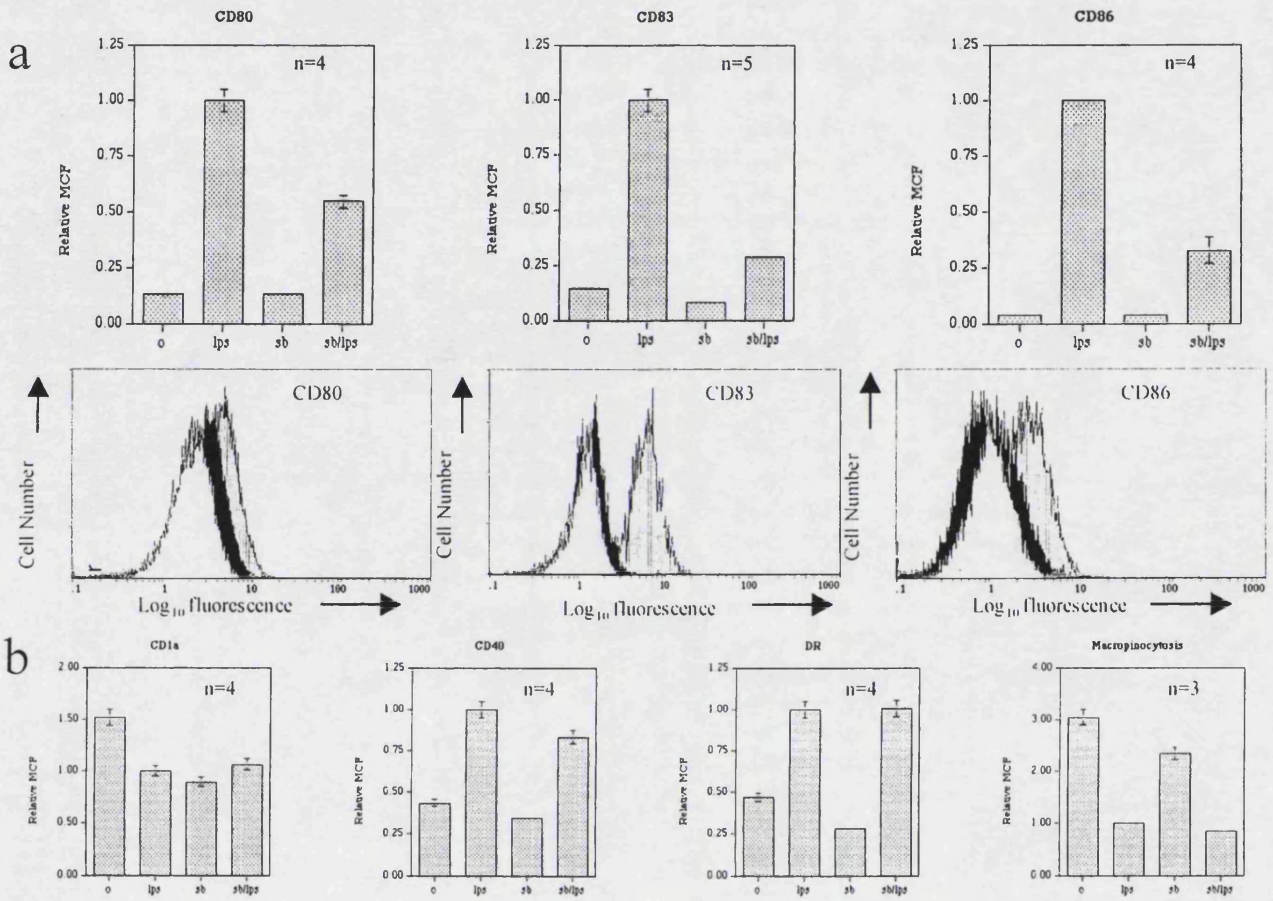


Figure 5.5 SB203580 inhibition of LPS-induced upregulation of CD80, CD83 and CD86 on monocyte derived DCs. Immature MoDCs were split on d7 of culture and either exposed to diluent control, LPS (100ng/ml), SB203580 (40μM) or SB203580 together with LPS for 48 hrs, then incubated with fluorochrome-conjugated antibodies directed against cell surface antigens and analysed using a flow cytometer. The data shown in the bar charts is the product of the percentage of cells expressing the different cell surface antigens and the MCF of the whole population of cells under scrutiny. The values obtained for cells exposed to LPS alone has been normalised to 1. Error bars indicate the SEM.

(a) The LPS-induced upregulation of CD80, CD83 and CD86 is inhibited by the p38 SAPK inhibitor SB203580. Representative single parameter histograms show the expression of CD80, CD83 and CD86 by d7 MoDCs 48 hrs after the addition of LPS (shaded grey), SB203580 plus LPS (shaded black) or nothing (unshaded).

(b) Inhibition of p38SAPK has little or no effect on the LPS-induced changes with regards to CD1a, CD40, HLA DR and macropinocytosis.

Variable		Mean % Positive (Range)	Mean MCF (Range)	Mean % Positive x Mean MCF
CD80 n=4	o	31.5 (3.0-64.2)	1.4 (0.2-3.1)	42.8
	lps	74.5 (45.0-88.5)	2.7 (1.1-4.1)	203.1
	sb	32.3 (5.0-62.9)	1.5 (0.2-4.5)	50.0
	sb/lps	61.3 (15.0-89.4)	2.2 (0.7-4.2)	137.0
CD83 n=5	o	39.2 (11.0-82.3)	0.7 (0.2-1.9)	29.2
	lps	81.7 (70.6-96.8)	1.9 (1.0-5.0)	156.7
	sb	34.1 (8.0-77.6)	0.5 (0.1-1.1)	17.0
	sb/lps	53.3 (20.0-88.9)	0.9 (0.4-2.0)	47.9
CD86 n=4	o	8.7 (0.7-20.2)	1.0 (0.2-2.7)	8.6
	lps	67.6 (40.9-77.8)	2.4 (1.0-4.9)	161.1
	sb	8.6 (1.3-17.4)	1.0 (0.1-2.8)	8.4
	sb/lps	43.6 (27.3-57.7)	1.3 (0.4-3.2)	57.9
DR n=4	o	96.7 (94.6-100)	53.8 (10.8-108)	5199.8
	lps	96.0 (94.0-100)	114.5 (19.7-187.1)	10987.7
	sb	96.2 (94.8-100)	31.1 (7.4-61.1)	299.4
	sb/lps	96.2 (94.8-100)	113.2 (24.5-235)	10896.4
CD40 n=4	o	90.9 (87.1-93.6)	8.4 (2.0-18.8)	767.1
	lps	90.7 (87.6-94.0)	20.0 (5.7-47.7)	1815.8
	sb	92.7 (90.9-94.7)	6.4 (1.2-12.7)	590.6
	sb/lps	93.4 (91.6-95.0)	15.8 (3.6-34.7)	1471.9
CD1a n=4	o	66.6 (47.1-96.1)	4.8 (1.2-13.3)	316.9
	lps	65.1 (32.7-84.8)	4.2 (1.0-12.2)	270.5
	sb	60.1 (42.6-84.5)	3.4 (0.8-9.7)	202.1
	sb/lps	63.7 (43.2-86.9)	4.2 (1.1-13.1)	267.4
MP n=3	o	-	227.3 (161.8-272)	-
	lps	-	102.0 (43-171)	-
	sb	-	195.1 (121.4-292)	-
	sb/lps	-	80.7 (41-128)	-

Table 5.3 LPS-induced upregulation of CD80. CD83 and CD86 on monocyte derived dendritic cells is inhibited by SB203580. Cells were treated as outlined in figure 5.5
The table displays the mean percentage of cells which expressed the antigen under consideration together with the mean MCF of the whole population of cells. In the final column is the product of these two readings. No normalisation has been performed. The final variable, macropinocytosis (MP), shows the mean MCF of the monocyte derived DCs after incubation in FITC-dextran for 60 minutes.

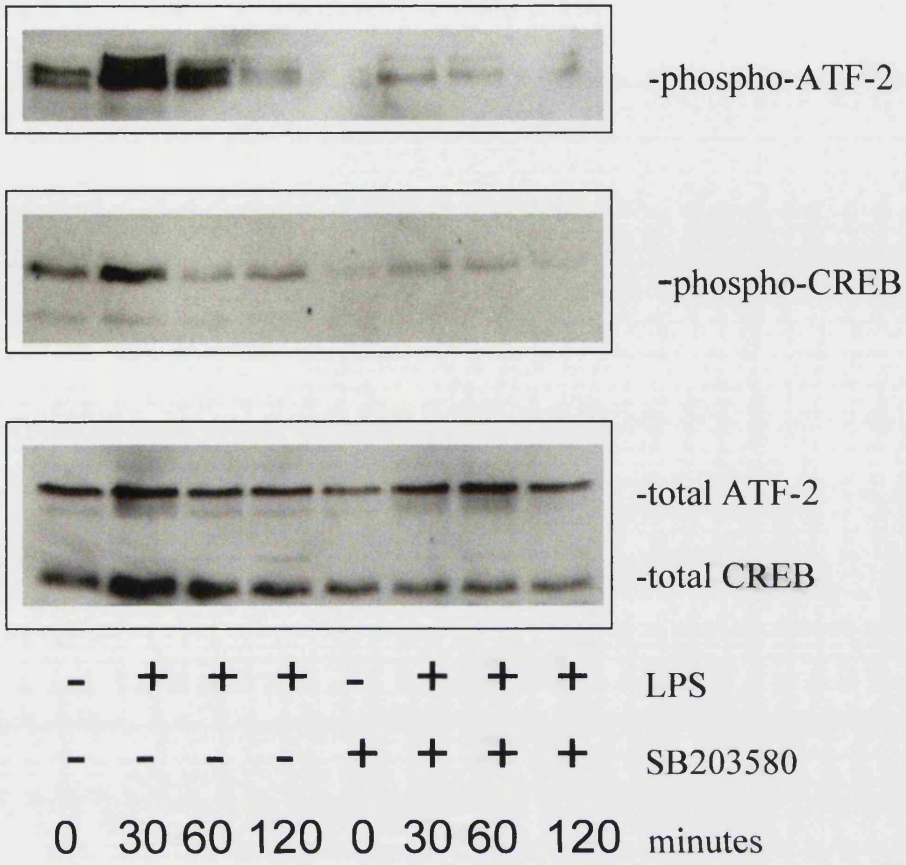


Figure 5.6 LPS induces phosphorylation of the transcription factors ATF-2 and CREB in a p38 SAPK-dependent manner.
Peripheral blood monocytes cultured with GM-CSF and IL-4 for 7 days (immature monocyte derived DCs) were exposed to LPS (100ng/ml) and after variable lengths of time, samples were removed and separated by SDS-PAGE and then probed with phospho- specific antibodies to the transcription factors CREB & ATF-2 . In some cases the d7 monocyte derived DCs were preincubated with the p38SAPK inhibitor SB203580 for 30 minutes prior to exposure to LPS. As SB203580 is dissolved in DMSO, monocyte derived DCs which had been incubated with DMSO alone were used as controls. The faint bands below the phospho CREB bands in the middle panel are due to cross reactivity of the anti-phospho ATF-2 antibody with ATF-1 which runs in this position. The lowest panel shows the blot probed for total ATF-2 and CREB to demonstrate loading of samples. Similar results were obtained in 3 separate experiments.

phosphorylated within 30 minutes of the addition of LPS to immature monocyte derived DCs. Furthermore, inhibiting the p38SAPK with SB203580 prior to the addition of LPS prevented this activation of CREB and ATF-2.

5.2.6 Inhibition of NF-κB signaling prevents monocyte derived DC maturation in response to LPS.

NF-κB knockout mice are known to have defective DCs (Weih *et al.*, 1997). In addition, NF-κB plays a significant role in LPS-induced signaling in macrophages and there is growing evidence that p38SAPK can interact with signaling by the NF-κB pathway (Legrand-Poels *et al.*, 1997; Shakhov *et al.*, 1990). The role of NF-κB in LPS-induced maturation in monocyte derived DCs was therefore investigated. The transcription factor NF-κB is bound to Iκ–Bα in the cytoplasm and retained there in an inactive form. Various stimuli result in the phosphorylation and subsequent ubiquitination of Iκ–Bα, leading to it being targeted to the proteasome for destruction. Free NF-κB is able to translocate to the nucleus and activate transcription of various genes. Day 7 immature monocyte derived DCs were exposed to LPS and at various lengths of time, up to 4 hours, samples were removed and lysates prepared. Western blotting revealed that Iκ–Bα was rapidly degraded upon addition of LPS to immature monocyte derived DCs, (Fig 5.7).

To assess the role of LPS-induced release of NF-κB in monocyte derived DC maturation, the cell-permeable SN50 peptide was utilised (Lin *et al.*, 1995). The SN50 peptide contains the nuclear localisation sequence of NF-κB and is postulated to block the receptors used to take up NF-κB into the nucleus thus inhibiting the nuclear translocation of NF-κB,. Nuclear extracts were prepared from unstimulated monocyte derived DCs and monocyte derived DCs which were stimulated with LPS, either in the presence or absence of the SN50 peptide. The nuclear extracts were incubated for 1 hour with agarose-bound oligonucleotides containing the consensus binding motifs for NF-κB. Any NF-κB which had translocated to the nucleus bound to the oligonucleotide-agarose conjugate and was detected by probing a Western blot with an antibody directed against NF-κB p65. It can be seen in figure 5.8 that the addition of LPS to monocyte derived DCs resulted in the nuclear translocation of NF-κB and that this was prevented by the addition of the SN50 peptide.

Addition of SN50, but not the control peptide SN50M, resulted in the partial inhibition of the LPS-induced upregulation of CD80, CD83, CD86 and HLA-DR. (Fig 5.9). To assess any potential interaction between p38SAPK and NF-κB the effect of inhibiting

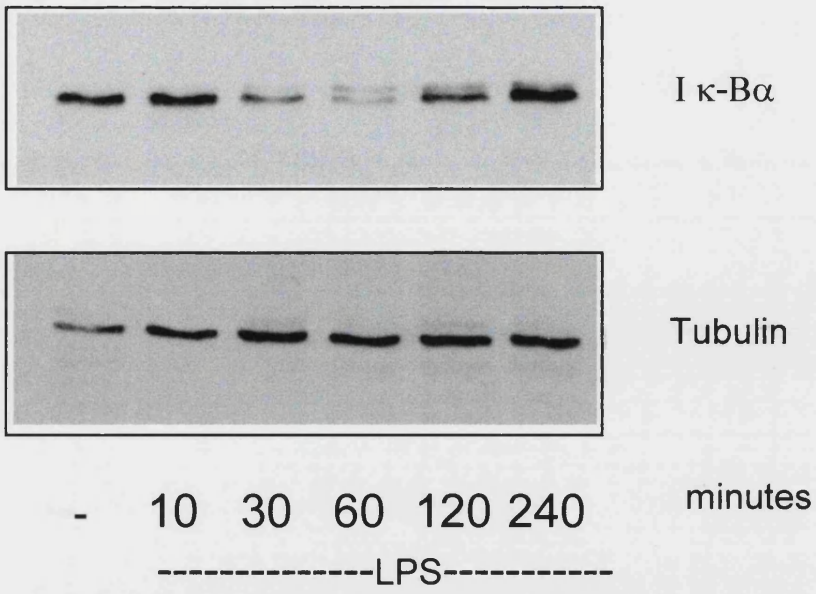


Figure 5.7 LPS induces the degradation of Iκ-Bα in MoDCs.
Day 7 immature MoDCs were exposed to LPS (100ng/ml) for variable lengths of time after which samples were removed and analysed by western blotting with an antibody directed against Iκ-Bα. The blot shows the degradation of Iκ-Bα induced in MoDCs by the addition of LPS. The lower panel shows the blot probed for tubulin to demonstrate equal loading of samples. Similar results were obtained in 3 separate experiments.

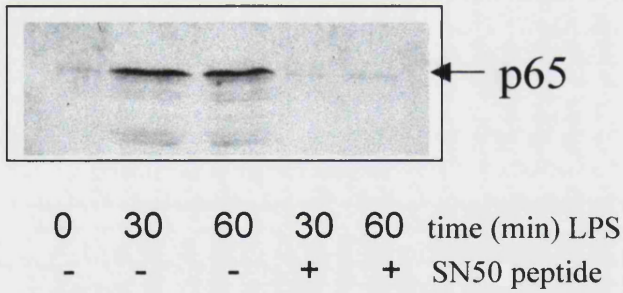


Figure 5.8 SN50 peptide inhibits the LPS-induced nuclear translocation of NF-κB.
Day 7 immature MoDCs were stimulated with LPS (100ng/ml) for varying times, in some cases after preincubation with SN50 peptide (50μg/ml). After this nuclear extracts were prepared. Oligonucleotides containing the consensus binding sequence for NF-κB bound to agarose beads were used to pull down nuclear NF-κB. The resulting sample were analysed by SDS-PAGE and immunoblotting using a polyclonal anti-p65 NF-κB antibody. LPS can be seen to induce the nuclear translocation of NF-κB within 30 minutes of its addition to MoDCs. This is prevented by the addition of the SN50 peptide.

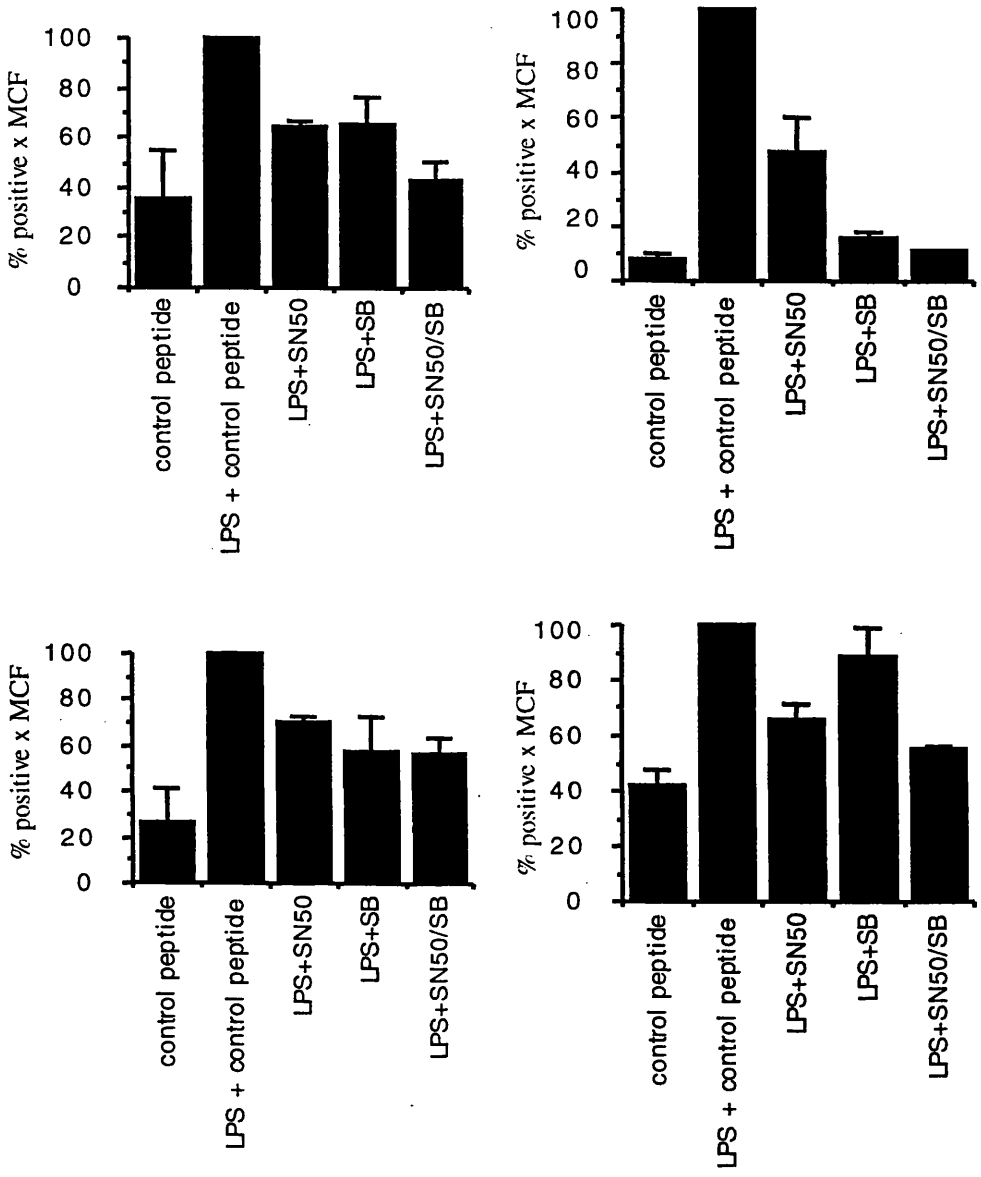


Figure 5.9 Graphs showing the effect of inhibiting the NF-κB and/or p38SAPK pathways on the LPS-induced upregulation of CD80, CD83. CD86 and HLA DR.
Day 7 immature MoDCs were pretreated either with nothing, a cell permeable peptide that inhibits NF-κB nuclear translocation (SN50 peptide (50μg/ml)), the p38 SAPK inhibitor SB20358(40μM) or both for 2 hours before the addition of LPS (100ng/ml) for 24 hours. Control cells were pretreated with a control peptide plus or minus LPS. The cell surface expression of CD80, CD83, CD86 and HLA DR were then measured using flow cytometry. Figures obtained are the product of the percentage of cells expressing the various cell surface antigens and the MCFof the whole population of cells. The value obtained for cells exposed to LPS alone has been normalised to 100%. Mean ± SEM of 3-4 experiments is shown.

both pathways simultaneously was investigated. This appeared to have varying effects depending on the phenotypic marker examined. For example, blocking both pathways virtually abolished the LPS-induced upregulation of CD80, whereas either pathway only partially blocked it. In the case of CD86, inhibiting both pathways did not appear to be additive, nor was the upregulation of this molecule entirely abolished. In contrast to the minimal effect seen with blocking p38SAPK signaling, inhibiting NF-κB significantly reduced the LPS-induced upregulation of HLA-DR (Figure 5.9).

5.3 Discussion

The maturation process is fundamental to the specialised function of the DC, during which the cell changes from being highly efficient at taking up exogenous antigen to being specialised in antigen presentation (Banchereau & Steinman, 1998). This maturation process is multifaceted. Antigen uptake mechanisms such as mannose receptor and Fcγ receptor mediated uptake, macropinocytosis and phagocytosis are all downregulated. There is upregulation of cell surface MHC molecules, which in the case of both MHC I and II is due to increased biosynthesis, and in the case of MHC II to a prolongation of the half-life of MHC-peptide complexes. The co-stimulatory molecules CD80, CD86 and CD40 are also upregulated, as is the DC-specific molecule CD83, to which no function has currently been assigned (Zhou & Tedder, 1995). Clearly, any antigen which is encountered in the peripheral tissues must be presented to T cells in the lymph nodes, thus the maturation process also must encompass the migration of DCs from the peripheral tissues to the paracortical area of lymph nodes through which large numbers of T cells circulate. This occurs by a rapid and coordinated switch in chemokine receptor expression after DCs receive a maturation stimulus (Sallusto *et al.*, 1998).

In the experiments described in this chapter, exposing immature monocyte derived DCs to LPS for 48 hours was shown to result in the development of a mature phenotype. That is, 1) FITC-Dextran uptake was reduced by 70±10% after incubation with LPS for 48 hours. 2) There was an increase of cell surface MHC class II molecules and the co-stimulatory molecules CD80, CD86 and CD40. 3) The marker for mature DCs, CD83, was also increased. These results are consistent with the findings of others (Banchereau & Steinman, 1998; Cella *et al.*, 1997; De Smedt *et al.*, 1996; Sallusto *et al.*, 1995; Sallusto & Lanzavecchia, 1994; Winzler *et al.*, 1997).

As little is known about the signal transduction pathways involved in the LPS-induced maturation of monocyte derived DCs, experiments were performed to investigate this further. The results showed that the classical MAP kinase pathway (MEK/ERK), the PI3 kinase/Akt pathway and the p38 SAPK pathway were all activated when immature monocyte derived DCs were exposed to LPS, implicating a role for these pathways in the maturation process. PI3 kinase activity was shown to be important for monocyte derived DC survival and, in addition, the experiments demonstrated for the first time that Akt is activated in DCs triggered with a maturation stimulus. Akt kinase, which is regulated by PI3-kinase, has been shown to control survival in many cell types including fibroblasts (Kauffmann-Zeh *et al.*, 1997), haemopoietic cells (Minshall *et al.*, 1996), epithelial cells (Khwaja *et al.*, 1997), and neuronal cells (Dudek *et al.*, 1997), and it is therefore likely to be involved in PI3 kinase mediated monocyte derived DC survival.

Inhibition of the MAPK/ERK pathway with PD 98059 did not have any effect on monocyte derived DC survival. This is in contrast to the findings of Rescigno *et al* (Rescigno *et al.*, 1998). Using a growth factor dependent murine DC cell line (D1 cells) that maintains its immature phenotype *in vitro*, they showed that LPS promoted the survival of D1 cells after growth factor withdrawal. LPS was shown to activate ERK in these cells, and inhibiting this pathway using PD 98059 abrogated the survival effect of the LPS. These differences may reflect the different biology of primary human cells compared with murine cell lines. In addition, in the experiments described here, inhibiting the MAPK/ERK pathway with PD 98059 did not affect the upregulation of CD83 or HLA DR induced by LPS.

Inhibition of the p38SAPK pathway with SB203580 was found to significantly reduce the LPS-induced upregulation of CD80, CD83 and CD86, but did not significantly affect the upregulation of CD40 or HLA-DR, nor the downregulation of CD1a or endocytic capacity. Thus it appears that some, but not all, aspects of DC maturation are regulated via the p38SAPK pathway. There are many known targets of p38SAPK. These include transcription factors such as ATF-2, growth arrest DNA damage (GADD)153, Elk-1, myogenic enhancer factor (MEF) 2C, and other kinases such as MAP kinase activating protein (MAPKAP) kinases 2 and 3, MAP kinase interacting kinases (Mnks) 1 and 2, and mitogen and stress activated protein kinase (Msk)-1. MAPKAP kinase 2 and Msk-1 in turn activate the transcription factors ATF-1 and CREB (Lu *et al.*, 1999). In the present studies, phosphorylation, and hence activation of the transcription factors ATF-2 and CREB, was detected soon after the monocyte derived DCs were exposed to LPS. This was shown to occur in a p38SAPK-dependent

manner. Using the MatInspector V2.2 database published by Quandt *et al.*, (1995) at least one binding site for ATF and CREB in the promoter sequence of CD86 was identified. The human CD80 promoter sequence also has one binding site for CREB (Fong *et al.*, 1996). Thus one possible mechanism by which LPS causes the upregulation of the co-stimulatory molecules is at the transcriptional level, mediated by the actions of CREB/ATF.

The p38SAPK pathway is involved in many aspects of immune cell function, being of importance in the innate immune response (Han *et al.*, 1994) as well as in the adaptive immune response. p38SAPK may play an important role in T cell development as it is found to be activated in T cells in the thymus (Sen *et al.*, 1996). The cytokines IL-2 and IL–7 also activate p38SAPK in T cells (Crawley *et al.*, 1997). In B cells it is activated during CD40-induced B cell proliferation (Craxton *et al.*, 1998). In macrophage cell lines p38SAPK is phosphorylated in response to LPS (Han *et al.*, 1994), and cytokine release by various cell types including IL-12 by DCs and macrophages (Lu *et al.*, 1999) and IFN-γ by T_{H1} cells (Rincon *et al.*, 1998), is mediated via the p38SAPK pathway. CpG-DNA specific activation of murine DCs is also mediated by p38SAPK (Hacker *et al.*, 1998), as is the IL-10-mediated selective repression of TNF-α–induced monocyte derived DC maturation (Sato *et al.*, 1999). Hence, the finding that the p38SAPK pathway is important in monocyte derived DC maturation is in keeping with its central role in immune cell signal transduction.

The activation of macrophages by LPS occurs via a Toll-like receptor and CD14 (Yang *et al.*, 1999). This, in turn, results in activation of NF-κB. Because of this, and in view of findings that RelB, a member of the NF-κB/Rel family, is highly expressed in DCs (Carrasco *et al.*, 1993) and that RelB knockout mice have greatly decreased numbers of DCs, the role of the NF-κB pathway in LPS-induced DC maturation was investigated.

LPS was shown to result in the activation of NF-κB pathway. Inhibition of NF-κB translocation to the nucleus with an inhibitory peptide decreased the upregulation of HLA-DR, as well as that of CD80, CD83 and CD86. It is of interest that inhibiting NF-κB had no effect on monocyte derived DC survival whereas in other cell types this pathway can regulate apoptosis (Beg & Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). Rescigno and colleagues (Rescigno *et al.*, 1998) have also shown that LPS activates NF-κB in the D1 cell line and that blocking nuclear translocation using the serine protease inhibitor TPCK (N-tosyl-L-phenylalanine chloromethyl ketone), which prevents Iκ–Bα degradation, reduces LPS-induced upregulation of HLA-DR and CD86.

It thus appears that the upregulation of CD80, CD83 and CD86 by LPS is controlled by at least two signal transduction pathways. The upregulation of HLA-DR, however, is NF-κB-dependent but not p38SAPK-dependent. Interestingly, blocking the NF-κB and p38SAPK pathways was at least additive for CD80, whereas for CD83, maximal inhibition was achieved by blocking p38SAPK alone. For CD86, blocking both NF-κB and p38SAPK did not completely abolish the effect of LPS, suggesting the existence of an unrelated regulatory pathway. The LPS-induced upregulation of CD40 and downregulation of CD1a and endocytosis did not appear to be mediated by the p38SAPK or NF-κB pathways and further work will need to be undertaken to dissect out the pathways involved in these processes. It will also be of interest to see whether other stimuli which result in monocyte derived DC maturation (such as TNF-α, IL-1β or monocyte conditioned medium) also use the same pathways. In keeping with this possibility, Sato *et al* have shown that TNF-α can activate the ERK2, JNK and p38SAPK pathways in monocyte derived DCs (Sato *et al.*, 1999).

Since different aspects of DC maturation appear to be regulated by different signal transduction pathways, it may be possible in the future to selectively block these pathways and thus manipulate the immune response towards anergy or activity. This could be useful in the treatment of autoimmune disease, malignancy or chronic infection.

Chapter 6

Preliminary Results of a Phase I Trial to Determine the Safety and Efficacy of Using CD34+ Derived DCs Pulsed with Tumour Lysate as a Treatment for non-Hodgkins Lymphoma.

6.0 Introduction

DCs have now been generated on a clinical scale from CD34+ progenitors from two patients with NHL using the serum-free method developed for this purpose (described in Chapter 3). These DCs were pulsed with a tumour lysate, as a source of tumour antigen, before reinfusion back into the patients. This method of antigen loading was chosen as it is a simple method, which would be generally applicable to all tumours, regardless of whether the tumour-specific antigens had been characterised or the HLA status of the patient is known.

These two patients were the first to be enrolled into a phase one study designed to determine the safety and efficacy of using tumour lysate pulsed, autologous, CD34+ derived DCs as a treatment for NHL. In this chapter the methods used and the clinical and immunological results available to date are presented, together with a discussion of the findings in the context of other DC vaccine trials published to date.

6.1 Materials and Methods

6.1.1 Patient Eligibility.

Patients were eligible for the study if they had relapsed or resistant B cell NHL and fulfilled the following criteria:

a) Either in relapse after previous high dose therapy/transplant procedure or in relapse and medically unsuitable for high dose therapy

b) Expected survival > 3 months

c) Age >16 yrs

d) Disease readily accessible to biopsy and able to be assessed clinically and/or radiologically.

Ethical approval was obtained from the local ethical committee (UCL ref no 96/3484).

6.1.2 Patient Characteristics.

At time of entry into the trial, RM was a 49 year old man with a long history of follicular NHL and CS was a 67 year old man with small cell lymphoma/ chronic lymphocytic leukaemia (though this was initially diagnosed as mantle cell lymphoma). Details of prior treatment is shown in Tables 6.1 and 6.2. After discussion of the study with the patients, which included written information, the patients' written consent was obtained.

6.1.3 Preparation of tumour cell lysates and cryopreservation of tumour cells.

Tumour samples were obtained from accessible sites. In the case of RM, circulating tumour cells were obtained from peripheral blood prior to his treatment with anti-CD20 antibody. Tumour cells were obtained from CS's bone marrow but this required 3 separate bone marrow aspirates. The samples were diluted 1:1 with PBS prior to processing using Ficoll-Hypaque density centrifugation (see chapter 2 section 2.0.5). Cells obtained from the interface were washed and the pellet resuspended in PBS, then lysed by 4 cycles of freeze-thawing. The resultant lysate was filtered through a 30μM filter followed by a 0.45μM filter in order to remove any cellular debris. The protein content of the lysate was assessed using a standard Bradford assay (Bradford, 1976). A small aliquot of tumour cells ($5x10^6$) were cryopreserved for use as targets in cytotoxicity assays.

6.1.4 Culture Medium.

As previously described in chapter 2, the culture medium consisted of X-VIVO 10 without phenol red or gentamicin (Biowhittaker, Walkersville Maryland), 10% citrated autologous plasma (collected at the time of leukapheresis), GM-CSF 100ng/ml (Novartis, Basel Switzerland), recombinant TNF-α 2.5ng/ml (R+D systems, Minneapolis) and heparin 2U/ml. From day 7 of culture IL-4 100ng/ml was added to the medium.

6.1.5 Generation of Dendritic Cells.

Small Scale Closed System Experiments

An aliquot of $1x10^6$ CD34+ cells were set up in culture as described in chapter 2 section 2.0.9 except that the cells were grown in a small Stericell culture bag (Baxter Healthcare Corp, Deerfield, Illinois, USA) instead of a tissue culture flask.

DATE	TREATMENT	OUTCOME
July 1985	Diagnosed stage IVa follicular NHL treated 24 months with chlorambucil	Complete remission
February 1992	Relapsed stage III (no BM involvement) treated with chlorambucil	Complete remission
August 1994	Relapsed. treated with CVP x 8 (cyclophosphamide, vincristine and prednisolone)	Partial remission
June 1995	Followed by fludarabine x 5	Complete remission
February 1996	BEAM 200 (BCNU, etoposide, cytarabine & melphalan) Peripheral Blood Stem Cell Transplant	Complete remission
April 1998	Relapsed 2 infusions with CD20 antibody	No Response
June 1998	Followed by FMD x 5 (fludarabine, mitozantrone & dexamethasone).	Complete remission

Table 6.1 Previous treatment regimens of RM and their outcome.

DATE	TREATMENT	OUTCOME
June 1998	Diagnosed with stage IV NHL. Treated with CHOP x 6 (cyclophosphamide, daunorubicin, vincristine and prednisolone)	Partial remission

Table 6.2 Previous treatment regimens of CS and their outcome.

Pilot Large Scale Closed System Experiment.

A total of $25x10^6$ CD34+ cells were set up in culture in serum-free medium with growth factors as in previous experiments but at a starting concentration of $2.5x10^5$ cells /ml and in a 12"x 15" Stericell bag. The bag was placed horizontally in a 37°C 5% CO_2 incubator and was only agitated on days 5 and 10 when fresh medium was added and on day 7 when IL-4 was added. The bag has 6 ports, after being used once the ports were heat sealed in an attempt to prevent infection, especially as the cultures were devoid of antibiotics. The characteristics of the patient from whom these cells were obtained is shown in Table 6.9.

Generation of Dendritic Cells For Clinical Use.

Stem cells were mobilised in RM by means of cyclophosphamide $1.5g/m^2$ followed by 3 vials (263µg x 3) of lenograstim™ per day administered subcutaneously for 11 days. In the case of CS ESHAP chemotherapy was used (etoposide $40mg/m^2$ for 4 days, *cis*-platin $25mg/m^2$ over 4 days, cytarabine $2g/m^2$ on day 1 only and methylprednisolone 500mg daily for 5 days), followed by a single vial (263µg) of lenograstim™ subcutaneously daily for 18 days. Mobilised peripheral blood MNCs were harvested by leukapheresis 11 and 13 days after the end of the chemotherapy in the 2 patients respectively. CD34+ cells were selected using the Clinimacs device; $20x10^6$ viable cells were obtained from RM and $1.1x10^9$ from CS. A total of $10x10^6$ (RM) and $25x10^6$ (CS) CD34+ cells were set up in culture in the large Stericell bags as described above. The starting volume of culture medium was100ml for both patients. Fresh medium and growth factors were added on days 5 and 10 in the case of CS, whereas in the case of RM fresh medium was only added on day 11. Thus the final volume of culture medium was 200ml in the case of RM and 400ml in the case of CS. Recombinant TNF-α (2.5ng/ml)(R and D systems, Minneapolis, USA) and recombinant human GM-CSF (100ng/ml)(Schering-Plough, Cork, Eire) were present throughout the culture period, IL-4 (50ng/ml)(Schering-Plough) was added from day 7 onwards. Surplus CD34+ cells were cryopreserved. The initial plan had been that if no significant toxicity was observed by day 14 after the first dose of DCs, a further aliquot of CD34+ cells would be thawed and expanded. This would be repeated for a maximum of 4 cycles unless disease progression occurred in which case the patients would be withdrawn from the study. Repeated vaccinations were not possible in RM due to a lack of CD34+ cells, nor in CS due to insufficient tumour lysate. Samples of the culture medium and cells were sent for microbiological testing two days prior to administering the cells back to the patient to ensure sterility of the vaccine.

6.1.6 Cryopreservation.

CD4+ T cells, CD8+ T cells and CD14+ monocytes for cryopreservation were resuspended in 1-2 mls of medium and an equal volume of tissue culture grade PBS (Dulbecco's phosphate buffered saline without calcium or magnesium, Sigma, Dorset, UK), containing 15% DMSO and 8% human serum albumin added as cryopreservative. The cells were then transferred to 2ml cryopreservative vials (Nalgene, Rochester, NY) and left at -80^{0}C overnight in a controlled rate freezing device (Nalgene, "Cryo 1^{0}C freezing container"). The following day the vials were quickly transferred to liquid nitrogen.

6.1.7 Antigen loading, washing and administration of dendritic cells.

At the end of the culture period (RM,18d; CS,14d) the cells were divided into 2 aliquots using a transfer pack. Assessment of volume was by weight. The tumour lysate was added to one half, 20mg lysate to 100ml medium in the case of RM and 14mg to 200ml in the case of CS, whilst 25μg (1 vial) of Pneumovax II (Pasteur Merieux, Berkshire, UK) was added to the other half. Pneumovax II consists of polysaccharide from each of 23 capsular types of pneumococcus. The cells were then returned to 37°C 5%CO_2 and left for 6 hours. After this time the cells were pooled and washed thoroughly using the COBE 2991 closed system cell washer. A 2 litre wash was performed with PBS supplemented with 1% human serum albumin (Bio Products Laboratory, Herts UK) (HSA). The cells from RM were suspended in a final volume of 30ml PBS/1%HSA and 85ml for CS. The cells were administered back to the patient intravenously over 5-15 minutes and the pulse, blood pressure, temperature and respiratory rate were monitored closely over the next 12 hours. RM received his DCs on 1.2.99. CS received his DCs on 1.10.99.

6.1.8 Selection of CD4+ and CD8+ cells for in vitro testing.

Pre-vaccination CD4+ and CD8+ cells were collected from the unselected fraction following CD34+ selection. Post-vaccination samples were obtained from peripheral blood. The cell selection techniques were as described earlier Chapter 2 Section 2.0.8.

6.1.9 CD4+ T cell proliferation assays.

CD4+ T cells which had been collected, pre- and post- vaccination and cryopreserved, were thawed and set up in parallel cultures with varying numbers of autologous DCs as previously described in Chapter 3 section 3.1.1. These cells were either pulsed with tumour lysate (20μg/ml), Pneumovax (4.5μg/ml) or left unpulsed. After 6 days, 1μCi of

^{3}H-thymidine ws added and left for 18 hours before harvesting the cells and measuring the incorporation of tritiated thymidine.

6.1.10 Measurement of pneumococcal antibodies and autoantibodies.

Pneumococcal antibodies were measured by ELISA at the Reference Laboratory based at the Immunology Laboratory at City Hospital, Birmingham UK. Autoantibodies were measured by the Immunology Laboratory, University College London Hospitals UK.

6.1.11 T cell receptor (TCR) CDR3 spectratyping.

These studies were performed by Dr Karl Peggs, Dept of Haematology, UCL. RNA was extracted from pre- and post-vaccination MNCs using Ultraspec RNA (BiotecX Laboratories, Houston, USA) according to the manufacturer's protocol. cDNA was generated from 1 μg of RNA in a 20 μl reaction using random hexanucleotide primers with Superscript reverse transcriptase (GibcoBRL, Paisley, UK). Each of 22 functionally rearranged Vβ gene subfamilies was amplified across the constant/variable junctions using the 24Vβ subfamily-specific primers described previously by Maslanka (Maslanka *et al.*, 1995), and a fluorescent dye-conjugated Cβ region-specific primer (FAM, Perkin Elmer, Cambridge, UK). Some of the Vβ primers amplify short PCR products, and others longer PCR products. "Short" and "long" Vβ primers were combined in duplex PCR reactions as follows: Vβ5.1+1; Vβ2+12; Vβ8+3; Vβ4+5.4; Vβ13+7; Vβ9+14; Vβ11+20; Vβ17+15; Vβ16+21; Vβ18+23; Vβ24+22. Vβ6.1 and Vβ6.2 were used unpaired.

The total PCR volume was 20 μl containing 2 x Genamp PCR buffer (Perkin Elmer), 2 mM $MgCl_2$, 0.2 mM each dNTP, 1 mM of each primer and 1 μl cDNA (equivalent to approximately 25 000 cells). The reaction mix was denatured for 5 minutes at 98°C then 0.5 U of Amplitaq DNA polymerase (Perkin Elmer) was added. Optimal cycling conditions were 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds for 30 cycles, followed by a final extension at 72°C for 5 minutes. 1 μl of PCR product was denatured in 12 μl formamide and electrophoresed through Performance Optimized Polymer 4 (Perkin Elmer) on an *ABI 310* automated sequencer (Perkin Elmer) in the presence of Tamra 500 size standard (Perkin Elmer). Genescan software 2.1 (Perkin Elmer) was used to analyze the data.

6.2 Results - Feasibility of generating CD34+ derived DCs in a closed culture bag system and of increasing this to a clinical scale.

As a prelude to clinical scale studies, two experiments were performed culturing $1x10^6$ CD34+ cells in 10mls of medium (X-VIVO10/10% citrated autologous plasma) in small Stericell bags instead of tissue culture flasks. In these experiments there was a 24 and 36 fold expansion in cell number over 14 days, and 12% and 8.4% of these cells were CD1a+CD14- respectively.

Subsequently, a clinical scale experiment was performed using a 12"x15" Stericell bag. In this experiment 25 x 10^6 CD34+ cells were set up at a starting concentration of 2.5 $x10^5$/ml (initial volume 100ml). The cell expansion was 5.7 fold over 14 days with 8.2% (11.7 x 10^6) of the cells being CD1a+. These cells were shown to be potent stimulators in an MLR (Fig 6.1).

6.3 Results - Phase 1 clinical study - The feasibility of using *ex vivo* generated autologous CD34+ derived DCs pulsed with tumour lysate as a treatment for NHL

6.3.1 Patient 1 RM

6.3.1a Treatment history, stem cell collection, DC generation and antigen loading.

RM had had multiple courses of treatment over aperiod of 14 years prior to recruitment into this study with varying degrees of response. His treatment history is shown in Table 6.1. Prior to mobilisation and collection of stem cells a haematological, biochemical and radiological evaluation was performed, results of which are shown in Table 6.3. Stem cells were mobilised as outlined in section 6.1.5. $10x10^6$ viable CD34+ cells were expanded into DCs over the course of the next 18 days. There was a 10 fold expansion in total cell number, 18% of which were CD1a+. The full immunophenotype is shown in Table 6.4. These cells were split into two aliquots and pulsed with 20mg of irradiated tumour lysate or 25μg of pneumococcus polysaccharide for 6 hours before being pooled and thoroughly washed. After washing $63x10^6$ cells remained. These were suspended in 30ml of PBS/1% HSA and injected intravenously over 15 minutes into RM (who had been pre-medicated with piriton 4mg iv and hydrocortisone 100mg iv).

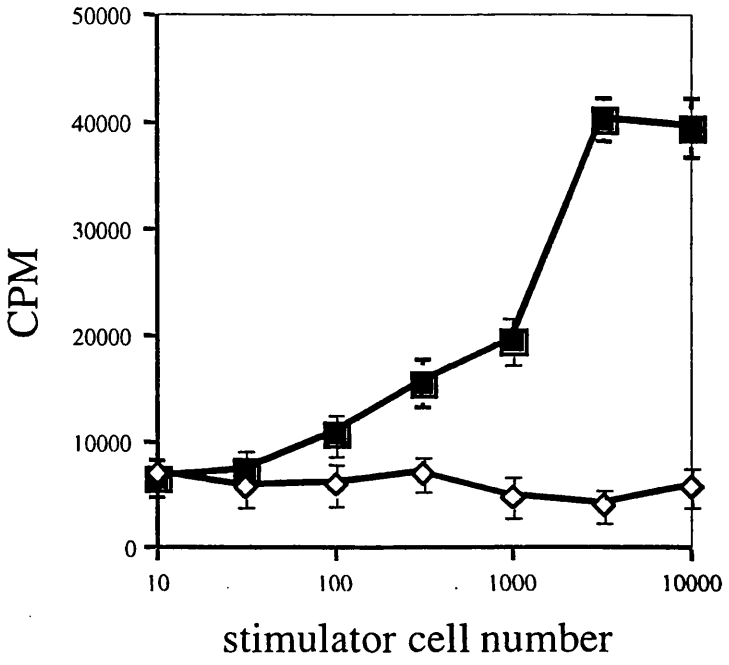


Figure 6.1 Mixed Lymphocyte Response of DCs generated from CD34+ cells in a large Stericell bag.
DC (■) or monocytes ◇) were used as stimulators and allogeneic MNCs were used as responders in this MLR. ^{3}H-thymidine incorporation, which is a measure of DNA synthesis and thus cell proliferation, was measured using a scintillation counter. DCs can be seen to induce a greater degree of proliferation of MNCs than monocytes. Each data point is the mean of 3 replicates. Error bars represent SEM.

WBC $3.5x10^9$/l	(3.0-10.0)	Sodium 145mmol/l	(136-145)
Hb $13.9x10^{12}$/l	(13-17)	Potassium 3.6 mmol/l	(3.5-5.1)
Plt $94x10^9$/l	(150-400)	Urea 5.2 mmol/l	(2.8-7.6)
Neut $2.8x10^9$/l	(2.0-7.5)	Creatinine 83 µmol/l	(80-133)
Lymph $0.7x10^9$/l	(1.5-4.0)		
		Bilirubin 8 µmol/l	(3-17)
ESR 5mm/hr		Alkaline phosphatase 72U/l	(45-122)
		Alanine transaminase 25U/l	(8-63)
Reticulocytes 1.4%	(<2%)	Aspartate transaminase 26U/l	(9-45)
		Albumin 50g/l	(35-50)
Vitamin B12 656pg/ml	(180-700)		
Red cell folate 682ng/ml	(150-650)	Calcium 2.4 mmol/l	(2.20-2.26)
		Magnesium 0.83 mmol/l	(0.6-1.1)
Blood group O rhesus negative		Urate 372µmol/l	(200-416)
No irregular antibodies detected		Lactate dehydrogenase 471U/l	(230-460)
Direct antiglobulin test negative			
Prothrombin time 12/11.5 secs			
Activated partial thromboplastin time 33/31 secs			
Thrombin time 11/15 secs			
Bone marrow aspirate: and trephine No evidence of lymphoma		CT scan chest, abdomen and pelvis: para-aortic lymphadenopathy present.	
Bone marrow cytogenetics: normal			

Table 6.3 Results of the haematological. biochemical and radiological evaluation of RM prior to vaccination.

Cell surface antigen	Percentage of output cells positive
CD40	20.9
CD86	6.1
CD1a	18.0
CD80	2.2
HLA DR	32.8
CD14	32.4
CD83	9.3

Table 6.4 Immunophenotype of the DCs generated from RM prior to pulsing with antigen.

6.3.1b Immediate side effects.

RM experienced no immediate side effects after DC administration, in particular there was no fever, chills, rigors or rashes, and his pulse, blood pressure and respiratory rate also remained normal. After 12 hours he was discharged from hospital.

6.3.1c Follow-up

RM was followed up at regular intervals 1) to determine if he was experiencing any untoward side effects and 2) to evaluate whether he had experienced any sort of response either clinically, radiologically or on immunological testing.

(i) Later side effects

On a clinical level, at no point to date (16 months after the vaccination) have any symptoms been experienced by RM, nor signs detected on examination, which could be considered a side effect of the DC therapy. His serum has also been tested repeatedly to look for the development of any autoantibodies, a possible side effect of DC treatment. Samples taken 15, 31, 48, 189 and 472 days after the DC infusion have all tested negative for gastric parietal cell, anti-nuclear, smooth muscle, mitochondrial, thyroglobulin and thyroid microsome antibodies. In addition, the direct antiglobulin test remains negative.

(ii) Clinical and radiological responses

At the time of DC administration no lymphoma was detectable clinically. This remains the case 16 months later. Radiologically, the para-aortic lymphadenopathy noted prior to the DC infusion has remained unchanged on CT scans performed 31 and 190 days later. In addition, the bone marrow when examined 87 days after the vaccination, was also clear of disease.

(iii) Immunological responses

Humoral response

In order to determine whether DC vaccination can prime an *in vivo* immunological response, some of the DCs infused into the patient had been pulsed with a control antigen in addition to being pulsed with tumour lysate. The control antigen used was the polyvalent pneumococcal vaccine (Pneumovax). This was chosen as a control antigen for several reasons; 1) it was one of the few vaccines available which did not contain phenol or aluminium hydroxide. 2) the titre of antibodies developing against pneumococcal antigens can be measured by the pneumococcal reference laboratory in Birmingham UK, and 3) *in vitro* pilot studies showed that CD34+ derived DCs could

present this antigen to autologous CD4+ cells and induce significant proliferation (Fig 6.2).

Accordingly, serum samples were analysed prior to, and at intervals after, vaccination for changes in anti-pneumococcal antibody titres. As can be seen in Table 6.5 there was no increase in anti-pneumococcal antibody titre in the first 46 days following the vaccination. However, when measured 157 days after the vaccination an increase in total IgG and IgG_2 was noted, though this still remained below the reference limit. The antibody level then reverted to its original levels by day 472.

Cellular responses

In order to determine whether the vaccination had induced CD4+ T cell responses to tumour and pneumococcal antigens. An aliquot of CD34+ cells which had been cryopreserved was thawed and used to generate DCs under standard conditions in a flask (Chapter 2, section 2.0.9). These were then pulsed with either tumour lysate (20μg/ml) or pneumococcal vaccine (4.5μg/ml) or left unpulsed. These cells were subsequently used as stimulators in proliferation assays with autologous CD4+ T cells which had been collected pre-vaccination and 15, 31 and 46 days after the vaccination and cryopreserved. The CD4+ T cells were thawed concurrently and all 4 assays were performed at the same time.

As can be seen in figure 6.3, prior to vaccination, CD4+ T cells when stimulated with DCs pulsed with tumour lysate did not proliferate to a greater degree than those stimulated with DCs alone. In fact, the level of proliferation of the CD4+ T cells when stimulated with DCs pulsed with tumour lysate appeared to be significantly below that of those stimulated with DCs alone. This was also the case for the CD4+ T cells stimulated with pneumococcal antigen.
By day 15 the situation was reversed for CD4+ cells stimulated with DCs that had been pulsed with tumour lysate; there was significantly more proliferation of CD4+ cells when stimulated with DCs pulsed with tumour lysate than of CD4+ cells stimulated with DCs alone. However, the level of proliferation of the CD4+ T cells stimulated with DCs pulsed with pneumococcal antigen remained significantly below that of those stimulated with DCs alone. At day 31, there was no apparent difference between the level of proliferation of CD4+ T cells stimulated with DCs alone or DCs pulsed with tumour antigen. The lower level of proliferation of the CD4+ T cells stimulated with DCs pulsed with pneumococcal antigen remained. On day 46 the pattern was the same as on day 15.

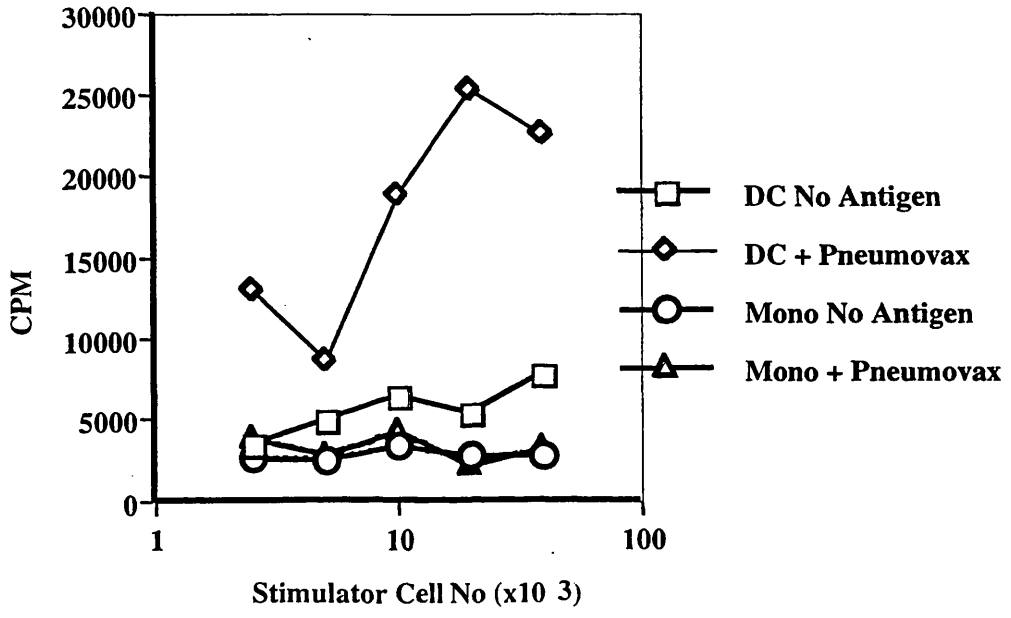


Figure 6.2 CD34+ derived DCs are able to present pneumovax antigens to autologous CD4+ T cells and induce their proliferation.Monocytes were used as a control cell.

	Reference	DAY 0	DAY 15	DAY 31	DAY 48	DAY 157	DAY 472
IgG	640	21	10	5	5	242	12
IgG_1	30	5	5	5	5	5	5
IgG_2	160	5	5	5	5	56	5

Table 6.5 Titre of anti pneumococcal antibodies pre and post vaccination in RM

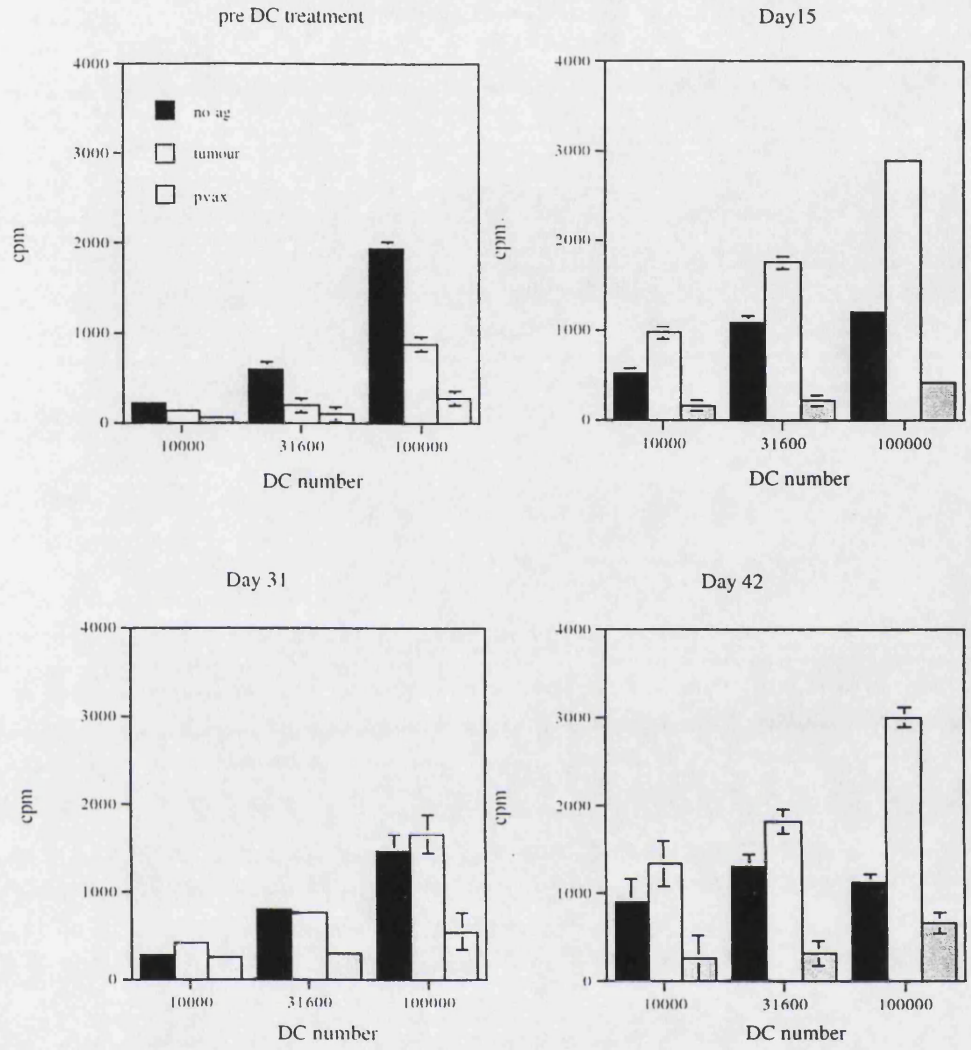


Figure 6.3 CD4+ T cell proliferative responses of RM pre and post vaccination. The proliferative response, as measured by ^{3}H-thymidine uptake, of RM's CD4+ T cells is shown after stimulation with varying numbers of irradiated DCs, either in the presence of tumour lysate, pneumovax (pvax) or no antigen. CD4+ T cells were collected prevaccination and at varying intervals afterwards. Each data point is the mean of 3 replicates. Error bars represent SEM.

These findings show that prior to the vaccination, both tumour and pneumococcal antigens suppressed the proliferation of CD4+ T cells. However following the vaccination the proliferation of the CD4+ T cells stimulated with DCs pulsed with tumour antigens was significantly above background on at least 2 of the days tested, implying that the vaccination may have resulted in the increased ability of CD4+ T cells to proliferate in response to tumour antigens. This was more striking as the proliferative response induced by DCs pulsed with pneumococcal antigen remains consistently low after the vaccination.

Cytotoxicity assays could not be performed as the target tumour cells which had been cryopreserved were not viable upon thawing.

TCR spectratype analysis was performed on peripheral blood collected pre and post-vaccination. During T cell development, TCR DNA rearrangement occurs. One member of the 22 Vβ family groups of genes is joined via a joining segment to the Cβ segment to give rise to the complete complementarity-determining region (CDR) 3 of the TCR β chain gene. During this process, a random number of nucleotides are inserted and deleted enzymatically at the junctions between gene segments. Since productive rearrangements are required for continued T cell development, functional CDR 3 lengths differ by multiples of three nucleotides amongst different T cell clones. The reverse transcriptase-polymerase chain reaction (RT-PCR) products spanning the CDR 3 regions can be separated according to size. The number of peaks, along with their size and distribution gives an indication of T cell repertoire diversity. The spectratypes obtained with each of the 24 different Vβ primers consisted of between 6-10 size class peaks each. The amount (peak size) was typically distributed in a normal Gaussian fashion, with most of the PCR product being in the medium size range (Fig 6.4). No significant difference was detected between most of the Vβ spectratypes of T cells obtained pre and post-vaccination. However the Vβ15 spectratype was altered, there being a significant increase in size of one of the spikes 157 days post-vaccination, indicating the expansion of at least one of the T cell clones following the vaccination (Fig 6.4). The long time interval between the vaccination and the sample being taken and the fact that only one sample was taken which showed this change, should however be noted.

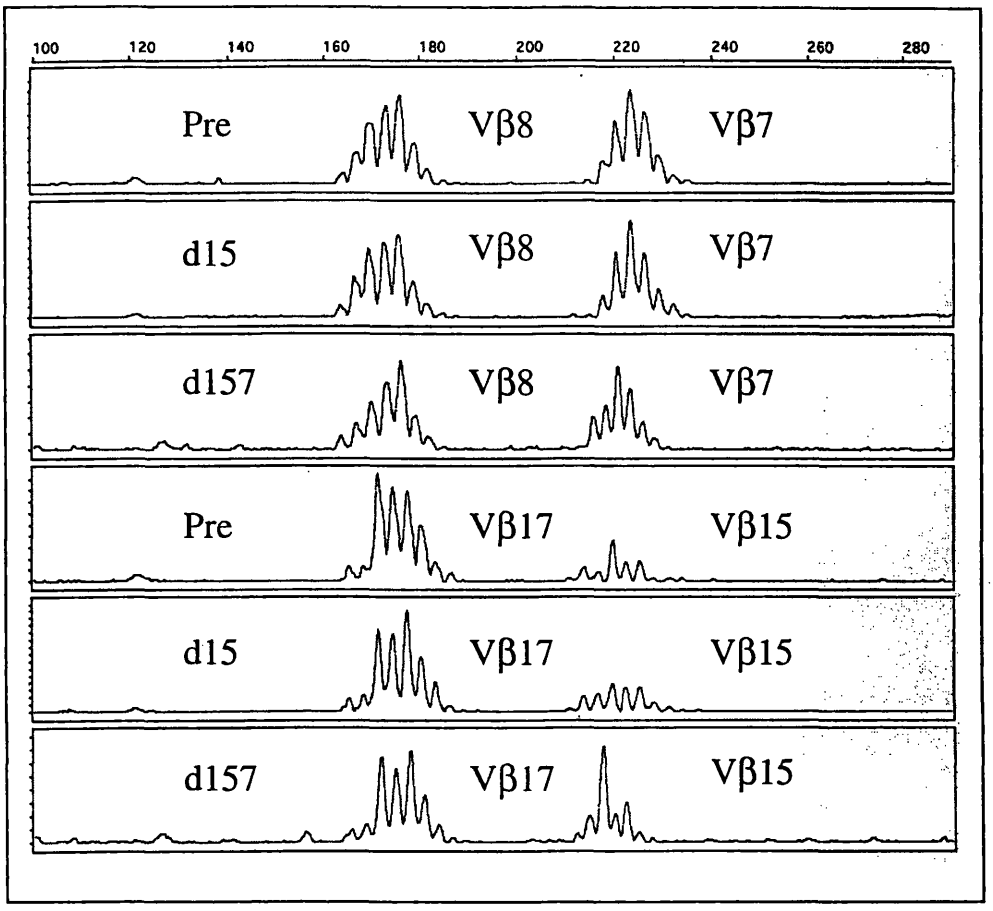


Figure 6.4 Representative results of T cell spectratyping performed on samples taken from RM pre and post vaccination. The upper 3 lines are representative of the majority of Vβ families analysed there being no change in the spectratype appearance pre and post vaccination. In the lower 3 lanes it can be seen that 157 days following the vaccination that one of the peaks in the Vβ15 family is more pronounced.

6.3.2 Patient 2 CS

6.3.2a Treatment history, stem cell collection, DC generation and antigen loading.

CS was initially treated with 6 cycles of CHOP (cyclophosphamide, daunorubicin, vincristine and prednisolone) chemotherapy and achieved a partial response. Prior to mobilisation and collection of stem cells a haematological, biochemical and radiological evaluation was performed, results of which are shown in Table 6.6. Stem cells were then mobilised as outlined in section 6.1.5. Cells were collected 18 days after the beginning of the mobilising regimen. This was 3 days later than had been predicted due to an episode of sepsis during the period of neutropenia, which required hospitalisation and treatment with antibiotics. A total of 25 $x10^6$ viable CD34+ cells were expanded into DCs over the course of the next 14 days. There was a 12.1 fold expansion in total cell number, 27.2% of which were CD1a+. The full immunophenotype is shown in Table 6.7. After treatment with tumour lysate or pneumococcal polysaccharides, as outlined in section 6.1.7, 208 $x10^6$ viable cells remained. These were suspended in 85ml of PBS/1%HSA and injected intravenously over 5 minutes into CS (who had received no premedication).

6.3.2b Immediate side effects.

CS experienced no immediate side effects after DC administration, in particular there was no fever, chills, rigors or rashes, and his pulse, blood pressure and respiratory rate also remained normal. After 12 hours he was discharged from hospital.

6.3.2c Follow-up

CS was followed up at regular intervals.

(i) Later side effects

CS has at no point to date (8 months after vaccination) had any symptoms or signs which could be considered a side effect of the DC therapy. His serum has also been tested repeatedly to look for the development of any autoantibodies. Samples taken 15, 28, 42 and 201 days after the DC infusion have all tested negative for gastric parietal cell, antinuclear, smooth muscle, mitochondrial, thyroglobulin and thyroid microsome antibodies. In addition the direct antiglobulin test remains negative.

WBC $4.0x10^9/l$	(3.0-10.0)	Sodium 142mmol/l	(136-145)
Hb $13.1x10^{12}/l$	(13-17)	Potassium 4.4 mmol/l	(3.5-5.1)
Plt $196x10^9/l$	(150-400)	Urea 5.8 mmol/l	(2.8-7.6)
Neut $2.2x10^9/l$	(2.0-7.5)	Creatinine 142 µmol/l	(80-133)
Lymph $1.2x10^9/l$	(1.5-4.0)		
		Bilirubin 7 µmol/l	(3-17)
ESR 7mm/hr		Alkaline phosphatase 74U/l	(45-122)
		Alanine transaminase 34U/l	(8-63)
Reticulocytes 1.1%	(<2%)	Albumin 45g/l	(35-50)
Vitamin B12 466pg/ml	(180-700)	Calcium 2.28 mmol/l	(2.20-2.26)
Red cell folate 263ng/ml	(150-650)	Magnesium 0.84 mmol/l	(0.6-1.1)
		Urate 341µmol/l	(200-416)
Blood group AB rhesus negative		Lactate dehydrogenase 526U/l	(230-460)
No irregular antibodies detected			
Direct antiglobulin test negative			
Prothrombin time 11/11.5 secs			
Activated partial thromboplastin time 27/34 secs			
Thrombin time 12/13 secs			
Bone marrow aspirate: lymphoid infiltrate 49% of nucleated cells		CT scan chest, abdomen and pelvis: Prior to ESHAP : para aortic and iliac lymphadenopathy.	
Bone marrow trephine: 70% infiltrated with lymphoma		Following ESHAP but before vaccination: para aortic and iliac nodes visible but not enlarged by CT criteria.	
Bone marrow cytogenetics: normal			

Table 6.6 Results of the haematological, biochemical and radiological evaluation of CS prior to vaccination.

Cell surface antigen	Percentage of output cells positive
CD40	56.1
CD86	6.6
CD1a	27.2
CD80	9.5
HLA DR	80.8
CD83	0.8

Table 6.7 Immunophenotype of the DCs generated from CS prior to pulsing with antigen.

(ii) Clinical and radiological responses

At the time of DC administration, no lymphoma was detectable on clinical examination. This remains the case 6 months later.

Radiologically, the para-aortic lymphadenopathy noted prior to the ESHAP chemotherapy had shrunk to within normal limits just prior to the DC infusion. This appearance remained unchanged by day 28. However, the scan performed 118 days after the infusion showed that there had been some disease progression with an increase in axillary, subclavian and retroperitoneal lymphadenopathy.

The bone marrow when examined 118 days after the infusion was still infiltrated with lymphoma (estimated at 70% on trephine biopsy).

(iii) Immunological responses

Humoral response

As can be seen in Table 6.8, there was no significant increase in anti-pneumococcal antibody titre in the first 201 days following the vaccination.

Cellular responses

Prior to vaccination, the proliferative response of autologous CD4+ T cells to DCs pulsed with tumour antigens was equivalent to, or less than, the proliferative response of the CD4+ T cells to DCs alone, i.e. the background level of proliferation. Once again, as in the case with RM, the proliferative response of the CD4+ cells pre-vaccination to DCs pulsed with pneumococcal antigens was significantly below background levels (Fig 6.5).

The proliferative response of CD4+ T cells collected 15, 28 and 42 days post-vaccination to DCs pulsed with tumour antigens was measured. On no occasion was this found to exceed background levels of proliferation. A similar finding was obtained when CD4+ T cells, obtained at the same time intervals post-vaccination, were stimulated with DCs pulsed with pneumococcal antigens (Fig 6.5). These findings indicate the failure of the vaccination to induce a significant CD4+ T cell response to tumour and pneumococcal antigens, at least when assessed in this way.

Attempts were made to perform cytotoxicity assays using CD8+ T cells collected pre and post-vaccination. The target cells were autologous cryopreserved tumour cells obtained from bone marrow aspirates prior to the vaccination. Unfortunately, these target cells did not label with ^{51}Cr and no result was obtained.

	Reference	DAY 0	DAY 28	DAY 42	DAY 118	DAY 201
IgG	640	341	394	260	242	333
IgG_1	30	40	31	10	5	26
IgG_2	160	97	65	48	56	84

Table 6.8 Titre of anti pneumococcal antibodies pre and post-vaccination in CS.

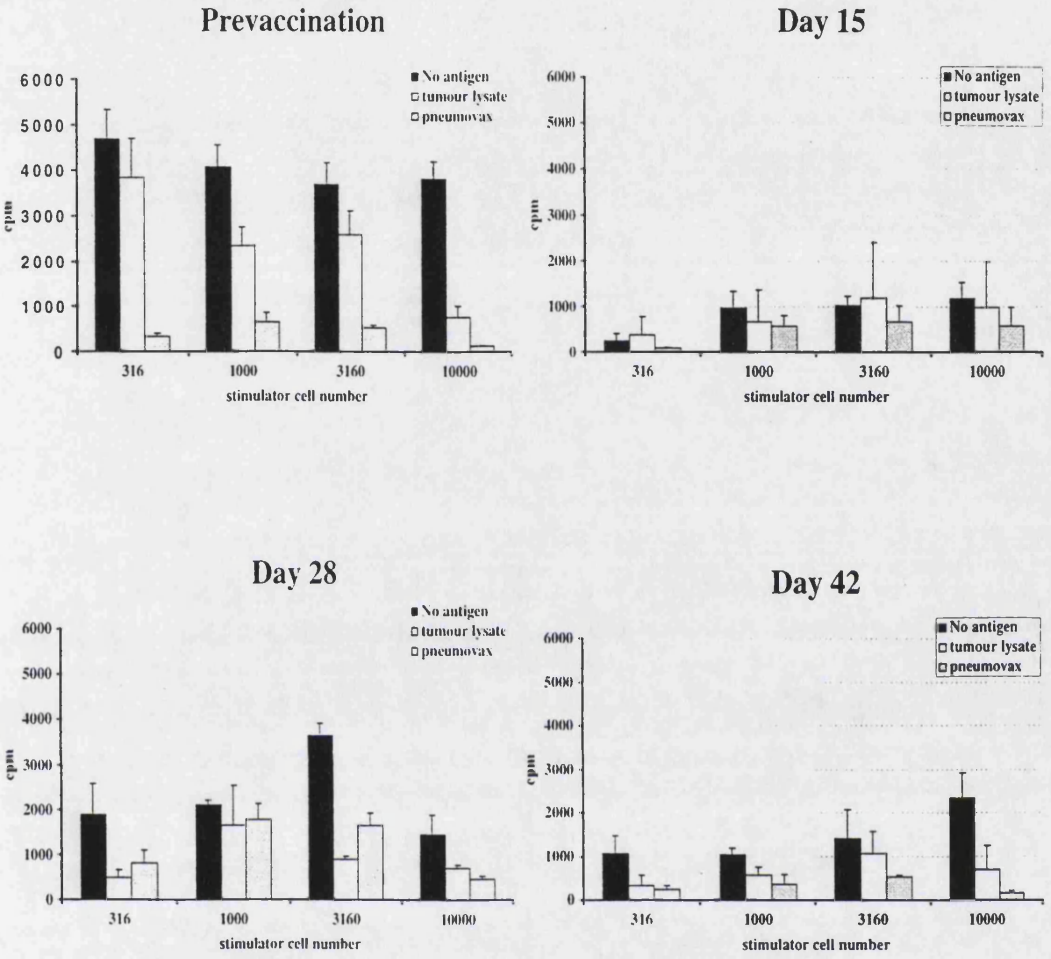


Figure 6.5 CD4+ T cell proliferative responses of CS pre and post vaccination.
The proliferative response, as measured by ^{3}H-thymidine uptake, of CS's CD4+ T cells, when stimulated with varying numbers of irradiated DCs either in the presence of tumour lysate, pneumovax or no antigen is shown. CD4+ T cells were collected prevaccination and at varying intervals afterwards. The experiments were run in parallel. Each data point is the mean of 3 replicates. Error bars represent SEM.

Spectratypes of the 22 different Vβ family groups of genes detected in the T cells obtained from CS pre-vaccination consisted of between 6-10 size class peaks each. The amount was again typically distributed in a normal Gaussian fashion (Fig 6.6). No significant difference was detected between most of the Vβ spectratypes of T cells obtained pre and post-vaccination. However the Vβ22 spectratype was altered, there being a significant increase in size of one of the spikes by day 15 post-vaccination, which persisted until the latest sample at day 42 at least, indicating an expansion of at least one of the T cell clones following the vaccination (Fig 6.6).

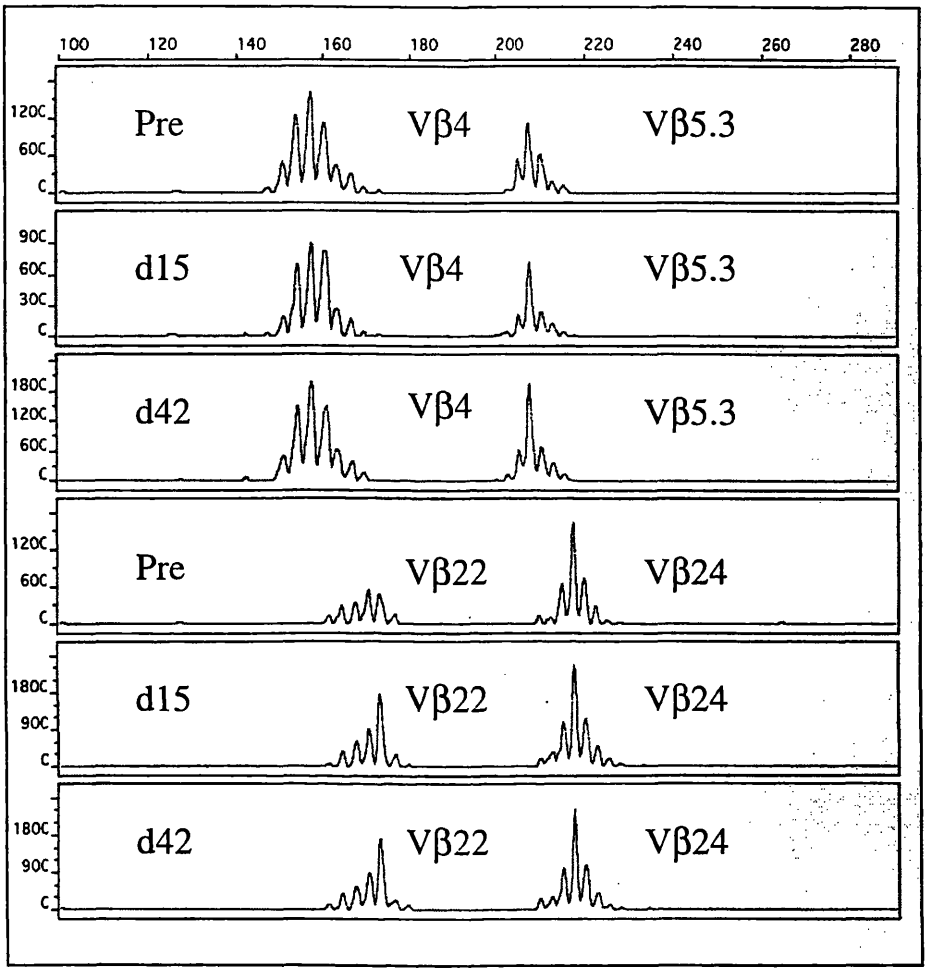


Figure 6.6 Representative results of T cell spectratyping performed on samples taken from CS pre and post vaccination. The upper 3 lines are representative of the majority of Vβ families analysed there being no change in the spectratype appearance pre and post vaccination. In the lower 3 lanes it can be seen that from 15 days following the vaccination that one of the peaks in the Vβ22 family is more pronounced.

6.4 Discussion

Having established a laboratory scale culture system for the generation of DCs suitable for clinical use, attempts were made to determine whether this system could be expanded to a clinical scale. Initial experiments, where cells were cultured in small closed system Stericell bags instead of flasks, appeared promising, there being no significant reduction in cell expansion (30 fold, n=2 vs 38 fold in Chapter 3 section 3.2.1) or in cells with the CD1a+CD14- phenotype (10.2%, n=2 vs 12.6% in Chapter 3 section 3.2.3). However, when the starting number of CD34+ cells was increased, with the concomitant increase in volume of culture medium and the use of larger closed system clinical scale Stericell bags, a marked reduction in cell expansion was noted (5.7 fold vs 30 fold). On a more encouraging note 8.2% (11.7 x 10^6) of these output cells were found to be CD1a+. They also proved to be potent stimulators in an MLR.

The number of DCs required to initiate immune responses in immunotherapy protocols is unknown, but clinical responses have been seen when as few as $1x10^6$ DCs have been used per vaccination (Nestle *et al.*, 1998). This level of expansion was achievable even on this larger scale. In view of this, and reassured by the functional integrity of the output cells, this clinical scale system was used to generate DCs from CD34+ cells for therapeutic use in two patients.

The cell expansion achieved in these two therapeutic clinical scale DC cultures was 10 fold (RM) and 12.1 fold (CS) respectively. Although it is tempting to ascribe this reduced cell proliferation to the fact that the CD34+ cells were derived from heavily pretreated patients, which is certainly the case with RM and the donor of the cells used in the pilot clinical scale experiment, this was not the case with CS (Table 6.9). More likely, the reduced cell expansion is due to either the increase in cell culture volume (with the concomitant reduction in aeration) or features peculiar to the larger Stericell bags (poor gas transfer, toxicity etc) or a combination of the two. A formal comparison between cells cultured on this large scale in flasks and in bags will be required to elucidate this further.

Despite the lower cell expansion in the two therapeutic clinical scale DC cultures, the percentage of cells which were CD1a+ (18.5% and 27.2%) was in the expected range when compared to results of the smaller scale cultures (12.2-32.9%, n=10)(Chapter 3 Table 3.2).

	Pre-treatment	Cell Expansion	Immuno-phenotype
Donor for pilot study	12 wk PACEBOM 1 x DexaBEAM 1x ESHAP	5.7	8.2% CD1a+CD14-
RM	Chlorambucil > 24 months, CVP x 8, Fludarabine x 5, BEAM SCT, 2 x CD20 Ab, FMD x 4 Cyclophosphamide.	10.0	18.0% CD1a+
CS	CHOP x 6	12.0	27.2% CD1a+

Table 6.9 Prior treatment of the 3 patients from whom CD34+ stem cells were obtained and differentiated into DCs using the large scale Stericell culture bags.
PACEBOM =prednisolone, adriamycin, cyclophosphamide, etoposide, bleomycin, vincristine & methotrexate. DexaBEAM = dexamethasone. BCNU, etoposide, cytarabine & melphalan. ESHAP = etoposide, cisplatin, cytarabine, methylprednisolone. CVP = cyclophosphamide, vincristine and prednisolone. BEAM SCT = BCNU, etoposide, cytarabine & melphalan stem cell transplant. FMD = fludarabine, mitozantrone & dexamethasone. CHOP = cyclophosphamide, daunorubicin, vincristine and prednisolone.

The cells which were generated in this closed bag system were divided into 2 aliquots and pulsed with either a tumour lysate or pneumococcal antigens for 6 hours before being pooled, washed thoroughly and infused into the patient.

A tumour lysate was used here as a source of tumour-specific antigens. In most DC trials reported to date, DCs have been loaded with tumour-specific peptides or proteins (Table 6.10). Using tumour-specific peptides and proteins as a source of tumour-specific antigens does have its limitations. These include the following: 1) Tumour-specific antigens and their relevant peptides are only known for a few tumours, thus restricting the range of tumours currently amenable to this form of DC therapy. 2) The tumour-specific peptides which are used are often only presented in the context of certain HLA haplotypes, e.g the melanoma peptide Mage-3A1, is only presented on patients cells expressing HLA A1. This restricts the use of DCs pulsed with this peptide to patients who are HLA A1+ (Thurner *et al.*, 1999). 3) Limiting the number of tumour-specific antigens presented by the DC increases the theoretical chance of immune escape by the tumour, or may result in immunoselection by the DC therapy for lesions which are negative for the tumour antigen targeted (Thurner *et al.*, 1999). 4) In the case of malignant lymphomas and multiple myeloma, each patient has their own unique tumour antigen in the form of the idiotypic determinant. Although in theory this could lead to patient-specific therapy, in practice to pulse DCs with this tumour-specific protein or peptidewould require much time and effort to purify the protein or sequence and synthesise the relevant peptide for each patient enrolled into a study.

Using tumour lysates can therefore broaden the range of tumours amenable to DC therapy to include those many tumours for which tumour-specific antigens are not known. The therapy is not HLA restricted and, by offering numerous antigens to the DC, the T cell immune response generated against the tumour is broad and the chance of immune escape is theoretically reduced. Particularly salient, in view of the time, effort and cost of labour involved, using tumour lysates may obviate the need to characterise the tumour-specific idiotypic determinant for each individual patient with lymphoma or myeloma.

Studies in mice (Fields *et al.*, 1998) and humans (Nestle *et al.*, 1998) have demonstrated the efficacy of DCs pulsed with tumour lysates in mediating anti-tumour immune responses. A potential disadvantage, however, of using tumour lysates is the possibility of inducing autoimmune reactivity to self antigens present in the tumour lysate, and indeed, murine studies have shown that repeated presentation of self antigens by DCs can result in the development of autoimmune disease (Ludewig *et al.*, 1998). In the

study by Nestle *et al* where melanoma patients were vaccinated with peptide or tumour lysate pulsed DCs, the development of autoantibodies was demonstrated in 4 patients (Nestle *et al.*, 1998). This was not accompanied by signs or symptoms of clinical disease. Paradoxically, those patients who developed autoantibodies were all treated with DCs pulsed with a cocktail of tumour-specific peptides and not tumour lysates.

Another method which has been used to load DCs with tumour antigens is the fusion of allogeneic DCs with autologous tumour cells. This method has several of the same advantages as using cell lysates as an antigen loading technique. Kugler *et al* (Kugler *et al.*, 2000) have recently used this technique to load DCs with antigen in a trial involving patients with metastatic renal cell carcinoma, with some promising results (Table 6.10).

The optimal length of time for which DCs should be pulsed with tumour antigens, the optimal concentration of the tumour antigens, the required dose of DCs, the maturation status of the DC, the most effective schedule of administration and the best route of vaccination are all unknown variables and differ widely in the studies published so far. In the study described here, a 6 hour period for antigen loading was used. This falls within the broad range used in other studies of between 2 hours and 48 hours. The DCs were pulsed with the tumour lysate at protein concentrations of 70 μg/ml (CS) and 200μg/ml (RM). The concentration at which antigens were used in the published series ranges from 1μg/ml to 100μg/ml. However, the control pneumococcal antigens were provided at much lower concentrations (125ng-250ng/ml), which may in part explain the lack of response to these antigens in both patients.

With respect to the dosing schedule, the initial aim in the study was to freeze down several aliquots of CD34+ cells at the time of stem cell collection, and at monthly intervals to thaw an aliquot and expand the cells into DCs. Each batch of DCs would then be pulsed with tumour lysate and infused into the patient, with a provisional maximum of 4 doses. Unfortunately it was not possible to adhere to this schedule. In the case of RM, only $20x10^6$ CD34+ cells were collected at the time of harvest; $10x10^6$ of these were set up in culture immediately and $10x10^6$ were set aside for *in vitro* testing, leaving no CD34+ cells for later expansions. In the case of CS, $1.11x10^9$ CD34+ cells were collected, but there was only enough tumour lysate for one vaccination.

The number of cells returned to RM with the single intravenous vaccination was 63 x 10^6, and to CS, 208 x 10^6. These cell doses are high when compared with other studies, the highest cell dose returned intravenously on any one occasion so far reported being100 x10^6 (Morse *et al.*, 1999b)(Table 6.10).

Many different routes of DC administration have been used in the clinical studies published to date. These range from intranodal, on the basis that this is the site at which antigen presentation will occur to T cells and therefore direct injection here will maximise the cell delivery to the node, to intradermal, with the argument that this more closely resembles the physiological process. In fact, several studies have used a combination of these different routes (Table 6.10). A potential disadvantage of the intravenous route is that large numbers of cells may be sequestered and destroyed in the lung and may even result in a compromise in lung function, though this latter potential adverse effect has not been reported to date. A study by Morse *et al* has shown that after intravenous injection indium-111 labelled DCs initially localise to the lungs and then redistributed to the spleen, liver and bone marrow. They did not localise in the lymph nodes or tumour masses. Following intradermal injection a small percentage of cells migrated to the local lymph nodes whereas following subcutaneous injection no lymph node localisation was detected (Morse *et al.*, 1999a).

The DCs used in this study, unlike any of the studies shown in table 6.10, have been generated from CD34+ progenitor cells. These DCs were not matured prior to being loaded with tumour antigens. There is controversy as to whether mature or immature DCs are more appropriate for use in DC vaccines. Indeed, different studies have used DCs of different maturation status (Table 6.10). Immature DCs have the advantage of being able to take up large amounts of antigen, whilst mature DCs are more potent stimulators of T cells. Studies by Labeur *et al* in mice (Labeur *et al.*, 1999) support the use of mature tumour antigen pulsed DCs as cancer vaccines. Morse *et al* have looked at the timing of maturation in relation to antigen loading in a murine system (Morse *et al.*, 1998). They have shown that, in the case of loading DCs with tumour peptides, maturation is best performed prior to loading, whilst if loading is to be achieved by transfecting the DCs with RNA, then the maturation step should follow the antigen loading procedure to ensure optimal cytotoxic lymphocyte (CTL) stimulation.

In both patients enrolled into the study described here, no immediate side effects were apparent, though in the case of RM his premedication with piriton and hydrocortisone may have masked potential adverse reactions. To date, which is 16 months post DC vaccination (RM) and 8 months (CS), no later side effects have been noted, in particular, there has been no evidence of any autoimmunity, which was a particular concern in view of the findings mentioned earlier.

CS did, however, become septic whilst neutropenic following the ESHAP chemotherapy used for mobilising his stem cells. This should currently be classified as a

toxicity of the protocol. Sepsis requiring hospitalisation occurs in 8% of patients following ESHAP chemotherapy (Watts *et al.*, 2000). If, however, as seems likely, DC therapy for lymphoma is used in the context of treating minimal residual disease following an autologous stem cell transplant, then the initial stem cell mobilisation procedure used to collect stem cells for the autograft would, in the majority of cases, provide enough stem cells to use to generate DCs as well. This would mean that, in most cases, no extra mobilisation procedure would be necessary to provide CD34+ cells to generate DCs and thus the toxicity associated with an additional mobilisation would be avoided.

In evaluating the response of a patient to DC therapy both clinical and immunological responses need to be considered, though it must be borne in mind that an immunological response may not necessarily lead to a clinical response. As regards RM, the para-aortic lymphadenopathy present prior to the DC vaccination remained unchanged and clinically there appeared to be no progression of his disease16 months later. Looking at this finding in isolation, one could attribute this to either the DC vaccination or to the effect of the cyclophosphamide used to mobilise his stem cells. However, the immunological findings, although not entirely consistent, do raise the possibility that the DC vaccine may have played some part in stabilising his disease. It can be seen that prior to vaccination, tumour antigens presented by DCs to CD4+ T cells suppressed the proliferation of CD4+ T cells below background levels. This situation was reversed following the vaccination, with increased responsiveness of CD4+ T cells to tumour antigens. This indicates that, at the very least, the vaccine had resulted in an immunological response, which was present for up to 46 days post-vaccination. The results of the TCR spectratyping showed that, post-vaccination there was an expansion of at least one T cell clone at the latest time point examined (day 157). Although it is tempting to ascribe this to the expansion of a T cell clone directed against tumour antigens, there was no direct evidence for this. Indeed, it must be emphasised that these immunological findings do not necessarily translate into the vaccination being the cause of his current stable clinical condition.

The fact that the pneumococcal antigens presented by the DCs consistently suppressed the proliferative responses of the CD4+ T cells below background levels cannot be ignored, but it is difficult to explain. It may be that the preservatives contained in the Pneumovax preparation inhibited T cell proliferation, but this was not found to be the case in pilot studies performed in exactly the same way which showed that DCs can present pneumococcal antigens to CD4+ cells and induce significant proliferation (Fig 6.1).

It was on the basis of these studies that the pneumococcal vaccine was chosen as a positive control antigen. In retrospect, the choice of this as the control antigen was flawed. Firstly, part of the reason to include a control antigen was to determine whether the vaccination induced a humoral response. However, the measurement of anti-pneumococcal antibodies is notoriously difficult and the results need to be interpreted with some caution. Secondly, no attempt was made to measure the titre of IgM antibody, which would be the isotype of antibody produced in the initial 6 weeks if the vaccination induced a primary antibody response. It must be said, however, that the majority of adults would have been exposed to at least the commoner of the 23 capsular polysaccharides included in the vaccine and therefore the antibody response mounted would not be a primary response. In the case of RM, it could in fact be postulated that the raised level of IgG_2 measured 157 days after the vaccination represented the tail of the IgG response to the vaccination, the peak of which had been missed. Thirdly, the antibody response to pneumococcal antigens is helper-independent and therefore does not represent the normal situation. Fourthly, the role of T cells in the immune response to pneumococcus is unclear. Finally, the pneumococcal antigens in Pneumovax are polysaccharides and therefore the immunological response to these may not be representative of the immunological responses to proteins. In particular, the presentation of polysaccharides by DCs may be occurring by means of the non-polymorphic CD1 molecules and not via the MHC molecules.

The response of CS to the vaccine is easier to interpret than that of RM, less than 4 months after the vaccination his disease had progressed, and on immunological testing there was no evidence of a response except the expansion of at least one T cell clone. The lack of response in this patient, as judged by the T cell proliferation assay, is likely to be multifactorial, but it is pertinent to note that in CLL there is a marked defect in T cell function (Bartik *et al.*, 1998). This may be relevant, as there is some suggestion that the response to vaccination correlates with the level of immune function present prevaccination (Lodge *et al.*, 2000).

Clearly, one of the endpoints of a cancer vaccine trial will be to determine whether a clinically relevant immune response has been generated. If a reliable, sensitive and specific assay, which required minimal clinical material was available to determine this, it would be a useful tool to allow optimisation of vaccination regimens. Unfortunately such a test is not available. Many of the trials to date have used delayed type hypersensitivity (DTH) tests, either as a global measure of immune status of the patients or to determine whether an immune response has been initiated. Some studies have

shown a correlation between a positive DTH test and a clinical response (Nestle *et al.*, 1998), whereas in others this is not the case (Kugler *et al.*, 2000; Morse *et al.*, 1999b). Standard cytotoxicity tests, which demonstrate the lysis of appropriate target cells, appear to lack the sensitivity required to detect the low number of CTLs which may be expanded by the vaccination. It is for this reason that many groups perform stimulations *in vitro* in order to expand the specific effector population to detectable thresholds. Generating target cells for these lytic assays is also cumbersome, requiring the generation of a cell line from the tumour cells, or pulsing cells with the appropriate HLA type with tumour antigens. In the study described here the aim had been to use Cr^{51} labelled tumour cells as targets in these assays. This failed, however, as in one case the tumour cells were no longer viable after thawing, and in the second case the tumour cells did not label with Cr^{51}. Other methods by which immune responses can be assessed include the ELISPOT test, antibody labelling of intracytoplasmic cytokines followed by flow cytometric analysis, and the use of HLA tetramers. Although these methods are very sensitive, they do not demonstrate functional lysis, and a correlation of positive results with clinical outcomes remains to be proven.

Recent studies by Dhodapkar *et al* have characterised the CD4+ and CD8+ T cell responses in normal human subjects following vaccination with DCs pulsed with both primary and recall antigens (Dhodapkar *et al.*, 2000; Dhodapkar *et al.*, 1999). They showed that a single subcutaneous injection of less than $3x10^6$ mature DCs pulsed with antigen could rapidly expand CD4+ and CD8+ T-cell immunity. Importantly, control immunisations of antigen alone and DCs alone were included. In a follow up study they showed that a booster injection of antigen primed mature DCs induced a CD8+ T cell response of greater magnitude and more rapidly than the first injection. In addition they were able to show that the CD8+ T cells generated had a higher functional avidity for their ligand than those elicited after the first injection.

In conclusion, the results obtained so far from the 2 patients entered into this study are promising. Apart from an episode of sepsis during stem cell mobilisation, no other toxicities have been noted. Administering *ex vivo* generated CD34+ derived DCs pulsed with tumour lysate can therefore be regarded as safe, though of course, further patients will need to be evaluated before this can be stated with certainty. The results obtained from one of the patients is suggestive of an immune response developing to the vaccination in an appropriate clinical context. This is certainly encouraging, especially after only one vaccination. However, there are clearly issues which will need to be addressed such as the use of an alternative control antigen and how to obtain larger amounts of tumour to make a lysate which would enable repeated vaccinations.

The use of DC based vaccines is in its infancy and many questions remain to be answered. These include questions such as: What cell dose is required? What is the best method of loading antigen? How long should the DCs be pulsed with antigen for? What is the best route of administration? How often should vaccinations be repeated? And how should responses be monitored? As well as these questions, the appropriate stage of a patient's treatment at which DC immunotherapy should be used will need to be determined, as this form of therapy will almost certainly be just one modality used in the fight against cancer.

Investigator Year	Source of DC	Type of antigen Loading method	Disease type and status	Pts HLA status	Infusion regimen and route	No of DCs infused	Mature DCs?	Side effects	Clinical Responses	Immunological responses
Reichardt et al 1999	PB DCs collected by leukapheresis & density gradient techniques	DCs pulsed with idiotype proteins for 36-48 hours.	Multiple myeloma post PBSCT. 2 CR, 9 PR, 1 SD	Not selected	3-7 months after PBSCT two iv infusions of Id pulsed DCs one month apart. Followed by 5 s/c boosts of Id/KLH at 4 wk intervals	$5 \pm 3 \times 10^6$ per infusion	No	Fever and chills. Throm -bophlebitis. Local erythema induration and soreness	12 patients 9 still alive: 2 CR 1 SD 6 PD	**11/12**-strong cellular proliferative responses to KLH. **2/12**-cellular prolif responses to idiotype -in the 2 pts still in CR **1/3**- Id specific CTL response detected for 1 month after 2nd DC-Id vacc'n in 1 pt still in CR
Morse et al 1999	Adherent PBMNC (collected at leukapheresis) cultured with GMCSF and IL4 for 7 days.	DCs pulsed with an HLA A2 restricted peptide fragment of CEA for 4hrs and then cryopreserved	Metastatic malignancies expressing CEA	HLA A2 + patients only	i.v. infusion of thawed peptide loaded DCs. 1)weekly intervals x4 2)then fornightly intervals ± intradermal peptide loaded DC x4	$1\text{-}10 \times 10^7$ per iv infusion. 1×10^6 per intra-dermal injection	No	Changes in autoantibody levels. ? flare up of Gauchers disease.	1 minor response 1 stable disease 17 progressive disease	DTH response to CEA peptide pre and post vaccination. -no consistent pattern of response **3/3** Skin biopsy of injection site of DCs loaded with CEA peptide show perivascular infiltrate.
Thurner et al 1999	Adherent PBMNC (collected at leukapheresis) cultured with GMCSF and IL4 for 6 days. Matured for 24h with monocyte conditioned medium ±TNFα	DC pulsed with tetanus toxoid (or tuberculin) as a recall antigen (10μg /ml; 24h) and the HLA A1 restricted peptide Mage-3A1. (10μM; 8h then 1hr)	Stage IV cutaneous malignant melanoma	HLA A1+ patients only . Mage 3 mRNA+ tumours	3 intracutaneous injections (both intradermal and subcutaneous) followed by 2 iv infusions. All at 2 week intervals	6×10^6 i/c then 6×10^6 i/c then 6×10^6 i/c then 6×10^6 i/v then 12×10^6 i/v	Yes	Local reactions and fever	13 patients entered 2 died after 2/3 vaccinations 11 alive after 5 vaccines 6/11 demonstrated complete regression of individual metastasis, but had overall disease progression.	Recall antigen-specific cellular proliferation detected in most patients. 5/5 immune response to tetanus measured by γ IFN-ELISPOT analysis. All ↑ after i/c injections but ↓ after iv injections. pCTL expansion to Mage in 8/11. γ IFN release by T cells stimulated by Mage detected by ELISPOT positive in 2/11.
Kugler et al 2000	Allogeneic DCs generated from PB monocytes cultured with GMCSF and IL4 for 7 days. TNFα added on days 6 and 7 to mature DCs	Electrofusion with autologous tumour cells obtained after surgery. These fusion hybrids were subsequently irradiated.	Metastatic renal carcinoma.	Not selected	2 x s/c vaccinations (close to lymph nodes) of fusion hybrids separated by 6 weeks. If no PD at 12 weeks then 3 monthly vaccinations thereafter. (maximum = 7)	5×10^7 tumour cells fused with 5×10^7 allogeneic DCs per vaccine	Yes	Mild fever, Transient erythema and induration		**2/4** HLA A2+ patients CD8+ T lymphocytes produced intracellular γIFN upon stimulation with the A2 peptide Muc1. In one case, lysis of HLA A2+ cell lines pulsed with Muc 1 was demonstrated. **11/17** DTH to tumour cells +ve not all responded clinically.

Table 6.10 Details of some of the DC based vaccine trials performed in humans to date. cont.,

Investigator Year	Source of DC	Type of antigen Loading method	Disease type and status	Pts HLA status	Infusion regimen and route	No of DCs infused	Mature DCs?	Side effects	Clinical Responses	Immunological responses
Hsu et al 1996	Fresh PB DC collected by leukapheresis & density gradient techniques	Pulsed with Id specific tumour peptide & KLH (24hr; 2µg/ml then16hr; 50µg/ml)	Follicular NHL 3/4 measurable disease (dx). 1/4 molecular evidence of dx	Not selected	iv Id/KLH pulsed DCs x4 (d0, d28, d56 and 5-6 mo) each followed 2wk later by s/c Id & KLH	Each infusion 2-32 x 10^6 (median 5 x 10^6)	No	Erythema at s/c injection site of KLH	1 CR (21mo post vacc) 1 PD 1Molecular remission 1 minor response to date	4/4 humoral and cellular proliferative responses to KLH 4/4 cellular proliferative responses to specific Id used in vaccination. 0/4 anti Id Ab 1/1 anti-Id CTL detected
Murphy et al 1996	Adherent PBMNC cultured with GMCSF and IL4 for 4-6 days	Pulsed with HLA A0201 specific PSMA peptides P1 and P2 (1µg/ml ; 2hrs)	Advanced hormone resistant prostate cancer.	Not selected but 30/51 are HLA A2+	Arms of trial		No	Mild to moderate hypotension 24/51 (worse with 1st infusion.) 3/51 Fatigue	PD / St / PR / n/e	Cellular proliferation in response to P1 or P2 1) overall: only modest response in DC+P2 group 2) analysing only HLA A2+ subjects; responses in DC+P1 and DC+P2 groups seen.
					P1 alone iv 4x/6-8wk	Nil			7 / 3 / 1 / 0	
					P2 alone iv 4x/6-8wk	Nil			5 / 3 / 1 / 0	
					DC alone iv 4x/68wk	1-20x10^6			10 / 2 / 0 / 0	
					DC+ P1 iv 3x/6-8wk	2-20x10^6			3 / 2 / 4 / 0	
					DC+ P2 iv 2x/6-8wk	2-20x10^6			7 / 1 / 1 / 1	
Nestle et al 1998	Adherent PBMNC cultured with GMCSF and IL4 for 7 days	12 pts pulsed with cocktail of haplotype dependent tumour specific peptides (50µg/ml;2hr) 4 pts tumour lysate (100µg/ml;4hr) All with KLH	Advanced melanoma	HLA type known and appro--priate peptides or lysate used.	Direct intranodal injection of DC. Initially 4 weekly vaccinations. 5th vaccination during week 6. Then at monthly intervals for a maximum of 10 vaccinations	1x10^6 per injection	No	Mild fever, LN swelling. 3 developed anti-TSH receptor Abs. 1 developed anti-nuclear Abs. No signs of autoimmunity	Peptide pulsed; 1 CR, 2 PR, 1 MR, 8 PD. Tumour lysate pulsed 1 CR, 1 PR, 2 PD.	All patients developed a DTH response to KLH. Significant DTH reactivity to DCs pulsed with tumour peptides in 5 patients. 4 of these had major clinical responses i.e. correlation of DTH response to tumour peptides and clinical outcome.

Table 6.10 cont., Details of some of the DC based vaccine trials performed in humans to date

cont.,

Investigator Year	Source of DC	Type of antigen Loading method	Disease type and status	Pts HLA status	Infusion regimen and route	No of DCs infused	Mature DCs?	Side effects	Clinical Responses	Immunological responses
Holtl et al 1999	Adherent PBMNC cultured with GMCSF and IL4 for 5 days. Then matured with TNFα & Prostaglandin E_2 for 24hrs.	Cell lysate from cultured autologous tumour cells + KLH (25µg/ml) for 1 h + 24hr with TNFα +PGE_2	Metastatic renal cell carcinoma	Not selected	Intravenous administration of tumour lysate and KLH pulsed DCs x 3 at monthly intervals.	15-95 x10^6	Yes	Moderate fever	1/4 PR	?/4 humoral and cellular proliferative responses to KLH ?/4 humoral & cellular proliferative responses to tumour cell lysate (and normal kidney lysate)
Murphy et al 1999	Adherent PBMNC (collected at leukapheresis) cultured with GMCSF and IL4 for 7 days.	Pulsed with HLA A0201 specific PSMA peptides P1 and P2 (10µg/ml ; 2hrs)	Hormone refractory metastatic prostate cancer.	Not selected but 14/25 evaluable patients are HLA A2+	i/v infusion of peptide pulsed DCs every 6 weeks for a max. of 6 infusions	5-24 x 10^6	No		2 CR (1/2 HLA A2+) 6 PR (4/6 HLA A2+) 16 PD 1 SD	DTH reactivity to a panel of recall antigens measured pre and post vaccination. Responders DTH reactivity 6/8 maintained, 2/8 decreased. Non-responders DTH reactivity 2/6 maintained, 4/6 decreased.
Thurner et al 1998	Adherent PBMNC cultured with GMCSF and IL-4 for 7 days.	Pulsed with tumour cell lysate and KLH with TNFα +PGE_2	Renal cell carcinoma	Not selected	i.v infusion of DCs every 4 weeks for up to six infusions	5-10 x 10^6 per infusion	Yes	Rhythmic fever	4 patients enrolled 2 completed treatment Results only given for 1 patient	DTH to KLH Lymphocytes proliferate in response to KLH and tumour lysates Antibodies against KLH and tumour antigens Partial response with regression of metastases

Table 6.10 cont., Details of some of the DC based vaccine trials performed in humans to date.

CEA=carcinoembryonic antigen, CR= complete remission, PR= partial response, SD=stable disease, Id+idiotype, KLH= keyhole limpet haemacyanin PD= progressive disease, DTH = delayed type hypersensitivity PSMA= prostate specific membrane antigen, PGE_2= Prostaglandin E_2, TSH= thyroid stimulation hormone, St= stable, n/e= not evaluable, MR= mixed response.

Chapter 7

Discussion and Conclusions

There have been many false dawns during the development of therapies which attempt to harness the power of the immune system to combat malignancy. With the recent advances in our understanding of the workings of the immune system, this goal once again appears to have become a real possibility. In particular, the realisation of the central role the dendritic cell plays in orchestrating an immune response and the recently gained knowledge of how to generate these cells *in vitro* has helped in designing potentially suitable cancer vaccines. It now appears clear that if an appropriate anti-tumour response is to be generated *in vivo*, then tumour-related antigens will need to be presented to the immune system by this potent antigen presenting cell.

Dendritic cells can be loaded with antigens *in vivo* or *in vitro*. In the former situation, tumour antigens or nucleic acids encoding these antigens are injected into the tissues in a formulation which would encourage their uptake by the DCs lying *in situ*. In the latter situation, DCs, which can be collected from the peripheral blood or generated *in vitro*, are loaded with antigen *ex vivo* and then returned to the patient. The approach favoured in this thesis has been the *ex vivo* generation of DCs and the subsequent loading of these cells with tumour antigens.

In this thesis

1) A clinically applicable method for the generation of DCs from human CD34+ progenitor cells has been described.
2) It has been shown to a high degree of certainty that proliferation does not accompany the differentiation of monocytes into monocyte derived DCs and that these monocyte derived DCs are therefore not susceptible to retroviral transduction.
3) It has also been shown that the PI3 kinase pathway is important in maintaining the survival of LPS-matured monocyte derived DCs, and that the p38SAPK and NF-κB pathways are important in the LPS-induced maturation of monocyte derived DCs.
4) The results of the first two patients enrolled into a phase I trial using CD34+ DCs pulsed with a tumour lysate as a treatment for relapsed or resistant NHL are also documented.

CD34+ cells were chosen as the preferred cell type from which DCs were generated. This was because the process is known to involve cell proliferation and thus renders

itself amenable to retroviral transduction. Retroviral transduction of DCs with genes encoding tumour-associated antigens is a particularly attractive method of loading DCs with antigen, especially as the resulting antigenic peptides would be directed to the MHC class I compartment and thus would be preferentially presented in association with MHC class I molecules. This, in turn, would result in the activation of CD8+ cytotoxic T cells which are the main effector cells implicated in tumour cell killing.

However, it is now clear that the generation of DCs from CD34+ cells is a lengthy, labour intensive and therefore expensive process, which results in a heterogeneous population of cells of which only a minority are DCs. In addition, it may not be possible in some heavily pre-treated patients to mobilise CD34+ progenitor cells. These findings, together with the fact that in the studies described here retroviral transduction of CD1a+ cells using the PG13 pseudotyped retrovirus was very inefficient, leads to the recommendation that in future clinical studies monocyte derived DCs should be used. These cells have the advantage that a homogenous population of monocyte derived DCs can be easily generated within seven days from monocytes collected from the resting peripheral blood of patients, with no requirement for mobilisation regimens or leukapheresis.

It has, however, been shown in this thesis that proliferation does not accompany the differentiation of monocytes into DCs, and that this process is therefore not amenable to the retroviral-mediated transduction of genes encoding tumour-associated antigens. Although this is a potential disadvantage, other methods more appropriate to insertion of genes into non-dividing cells need to be attempted. These should include both physical methods, e.g. lipofection and electroporation as well as methods involving other viral vectors, eg adenoviruses, lentiviruses and herpesviruses. A comparison of several different methods of gene transfer has been made by Arthur *et al.* They showed only low level transduction by the physical methods, whereas efficiencies of over 95% were achieved when recombinant adenovirus vectors were used (Arthur *et al.*, 1997). Herpesviruses have also been used successfully to transduce monocyte derived DCs (Coffin *et al.*, 1998).

Obviously monocyte derived DCs will need to be generated in a manner which is clinically applicable. X-VIVO10 containing 10% autologous resting serum or plasma and supplemented with IL-4 and GM-CSF would be a reasonable culture medium to be evaluated in the first instance.

In the clinical trial described here, DCs were loaded with tumour antigens by means of a tumour lysate. This method of antigen loading has the advantage of not needing to know the exact nature of the 'tumour-specific antigens' concerned and theoretically reduces the chance of immune escape, but it has the potential disadvantage of triggering autoimmune disorders. Although the follow up here is limited, no evidence of autoimmunity, or indeed any other side effect, has yet been noted in the two patients described here to date. Cell lysates have also been used by others as a means of loading DCs with tumour antigens and, encouragingly, complete and partial responses have been reported without any evidence of autoimmunity developing (Holtl *et al.*, 1999; Nestle *et al.*, 1998; Thurnher *et al.*, 1998).

The source of antigen, method of antigen loading, the route of DC administration, as well as the dose of DCs and the schedule of vaccination, are all variables which will need to be optimised. The results of trials which are currently underway will help to answer some of these questions, though a formal comparison of these variables will ultimately be needed. The setting in which DC vaccination will be most beneficial will also need to be determined, though it seems likely that its value will be greatest in minimal disease states as this appears to be the case in animal models (Mayordomo *et al.*, 1995).

In assessing whether the DC vaccine induced an immune response, it would be beneficial to include some of the newer immunological techniques outlined in chapter 6 in addition to those currently in use. It will still, however, remain necessary to determine if the immune responses detected are longlasting or require boosting and whether they do indeed translate into clinically meaningful responses. The inclusion of a more appropriate control antigen such as KLH would also be helpful in the interpretation of the immune responses induced by the vaccination.

The signal transduction pathways involved in the maturation process, which is central to the function of the DC, have only just begun to be elucidated. Further investigation here is warranted, as understanding these pathways will help with the fine tuning of immune responses.

In conclusion, the work presented in this thesis can be seen to have contributed to the advancement of our understanding of the biology of monocyte derived DCs. In addition, it has provided some useful data regarding the use of tumour lysate pulsed CD34+ derived DCs as a tumour vaccine in the context of NHL.

This new millenium promises much progress in the field of immunotherapy, and exciting times lie ahead, but it is safe to say that the advances which have been made since the medical student Paul Langerhans discovered the Langerhans cell 132 years ago could not have been imagined, and we too remain to be surprised.

References

Adema, G.J., Hartgers, F., Verstraten, R., de Vries, E., Marland, G., Menon, S., Foster, J., Xu, Y., Nooyen, P., McClanahan, T., Bacon, K.B. & Figdor, C.G. (1997). A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature*, **387,** 713-7.

Aicher, A., Westermann, J., Cayeux, S., Willimsky, G., Daemen, K., Blankenstein, T., Uckert, W., Dorken, B. & Pezzutto, A. (1997). Successful retroviral mediated transduction of a reporter gene in human dendritic cells: Feasibility of therapy with gene-modified antigen presenting cells. *Exp Haematol*, **25,** 39-44.

Albert, M.L., Pearce, S.F., Francisco, L.M., Sauter, B., Roy, P., Silverstein, R.L. & Bhardwaj, N. (1998a). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med*, **188,** 1359-68.

Albert, M.L., Sauter, B. & Bhardwaj, N. (1998b). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature*, **392,** 86-9.

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. & Saltiel, A.R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen- activated protein kinase kinase in vitro and in vivo. *J Biol Chem*, **270,** 27489-94.

Alters, S.E., Gadea, J.R., Sorich, M., O'Donoghue, G., Talib, S. & Philip, R. (1998). Dendritic cells pulsed with CEA peptide induce CEA-specific CTL with restricted TCR repertoire. *J Immunother*, **21,** 17-26.

Arthur, J.F., Butterfield, L.H., Roth, M.D., Bui, L.A., Kiertscher, S.M., Lau, R., Dubinett, S., Glaspy, J., McBride, W.H. & Economou, J.S. (1997). A comparison of gene transfer methods in human dendritic cells. *Cancer Gene Ther*, **4,** 17-25.

Ashley, D.M., Faiola, B., Nair, S., Hale, L.P., Bigner, D.D. & Gilboa, E. (1997). Bone marrow-generated dendritic cells pulsed with tumor extracts or tumor RNA induce antitumor immunity against central nervous system tumors. *J Exp Med*, **186,** 1177-82.

Avigan, D. (1999). Dendritic cells: development, function and potential use for cancer immunotherapy. *Blood Rev*, **13,** 51-64.

Bakker, A.B., Marland, G., de Boer, A.J., Huijbens, R.J., Danen, E.H., Adema, G.J. & Figdor, C.G. (1995). Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells in vitro. *Cancer Res*, **55,** 5330-4.

Banchereau, J. & Steinman, R.M. (1998). Dendritic cells and the control of immunity. *Nature*, **392,** 245-52.

Bartik, M.M., Welker, D. & Kay, N.E. (1998). Impairments in immune cell function in B cell chronic lymphocytic leukemia. *Semin Oncol*, **25,** 27-33.

Beg, A.A. & Baltimore, D. (1996). An essential role for NF-kappaB in preventing TNF-alpha-induced cell death [see comments]. *Science*, **274,** 782-4.

Bennett, S.R., Carbone, F.R., Karamalis, F., Flavell, R.A., Miller, J.F. & Heath, W.R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling [see comments]. *Nature*, **393,** 478-80.

Bernhard, H., Disis, M.L., Heimfeld, S., Hand, S., Gralow, J.R. & Cheever, M.A. (1995). Generation of immunostimulatory dendritic cells from human CD34+ hematopoietic progenitor cells of the bone marrow and peripheral blood. *Cancer Res*, **55,** 1099-1104.

Bhardwaj, N., Bender, A., Gonzalez, N., Bui, L.K., Garrett, M.C. & Steinman, R.M. (1994). Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8+ T cells. *J Clin Invest*, **94,** 797-807.

Boon, T. & Old, L.J. (1997). Cancer Tumor antigens. *Curr Opin Immunol*, **9,** 681-3.

Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H. & Yancopoulos, G.D. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, **65,** 663-75.

Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slaughter, C., Moomaw, C., Hsu, J. & Cobb, M.H. (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science*, **249,** 64-7.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72,** 248-54.

Brenner, M. & Porcelli, S. (1997). Antigen presentation: a balanced diet [comment]. *Science*, **277,** 332.

Carrasco, D., Ryseck, R.P. & Bravo, R. (1993). Expression of relB transcripts during lymphoid organ development: specific expression in dendritic antigen-presenting cells. *Development*, **118,** 1221-31.

Caux, C., DezutterDambuyant, C., Schmit, D. & Banchereau, J. (1992). GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature*, **360,** 258-261.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I. & Banchereau, J. (1994). Activation of human dendritic cells through CD40 cross-linking. *Journal of Experimental Medicine*, **180,** 1263-1272.

Caux, C., Vanbervliet, B., Massacrier, C., Dezutterdambuyant, C., Desaintvis, B., Jacquet, C., Yoneda, K., Imamura, S., Schmitt, D. & Banchereau, J. (1996). Cd34(+) Hematopoietic Progenitors From Human Cord-Blood Differentiate Along 2 Independent Dendritic Cell Pathways In Response to Gm- Csf+Tnf-Alpha. *J Exp Med*, **184,** 695-706.

Cavanagh, L., Saal, R., Grimmett, K. & Thomas, R. (1998). Proliferation In Monocyte-Derived Dendritic Cell Cultures Is Caused By Progenitor Cells Capable of Myeloid Differentiation. *Blood.*, **92,** 1598-1607.

Cella, M., Engering, A., Pinet, V., Pieters, J. & Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells [see comments]. *Nature*, **388,** 782-7.

Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I. & Lanzavecchia, A. (1999). Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med*, **189,** 821-9.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A. & Alber, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med*, **184,** 747-52.

Celluzzi, C.M., Mayordomo, J.I., Storkus, W.J., Lotze, M.T. & Falo, L.D., Jr. (1996). Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity [see comments]. *J Exp Med*, **183,** 283-7.

Chanarin, I. (1989). *Laboratory haematology : an account of laboratory techniques.* Churchill Livingstone: Edinburgh ; New York.

Chapuis, F., Rosenzwajg, M., Yagello, M., Ekman, M., Biberfeld, P. & Gluckman, J. (1997). Differentiation of human dendritic cells from monocytes in vitro. *Eur J Immunol.*, **27,** 431-441.

Coffin, R.S., Thomas, S.K., Thomas, N.S., Lilley, C.E., Pizzey, A.R., Griffiths, C.H., Gibb, B.J., Wagstaff, M.J., Inges, S.J., Binks, M.H., Chain, B.M., Thrasher, A.J., Rutault, K. & Latchman, D.S. (1998). Pure populations of transduced primary human cells can be produced using GFP expressing herpes virus vectors and flow cytometry. *Gene Ther*, **5,** 718-22.

Crawley, J.B., Rawlinson, L., Lali, F.V., Page, T.H., Saklatvala, J. & Foxwell, B.M. (1997). T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. *J Biol Chem*, **272,** 15023-7.

Craxton, A., Shu, G., Graves, J.D., Saklatvala, J., Krebs, E.G. & Clark, E.A. (1998). p38 MAPK is required for CD40-induced gene expression and proliferation in B lymphocytes. *J Immunol*, **161,** 3225-36.

De Smedt, T., Pajak, B., Muraille, E., Lespagnard, L., Heinen, E., De Baetselier, P., Urbain, J., Leo, O. & Moser, M. (1996). Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J Exp Med*, **184,** 1413-24.

Derijard, B., Hibi, M., Wu, I.H., Barrett, T., Su, B., Deng, T., Karin, M. & Davis, R.J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, **76,** 1025-37.

Dhodapkar, M.V., Krasovsky, J., Steinman, R.M. & Bhardwaj, N. (2000). Mature dendritic cells boost functionally superior CD8(+) T-cell in humans without foreign helper epitopes [see comments]. *J Clin Invest*, **105,** R9-R14.

Dhodapkar, M.V., Steinman, R.M., Sapp, M., Desai, H., Fossella, C., Krasovsky, J., Donahoe, S.M., Dunbar, P.R., Cerundolo, V., Nixon, D.F. & Bhardwaj, N. (1999). Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J Clin Invest*, **104,** 173-80.

Dieu, M.C., Vanbervliet, B., Vicari, A., Bridon, J.M., Oldham, E., Ait-Yahia, S., Briere, F., Zlotnik, A., Lebecque, S. & Caux, C. (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med*, **188,** 373-86.

Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. *Curr Opin Cell Biol*, **10,** 262-7.

Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R. & Greenberg, M.E. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt [see comments]. *Science*, **275,** 661-5.

Ferlazzo, G., Wesa, A., Wei, W.Z. & Galy, A. (1999). Dendritic cells generated either from CD34+ progenitor cells or from monocytes differ in their ability to activate antigen-specific CD8+ T cells. *J Immunol*, **163,** 3597-604.

Fields, R.C., Shimizu, K. & Mule, J.J. (1998). Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. *Proc Natl Acad Sci U S A*, **95,** 9482-7.

Fisch, P., Kohler, G., Garbe, A., Herbst, B., Wider, D., Kohler, H., Schaefer, H.E., Mertelsmann, R., Brugger, W. & Kanz, L. (1996). Generation Of Antigen-Presenting Cells For Soluble-Protein Antigens Ex-Vivo From Peripheral-Blood Cd34(+) Hematopoietic Progenitor Cells In Cancer-Patients. *Eur J Immunol*, **26,** 595-600.

Fong, T.C., Wu, Y. & Kipps, T.J. (1996). Identification of a promoter element that regulates tissue-specific expression of the human CD80 (B7.1) gene. *J Immunol*, **157,** 4442-50.

Gong, J., Chen, D., Kashiwaba, M. & Kufe, D. (1997). Induction of anti tumour activity by immunisation with fusions of dendritic and carcinoma cells. *Nature Medicine*, **3,** 558-561.

Grouard, G., Rissoan, M.C., Filgueira, L., Durand, I., Banchereau, J. & Liu, Y.J. (1997). The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med*, **185,** 1101-11.

Hacker, H., Mischak, H., Miethke, T., Liptay, S., Schmid, R., Sparwasser, T., Heeg, K., Lipford, G.B. & Wagner, H. (1998). CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J*, **17,** 6230-40.

Hambleton, J., Weinstein, S.L., Lem, L. & DeFranco, A.L. (1996). Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide- stimulated macrophages. *Proc Natl Acad Sci U S A*, **93,** 2774-8.

Han, J., Lee, J.D., Bibbs, L. & Ulevitch, R.J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*, **265,** 808-11.

Herbst, B., Kohler, G., Mackensen, A., Veelken, H., Kulmburg, P., Rosenthal, F.M., Schaefer, H.E., Mertelsmann, R., Fisch, P. & Lindemann, A. (1996). In-Vitro Differentiation Of Cd34(+) Hematopoietic Progenitor Cells Toward Distinct Dendritic Cell Subsets Of the Birbeck Granule and Miic-Positive Langerhans Cell and the Interdigitating Dendritic Cell- Type. *Blood*, **88,** 2541-2548.

Hock, B.D., Starling, G.C., Daniel, P.B. & Hart, D.N. (1994). Characterization of CMRF-44, a novel monoclonal antibody to an activation antigen expressed by the allostimulatory cells within peripheral blood, including dendritic cells. *Immunology*, **83,** 573-81.

Holtl, L., Rieser, C., Papesh, C., Ramoner, R., Herold, M., Klocker, H., Radmayr, C., Stenzl, A., Bartsch, G. & Thurnher, M. (1999). Cellular and humoral immune responses in patients with metastatic renal cell carcinoma after vaccination with antigen pulsed dendritic cells. *J Urol*, **161,** 777-82.

Hsu, F.J., Benike, C., Fagnoni, F., Liles, T.M., Czerwinski, D., Taidi, B., Engleman, E.G. & Levy, R. (1996). Vaccination Of Patients With B-Cell Lymphoma Using Autologous Antigen-Pulsed Dendritic Cells. *Nature Medicine*, **2,** 52-58.

Huang, A.Y., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D. & Levitsky, H. (1994). Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science*, **264,** 961-5.

Katz, S.I., Tamaki, K. & Sachs, D.H. (1979). Epidermal Langerhans cells are derived from cells originating in bone marrow. *Nature*, **282,** 324-6.

Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J. & Evan, G. (1997). Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature*, **385,** 544-8.

Kellermann, S.A., Hudak, S., Oldham, E.R., Liu, Y.J. & McEvoy, L.M. (1999). The CC chemokine receptor-7 ligands 6Ckine and macrophage inflammatory protein-3 beta are potent chemoattractants for in vitro- and in vivo-derived dendritic cells. *J Immunol*, **162,** 3859-64.

Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P.H. & Downward, J. (1997). Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J*, **16,** 2783-93.

Kleijmeer, M.J., Raposo, G. & Geuze, H.J. (1996). Characterization of MHC Class II Compartments by Immunoelectron Microscopy. *Methods*, **10,** 191-207.

Kugler, A., Stuhler, G., Walden, P., Zoller, G., Zobywalski, A., Brossart, P., Trefzer, U., Ullrich, S., Muller, C.A., Becker, V., Gross, A.J., Hemmerlein, B., Kanz, L., Muller, G.A. & Ringert, R.H. (2000). Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids [see comments]. *Nature Medicine*, **6,** 332-6.

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J. & Woodgett, J.R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, **369,** 156-60.

Labeur, M.S., Roters, B., Pers, B., Mehling, A., Luger, T.A., Schwarz, T. & Grabbe, S. (1999). Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J Immunol*, **162,** 168-75.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227,** 680-5.

Langerhans, P. (1868). *Virchows Archiv fur pathologische Anatomie und Physiologie und fur klinische Medizin*, **44,** 325-337.

Lanzavecchia, A. (1997). Understanding the mechanisms of sustained signaling and T cell activation [comment]. *J Exp Med*, **185,** 1717-9.

Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Heys, J.R., Landvatter, S.W. & et al. (1994). A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, **372,** 739-46.

Legrand-Poels, S., Maniglia, S., Boelaert, J.R. & Piette, J. (1997). Activation of the transcription factor NF-kappaB in lipopolysaccharide- stimulated U937 cells. *Biochem Pharmacol*, **53,** 339-46.

Lin, Y.Z., Yao, S.Y., Veach, R.A., Torgerson, T.R. & Hawiger, J. (1995). Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide

containing a cell membrane-permeable motif and nuclear localization sequence. *J Biol Chem*, **270,** 14255-8.

Lodge, P.A., Jones, L.A., Bader, R.A., Murphy, G.P. & Salgaller, M.L. (2000). Dendritic cell-based immunotherapy of prostate cancer: immune monitoring of a phase II clinical trial. *Cancer Res*, **60,** 829-33.

Lotze, M.T. & Thomson, A.W. (1999). *Dendritic cells : biology and clinical applications*. Academic Press: San Diego.

Lu, H.T., Yang, D.D., Wysk, M., Gatti, E., Mellman, I., Davis, R.J. & Flavell, R.A. (1999). Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. *EMBO J*, **18,** 1845-57.

Ludewig, B., Odermatt, B., Landmann, S., Hengartner, H. & Zinkernagel, R.M. (1998). Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue. *J Exp Med*, **188,** 1493-501.

Mackensen, A., Herbst, B., Kohler, G., WolffVorbeck, G., Rosenthal, F.M., Veelken, H., Kulmburg, P., Schaefer, H.E., Mertelsmann, R. & Lindemann, A. (1995). Delineation of the dendritic cell lineage by generating large numbers of Birbeck granule-positive Langerhans cells from human peripheral blood progenitor cells in vitro. *Blood*, **86,** 2699-2707.

MacPherson, G., Kushnir, N. & Wykes, M. (1999). Dendritic cells, B cells and the regulation of antibody synthesis. *Immunol Rev*, **172,** 325-34.

Mannering, S.I., McKenzie, J.L., Fearnley, D.B. & Hart, D.N. (1997). HLA-DR1-restricted bcr-abl (b3a2)-specific CD4+ T lymphocytes respond to dendritic cells pulsed with b3a2 peptide and antigen-presenting cells exposed to b3a2 containing cell lysates. *Blood*, **90,** 290-7.

Maslanka, K., Piatek, T., Gorski, J. & Yassai, M. (1995). Molecular analysis of T cell repertoires. Spectratypes generated by multiplex polymerase chain reaction and evaluated by radioactivity or fluorescence. *Hum Immunol*, **44,** 28-34.

May, M.J. & Ghosh, S. (1998). Signal transduction through NF-kappa B. *Immunol Today*, **19,** 80-8.

Mayordomo, J.I., Zorina, T., Storkus, W.J., Zitvogel, L., Celluzzi, C., Falo, L.D., Melief, C.J., Ildstad, S.T., Kast, W.M., Deleo, A.B. & Lotze, M.T. (1995). Bone-Marrow-Derived Dendritic Cells Pulsed With Synthetic Tumor Peptides Elicit Protective and Therapeutic Antitumor Immunity. *Nature Medicine*, **1,** 1297-1302.

McLellan, A.D., Sorg, R.V., Williams, L.A. & Hart, D.N.J. (1996). Human Dendritic Cells Activate T-Lymphocytes Via a Cd40-Cd40 Ligand- Dependent Pathway. *Eur J Immunol*, **26,** 1204-1210.

Mehta, D., Markowicz, S. & Engelman, E. (1994). Generation of antigen specific CD8 CTLs from naive precursors. *J Immunol*, **153,** 996-1003.

Mehta, D., Markowicz, S. & Engelman, E. (1995). Generation of antigen specific CD4+ T cell lines from naive precursors. *Eur J Immunol*, **25,** 1206-1211.

Meierhoff, G., Krause, S.W. & Andreesen, R. (1998). Comparative analysis of dendritic cells derived from blood monocytes or CD34+ hematopoietic progenitor cells. *Immunobiology*, **198,** 501-13.

Mellman, I., Turley, S.J. & Steinman, R.M. (1998). Antigen processing for amateurs and professionals. *Trends Cell Biol*, **8,** 231-7.

Minshall, C., Arkins, S., Freund, G.G. & Kelley, K.W. (1996). Requirement for phosphatidylinositol 3'-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. *J Immunol*, **156,** 939-47.

Morse, M.A., Coleman, R.E., Akabani, G., Niehaus, N., Coleman, D. & Lyerly, H.K. (1999a). Migration of human dendritic cells after injection in patients with metastatic malignancies. *Cancer Res*, **59,** 56-8.

Morse, M.A., Deng, Y., Coleman, D., Hull, S., Kitrell-Fisher, E., Nair, S., Schlom, J., Ryback, M.E. & Lyerly, H.K. (1999b). A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. *Clin Cancer Res*, **5,** 1331-8.

Morse, M.A., Lyerly, H.K., Gilboa, E., Thomas, E. & Nair, S.K. (1998). Optimization of the sequence of antigen loading and CD40-ligand-induced maturation of dendritic cells. *Cancer Res*, **58,** 2965-8.

Nestle, F.O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G. & Schadendorf, D. (1998). Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells [see comments]. *Nature Medicine*, **4,** 328-32.

O'Doherty, U., Peng, M., Gezelter, S., Swiggard, W.J., Betjes, M., Bhardwaj, N. & Steinman, R.M. (1994). Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology*, **82,** 487-93.

Oehler, L., Majdic, O., Pickl, W.F., Stockl, J., Riedl, E., Drach, J., Rappersberger, K., Geissler, K. & Knapp, W. (1998). Neutrophil granulocyte-committed cells can be driven to acquire dendritic cell characteristics. *J Exp Med*, **187,** 1019-28.

Paglia, P., Chiodoni, C., Rodolfo, M. & Colombo, M.P. (1996). Murine Dendritic Cells Loaded In-Vitro With Soluble-Protein Prime Cytotoxic T-Lymphocytes Against Tumor-Antigen In-Vivo. *J Exp Med*, **183,** 317-322.

Peters, P.J., Raposo, G., Neefjes, J.J., Oorschot, V., Leijendekker, R.L., Geuze, H.J. & Ploegh, H.L. (1995). Major histocompatibility complex class II compartments in human B lymphoblastoid cells are distinct from early endosomes. *J Exp Med*, **182,** 325-34.

Porgador, A. & Gilboa, E. (1995). Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. *J Exp Med*, **182,** 255-60.

Quandt, K., Frech, K., Karas, H., Wingender, E. & Werner, T. (1995). MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res*, **23,** 4878-84.

Raychaudhuri, S. & Rock, K.L. (1998). Fully mobilizing host defense: building better vaccines. *Nat Biotechnol*, **16,** 1025-31.

Reddy, A., Sapp, M., Feldman, M., Subklewe, M. & Bhardwaj, N. (1997). A monocyte conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells. *Blood*, **90,** 3640-6.

Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P. & Amigorena, S. (1999). Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med*, **189,** 371-80.

Reid, C.D., Fryer, P.R., Clifford, C., Kirk, A., Tikerpae, J. & Knight, S.C. (1990). Identification of hematopoietic progenitors of macrophages and dendritic Langerhans cells (DL-CFU) in human bone marrow and peripheral blood. *Blood*, **76,** 1139-49.

Reid, C.D.L. (1997). The Dendritic Cell Lineage in Haemopoiesis. *Brit J Haematol*, **96,** 217-223.

Reid, C.D.L., Stackpoole, A., Meager, A. & Tikerpae, J. (1992). Interactions of tumor necrosis factor with granulocyte-macrophage colony- stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bipotent CD34+ progenitors in human bone marrow. *J Immunol*, **149,** 2681-2688.

Rescigno, M., Martino, M., Sutherland, C.L., Gold, M.R. & Ricciardi-Castagnoli, P. (1998). Dendritic cell survival and maturation are regulated by different signaling pathways. *J Exp Med*, **188,** 2175-80.

Ridge, J.P., Di Rosa, F. & Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell [see comments]. *Nature*, **393,** 474-8.

Rincon, M., Enslen, H., Raingeaud, J., Recht, M., Zapton, T., Su, M.S., Penix, L.A., Davis, R.J. & Flavell, R.A. (1998). Interferon-gamma expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway. *Embo J*, **17,** 2817-29.

Rock, K.L., Rothstein, L., Gamble, S. & Fleischacker, C. (1993). Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J-Immunol*, **150,** 438-46.

Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P. & Amigorena, S. (1999). Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat Cell Biol*, **1,** 362-368.

Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P.O., Steinman, R.M. & Schuler, G. (1994). Proliferating dendritic cell progenitors in human blood. *J Exp Med*, **180,** 83-93.

Rosenberg, S.A., Kawakami, Y., Robbins, P.F. & Wang, R. (1996). Identification of the genes encoding cancer antigens: implications for cancer immunotherapy. *Adv Cancer Res*, **70,** 145-77.

Rosenzwajg, M., Canque, B. & Gluckman, J.C. (1996). Human Dendritic Cell-Differentiation Pathway From Cd34(+) Hematopoietic Precursor Cells. *Blood*, **87,** 535-544.

Russo, V., Sartirana, C., Dalerba, P., Rossini, S., Traversari, C. & Bordignon, C. (1997). Transfer of tumour-associated antigen genes into dendritic cells for active immunotherapy of neoplastic diseases. *Blood*, **90,** 405a.

Sallusto, F., Cella, M., Danieli, C. & Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: Downregulation by cytokines and bacterial products. *J Exp Med*, **182,** 389-400.

Sallusto, F. & Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med*, **179,** 1109-1118.

Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C.R., Qin, S. & Lanzavecchia, A. (1998). Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol*, **28,** 2760-9.

Sato, K., Nagayama, H., Tadokoro, K., Juji, T. & Takahashi, T.A. (1999). Extracellular signal-regulated kinase, stress-activated protein kinase/c-Jun N-terminal kinase, and p38mapk are involved in IL-10- mediated selective repression of TNF-alpha-induced activation and maturation of human peripheral blood monocyte-derived dendritic cells. *J Immunol*, **162,** 3865-72.

Schoenberger, S.P., Toes, R.E., van der Voort, E.I., Offringa, R. & Melief, C.J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions [see comments]. *Nature*, **393,** 480-3.

Schuler, G. & Steinman, R.M. (1985). Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J Exp Med*, **161,** 526-46.

Schuler, G. & Steinman, R.M. (1997). Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J Exp Med*, **186,** 1183-7.

Schumm, M., Lang, P., Taylor, G., Kuci, S., Klingebiel, T., Buhring, H.J., Geiselhart, A., Niethammer, D. & Handgretinger, R. (1999). Isolation of highly purified autologous and allogeneic peripheral CD34+ cells using the CliniMACS device. *J Hematother*, **8,** 209-18.

Sen, J., Kapeller, R., Fragoso, R., Sen, R., Zon, L.I. & Burakoff, S.J. (1996). Intrathymic signals in thymocytes are mediated by p38 mitogen-activated protein kinase. *J Immunol*, **156,** 4535-8.

Shakhov, A.N., Collart, M.A., Vassalli, P., Nedospasov, S.A. & Jongeneel, C.V. (1990). Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J Exp Med*, **171,** 35-47.

Shapiro, H.M. (1995). *Practical flow cytometry*. Wiley-Liss: New York.

Siena, S., Dinicola, M., Bregni, M., Mortarini, R., Anichini, A., Lombardi, L., Ravagnani, F., Parmiani, G. & Gianni, A.M. (1995). Massive Ex-Vivo Generation Of Functional Dendritic Cells From Mobilized Cd34(+) Blood Progenitors For Anticancer Therapy. *Exp Hematol*, **23,** 1463-1471.

Song, W., Kong, H.L., Carpenter, H., Torii, H., Granstein, R., Rafii, S., Moore, M.A. & Crystal, R.G. (1997). Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J Exp Med*, **186,** 1247-56.

Sozzani, S., Allavena, P., Vecchi, A. & Mantovani, A. (1999). The role of chemokines in the regulation of dendritic cell trafficking. *J Leukoc Biol*, **66,** 1-9.

Specht, J.M., Wang, G., Do, M.T., Lam, J.S., Royal, R.E., Reeves, M.E., Rosenberg, S.A. & Hwu, P. (1997). Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. *J Exp Med*, **186,** 1213-21.

Stahl, P.D. (1992). The mannose receptor and other macrophage lectins. *Curr Opin Immunol*, **4,** 49-52.

Steinman, R. (1991). The dendritic cell system and its role in immunogenicity. *Ann RevImmunol*, **9,** 271-296.

Steinman, R.M. (1996). Dendritic Cells and Immune-Based Therapies. *Exp Hematol*, **24,** 859-862.

Steinman, R.M. & Cohn, Z.A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*, **137,** 1142-62.

Steinman, R.M. & Swanson, J. (1995). The Endocytic Activity of Dendritic Cells. *J. Exp. Med.*, **182,** 283-288.

Strunk, D., Rappersberger, K., Egger, C., Strobl, H., Kromer, E., Elbe, A., Maurer, D. & Stingl, G. (1996). Generation of human dendritic cells/Langerhans cells from circulating CD34+ hematopoietic progenitor cells. *Blood*, **87,** 1292-302.

Swanson, J. & Watts, C. (1995). Macropinocytosis. *Trends in Cell Biology*, **5,** 424-428.

Swanson, J.A. (1989). Phorbol esters stimulate macropinocytosis and solute flow through macrophages. *J Cell Sci*, **94,** 135-42.

Szabolcs, P., Gallardo, H.F., Ciocon, D.H., Sadelain, M. & Young, J.W. (1997). Retrovirally transduced human dendritic cells express a normal phenotype and potent T-cell stimulatory capacity. *Blood*, **90,** 2160-7.

Szabolcs, P., Moore, M.A.S. & Young, J.W. (1995). Expansion of immunostimulatory dendritic cells among the myeloid progeny of human CD34+ bone marrow precursors cultured with c-kit ligand, granulocyte-macrophage colony-stimulating factor, and TNF-alpha. *J Immunol*, **154,** 5851-5861.

Tan, M.C., Mommaas, A.M., Drijfhout, J.W., Jordens, R., Onderwater, J.J., Verwoerd, D., Mulder, A.A., van der Heiden, A.N., Scheidegger, D., Oomen, L.C., Ottenhoff, T.H., Tulp, A., Neefjes, J.J. & Koning, F. (1997). Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. *Eur J Immunol*, **27,** 2426-35.

Thomas, N.S. (1989). Regulation of the product of a possible human cell cycle control gene CDC2Hs in B-cells by alpha-interferon and phorbol ester. *J Biol Chem*, **264,** 13697-700.

Thomas, N.S.B. (1999). *Cell Cycle Regulation: chapter 8 in Textbook of malignant haematology*. Martin Dunitz ;
Distributed in the United States by Blackwell Science: London
Malden, MA, USA.

Thomas, R. & Lipsky, P.E. (1994). Human peripheral blood dendritic cell subsets. Isolation and characterization of precursor and mature antigen-presenting cells. *J-Immunol*, **153,** 4016-28.

Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P., Brocker, E.B., Steinman, R.M., Enk, A., Kampgen, E. & Schuler, G. (1999). Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med*, **190,** 1669-78.

Thurnher, M., Rieser, C., Holtl, L., Papesh, C., Ramoner, R. & Bartsch, G. (1998). Dendritic cell-based immunotherapy of renal cell carcinoma. *Urol Int*, **61,** 67-71.

Tong, L., Pav, S., White, D.M., Rogers, S., Crane, K.M., Cywin, C.L., Brown, M.L. & Pargellis, C.A. (1997). A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. *Nat Struct Biol*, **4,** 311-6.

Triozzi, P.L. & Aldrich, W. (1997). Phenotypic and functional differences between human dendritic cells derived in vitro from hematopoietic progenitors and from monocytes/macrophages. *J Leukoc Biol*, **61,** 600-8.

Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R. & Verma, I.M. (1996). Suppression of TNF-alpha-induced apoptosis by NF-kappaB [see comments]. *Science*, **274,** 787-9.

Van Parijs, L. & Abbas, A.K. (1998). Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science*, **280,** 243-8.

Vanderbruggen, P., Traversari, C., Chomez, P., Lurquin, C., Deplaen, E., Vandeneynde, B., Knuth, A. & Boon, T. (1991). A Gene Encoding an Antigen Recognized By Cytolytic Lymphocytes-T On a Human-Melanoma. *Science*, **254,** 1643-1647.

Vlahos, C.J., Matter, W.F., Hui, K.Y. & Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4- morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem*, **269,** 5241-8.

Wang, C.Y., Mayo, M.W. & Baldwin, A.S., Jr. (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB [see comments]. *Science*, **274,** 784-7.

Watts, C. (1997). Immunology. Inside the gearbox of the dendritic cell [news; comment]. *Nature*, **388,** 724-5.

Watts, M.J., Ings, S.J., Leverett, D., MacMillan, A., Devereux, S., Goldstone, A.H. & Linch, D.C. (2000). ESHAP and G-CSF is a superior blood stem cell mobilizing regimen compared to cyclophosphamide 1.5 g m(-2) and G-CSF for pre-treated lymphoma patients: a matched pairs analysis of 78 patients. *Br J Cancer*, **82,** 278-82.

Watts, M.J., Sullivan, A.M., Ings, S.J., Leverett, D., Peniket, A.J., Perry, A.S., Williams, C.D., Devereux, S. & Linch, D.C. (1997). Evaluation of Clinical Scale CD34+ cell purification: Experience of 71 Immunoaffinity column procedures. *Bone Marrow Trans*, **20,** 157-162.

Weih, F., Warr, G., Yang, H. & Bravo, R. (1997). Multifocal defects in immune responses in RelB-deficient mice. *J Immunol*, **158,** 5211-8.

Weinstein, S.L., Sanghera, J.S., Lemke, K., DeFranco, A.L. & Pelech, S.L. (1992). Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. *J Biol Chem*, **267,** 14955-62.

Westermann, J., Aicher, A., Qin, Z., Cayeux, S., Daemen, K., Blankenstein, T., Dorken, B. & Pezzutto, A. (1998). Retroviral interleukin-7 gene transfer into human dendritic cells enhances T cell activation. *Gene Therapy*, **5,** 264-271.

Williams, S.F., Lee, W.J., Bender, J.G., Zimmerman, T., Swinney, P., Blake, M., Carreon, J., Schilling, M., Smith, S., Williams, D.E., Oldham, F. & Van Epps, D. (1996). Selection and expansion of peripheral blood CD34+ cells in autologous stem cell transplantation for breast cancer. *Blood*, **87,** 1687-91.

Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adorini, L., Zimmermann, V.S., Davoust, J. & Ricciardi-Castagnoli, P. (1997). Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp Med*, **185,** 317-28.

Yang, R.B., Mark, M.R., Gurney, A.L. & Godowski, P.J. (1999). Signaling events induced by lipopolysaccharide-activated toll-like receptor 2. *J Immunol*, **163,** 639-43.

Young, J.W., Szabolcs, P. & Moore, M.A.S. (1995). Identification of dendritic cell colony-forming units among normal human CD34+ bone marrow progenitors that are expanded by c-kit-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha. *J Exp Med*, **182,** 1111-1120.

Zhou, L.J. & Tedder, T.F. (1995). Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J-Immunol*, **154,** 3821-35.

Zitvogel, L., Mayordomo, J.I., Tjandrawan, T., DeLeo, A.B., Clarke, M.R., Lotze, M.T. & Storkus, W.J. (1996). Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines [see comments]. *J Exp Med*, **183,** 87-97.