Immunoregulation by Hepatocyte Growth Factor in Malignant Mesothelioma

By

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Should we meet again - *Elen síla lúmenn' omentielvo.*

This thesis is dedicated

to my family for their great support and understanding

To

My parents Karen and Graham Cook

My sisters Natalie Briant and Tabitha Cook

iii. Publications and presentations

Publications

Tumour associated hepatocyte growth factor modulates DC differentiation towards tolerogenic phenotype. Manuscript to be submitted to Immunology, Cook N, Coleman S, Jiang W G, Clayton A, Mason M D, and Tabi Z.

Molecular Targets in Cancer Therapy, 4th Biannual meeting, Florida, 2007. Hepatocyte growth factor-induced dysregulation of monocyte-derived dendritic cell differentiation and function. Cook N, Mason M D, and Tabi Z.

Characterisation of newly established human mesothelioma cell lines. British Society of Immunology Annual Congress, Harrogate, 2005, Abstract in Immunology, 5116:89. Suppl. 1. 2005. Cook N, Coleman S, Butchart E, Gibbs A, Jasani B, Navabi H, Reece A, Clayton A, Mason M D, and Tabi Z.

Presentations

HGF and immune dysfunction. University of Wales Immunology, infection and immunity IRG annual meeting, Gregynog, 2007. Cook N.

Hepatocyte growth factor-induced dysregulation of monocyte-derived dendritic cell differentiation and function. 21st Annual Cardiff University Postgraduate day 2006 - Poster - Cook N, Coleman S, Jiang W G, Clayton A, Mason M D, and Tabi Z.

Hepatocyte growth factor-induced dysregulation of monocyte-derived dendritic cell differentiation and function. Inaugural Velindre NHS trust R&D meeting, 2006. Cook N, Coleman S, Jiang W G, Mason M D, and Tabi Z.

Characterisation of newly established human mesothelioma cell lines. 20th Annual Cardiff University Postgraduate day 2005. Cook N, Coleman S, Butchart E, Gibbs A, Jasani B, Navabi H, Reece A, Clayton A, Mason M D, and Tabi Z.

iv. Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen Presenting Cells
CD	Cluster of differentiation,
CCL19	Chemokine (C-C motif) ligand 19
CLIP	Class II associated invariant chain peptide
CTL	Cytotoxic T Lymphocyte
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DAMP	Damage associated molecular patterns
DISC	Death-inducign signalling complex
DC	Dendritic Cell
ELISA	Enzyme-Linked ImmunoSorbent Assay
ERp57	Glucose regulated protein 58 kDa
ERK1/2	Extracellular signal-regulated kinase 1/2
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine serum
FITC	Fluorescein IsoThioCyanate
GITR	Glucocorticoid-Induced Tumour necrosis factor Receptor
GM4	GM-CSF and IL-4
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
h	hour
HMGB1	High mobility group box 1
HGF/SF	Hepatocyte Growth Factor/scatter factor
H-iDC	HGF co-treated immature DC
ICAM	intracellular adhesion molecule
iDC	immature Dendritic Cell
IFN	Interferon
IFNα	Interferon α
IFNγ	Interferon γ
Ig	Immunoglobulin
IκB	Inhibitor of kappa B
IL	Interleukin
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17	Interleukin 17
IL-4	Interleukin 4
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
JNK	Jun N-terminal Kinase
LPS	Lipopolysaccharide

mAb	monoclonal Antibody
MAPK	Mitogen-activated protein kinase
MDDC	Monocyte derived dendritic cell
M-CSF	Macrophage Colony Stimulating Factor
MHC	Major Histo-compatibility Complex
mDC	mature Dendritic Cell
MFI	Mean Fluorescent Intensity
min	minute
ml	millilitre
MLR	Mixed Lymphocyte Reaction
MPM	Malignant Pleural Mesothelioma
NF-κB	Nuclear Factor-kappa B
NK	Natural Killer Cell
PAMP	Pathogen associated molecular patterns
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pC-iDC	Pre-Treated Control Immature DC
pC-mDC	Pre-Treated Control Mature DC
PCR	Polymerase Chain Reaction
PD-1	Programmed Death 1 / CD279
PD-L1	Programmed Death-Ligand 1 / CD274
PD-L2	Programmed Death-Ligand 2 / CD273
PE	Phycoerythrin
pH-iDC	HGF Pre-Treated immature DC
pH-mDC	HGF Pre-Treated mature DC
PRR	Pattern recognition receptors
R-PE	Phycoerythrin
rpm	Revolution Per Minute
RT	Room temperature
RTK	Receptor Tyrosine Kinase
SD	Standard Deviation
SEM	Stand Error of Mean
TAP	Transporter associated with antigen processing
TCR	T Cell Receptor
TRAIL	Tumour Necrosis Factor Related Apoptosis Inducing Ligand
TGF	Transforming Growth Factor
TLR	Toll-like Receptor
T_h	T Helper Cell
T_H1	T helper cell type 1
T_H2	T helper cell type 2

TNF	Tumour Necrosis Factor
U	units
µl	microlitre

v. Summary

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Summary of Thesis:

This thesis is an investigation into effect of elevated levels of hepatocyte growth factor (HGF) on immune responses.
High levels of HGF are found in cancer such as in MPM patient serum and pleural fluid. However, little is known about the consequence of high HGF levels on the development and function of dendritic cells and contribution of these effects to tumour immune evasion.

A pre-treatment *in vitro* model system was applied to study the effects of high HGF concentrations on the development of dendritic cells (DC) from monocytes. The effects of HGF on the phenotype of dendritic cell, functional characteristics, including migration, phagocytosis and T cell stimulation were analysed.

Using this model system I discovered a previously un-reported immature DC-like phenotype, caused by the pre-treatment of monocytes for 24 h prior to induction of iDC development by GM-CSF/IL-4: Delayed differentiation of DC alone generates a T_H2 bias, which is further enhanced by the presence of HGF.

HGF pre-treated DCs express both monocyte marker CD14 and DC marker CD209 (DC-SIGN). They are able to take up antigen by phagocytosis. However, they produce increased levels of IL-10 and express elevated levels Programmed Death 1 (PD-1) ligand, PD-L1. HGF-pre-treated DC also display impaired ability to stimulate allo-T cell proliferation and antigen-specific IFN-γ production. HGF pre-treated DC induces increased IL-10 production by T cells.

Blocking IL-10 with a neutralizing antibody restores normal DC differentiation, partially reduced PD-L1 levels and restored T cell stimulatory capacity of DC. The physiological relevance of these findings was demonstrated by similar effects on DC developed in the presence of mesothelioma pleural fluid, in a HGF dependent manner. This thesis demonstrates that HGF is an immunosuppressive factor that can contribute to tumour-induced regulation of DC function and T cell responses.

vi. Contents

Chapter - 1 Introduction

1.1 Cell Mediated Immunity in Cancer

The immune system is primarily responsible for protection against pathogens, and is divided into two parts, the innate and the adaptive systems. The innate arm of the immune system is fast acting and recognises types of molecules peculiar to frequently encountered pathogens. It includes physical and chemical barriers (e.g. skin, mucosal membranes and stomach acids), phagocytic cells (neutrophils) and complement. The innate immune system can respond in hours, and once phagocytic cells encounter pathogens results in initiation of inflammatory responses, which is the first step towards a specific responses against the pathogen.

This specific response is the purview of the adaptive immune system, which in terms of cancer is regarded as the more important of the two arms of the immune system. Although some innate components, such as NK cells, can recognise cancer cells the majority of the innate immune system cannot. This is due the to the nature of cancer since it arises from host cells and it is therefore not a foreign pathogen, it bypasses much of the innate immune system. The adaptive immune system is able to recognise both foreign pathogens and self antigens (cancer/precancerous cells). It comprises of B cells, antigen presenting cells (APC) and T cells.

1.2 T Cells

T lymphocytes are composed of two subsets, based on the expression of CD4 and CD8 molecules. $CD8^+$ T cell function is to recognise and eliminate transformed and virus infected cells, while the $CD4^+$ subset is primarily responsible for providing

'help' to $CD8^+$ T cells via secretion of cytokines or cell to cell contact and are therefore commonly called T helper cells. $CD4^+$ T cells play an important role in the induction and control of inflammatory responses and generation of $CD8^+$ T cell responses. The importance of T cells in the control of immunity to tumours is supported by various studies using mouse models such as SCID or Rag2-/- mice, which lack T and B cells, or nude mice. These mice have increased susceptibility to tumour growth (Bosma, Custer et al. 1983; Svane, Engel et al. 1996; shankaran, Ikeda et al. 2001) while in humans immuno deficiency such as that seen in AIDS or transplant patients can also increase the susceptibility to tumour development (Weiss 1999; Goedert 2000).

1.2.1 CD8+ T Cell Responses

T cells expressing $CD8^+$ molecules recognise MHC Class I molecules presenting peptides derived from intracellular proteins. This endogenous antigen presentation by DC will be discussed in detail later. Briefly; proteins are degraded in cytostolic proteosomes; this typically produces 7-10 amino acid long peptides. These peptides are transported to the ER (via transporter proteins such as TAPs and LMPs) and bind to MHC Class I molecules, which are transported via the Golgi apparatus to the cell surface, displaying these peptide antigens for immune recognition. Specific recognition of antigens ensures lysis of infected, foreign, transformed or malignant cells.

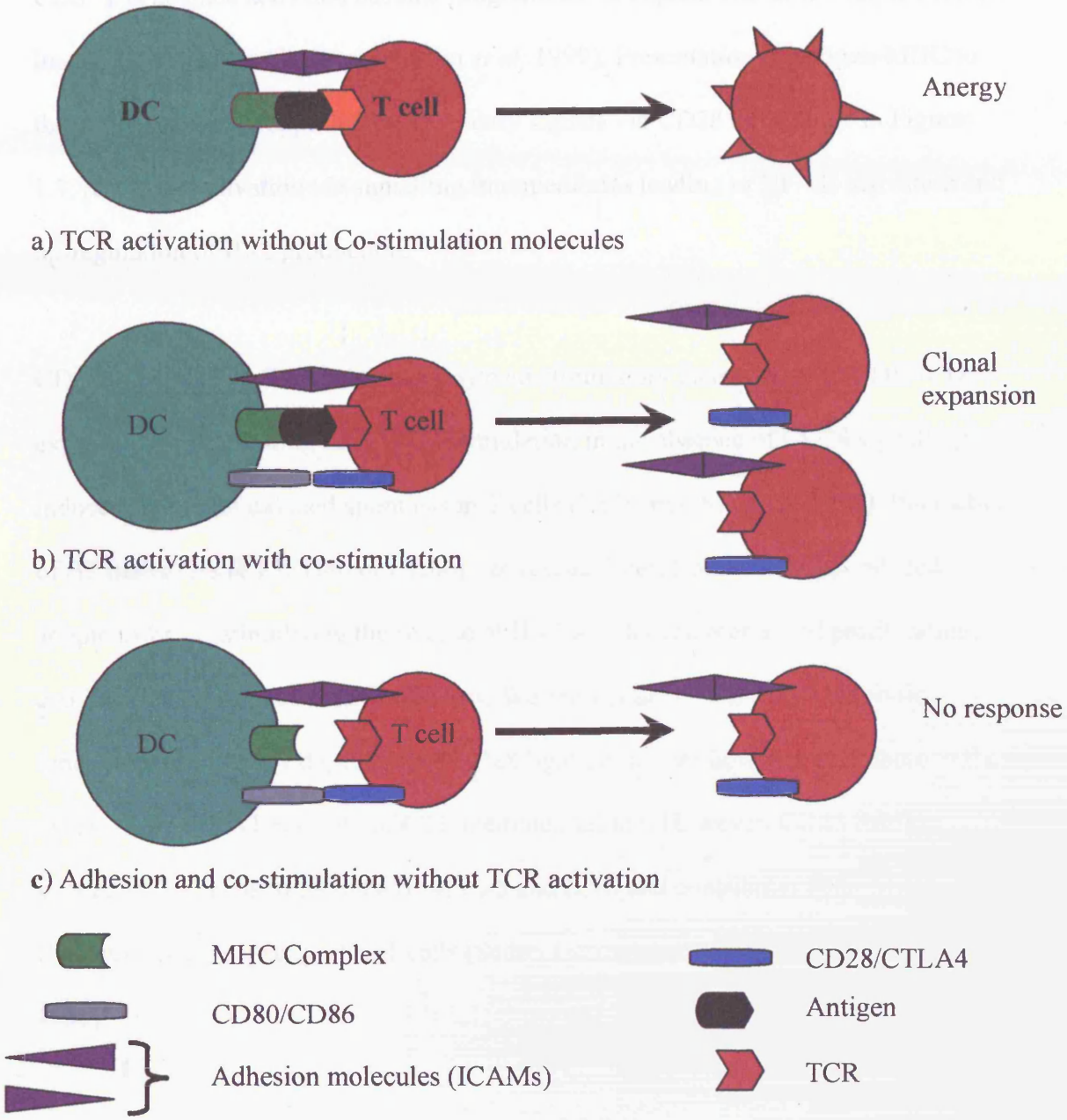


Figure 1.1 Co-Stimulatory Interactions in T Cell Stimulation

Outcomes of interactions between APC and T cells

a) Induction of anergy due to lack of appropriate co-stimulatory molecule:CD28 interactions. b) Clonal expansion due to appropriate TCR activation and co-stimulatory interactions. c) No response when no antigen is present to induce the primary signal via TCR.

$CD8^+$ T cells once activated become programmed to expand and do not require further interaction with DC (Zarling, Johnson *et al.* 1999). Presentation of antigen-MHC to the T cell receptor, supported by secondary signals via CD28 as outlined in Figure 1.1, results in activation via signalling intermediaries leading to NF-κB activation and up-regulation of IL-2 production.

CD28 expressed on T cells interacts with co-stimulatory factors such as CD80/CD86 expressed on stimulating cells. TCR stimulation in the absence of CD28 signalling induces activation induced apoptosis in T cells (Radvanyi, Shi *et al.* 1996). Interaction of B7 molecules (e.g. CD80 or CD86) can rescue T cell from activation induced apoptosis by co-stimulating the release of IL-2 which promotes T cell proliferation and inhibits T cell apoptosis (Lenschow, Walunas *et al.* 1996). While cytolytic function of T cells is independent of CD28 ligation, it does however contribute to the overall strength and intensity of CTL mediated killing. However, CD28 ligation stimulates T_H2 cells to produce IL-4, IL-5 and IL13 and contributes somewhat to the IFNγ producing capacity of T_H1 cells (Seder, Germain *et al.* 1994; King, Stupi *et al.* 1995).

Interactions of B7 molecules with CD28 support can also suppress T cell activation via other receptors. CTLA4, a ligand for CD80 and CD86 molecules, which is expressed on T cells late on during activation and inhibits T cell activation (Chambers, Krummel *et al.* 1996). CTLA4 signalling limits the number and activity of CD4 and CD8 T cells that respond to antigen. CTLA affects both T_H1 and T_H2 cells. CTLA-4 does not just have these affects by competing with CD28 for CD80/CD86 binding, but also actively suppresses T cell activation. CTLA-4

stimulation prevents cytokine production (IL-2), T cell proliferation and also increases apoptosis (Carter and Carreno 2003). Therefore CTLA-4 has been implicated in control of T cell activation, and is implicated in control of autoimmunity (Chambers, Krummel et al. 1996; Peggs, Quezada et al. 2006).

Lack of co-stimualtion via CD28 results in lower NF-κB activation leading to induction of anergy or activation induced apoptosis. $CD8^+$ T cells can autonomously divide multiple times and acquire cytotoxic effector functions, without requiring further antigen stimulation (Kaech and Ahmed 2001; Van Stipdonk, Lemmens *et al.* 2001). Cytotoxic T lymphocytes (CTL) do not stay in secondary lymphoid organs but migrate to sites of inflammation for delivery of their effector function. These CD8+ effector T cells have four main mechanisms for eliminating target cells.

A. Cytotoxic Cytokine Secretion

Cytokines, such as TNFα and IFNγ, inhibit virus replication and increase MHC Class I-antigen presentation. They can also cause increase cell death by targeting cells susceptible to lysis (via up-regulation of death receptors such as Fas) or to programmed cell death (apoptosis). $CD8^+$ T cell derived cytokines can remove or control the growth of malignant cells, which is demonstrated by the fact that mice lacking IFNγ genes develop spontaneous tumours (Street, Trapani *et al.* 2002).

B. Perforin-Dependent or Calcium Dependent Cytotoxicity

Lytic granules, are stored by CTL and are the main killing mechanism employed by these cells. The granules are modified lysozomes containing pore-forming proteins (perforin) and enzymes such as granzymes. In combination, these proteins work

together with perforins polymerising (in the presence of Ca^{2+}) and inserting pores into the membrane which allow entry of granzymes and other proteins that can induce apoptotic cell death. Perforin can also cause cell death by reducing the integrity of the plasma membrane causing osmotic lysis (Ojcius and Young 1990; Podack 1995; Bolitho, Voskoboinik *et al.* 2007); mouse models lacking IFNγ and perforin genes develop spontaneous tumours (Smyth, Thia et al. 2000; Street, Trapani et al. 2002).

C. FAS-Dependent or Calcium Independent Cytotoxicity

Activated T cells, (mainly $CD8^+$ T cells) can also kill via Fas:FasL interactions. Fas expression is widespread. However, its natural ligand has limited expression. Fas on tumour cells interacts with Fas-ligand on effector T cells causing aggregation of intracellular death domains to form the death-inducing signalling complex (DISC) in tumour cells. This leads to the activation of caspases, causing apoptotic cell death (Kagi, Vignaux *et al.* 1994; Walsh, Matloubian *et al.* 1994). However, it has also been suggested that Fas could be involved in tumour counterattack on the immune system as Fas is also expressed by activated T cells (Wajant 2006).

D. TRAIL (Tumour Necrosis Factor Related Apoptosis Inducing Ligand) Cytotoxicity

Activated T cells up-regulate another apoptosis-inducing ligand, TRAIL, which is a TNF-related ligand (Wiley, Schooley *et al.* 1995). TRAIL receptors implicated in cell death are widely expressed and can be up-regulated in cancer (Walczak, Degli-Esposti *et al.* 1997; Fanger, Maliszewski *et al.* 1999; Hoskin 2000). Engagement of TRAIL on T cells with its receptors TRAIL-R1 or TRAIL-R2 on cancer cells, like the Fas:FasL interaction, leads to the activation of DISC and inhibition of Bcl-2 and induction of

caspase-dependent apoptotic cell death (Wiley, Schooley et al. 1995; Hoskin 2000). $CD4^+$ cytolytic cells have been shown to kill TRAIL-R expressing tumours via the TRAIL/TRAIL-R system (Thomas and Hersey 1998; Wang, Boonman *et al.* 2003). In mice lack of TRAIL expression causes increased growth and incidence of tumours (Takeda, Smyth *et al.* 2002; Zerafa, Westwood *et al.* 2005).

1.2.2 CD4+ T Cell Responses

While $CD8^+$ T cells or cytotoxic T lymphocytes (CTL), are the usual effector cells eliminating MHC Class I+ target cells $CD4^+$ T cells are required for the generation of $CD8^+$ T cell responses. $CD4^+$ T cells provide help in the generation of $CD8^+$ T cell responses, either directly through cell-cell contact or by secretion of cytokines.

$CD4^+$ T cells require antigen to be presented in the context of MHC Class II molecules, which are only expressed by antigen presenting cells. DC and other antigen presenting cells internalise foreign pathogens by phagocytosis, micropinocytosing soluble and endocytosing particulate proteins. These exogenous proteins are degraded in endosomes to produce peptides of 12-25 amino acid long and presented as discussed later.

$CD4^+$ T cell priming by DC is delivered by three signals;

1) antigen presented on MHC Class II molecules,

2) co-stimulatory molecules CD80/CD86

3) cytokine secretion by DC (Fagnoni, Takamizawa *et al.* 1995; Dilioglou, Cruse *et al.* 2003; Shin, Kennedy *et al.* 2003). Naïve $CD4^+$ T cells can receive polarising

signals which induce their differentiation into distinct T helper populations usually categorised into either T_H1, T_H2 or T_H17 type responses.

Generally, pro-inflammatory cytokines/signals such as IFNγ and IL-12 induce the development of T_H1 type responses (Caminschi, Venetsanakos *et al.* 1998; Caminschi, Venetsanakos *et al.* 1999; Rissoan, Soumelis *et al.* 1999; Luft, Maraskovsky *et al.* 2004; Bellone, Carbone *et al.* 2006). In the absence of IL-12, other cytokines, such as IL-10 and IL-4, can differentiate $CD4^+$ T cells into T_H2 type cells (Lucey, Clerici *et al.* 1996). The distinction between T_H1 and T_H2 type responses is based on the expression of cytokines, which induce different types of immune responses. Type I cytokines in the main are IFNγ, IL-12 and TNFα while Th2 cytokines are IL-4, IL-5 and IL-10 (Lucey, Clerici et al. 1996). T_H1 cytokines induce cell-mediated immunity, increase cell susceptibility to lysis, activate CTL and Natural Killer (NK) cells, while T_H2 cytokines activate B cells and are fundamental to the generation of humoral responses. T_H17 type T cells are mainly characterised by production of IL-17 and IL-22, and are implicated in autoimmune and inflammatory responses and are critical for protection against microbial infection, especially extracellular bacteria and fungi (Zhu and Paul 2008).

$CD4^+$ T cells participate in the generation of $CD8^+$ CTL responses to exogenous antigen presented by APC, where $CD4^+$ T cell assistance is required (Friedman, Green *et al.* 1988; Bennett, Carbone *et al.* 1997; Kennedy and Celis 2006; Gupta, Boppana *et al.* 2008). $CD4^+$ T cells help in this process by recognising antigens expressed on DC and secreting cytokines (particularly IL-2) during interaction of APC with naïve $CD8^+$ T cells, or aiding the functional maturation of DC (increased costimulatory

molecule and cytokine expression), prior to interaction of APC with naïve $CD8^+$ T cells (see figure 1.2). The interaction of $CD4^+$ T cells with DC is via CD40 molecules (DC) and CD40L (CD154 on T cells) (Caux, Massacrier et al. 1994; Bennett, Carbone et al. 1997; Danese, Scaldaferri et al. 2007). The importance of this interaction can be seen in *in vivo* experiments; CD40 ligation can completely restore $CD8^+$ CTL activity in mice depleted of $CD4^+$ T helper cells. (Bennett, Carbone *et al.* 1998; Schoenberger, Toes *et al.* 1998). Cancer immunotherapy approaches include CD40 stimulation using autologous tumour cell vaccines, CD40 gene therapy and CD40 agonistic antibodies (Bennett, Carbone et al. 1998; Schoenberger, Toes et al. 1998; Schultze, Anderson et al. 2001; Dzojic, Loskog et al. 2006; Loskog and Totterman 2007; Wu, Zhao et al. 2007; Llopiz, Dotor et al. 2008).

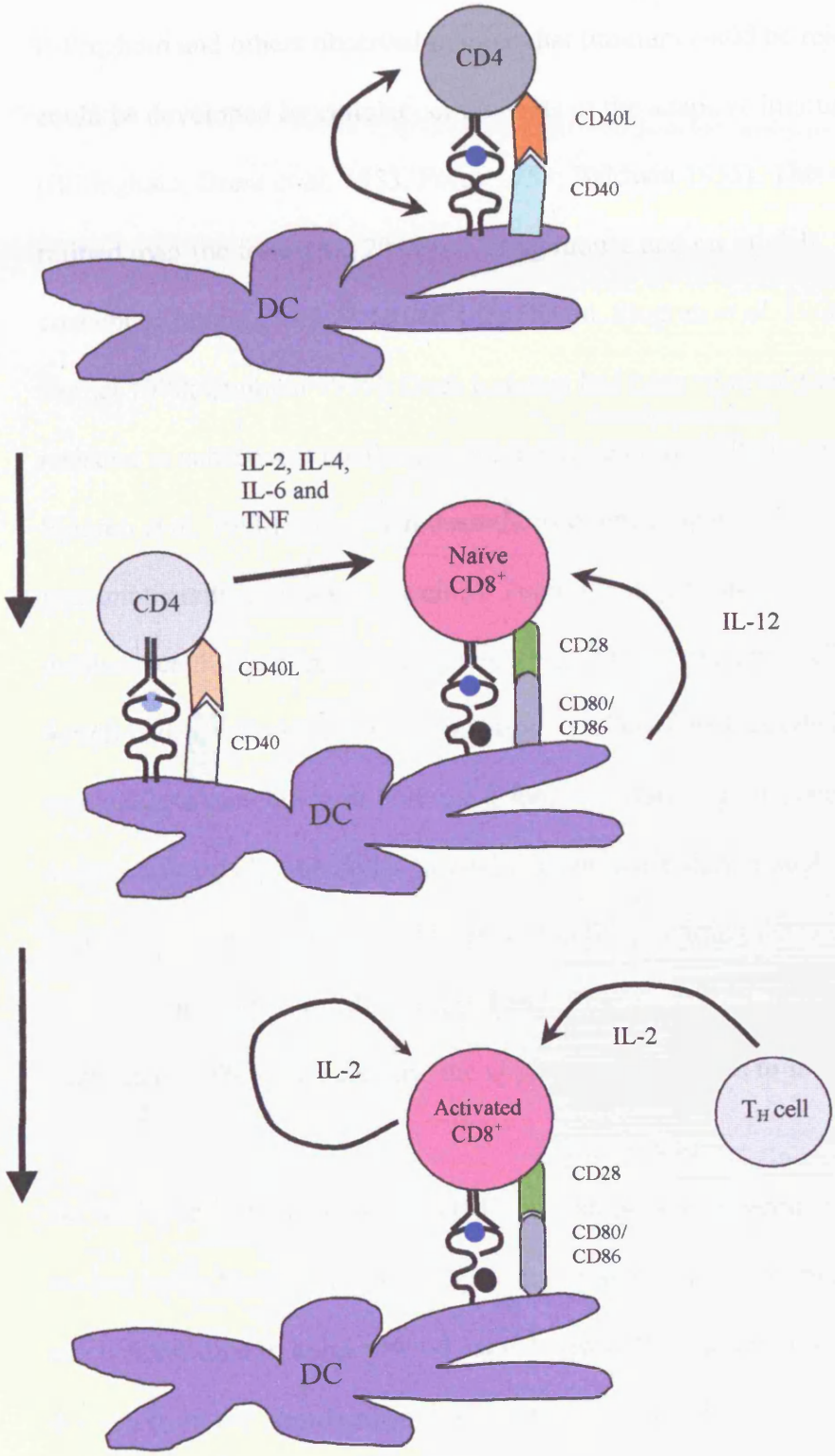


Figure 1.2 CD4 T Helper Interactions with DC

1.3 Dendritic Cells

Billingham and others observed in mice that tumours could be rejected and immunity could be developed by cellular components of the adaptive immune system (Billingham, Brent *et al.* 1953; Foley 1953; Baldwin 1955). This discovery was refined over the following 20 years, using mouse and rat models, culminating in the concept of immunological surveillance (Klein, Sjogren *et al.* 1960; Burnet 1964; Burnet 1970; Stutman 1975). Once tumours had been rejected these animals were resistant to subsequent challenge with the same tumour (Prehn and Main 1957; Klein, Sjogren et al. 1960). These demonstrations opened the way for developing both immunotherapy and cancer vaccines. Tumour antigens are carried by APC, which are dendritic cells, macrophages and monocytes. DC are the only cells able to migrate to lymph nodes, where the naïve population of $CD8^+$ T cells reside in an environment conducive to generation of immune responses. Maturing DC, containing antigen, migrate towards lymph nodes following chemokine signals such as CCL19. In the lymph nodes DC can present antigen to T cells, providing the necessary signals such as antigen in context of MHC Class I and II molecules, co-stimulatory signalling via CD80 and CD86 molecules and the appropriate cytokines to induce T cell activation.

DC derive from bone marrow $CD34^+$ haematopoietic progenitor stem cells and are present in the blood, lymphoid organs and tissues. Two main subsets of dendritic cells can be identified in human blood using known DC markers (Figure 1.3). The cells derived from lymphoid progenitors (CD11c) *in vitro* differentiate into plasmacytoid DC (pDC) after stimulation with IL-3 and CD40 ligand (CD40L). The ones derived from myeloid progenitors ($CD11^+$) develop into other cells such as granulocytes, monocytes, macrophages and myeloid DC and Langerhans cells, which reside in the

epithelium and provide a sentinel role for infection. Plasmacytoid DC (pDC) produce large amounts of cytokines, particularly type IFNα and regulate inflammation (Colonna, Trinchieri *et al.* 2004). pDC provide a link between innate and adaptive immunity. IFN released by human pDC activate NK cell cytolytic activity. pDCs are also activated by virus infections, and may be particularly important in inducing antiviral antibodies (Colonna, Trinchieri et al. 2004).

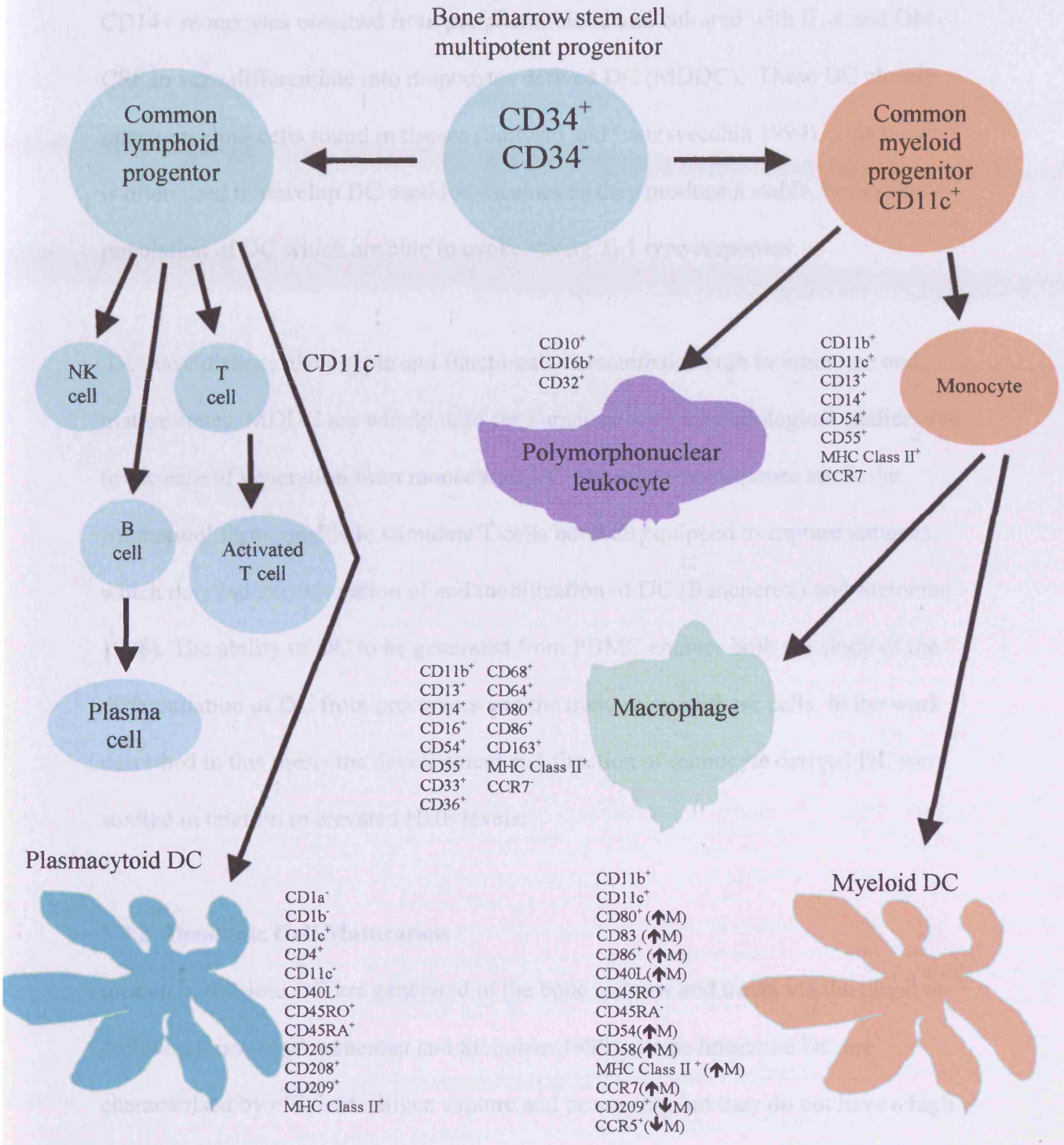


Figure 1.3 Dendritic Cell Development and Markers.

Molecules down-regulated during myeloid DC maturation (↓M), up-regulated during myeloid DC maturation (↑M)

CD14+ monocytes obtained from peripheral blood and cultured with IL-4 and GM-CSF *in vitro* differentiate into monocytes derived DC (MDDC). These DC closely match myeloid cells found in tissues (Sallusto and Lanzavecchia 1994). This method is often used to develop DC used for vaccines as they produce a stable, homogenous population of DC which are able to evoke strong T_H1 type responses.

DC have distinct phenotypic and functional characteristics both in immature and mature states. MDDC are widely used for human *in vitro* immunological studies, due to the ease of generation from monocytes. DC present in most tissues are in the 'immature' form, unable to stimulate T cells but well equipped to capture antigens, which may induce maturation of and mobilization of DC (Banchereau and Steinman 1998). The ability of DC to be generated from PBMC enables both the study of the differentiation of DC from precursors and the maturation of these cells. In the work described in this thesis the development and function of monocyte derived DC were studied in relation to elevated HGF levels.

1.3.2 Dendritic Cell Maturation

Immature myeloid DC are generated in the bone marrow and travel via the blood to peripheral tissues (Banchereau and Steinman 1998). These immature DC are characterised by efficient antigen capture and processing but they do not have a high capacity to stimulate T cells. Phenotypically, they express low levels of co-stimulatory molecules (CD40, CD86, CD80) and do not express CD83. They can express other markers such as CD209, CD1a, but do not express high levels of CD14. In peripheral tissues these DC can account for up to 1-2% of total cells (Guermonprez, Valladeau *et al.* 2002). Their ability to capture antigens by various mechanisms

(micropinocytosis and endocytosis) enables them to function as peripheral sentinels for pathogens and damaged/abnormal cells.

Activation of DC induces the release of inflammatory chemokines and cytokines, such as IFNγ, IL-12, TNFα, MIP-1α and RANTES, to regulate leukocyte recruitment (Sozzani, Allavena *et al.* 1998; Park, Kim *et al.* 1999; Sallusto, Palermo *et al.* 1999; Sozzani 2005). DC also undergo coordinated maturation, transforming from antigen processing to antigen presenting cells, enabling increased stimulation of memory and naïve T cells (Sallusto, Cella *et al.* 1995; Tschoep, Manning *et al.* 2003). DC possess receptors that recognise conserved molecules in pathogens or pathogen associated molecular patterns (PAMP). Toll-like receptors (TLR) are one example of these pattern recognition receptors(PRR) (Janeway Jr 1989). As PAMPs are absent from the host, they are not likely to be involved in the maturation of DC on encountering tumour cells. An alternative DC activation mechanism proposes that DC respond to endogenous signals (so called damage associated molecular patterns, DAMP) released by necrotic and damaged cells and cells under stress, in the absence of PAMP (Bianchi 2007).

Examples of PAMPs are: LPS, bacterial DNA and double stranded DNA (De Smedt, Pajak *et al.* 1996; Akbari, Panjwani *et al.* 1999; Cella, Salio *et al.* 1999; Hartmann, Weiner *et al.* 1999), while DAMPs can be signals from stressed or dying cells, such as TNFα, IL-1, IL-6, TGF-β and HMGB1 and heat shock proteins (Gallucci, Lolkema *et al.* 1999; Bianchi 2007; Foell, Wittkowski *et al.* 2007).

T cell-derived signals, especially CD40L interaction with CD40, induce terminal DC differentiation (Caux, Massacrier et al. 1994; Schoenberger, Toes et al. 1998;

Vidalain, Azocar et al. 2000; Onaitis, Kalady et al. 2003). These mechanisms of inducing DC maturation have been investigated as part of possible immunotherapy approaches via gene therapy, cytokine therapy and tumour cell vacines (Friedlander, Delaune *et al.* 2003; Gregoire, Ligeza-Poisson *et al.* 2003; Koya, Kasahara *et al.* 2003; Bhardwaj 2007).

Maturation signals, such as LPS, lead in DC to the activation of the NF-κB pathway (Neumann, Fries *et al.* 2000). In response to stimulation the IκB proteins which bind to and sequester NF-κB in the cytoplasm are broken down in proteosomes releasing active NF-κB. NF-κB translocates into the nucleus where it binds to specific sequences in the promoter/enhancer regions of genes, inducing transcription. Such activated genes, like MHC Class I and Class II molecules, co-stimulatory molecules, cytokines and cell adhesion receptors are important in the function of DC in immune response induction. The requirement of NF-κB for DC maturation has been demonstrated by blocking NF-κB using IκBα transfection, which results in down regulation of MHC Class II, CD80, CD86, CD40 and pro-inflammatory cytokine expression such as IL-12 and TNFα (Yoshimura, Bondeson *et al.* 2001).

Maturing DC also up-regulate molecules such as CCR7, a receptor for CCL19 (a chemokine responsible for migration towards lymph nodes). Furthermore, they lose endocytic/phagocytic receptors and the functional capacity to capture antigen (De Smedt, Pajak et al. 1996; Jonuleit, Kuhn et al. 1997; Rovere, Vallinoto et al. 1998; Sozzani, Allavena et al. 1998; Cella, Salio et al. 1999). Additionally, CD83, a DC maturation marker is up-regulated. Cytokine production is increased, particularly the

production of IL-12 and TNFα. LPS induces NF-κB pathway activation and is widely used to study DC maturation, it has been used in this thesis to mature DC. Molecules, expressed on DC and with the greatest relevance to the generation of adaptive immune responses, are discussed below.

1.3.3 MHC Class I and II

'Classical' $CD8^+$ T cell stimulation by peptide antigen requires that these proteins are synthesised within the antigen presenting cells (APC). This may represent a disadvantage for tumour antigen recognition associated with solid tumours, as these antigens are not endogenously synthesised in APC. Dendritic cells, as APC, support an alternative antigen presentation pathway that induces primary $CD8^+$ T cells responses via the exogenous antigen presentation pathway. This process is called 'cross-presentation' and the priming of CD8 T cells is referred to as 'cross-priming' (Brossart and Bevan 1997). This pathway is of particular interest in cancer immunology, as it allows presentation of antigens derived from solid tumours. Cross-presentation has been described when high concentration of exogenous antigen, either soluble or particulate (Bevan 1987) and from either apoptotic or necrotic tumour cells is presented by DC (Herr, Ranieri *et al.* 2000; Sauter, Albert *et al.* 2000). Exogenous antigen is taken up by DC, and processed for MHC Class I-restricted presentation, as described by (Brossart and Bevan 1997). Activation of T cell cytotoxic responses by cross presentation requires the assistance or 'help' of $CD4^+$ T cells (Bennett, Carbone et al. 1997).

MHC Class I and II molecules bind endogenous or exogenous peptides and present these peptide antigens for recognition by T cells. Peptide antigens result from

degradation of proteins into peptides within DC. Peptide antigens are transported to the cell surface by one of two routes; the endogenous pathway (for expression with MHC Class I) or the exogenous pathway (for expression on MHC Class II, see figure 1.3).

The endogenous pathway presents peptide fragments, produced by the degradation of endogenous protein in proteosomes, which are then transported via TAP proteins to the rough endoplastic reticulum. Here they bind to MHC Class I/$\beta_2$microglobulin complexed with chaperone proteins Calreticulin, Tapasin and ERp57. Binding of antigen to this complex releases the chaperones; MHC Class I-peptide complex is able to translocate via the Golgi apparatus to the cellular surface (Cresswell, Ackerman *et al.* 2005).

The exogenous pathway presents exogenous proteins phagocytosed and degraded in endocytic compartments. MHC Class II molecules, complexed with the invariant chain/CLIP fragment (to stop non-specific protein binding), bind to these compartments causing disassociation of CLIP fragments and binding of exogenous peptides to MHC Class II molecules. These complexed antigen–MHC Class II molecules are then transported to the cells' surface. The up-regulation of MHC-antigen complexes increases the efficiency of presentation of antigen to TCR on effector T cells.

1.3.4 CD80 /CD86

The importance of DC providing the "second signal" via CD80 and CD86 molecules during the induction of primary T cell responses is well established. In some cases co-

stimulation via CD28 molecules on T cells is not required for generation of cytotoxic T lymphocytes (CTL), as high levels of IL-12 and IL-2 can replace CD28 signalling (Makrigiannis, Musgrave *et al.* 2001).

CD80 (B7-1) and CD86 (B7-2), are part of the B7 family of co-stimulatory molecules and are in turn members of the immunoglobulin superfamily of receptors. They are almost exclusively expressed on professional APC such as DC, monocytes and activated B cells. CD80/CD86 molecules interact with CD28, expressed on T cells, providing a second signal that in conjunction with the signal from TCR interaction with antigen-MHC complexes, results in prolonged and enhanced IL-2 production. This causes prolonged and strong T cell activation (Van Gool, Vandenberghe *et al.* 1996; Kuroki, Shibaguchi *et al.* 2004). Mouse models studying CD80 and CD86 suggest that although co-stimulatory signals from these molecules are similar, CD80 provides a quantifiably stronger stimulation than CD86, in terms of naïve $CD8^+$ T cell proliferation and IL-2 production (Creery, Diaz-Mitoma *et al.* 1996; Gajewski 1996; Saito, Yagita *et al.* 1996).

Interaction between MHC-antigen complexes on APC with TCR on T cells can result in functional immune responses, anergy or apoptosis (Greenwald, Freeman *et al.* 2005). Interactions between CD80/CD86 with CD28 on T cells can prevent activation induced apoptosis (Shi, Radvanyi *et al.* 1995). While T cell stimulation with little or absent CD80 or CD86 signalling results in T cell anergy (Schwartz 1990; Radvanyi, Shi et al. 1996) this may be the result of lack of IL-2 production (Becker and Brocker 1994; Coughlin, Wysocka *et al.* 1995). The role of the B7 family on regulation of T cell activation or tolerence, via CD28 or CTLA4 respectively, make modulation of B7

family members interactions of importance in the control of T cell activation (like PD-L1 and 2) in therapy (Greenwald, Freeman et al. 2005).

1.3.5 CD83

CD83 is often used as a marker of DC maturation due to its selective expression by mature DC (Zhou and Tedder 1995). CD83 expressed on DC surface acts as an adhesion receptor binding blood monocytes and activated or stressed $CD8^+$ T cells (Scholler, Hayden-Ledbetter *et al.* 2001). However CD83 is also found in soluble form (CD83s), released from activated DC by proteolytic shedding of the ecto domain of membrane CD83. sCD83 Ig domain has been shown to inhibit DC-mediated primary allogenic T cell proliferation as well as peptide specific T cell proliferation *in vitro*. sCD83 also binds immature DC, blocking maturation and can down-regulate CD83 expression on mature DC (Lechmann, Krooshoop *et al.* 2001; Lechmann, Zinser *et al.* 2002; Fujimoto and Tedder 2006). CD83 expressed by DC seems to play a role in intercellular T cell and DC communication, while sCD83 may serve as an immuno-regulatory molecule (Lechmann, Zinser et al. 2002; Fujimoto and Tedder 2006). CD83 ligand engagement, following TCR and CD28 signalling, supports the generation of long-lived antigen specific cytotoxic T cells, by inducing proliferation and inhibiting apoptosis (Hirano, Butler *et al.* 2006).

1.3.6 CD40

CD40, a member of the TNFα receptor family, is expressed on all APC. CD40 ligand (CD40L or CD154) is also a member of this family, and its expression is mainly restricted to activated $CD4^+$ T cells (Armitage, Tough *et al.* 1993; Cayabyab, Phillips *et al.* 1994; Grewal and Flavell 1996). As discussed earlier, CD40 ligation induces

terminal differentiation of DC characterised by increased expression of other co-stimulatory molecules and adhesion molecules including CD80, CD86, CD54 and CD58. Additionally, stimulation via CD40 induces increased IL-12 production by DC (Armant, Armitage *et al.* 1996; Cella, Scheidegger *et al.* 1996; Kato, Hakamada *et al.* 1996). Therefore CD40 activation of DC is critical for effective antigen presentation and effective induction of T cell responses.

1.3.7 PD-L1 and PD-L2

Program death ligand 1 and 2 (PD-L1 and PD-L2) are recently discovered members of the B7 family of cell surface receptors, and are expressed on a number of immune cells including B cells, macrophages, T cells and dendritic cells, mesenchymal stem cells, bone marrow-derived mast cells and DC in mice and some cancer cells (Yamazaki, Akiba *et al.* 2002). PD-L1 and PD-L2 are ligands of programmed death 1 molecule (PD-1), which itself closely resembles CTLA-4. PD-1/PD-L1 or PD-L2 interactions may result in cell cycle arrest of T cells (Brown, Dorfman *et al.* 2003). PD-1 is inducibly expressed on activated T cells and ligation of PD-1 by PD-L1 has been shown to dampen T cell activation (Ozkaynak, Wang *et al.* 2002).

Expression of PD-L1 has been shown as an indicator of poor prognosis in cancer (Hamanishi, Mandai *et al.* 2007; Noami, Sho *et al.* 2007; Wang, Han *et al.* 2007). PD-L1 on tumour associated myeloid DC can be up-regulated by factors in the tumour microenvironment, and its antibody blockade can enhance DC/T cell activation. PD-1 blockade does not change levels of apoptotic cells but by decreasing proliferation, as signalling via PD-1 inhibits both IL-2 and IFNγ production by T cells. However as PD-1 and PD-L1 are also expressed on regulatory T cells they may control Treg

regulatory effects. PD-L2 expression is more restricted as is inducibly expressed on DC, macrophages and bone marrow derived stem cells (Zhong, Tumang *et al.* 2007).

The role of PD-1 and its ligands in regulating the balance between T cell activation and tolerance, how this mechanism has been used by tumours to aid in immune escape and the possible uses in therapy, have been thoroughly reviewed recently by *Keir et al* (Keir, Butte *et al.* 2008). Signalling via PD-1 interacts with early B7:CD28 signalling by blocking the activation of PI3K via CD28 leading to down-regulation of IL-2 , IFNγ and a decrease in Bcl-2 family of anti-apoptotic proteins. Tumour associated APC can also use the PD-1 pathway to control T cell immune responses (Curiel, Wei *et al.* 2003). PD-1 interactions exert vital and diverse immuno-regulatory roles on T cell activation and tolerance, by controlling pathogenic effector T cells. PD-L1/PD-L2 are therefore fundamentally and therapeutically important in cancer immunology and merit further study

1.3.8 CD209

CD209, also known as DC-SIGN (DC - Specific ICAM-3 Grabbing Non-integrin), is a member of the mannose receptor family. The expression of CD209 is restricted to immature DC (Bleijs, Geijtenbeek *et al.* 2001), and is down-regulated during maturation (Relloso, Puig-Kroger *et al.* 2002). CD209 is not expressed by monocytes, activated monocytes, T cells, B cells, activated B cells, thymocytes and $CD34^+$ bone marrow cells. It is expressed by cells in the T cell area of lymph nodes and other lymphoid organs; it is not expressed by CD1a Langerhans cells in the skin, but is expressed by DC like cells in mucosal tissues (Geijtenbeek, Torensma *et al.* 2000). Additionally, recent studies suggest that CD209 may be expressed by macrophages in

fetal tissues, and also on endothelial cells in the hepatic sinusoid and lymphatic sinus (Bleijs, Geijtenbeek et al. 2001). CD209 up-regulation during development of DC from monocytes using GM-CSF-CSF and IL-4 (GM4) is dependent on IL-4 (Relloso, Puig-Kroger et al. 2002).

CD209 on DC progenitors, immature and maturing DC can facilitate trans-endothelial migration by binding with ICAM-2 molecules on endothelial cells. Up-regulation of ICAM-1 during inflammation can strengthen this interaction and coupled with localised expression of chemokines this can result in the specific migration of DC into sites of inflammation. This allows these DC to carry out their immunological functions such as take up and processing of antigen, in the periphery. (Zhou, Chen *et al.* 2006). In the lymph node, CD209 can stabilise the binding of DC to T cells by interactions with ICAM-3. This binding also activates LFA-1 on T cells which stabilises the immunological synapse due to high affinity binding with ICAM-1 (Zhou, Chen *et al.* 2006).

In addition to stabilising T cell binding, CD209 can contribute directly to T cell activation by promoting T cells to produce IL-2 and increasing TCR signalling. Inhibition of CD209 expression has been shown to affect expression of CD11c, CD83, CD80 and CD86; it is believed that this occurs via the NF-κB pathway, but the mechanism is still unclear (Zhou, Zhang *et al.* 2006). CD209 has however, also been implicated in viral infections. Virus can bind to and then infect DC, as CD209 can recognise and bind to glycosylated viral proteins such as gp120 on HIV. This allows transportation of the virus to lymphoid tissues by the DC (Geijtenbeek, Torensma et al. 2000; Alvarez, Lasala et al. 2002; Engering, Geijtenbeek et al. 2002; Caparros,

Serrano et al. 2005; Aarnoudse, Vallejo et al. 2006; Caparrós, Munoz et al. 2006). Additionally, CD209 cross-linking bacteria such as Lewis$^+$ Heliobacter pylori has been shown to mediate induction of T_H2 type responses, allowing immune escape (Bergman, Engering *et al.* 2004).

Recently CD209 has been linked to immune escape by tumours, but is poorly understood; it has however been demonstrated that there is cross-talk between CD209 and Toll like receptors, which can interfere with TLR signalling and induction of DC maturation (Koppel, van Gisbergen et al. 2005; van Gisbergen, Aarnoudse et al. 2005; Zhou, Chen et al. 2006).

It has also been shown that cancer antigens such as CEA in colorectal cancer interact with DC via CD209 (van Gisbergen, Aarnoudse et al. 2005). CD209 cross-linking increases IL-10 production by LPS-stimulated DC, and can alter the balance of T_H1/T_H2 type responses and can induce regulatory cells (Caparros, Munoz *et al.* 2006; Nonaka, Izumo *et al.* 2008). In acute lymphoblastic leukemia interaction of cancer cells with CD209 has been shown to correlate with poor prognosis and tolerance induction (Gijzen, Raymakers *et al.* 2008).

1.3.9 CD14

CD14 is a 55 kDa cell surface LPS receptor. It lacks a transmembrane domain and is anchored to the plasma membrane via a GPI anchor (Ziegler-Heitbrock and Ulevitch 1993; Antal-Szalmas 2000). CD14 forms part of the LPS receptor complex. LPS binds to CD14 and this binding is increased greatly by the soluble plasma protein, LPS binding protein (LBP) (Ziegler-Heitbrock and Ulevitch 1993; Hailman,

Lichenstein *et al.* 1994; Fenton and Golenbock 1998; Triantafilou and Triantafilou 2002). The complex of CD14-LPS-LBP is likely to be stabilised by other membrane proteins which play a role in internalisation and signalling. CD14 is implicated in the binding and internalisation of apoptotic bodies and the programmed cell death of monocytes. Many of the intracellular signalling proteins that are activated by LPS in a CD14 dependent manner are associated with caveolae/GPI-microdomains and these may have a role in CD14 signalling (Lisanti, Tang *et al.* 1995; Diks, Richel *et al.* 2004).

CD14 is present on the surface of different myeloid cells and at very low levels on B cells, basophils, mammary cells, placental trophoblasts and gingival fibroblasts. The earliest myeloid precursors are CD14 negative but CD14 expression is up-regulated during differentiation (Landmann, Wesp *et al.* 1991; Ziegler-Heitbrock and Ulevitch 1993; Zhou and Tedder 1996). CD14 expression on macrophages differentiated from monocytes differs depending on tissues types. In monocytes cytokines such as IFNα, IFNγ, IL-2, IL-4 and IL-13 decrease CD14 expression (Landmann, Ludwig *et al.* 1991). LPS, the ligand for CD14, can alter expression on monocytes, causing rapid up-regulation followed by a decrease in expression after a few hours; this is due to the mobilisation of intracellular stores of CD14 (Landmann, Knopf *et al.* 1996). A marked increase in CD14 expression is observed after 1 day, due to *de novo* synthesis, and possibly correlates with monocyte differentiation (Landmann, Knopf et al. 1996).

CD14 is typically used as a marker of monocytes and its expression is usually lost during DC development (Ruppert, Schutt *et al.* 1993; Xu, Kramer *et al.* 1995; Kiertscher and Roth 1996; Pickl, Majdic *et al.* 1996; Zhou and Tedder 1996; Chapuis,

Rosenzwajg *et al.* 1997). CD14 shedding has been shown to be responsible for controlling surface expression of CD14 and generates soluble CD14 (sCD14) (Bazil and Strominger 1991; Rokita and Menzel 1997). sCD14 is required by DC for LPS mediated activation and maturation (Verhasselt, Buelens *et al.* 1997). While CD14 is usually down-regulated on DC, Katoh *et al* demonstrated that histamine can prevent the loss of CD14 during GM4 induced DC development, resulting in DC expressing CD14 (Katoh, Soga *et al.* 2005). These CD14+CD1a- DC had enhanced capacity to induce pro-inflammatory cytokines and chemokines and showed increased phagocytic capacity while having reduced antigen-presenting capacity (Katoh, Soga et al. 2005). Additionally, IL-10 produced by these histamine treated DC induced T cells with regulatory properties (Katoh, Soga et al. 2005).

1.4 NK Cells

Natural killer (NK) cells are a heterogeneous population of cells comprising about 10-20% of all peripheral blood lymphocytes (PBL). Human NK cells are defined phenotypically by surface expression of CD56 and the lack of surface CD3 expression and functionally by their ability to lyse targets independent of MHC antigens and without prior sensitisation (Robertson and Ritz 1990; Farag and Caligiuri 2006). Due to these functional properties NK cells are an important part of the innate immune system, providing a first line defence against infected, stressed or cancerous cells (Robertson and Ritz 1990; Whiteside and Herberman 1995; O'Byrne and Rusch 2006). NK cells are able to distinguish between normal and abnormal cells, using stimulatory receptors such as the NKR-P1 receptor, which can recognise oligosaccharide moieties on target cells, NKG2D which recognises MIC-A or B and ULBP molecules. Other inhibitory receptors such as KIR system recognise MHC

class I molecules and down-regulate NK cytotoxic activity. However, unlike cells of the adaptive immune system they are not sensitised to specific antigens, nor can they develop long lasting memory responses (Robertson and Ritz 1990; Hallett and Murphy 2004). NK cells similar to CTL, can kill via expression of perforin and granzyme, Fas ligand or TRAIL (Robertson and Ritz 1990; Zamai, Ponti *et al.* 2007; Klingemann and Boissel 2008). NK cells can additionally produce a wide range of inhibitory or stimulatory cytokines upon activation, such as IFNγ, TGF-β, GM-CSF and G-CSF (Robertson and Ritz 1990; Zamai, Ponti et al. 2007; Klingemann and Boissel 2008).

NK cell killing of tumour cells can contribute to tumour antigen uptake by DC making tumour antigenic material from dead and apoptotic tumour cells available. Recently it has been discovered that while NK cells are the major innate immune systems effector cells, they can also contribute to the development of adaptive immune responses (Kalinski, Mailliard *et al.* 2005). NK cells provide help to the adaptive immune system by being able to mature DC and increase DC ability to produce pro-inflammatory cytokines such as IL-12, and induce DC maturation into stable type 1 cells (Cooper, Fehniger *et al.* 2004; Ferlazzo 2005; Kalinski, Giermasz *et al.* 2005; Woo, Clay *et al.* 2006). These "polarised" DC stimulate T_H1 and CTL responses in tumour specific $CD4^+$ and $CD8^+$ T cells (Kalinski, Mailliard *et al.* 2005).

In cancer, NK cells play an important role, as according to the 'missing self' hypothesis, tumour cells with low levels of MHC Class I molecule expression can be targeted by NK cells (Hallett and Murphy 2004). The observation that nude mice (which lack T and B cells but not NK cells) do not develop tumours more frequently

than wild type mice supports the role of NK cells in control of tumour growth (Talmadge, Meyers *et al.* 1980; Stanbridge and Ceredig 1981; Malygin 1985; Frydecka 1988). Additionally, patients with Chediak-Higashi syndrome (who have abnormal NK function) have a 200-fold increased risk of developing cancer (Abo, Roder *et al.* 1982).With this and other evidence for the role of NK cells in cancer, serious interest began to turn towards the use of NK cells in immunotherapy (Hallett and Murphy 2004; Kalinski, Giermasz et al. 2005; Kalinski, Mailliard et al. 2005; Bhardwaj 2007; Zamai, Ponti et al. 2007).

NK cell-based immunotherapy in animal models gives encouraging results, but the translation of this into clinical applications has only been moderately effective (Herberman 2002; Hallett and Murphy 2004; Klingemann and Boissel 2008). The limited clinical effectiveness of NK therapies is thought to be due to tumour NK escape mechanisms as discussed later. Many of these may utilise existing control mechanisms to control or escape from NK cell killing (Klingemann and Boissel 2008). Effective treatment will result therefore only if inhibitory controls on NK killing are bypassed. However, as the immune system and conventional therapies increase the selective pressure on tumours cells there is a risk of resistance developing to both chemotherapy and immunotherapy (when using NK cells) as cells naturally express molecules, such as MHC Class I molecules and KIR ligands, that prevent them being killed by NK cells (Herberman 1986; Timonen and Helander 1997; Herberman 2002). NK therapies are expected to be beneficial mainly in an anti-metastatic role and in haematological malignancies (Herberman 2002; Hallett and Murphy 2004).

1.5 Cancer and the Immune System

Cancer is a multistep process and the steps required for the development of cancer (as defined by Hanahan and Weinberg) are shown in Figure 1.1 (Hanahan and Weinberg 2000). The immune system is primarily responsible for protection against foreign pathogens; however in the 1950s, this understanding of the role of the immune system was widened to include elimination of tumour or precancerous cells. Tumour immuno-surveillance has been reviewed many times but the evidence in the main comes from three types of observations (Dunn, Bruce *et al.* 2002; Zitvogel, Tesniere *et al.* 2006; Bhardwaj 2007; Swann and Smyth 2007);

1) Spontaneous cancer regression in patients, associated with elevated immune responses, such as those reported in mesothelioma (Fischbein, Suzuki *et al.* 1978; Robinson, Robinson *et al.* 2001).

2) Increased incidence of cancer in immuno-suppressed individuals, ranging from organ transplanted patients, individuals with primary immunodeficiency disorders and in recent years the prevalence of tumours in patients infected with HIV who have developed AIDS (Scully, Cawson *et al.* 1986; Kahn, Northfelt *et al.* 1992; Weiss 1999).

3) Survival advantage of patients with activated $CD8^+$ T cells infiltrating the tumour (Galon, Costes *et al.* 2006; Anraku, Cunningham *et al.* 2008; Han, Fletcher *et al.* 2008).

These observations however only indirectly prove the existence of tumour immuno-surveillance.

Since it was discovered that the immune system could mediate rejection and resistance to tumours, research has mainly been focused on understanding tumour

antigen recognition, tumour antigen processing, antigen presentation and stimulation of effector cells (e.g. T cells) (Burnet 1964; Kavanaugh and Carbone 1996; Banchereau, Palucka et al. 2001; Dunn, Bruce et al. 2002; Ohm and Carbone 2002; Parish 2003; Gajewski, Meng et al. 2006; Zitvogel, Tesniere et al. 2006; Swann and Smyth 2007). Recently an addition to the hallmarks of cancer to include immune escape (see Figure 1.4) has been proposed by (Zitvogel, Tesniere et al. 2006).

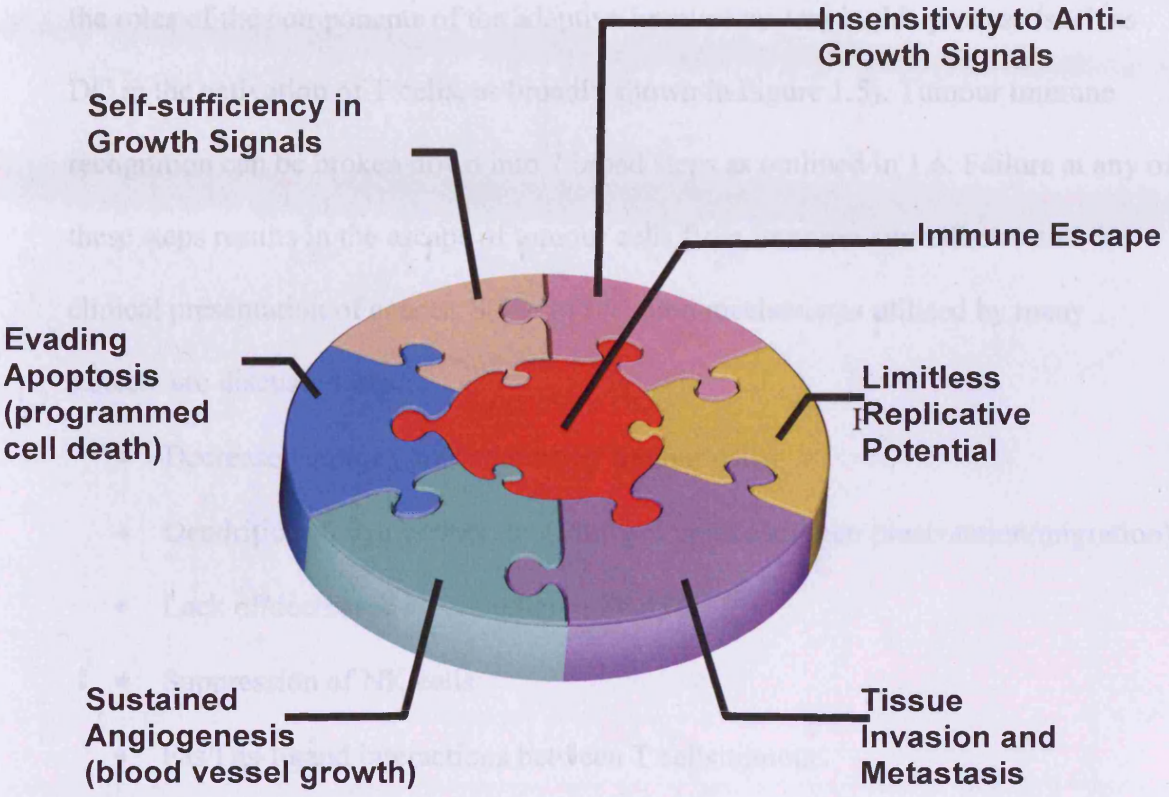


Figure 1 Steps in Cancer Development

(Hanahan and Weinberg 2000; Zitvogel, Tesniere et al. 2006)

The goal of treating cancer via immunotherapeutic methods or developing cancer vaccines requires an understanding of the process of tumour immune recognition and the roles of the components of the adaptive immune system in this process (such as DC in the activation of T cells, as broadly shown in Figure 1.5). Tumour immune recognition can be broken down into 7 broad steps as outlined in 1.6. Failure at any of these steps results in the escape of tumour cells from immuno-surveillance and the clinical presentation of cancer. Some of the main mechanisms utilised by many cancers are discussed here;

- Decreased antigen presentation by tumour cells.
- Dendritic cell dysfunction (e.g. antigen uptake/antigen presentation/migration)
- Lack of/decreased co-stimulation by APC.
- Suppression of NK cells
- Fas/Fas-ligand interactions between T cells:tumour.
- Suppressor cells (Tregs, Regulatory DC and MDSC)
- Immunosuppressive cytokines

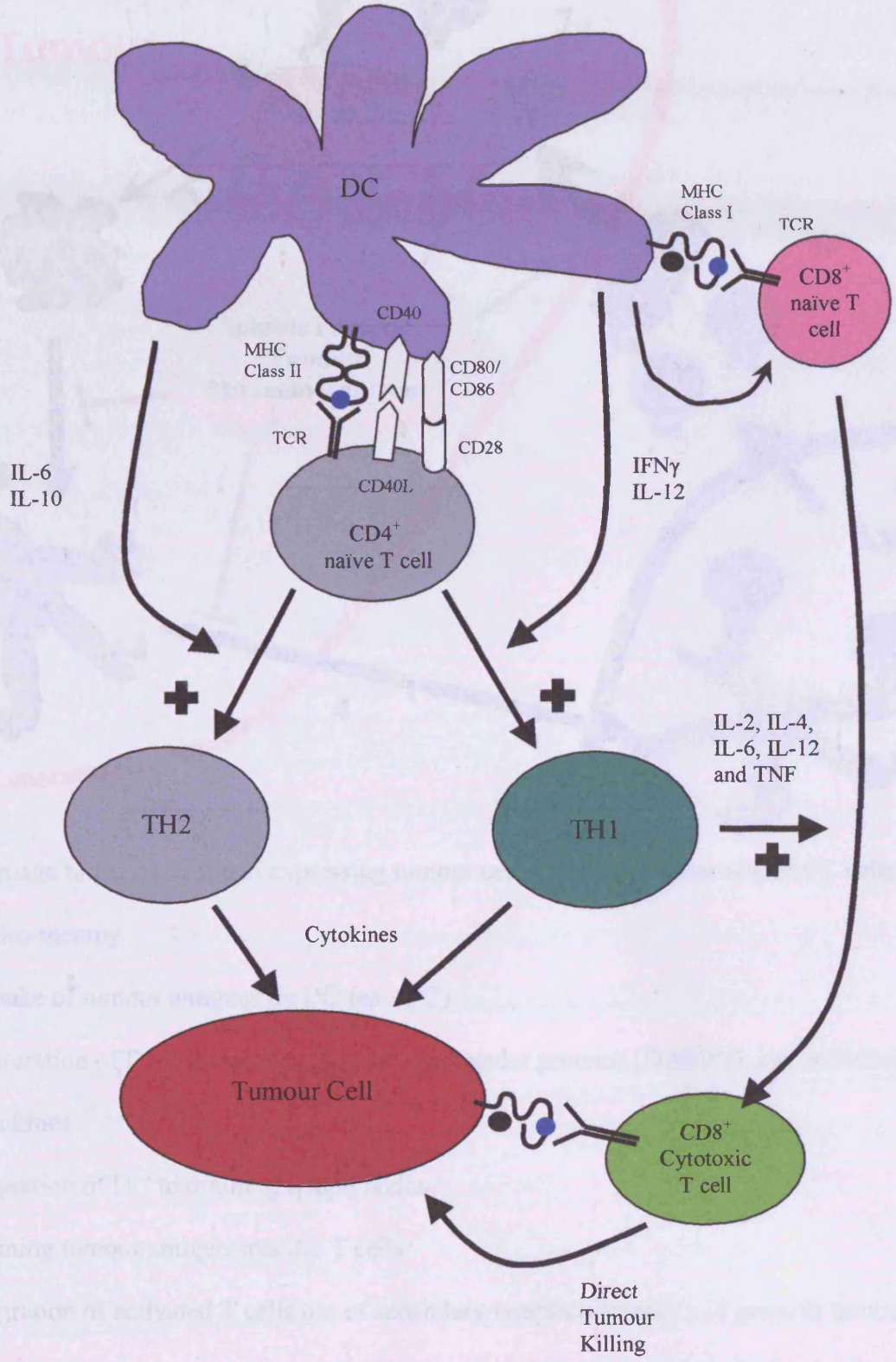
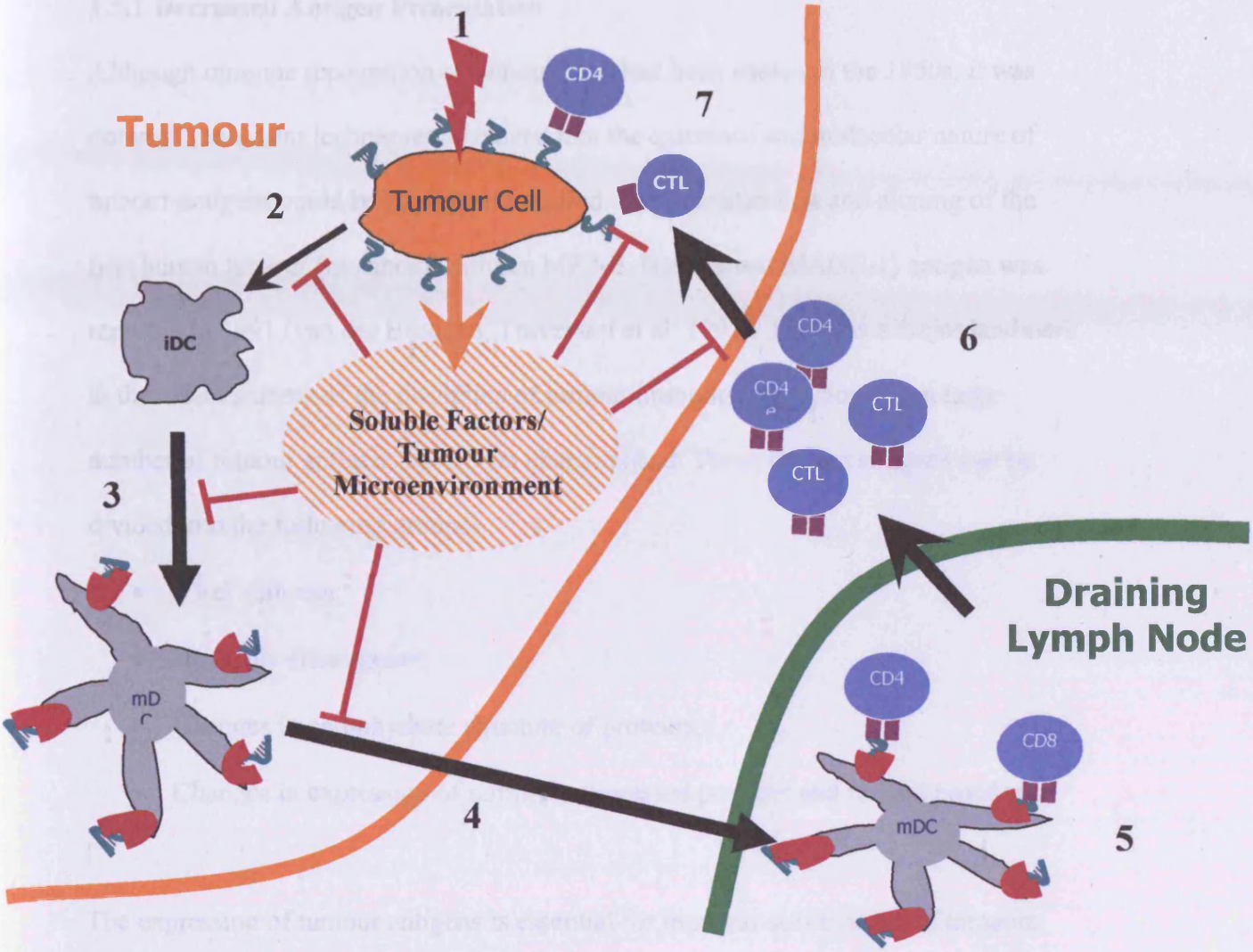


Figure 1.5 Anti Tumour T Cell Responses, The Role of DC in T Cell Activation



1. Damage to tumour antigen expressing tumour cells (NK cells, cross-reactive T cells and chemo-/radio-therapy
2. Uptake of tumour antigens by DC (as APC)
3. Maturation of DC by damage associated molecular proteins (DAMPs), and inflammatory cytokines
4. Migration of DC to draining lymph nodes.
5. Priming tumour antigen specific T cells
6. Migration of activated T cells out of secondary lymphoid organs and towards tumours.
7. Recognition of tumour cells, by specific T cells and T cell mediated tumour lysis

Figure 1.6 Tumour-Immune System Interactions

1.5.1 Decreased Antigen Presentation

Although immune recognition of tumour cells had been shown in the 1950s, it was not until molecular techniques advanced that the existence and molecular nature of tumour antigens could be proven and studied. The identification and cloning of the first human tumour (melanoma antigen MZ2-E, later named MAGE-1) antigen was reported in 1991 (van der Bruggen, Traversari et al. 1991). This was a major landmark in the establishment of the discipline of tumour immunology. Since then a large number of tumour antigens have been characterised. These tumour antigens can be divided into the following groups;

- Viral antigens;
- Normally silent genes;
- Changes in carbohydrate structure of proteins;
- Changes in expression of normally expressed proteins and mutant proteins.

The expression of tumour antigens is essential for immuno-surveillance of tumours; conversely, tumours need to escape from this immuno-surveillance to survive. MHC expression is required for recognition of tumour cells by T cells. Down-regulation of the expression of MHC molecules on APC prevents the activation /priming of T cells to TAA. Down-regulation of MHC on the tumour cell surface also reduces the probability of activated T cells recognising tumour cells(Festenstein 1987; Garrido, Cabera *et al.* 1993; Garrido, Ruiz-Cabello *et al.* 1997).

The following types of HLA loss have been identified:

- Total loss of HLA class (MHC Class I),
- Loss of HLA haplotype,
- HLA-A locus specific loss,
- HLA-B locus specific loss and
- Single HLA allelic loss (Garrido, Ruiz-Cabello et al. 1997).

Changes in HLA expression are not infrequent and may affect 39%-88% of tumours derived from epithelial cells, such as colorectal carcinomas, gastric carcinomas (Lopez Nevot, Esteban *et al.* 1989; Algarra, Gaforio *et al.* 1999; Sette, Chesnut *et al.* 2001; Ordemann, Jacobi *et al.* 2002; Mazzoccoli, Grilli *et al.* 2003; Rajendra, Ackroyd *et al.* 2006; Ferrone and Whiteside 2007). It has been suggested that selective pressure by immuno surveillance of tumours leads to immuno-editing, producing tumours where those cells that have high expression of MHC Class I, increased TAA expression or both, are selected out with only cells with defects in MHC Class I/TAA expression surviving (Dunn, Bruce et al. 2002; Parish 2003; Swann and Smyth 2007). The down-regulation of antigen/antigen presentation can occur by transcriptional defects, mutations of genes' HLA subunits or defects in the MHC processing machinery genes, including functional loss of β2-microglobulin and peptide transporter defects (TAP-1 and 2, LMP-2, LMP-7) (Ochoa 2003).

1.5.3 Dendritic Cell Dysfunction

DC, as the backbone of immune responses, have become the focus of research in cancer immunotherapy, since they are able to induce both primary and secondary T cell responses.

In healthy individuals it is believed that DC in the tissues take up tumour antigens from damaged, apoptotic or necrotic e.g. due to NK killing or cross reactive T cell responses to abnormal self proteins (Cramer, Titus-Ernstoff *et al.* 2005; Oppenheim, Dong *et al.* 2005). DC process these antigens via cross presentation or the exogenous pathways and present antigen on both MHC Class I and II molecules (Finn 2004). During maturation MHC-antigen complexes and co-stimulatory molecules are up-regulated and DC migrate into lymph nodes where they interact with naive $CD4^+$ and $CD8^+$ T cells and generate both T helper and CTL responses. These effector cells migrate to the tissues and target and eliminate neoplastic cells. This process is briefly outlined in Figure 1.6 which also shows the possible stages where the tumour microenvironment can influence DC and T cell function.

Gabrilovich recently reviewed differentiation of myeloid cells in cancer and concluded that abnormal differentiation of DC from precursors results in three main consequences; decreased production of mature, functionally competent DC; the accumulation of iDC (which cannot up-regulate MHC Class II and co-stimulatory molecules); and increased production of immature myeloid cells (Gabrilovich 2004). Investigations of the functional activity of DC in cancer have reported defects in DC function such as poor expression of co-stimulatory molecules, inhibition of migration, inhibition of maturation of DC and defective antigen presentation (Chaux, Moutet *et al.* 1996; Thurnher, Radmayr *et al.* 1996; Chaux, Favre *et al.* 1997; Gabrilovich, Corak *et al.* 1997; Nestle, Alijagic *et al.* 1998).

Stimulation of tumour bearing host T cells by competent DC can result in anti-tumour responses (Spisek, Chevallier et al. 2002; Gregoire, Ligeza-Poisson et al. 2003). Therefore T cell responses seem to be affected only as a result of defective stimulation, as even CD3-antibody stimulation does not demonstrate detectable T cell defects, while *in vitro* functionally competent DC can be generated from tumour host DC-precursors. It has been shown that tumour cell conditioned supernatants can impair DC development from progenitors, affecting expression of molecules involved in antigen presentation and functions of DC (Gabrilovich, Ciernik *et al.* 1996; Gabrilovich, Nadaf *et al.* 1996). Tumour cell supernatants can also skew DC differentiation towards monocytic cells with diminished APC function (Menetrier-Caux, Montmain *et al.* 1998).

In tumour bearing hosts DC defects, including reduced stimulatory capacity, affect DC of myeloid origin, while inhibition of DC differentiation results in the generation of immature myeloid cells and monocytes. Several factors have been implicated in DC dysfunction, including M-CSF,TGF-β, IL-6 and IL-10. M-CSF and IL-6 have been identified by use of neutralising antibodies (Vuckovic, Clark *et al.* 2002; Zou and Tam 2002; Panoskaltsis, Reid *et al.* 2004; Sikora, Dworacki *et al.* 2004). IL-10 is probably the best known of these inhibitory factors. IL-10 has been shown to block monocyte to DC differentiation and DC maturation and can skew development of monocytes towards macrophages or macrophage like subsets, induce DC capable of generating suppressive T cells (Tregs) and induce autocrine IL-10 production (Allavena, Piemonti *et al.* 1998; Fortsch, Rollinghoff *et al.* 2000; Corinti, Albanesi *et al.* 2001). IL-10 is rarely produced by tumour cells, and neutralisation of IL-10, using neutralising Ab, in tumour cells does not totally abrogate inhibitory effects of tumour

cell supernatants (Sica, Saccani *et al.* 2000; Specht, Bexten *et al.* 2001; Steinbrink, Graulich *et al.* 2002). Other tumour-related factors, such as VEGF, may also effect DC development by acting on DC precursors, blocking their development into DC (Gabrilovich, Ishida *et al.* 1998). There is much work to be done in identifying the mechanisms by which tumour-induced factors affect DC development.

1.5.4 Lack of /decreased Co-Stimulatory Molecule Expression.

Stimulation of naïve T cells into effector cells requires interactions with professional antigen presenting cells of which DC are the most potent. These APC are able to provide the three signals required to activate naïve T cells, such as MHC-antigen complexes, co-stimulatory molecules, and cytokines. APC-T cell interactions include adhesion, recognition of MHC-antigen complex by the TCR and co-stimulation. The results of these interactions are illustrated in figure 1.5. MHC- TCR interactions without antigen even in the presence of both adhesion molecules and appropriate costimulatory molecules does not result in activation, while interaction of the MHC-antigen complex and TCR without costimulatory molecules induces T cell anergy or activation induced cell death. As shown on Figure 1.7, co-stimulation induces multiple transcription factors, whereas without co-stimulation activation of Nf-κB is inhibited resulting in reduced IL-2 production and activation of T cell

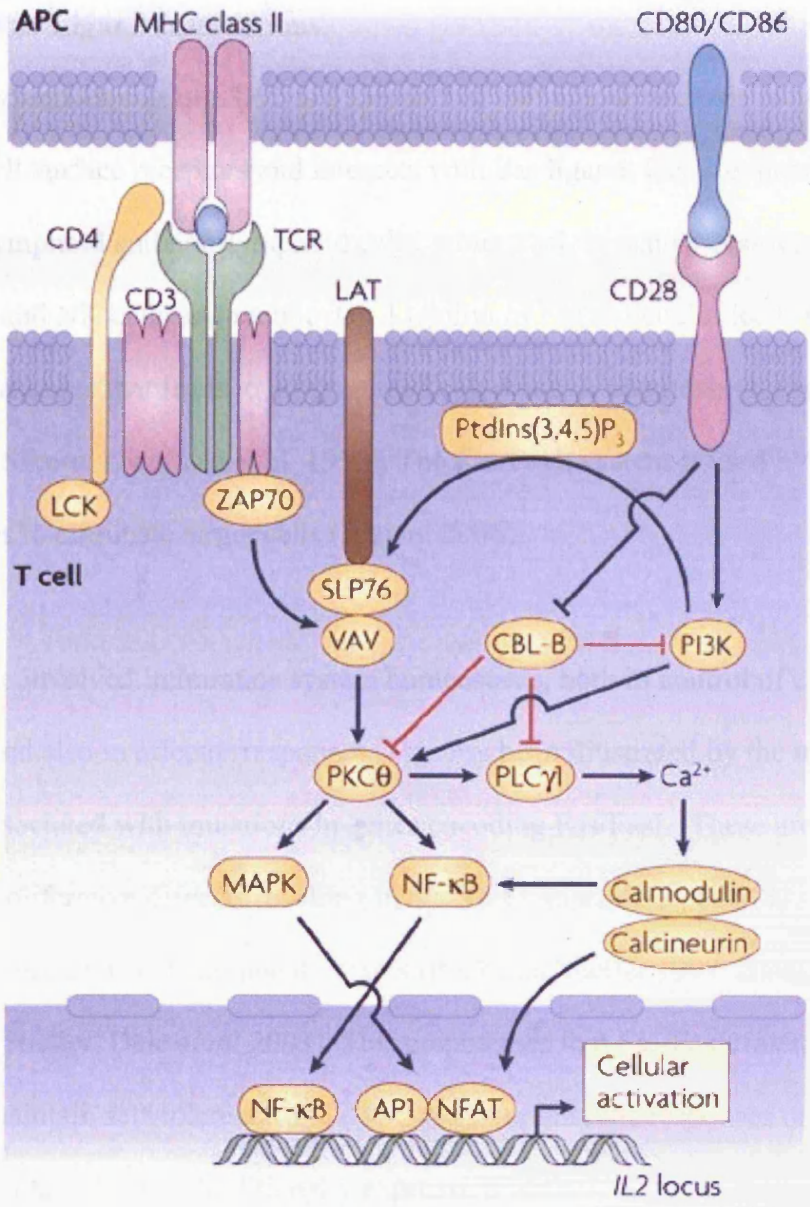


Figure 1 Outline of Co-stimulatory Interactions in T cell Stimulation

(Miller, Turley *et al.* 2007)

Outline of signalling by CD28:CD80/CD86 interactions supporting TCR activation and inducing IL-2 production and T cell activation, taken from (Miller, Turley et al. 2007)

1.5.5 Fas/Fas-Ligand Interactions.

As described earlier, Fas or CD95 is a member of the tumour necrosis factor receptor family of cell surface receptors and interacts with Fas ligand. Fas is expressed on a variety of lymphoid and non-lymphoid cells, while FasL is mainly restricted to activated T and NK cells and monocytes. Ligation of Fas molecules leads to signalling cascades that leads to programmed cell death or apoptosis (Kagi, Vignaux et al. 1994; Sikora, Dworacki et al. 1998). The Fas/FasL system is used by lymphocytes to eliminate target cells (Wajant 2006).

Fas/FasL are involved in immune system homeostasis, both in control of clonal expansion and also in effector responses. This has been illustrated by the immune disorders associated with mutations in genes encoding Fas/FasL. These are manifested in lymphoproliferative disease, resulting in massive lymphadenopathy, altered and enlarged T cells, and autoimmune disorders (Puck and Sneller 1997; Bleesing, Straus *et al.* 2000; Sneller, Dale *et al.* 2003). This emphasises that Fas/FasL interactions shape and maintain self-tolerence and down-regulate immune responses once the antigen stimulus subsides. Additionally expression of FasL on testes and eye tissues induces immune protection by inducing apoptosis in invading cells. Many tumour cells have been shown to express FasL, while hematopoietic malignancies and numerous non-hematopoietic tumours have been reported to express Fas (Wajant 2006). This has given rise to the hypothesis that the Fas/FasL system allows immune escape by allowing FasL expressing tumour cells to counter-attack infiltrating lymphocytes (O'Connell, O'Sullivan et al. 1996). Reduced tumour formation in a

mouse model was reported where native FasL expression was silenced in tumours, supporting the FasL counterattack hypothesis (Ryan, Shanahan *et al.* 2005).

Tumour cells have also been reported to develop resistance to Fas mediated apoptosis. This may result from immunoediting, as lack of expression or functionality of Fas confers a survival advantage to tumour cells (Sikora, Dworacki et al. 1998). Surface expression of Fas is requisite for FasL mediated induction of apoptosis. IFNγ can revert tumour cells to a Fas-sensitive type in melanoma, although the mechanism is not well defined and not thought to be a common phenomenon (Ochoa 2003). The emergence of a Fas-apoptosis resistant phenotype seems to be a common immune escape by tumours.

The Fas/FasL system also offers the possibility of using this system for therapeutic intervention (Wajant 2006). This would be two-fold, such as increasing Fas expression/sensitivity on tumour cells, while decreasing FasL expression on tumour cells and decreasing Fas expression/sensitivity of CTL. However, due to the function of Fas in controlling immune cells and the fact that lack of Fas/FasL leads to autoimmunity this area of therapy will need much more study before it is ready for clinical use (Wajant 2006).

1.5.6 Suppression of NK Cells

Down-regulation of MHC Class I as described above is a mechanism allowing escape of cells from target recognition by $CD8^+$ T cells. Lack of MHC Class I expression would normally target cells for elimination by NK cells. However allelic losses will not be susceptible to NK cells as HLA molecules are still expressed by tumour cells,

but have impaired antigen presentation. Furthermore, several surface molecules such as NKp44 and other members of the HLA family have been shown to mediate suppression of NK lysis and may be used by tumours to facilitate immune escape. Soluble MIC-A and -B in tumour can down-regulate NK activity via blocking NKG2D (Raffaghello, Prigione *et al.* 2004; Clayton, Mitchell *et al.* 2008)

1.5.7 Immunosuppressive Cytokines

Tumours secrete a number of soluble factors that support tumour cell growth, induce angiogenesis and cell invasiveness (e.g. VEGF, HGF). Factors secreted by tumours may also affect the induction of immune responses. IL-10, GM-CSF, M-CSF, IL-6 are all factors secreted by tumours that have been shown to modulate immune cells. Mesothelioma cell lines and tumours have been shown to produce cytokines including GM-CSF, IL-6 and IL-10 (Gottehrer, Taryle et al. 1991; Schmitter, Lauber et al. 1992; DeLong, Carroll et al. 2005).

GM-CSF can, in appropriate, amounts induce DC development and stimulate immune functions. Tumour cells modified to producing GM-CSF, irradiated and used as vaccines, increased anti-tumour responses. However, in mice, GM-CSF has been implicated in generation of immuno-suppressive myeloid cells with Gr-1^{+}CD$11b^{+}$ phenotype, following vaccination of mice with cells expressing large amounts of GM-CSF (Steptoe, Ritchie *et al.* 2005).

IL-10 has been shown to affect DC development and it appears that IL-10 affects more mature myeloid cells. As also discussed earlier, in mouse models IL-10 was found to be responsible for DC dysfunction (Kusmartsev and Gabrilovich 2006). DC

exposed to IL-10 during their development have decreased allogenic T cell stimulatory capacity, reduced CTL responses and IL-12 production. Additionally, IL-10 induces CD4 and CD8 T cells that suppress antigen specific T cell proliferation. IL-10 may also mediate PGE2 and possibly other immune suppressive factors, as the immune suppressive effect of PGE2 may be reversed by administration of anti-IL-10 antibodies (Misra, Selvakumar et al. 1995; van der Pouw Kraan, Boeije et al. 1995; Kim, Emi et al. 2006).

M-CSF and IL-6 modulate myeloid cell differentiation. Cancer cells expressing large amounts of M-CSF and IL-6 induce myeloid progenitor cells to differentiate into monocytic cells and can inhibit DC development (Menetrier-Caux, Montmain et al. 1998; Zou and Tam 2002). These cells have characteristics of macrophages, with better phagocytic but poorer antigen presentation capacity (Kusmartsev and Gabrilovich 2006). *In vitro* pro-inflammatory cytokines such as IL-4 and IL-13 can reverse the effects of IL-6 and M-CSF (Park, Nakagawa *et al.* 2004).

Soluble factors secreted by tumours can, by affecting DC, amplify immuno suppressive effects since DC play a central role in T cell stimulation. Immuno suppressive cytokines can affect DC by modulating their development from precursors or by altering their functional ability; these effects have been discussed previously. Regulatory DC express lower levels of MHC Class II, CD86 but have higher expression of CD80, CD40 molecules; they also secrete more IL-10 and less IL-12, than normal DC (Zhang, Tang *et al.* 2004). Regulatory DC cells did not mediate the suppression of T cell by differentiation of $CD4^+$ T cells into Tregs(Zhang, Tang et al. 2004).

1.5.8 Regulatory Cells (Tregs, Regulatory/Tolerogenic DC and MDSC)

Tumour cells can also evade the immune system by inducing regulatory T cells, DC and myeloid cells.

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of myeloid cells comprising immature macrophages, immature DC and immature myeloid cells, among others. These cells are at the early stages of differentiation, and in mice are defined as Gr-1$^+$CD11b$^+$ cells. In tumour bearing mice MDSC express MHC Class I molecules but little MHC Class II and co-stimulatory molecules (Rabinovich, Gabrilovich *et al.* 2007).

MDSC are characterised functionally by the inhibition of IFNγ production by CD8$^+$ T cells, stimulated by antigen-MHC Class I complexes on MDSC. The inhibitory effect is dependent on MHC Class I expression, requires cell:cell contact and is not mediated by soluble factors, but is mediated by reactive oxygen species. In a mouse model MDSC (but not iMC) induce antigen specific T cell tolerence (Watanabe, Deguchi *et al.* 2008). MDSC are capable of taking up antigen, processing and presenting it on the cell surface, and inducing antigen specific T cell anergy and suppression of T cell proliferation (Huang, Pan *et al.* 2006).

In humans, MDSC are defined as cells that express CD33 (a common myeloid marker) but lack expression of mature myeloid and lymphoid cell markers and HLA-DR (Almand, Clark *et al.* 2001). In advanced cancer these cells can accumulate in the blood (Almand, Clark et al. 2001; Danna, Sinha et al. 2004). MDSC from advanced

cancer patients were functionally similar to mouse MDSC in inhibiting IFNγ production by $CD8^+$ T cells (Almand, Clark et al. 2001). Impaired cytokine production by T cells was abrogated by addition of catalase as a reactive oxygen scavenger (Kusmartsev and Gabrilovich 2003). MDSC can also induce T regulatory cells (Tregs) *in vitro*. This is dependent on IFNγ and IL-10 (Kusmartsev and Gabrilovich 2006).

Recently much interest has been focussed on the role of Tregs in cancer-mediated immune suppression/escape. Tregs cells were identified as $CD4^+CD25^+$ T cells, a naturally occurring T cell subset that comprises about 5-10 % of all peripheral T cells, and which are capable of suppressing T cell responses *in vivo* reviewed by (Rabinovich, Gabrilovich et al. 2007). The transcription factor FoxP3 is used as an intracellular marker for Tregs in combination with other markers such as CD4 and CD25 and glucocorticoid-induced tumour necrosis factor receptor (GITR) (Zou 2005). Tregs play a pivotal role in the suppression of tumour immunity. In many cancer patients, including breast, ovarian and lung cancer patients elevated frequencies of $CD4^+CD25^+FoxP3^+$ cells are found either in the circulation or in the tumour itself (Banham, Powrie *et al.* 2006; Betts, Clarke *et al.* 2006; Wolf, Rumpold *et al.* 2006; Curiel 2007; Wang and Wang 2007). Large numbers of $CD4^+CD25^+FoxP3^+$ cells in tumour/tumour microenvironment correlate with poor prognosis (Curiel, Coukos *et al.* 2004). Removal or depletion of Tregs by using anti-CD25 antibodies results in increased T cell mediated tumour rejection (Sutmuller, van Duivenvoorde et al. 2001; Grauer, Sutmuller et al. 2008; Kline, Brown et al. 2008). Strategies to inhibit/deplete Tregs, such as the engagement of toll-like receptors which can reverse the function of Tregs and drug mediated selective depletion of

$CD4^{+}CD25+$ T cells are being investigated as cancer therapies (Rabinovich, Gabrilovich et al. 2007). Recent work has also shown that DC exposed to IL-10 can promote Treg development (Bellinghausen, Konig *et al.* 2006).

The expression of functionally active IDO, a factor that causes inhibition of T cell proliferation by depletion of extracellular tryptophan, has been reported by IDO-competent DC developed in the presence of IFNγ (Orabona, Puccetti *et al.* 2006). These cells are functional variable, causing difficulties in positively defining the IDO-competent cells able to suppress T cells (Rabinovich, Gabrilovich et al. 2007). Immune cells with suppressive function, especially Treg cells, have been of increased interest in cancer immune therapy approaches. (Chattopadhyay, Chakraborty et al. 2005; von Boehmer 2005; Betts, Clarke et al. 2006; Wolf, Rumpold et al. 2006; Rabinovich, Gabrilovich et al. 2007; Wang and Wang 2007).

1.6 HGF

Hepatocyte Growth Factor (HGF) or scatter factor (SF) was first discovered in the 1980s as a growth factor for hepatocytes (Nakamura, Nawa *et al.* 1984; Nakamura, Nishizawa *et al.* 1989). It became clear that the biological activity of HGF was not solely limited to stimulation of cell growth, nor confined to a single cell type (Zarnegar and Michalopoulos 1995). HGF is now well known to be a pleiotropic growth factor inducing mitogenic, morphogenic and motogenic activities in target cells. HGF is stromally derivred growth factor, but has been detected in a variety of cell types including; alveolar epithelial cells, hepatocytes, mesenchymal cells, neutrophils, macrophages, hematopoietic stem cells, endothelial cells, DC, myofibroblasts, and cancer cells. HGF is secreted as a single chain precursor that is

biologically inert, called pro-HGF, and requires processing by proteases, such as urokinase-type plasminogen activator or a factor XII-like enzyme called HGF-activator. These enzymes cleave pro-HGF into active HGF, which is an 80kDa hetero-dimeric protein, consisting of two subunits; α and β (65 and 35 kDa respectively), linked by a single disulphide bond.

The amino acid sequence of HGF closely resembles several coagulation/fibrinolytic proteins, including prothrombin and plasminogen, due to the presence of kringle domains and a serine protease domain. HGF however, has no clotting or fibrinolytic activity; the converse is also true with related proteins showing no growth potentiating properties. Another property of HGF is its ability to bind heparin sulphate proteogylcans; this results in sequestration of HGF in the vicinity of HGF secreting cells. This then leads to high localised concentrations of HGF promoting a localised response, reviewed by (Zarnegar and Michalopoulos 1995).

HGF has only a single known receptor, c-Met; this receptor was first characterised as an onco-protein (Cooper, Park *et al.* 1984). The association and relevance of HGF and c-Met in cancer and HGF have been reviewed by several people discussing the migration and motility effects of c-Met signalling, and how HGF can promote cell invasion and growth, in particular by the disruption of cell:cell structures (Giordano, di Renzo et al. 1992; Zarnegar and Michalopoulos 1995; Corso, Comoglio et al. 2005; Jiang, Martin et al. 2005; Peruzzi and Bottaro 2006). The therapeutic opportunities and implications for treatment of cancer by targeting HGF have also been discussed (Stella and Comoglio 1999; Maulik, Shrikhande et al. 2002; Corso, Comoglio et al. 2005; Jiang, Martin et al. 2005; Peruzzi and Bottaro 2006). However, little work has

been done to investigate the high levels of HGF and its potential effects on immune responses. Indeed little work has been carried out even to characterise c-Met expression on human immune cells, apart from *Galimi et al* demonstrating that c-Met is expressed on monocytes (Galimi, Cottone *et al.* 2001). As part of the work for this thesis I have demonstrated functional expression of c-Met on human monocytes and dendritic cells and the lack of its expression on lymphocytes.

1.6.1 HGF in Health

HGF is a growth factor supporting the development and function of hepatocytes. Research on lung fibroblasts highlighted the effect of HGF, causing the dispersal of epithelial cells (Matsumoto and Nakamura 1993). Studies after this demonstrated that HGF induces morphogenesis in several types of epithelial cells. HGF added to cultures of various epithelial cells leads to formation of tubules, ducts and other structures, depending on the origin of the epithelial cells. Studies on mice have also contributed to the understanding of HGF. Knock out of HGF or c-Met genes causes pathological defects in animals leading to death *in utero* on day 15 of gestation. These embryos exhibit major defects in the placenta, liver and muscle (Uehara, Minowa *et al.* 1995). These and other studies, including those on organ regeneration, demonstrated that HGF and its receptor c-Met are mediators of epithelial-mesenchymal interactions and organ morphogenesis, and are critical in these processes during embryogenesis. However, while HGF's role in the processes of cell growth and motility are understood, it remains unclear what role if any HGF and c-Met play in processes such as neuronal outgrowth, muscle migration and growth, angiogenesis, hematopoiesis, lympocyte adhesion and migration.

1.6.2 HGF in Cancer

Elevated levels of HGF have been reported in several clinical conditions including cancer where high levels in plasma and in the tumour environment have been found (Giordano, di Renzo et al. 1992; Sheen-Chen, Liu et al. 2005; Jagadeeswaran, Ma et al. 2006; De Herdt and Baatenburg de Jong 2008). These high levels are associated with poor prognosis which is often attributed to the role of HGF in metastatic spread of cancer (Taniguchi, Kitamura *et al.* 1997; Zhu and Humphrey 2000; To, Seiden *et al.* 2002).

In cancer, as described and illustrated in Figure 1.4, control of growth is dysregulated and invasive growth/metastasis is promoted. c-Met activation by HGF induces neoplastic/tumour cells to grow invasively by eroding the basement membrane and infiltrating stromal layers, which eventually leads to metastatic spread of these cells (Vande Woude, Jeffers *et al.* 1997; Stella and Comoglio 1999; Gao, Xie *et al.* 2005).

c-Met activation or dysregulation of activation can be both HGF dependent and independent, which has to be considered when searching for targets in cancer therapy. c-Met over-expression in cancer is the most frequent alteration noted in human tumours. This over-expression enables receptors to spontaneously dimerise causing activation. c-Met can also be affected by mutations, which cause increased kinase activity. Although it is still dependent on HGF, it correlates with increased invasiveness and metastatic spread of tumour cells (Danilkovitch-Miagkova and Zbar 2002). Increased secretion of HGF by tumour cells can lead to autocrine c-Met activation.

Over-expression of HGF and c-Met have been reported in many cancers, such as bladder, lung, pancreas, thyroid, colon, stomach and breast cancer, and in some cases it serves as a negative prognostic factor (Di Renzo, Narsimhan *et al.* 1991; Yamashita, Ogawa *et al.* 1994; Di Renzo, Olivero *et al.* 1995; Di Renzo, Poulsom *et al.* 1995). Mouse models using cells transfected with HGF and c-Met demonstrate that co-expression of these genes transforms transfected cells into tumourigenic cells (Rong, Bodescot *et al.* 1992). The fact that tumour cells, expressing both HGF and c-Met, also express epithelial and mesenchymal markers suggests that HGF maybe relevant in malignant pleural mesothelioma (MPM). This was studied by Harvey *et al* where they concluded that HGF is involved in MPM development (Eagles, Warn *et al.* 1996; Harvey, Warn *et al.* 1996).

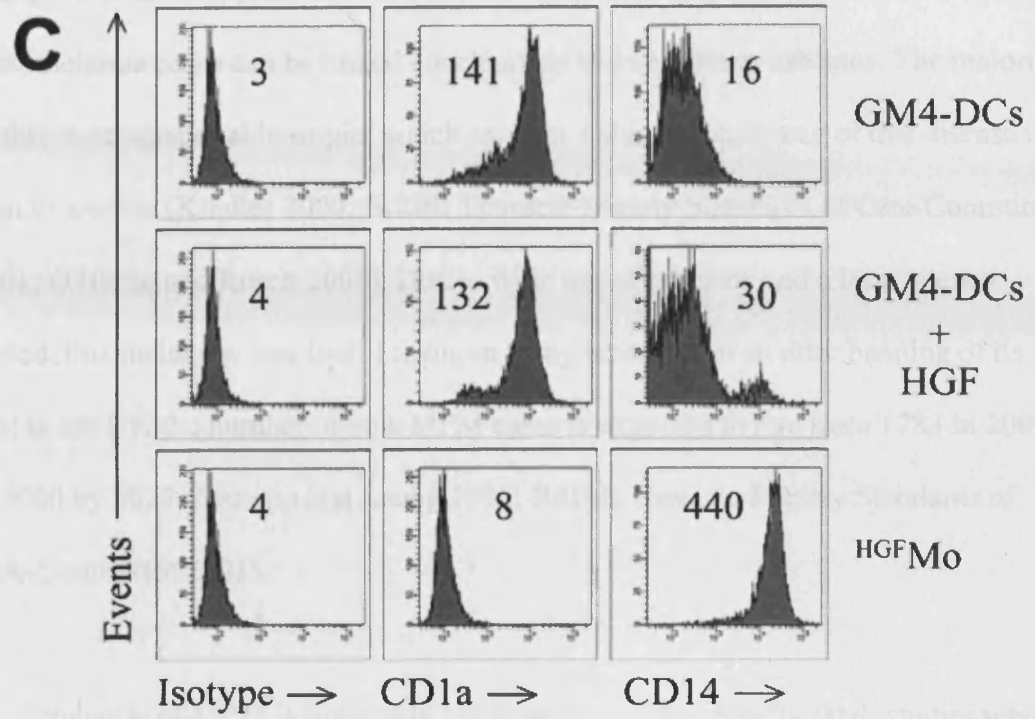
1.6.3 Immunological Aspects of HGF

While traditionally thought of as a specific growth factor of epithelial cells, it has become clear recently that HGF can act as a regulator of immune function. HGF is known to stimulate the invasiveness of monocytes and alter the gene expression profile which suggests a pro-inflammatory role for HGF stimulated monocytes (Beilmann, Vande Woude *et al.* 2000). HGF has also been implicated in regulation of monocyte-macrophage function such as IL-6 production, c-Met up-regulation and HGF production (Galimi, Cottone *et al.* 2001).

Mouse models of bone marrow transplantation showed that HGF gene transfection reduced graft-versus-host disease (GVHD), suggesting that in transplanted mice immunological tolerance to host antigens was established (Imado, Iwasaki *et al.* 2004). HGF has also been reported to alleviate airway inflammation, collagen induced

arthritis and autoimmune nephritis in mouse models (Ito, Kanehiro *et al.* 2005; Okunishi, Dohi *et al.* 2005; Kuroiwa, Iwasaki *et al.* 2006; Okunishi, Dohi *et al.* 2007) Skibinski discussed, in a brief review, the interactions of HGF with immune cells such as neutrophils, B cells and its effects on adhesion and transmigration, and suggested that HGF's potential role in immune modulation via DC should be studied (Skibinski 2003).

Rutella *et al* have recently reported that HGF treated monocytes could induce Treg development and had increased IL-10 production. HGF was also observed to alter gene expression in monocytes, as discussed later (Rutella, Danese *et al.* 2006). Differentiation of HGF treated monocytes into DC was not studied apart from CD14 and CD1a levels, as shown in their figure below. As the authors concluded, that "When HGF was added to GM-CSF and IL-4, the phenotype of DCs was similar to that described for GM4-DCs", we assessed whether my project will result in novel information. We decided to continue the ongoing studies as it addressed a different question from that discussed in the paper by *Rutella et al.*



'Figure 1.C - (Rutella, Bonanno *et al.* 2006)'

Furthermore, my experimental approach extended that used by *Rutella et al*, as in my model monocytes were not only simultaneously treated with HGF and GM4 but also pre-treated with HGF before they enter DC differentiation induced by GM4. This is likely to mimic physiological conditions more closely, as HGF is likely to be present before and during DC development.

1.7 Mesothelioma

Malignant pleural mesothelioma (MPM) is an uncommon cancer arising from the mesothelial cells lining the membranes of the pleura. The incidence of this cancer in more developed areas of the world, such as Europe and Australia, is roughly the same as cancers of the liver, bone and bladder. Wagner et al first published observations linking mesothelioma with exposure to asbestos fibres in the 1970's (Wagner,

Campbell *et al.* 1979). Since then a number of studies have shown that 70-90% of mesothelioma cases can be linked conclusively to exposure to asbestos. The majority of this is occupational in origin, which leads to a skewed incidence of this disease in men to women (Kindler 2000; British Thoracic Society Standards of Care Committee 2001; O'Byrne and Rusch 2006). Due to wide use of asbestos and a long latency period, this incidence rate is still rising in many countries even after banning of its use; in the UK the number of new MPM cases is expected to rise from 1783 in 2003 to 3000 by 2025 (Garlepp and Leong 1995; British Thoracic Society Standards of Care Committee 2001).

The prognosis of MPM is invariably bleak, and it is often rapidly fatal: studies report median survival times of between 8 to 14 months (Berry, Musk *et al.* 2003). MPM is resistant to radiotherapy and chemotherapy and often unsuitable for surgery: it is also often diagnosed late, which results in poor survival rates (Kindler 2000; O'Byrne and Rusch 2006). Patients with cancer of comparable incidence (in UK) such as bladder or bone cancer show 5 year survival rates of 20-60% (CRUK). While multimodal forms of therapy offer MPM patients some hope, only up to 10% of patients are suitable for treatment, increasing the need for new treatments to be developed (British Thoracic Society Standards of Care Committee 2001). Increasing interest and research is therefore focussing on new treatment options such as gene therapy and immunotherapy. Recently it has been reported that HGF and c-Met are up-regulated in mesothelioma tumours and that targeting of this pathway in MPM maybe useful in future clinical trials (Jagadeeswaran, Ma et al. 2006).

Although not generally considered as a classically immunogenic cancer, there is evidence for immune involvement in MPM including: a relationship between presence of tumour infiltrating lymphocytes (TIL) and prognosis (Leigh and Webster 1982; Anraku, Cunningham *et al.* 2008); a case of spontaneous regression associated with lymphocyte infiltration (Robinson, Robinson *et al.* 2001); a case of long term survival linked to normal T and B cell functions (Fischbein, Suzuki *et al.* 1978); murine MPM models demonstrating responses to immunotherapy (Jackaman, Bundell *et al.* 2003; Kruklitis, Singhal *et al.* 2004; Hegmans, Hemmes *et al.* 2005). Interest is focussing on the stimulation of specific responses by the activation of cytotoxic T lymphocytes (CTL), which has initially been demonstrated in vitro for MPM (Ebstein, Sapede *et al.* 2004).

Immune deficiency is not well understood in MPM or other cancers, but is generally thought to be due to soluble factors released by tumours. Mesotheliomas produce high levels of soluble factors including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), interleukin-6 (IL-6) among others. Most of these factors are studied in cancer as they are required for tumour progression via angiogenesis, cell proliferation and metastatic invasion.

Immune suppression in cancer patients may contribute to tumour progression and the limited success of immunotherapy which relies on the patients' immuno-competence (Lew, Tsang *et al.* 1986). Therefore establishing if HGF and how it may contribute towards tumour immune suppression and can contribute to mesothelioma immune-escape would be valuable for the development of effective immunotherapy regimes for mesothelioma patients.

Therefore in this thesis I aim to:

- Establish levels of HGF in mesothelioma (including differences between mesothelioma subtypes) and investigate if the tumour micro environment differs from circulating plasma with respect to HGF levels in mesothelioma.
- Investigate if impairment of mesothelioma patients (previously measured) immune responses correlate with systemic HGF levels.
- Develop models for investigating the effects of DC development.
- Analyse the effects of HGF on DC phenotype and function including migration and phagocytosis.
- Investigate effects of HGF on DC:T cell interations.
- Investigate mechanisms by which HGF may inhibit DC ability to stimulate T cell proliferation/function.
- Establish if *in vitro* effects can be observed in mesothelioma and the physocigcal relevance of *in vitro* observations.

Chapter 2 - Materials and Methods

2.1 Donors

Peripheral blood was obtained by trained phlebotomists from healthy volunteers after informed consent had been obtained, using vacutainers (BD Pharmingen, San Diego, CA, USA) containing EDTA preservative to prevent coagulation. Blood and pleural fluid samples from mesothelioma patients used in this study were obtained from patients attending clinic at Llandough hospital (Cardiff and Vale NHS Trust). Ethical approval was obtained from the South East Wales Local Research Ethics Committee (Ref: 05/WSE02/177) and informed consent was provided. HLA types were determined by PCR single strand conformational polymorphism method, carried out by the Welsh Blood Service, Cardiff UK.

2.2 Tissue Culture Reagents

RPMI (RPMI 1640, Cambrex), was supplemented with 5% fetal bovine serum (FBS, PAA laboratories, Austria) with 100 IU/ml penicillin (Gibco, Grand Island, NY, USA), 100 μg/ml streptomycin (Gibco) 2 mM L-glutamine (Gibco), 25 mM HEPES buffer (Sigma, Poole, Dorset, UK) and 2 mM sodium pyruvate (Sigma). Freezing cocktail for storing cells in vapour phase N_2 contained 10% dimethyl sulphoxide (DMSO, Sigma) 20% FBS and 70% RPMI. Cells were centrifuged at 250 g for 3 min and resuspended on ice in cold freezing medium, prior to freezing at -80°C overnight and subsequent transfer to vapour phase N_2 for long term storage.

2.3 Mesothelioma Cell Lines

Primary cell lines have been isolated in the department as part of another project from tumour biopsies as follows. Tumour samples were cut into approximately 2-4mm pieces and cultured for 1-2 days in RPMI + 10% FBS in 75 cm^2 culture flasks(Corning Inc., Corning, NY, USA) . The non adherent material was then removed and the adherent layer was washed and cultured in fresh RPMI + 5% FBS.

Primary cell lines were isolated from pleural fluid samples as follows. The cellular fraction of pleural fluid pelleted at 400 g and further isolated using Histopaque as described for PBMC. Cells were then resuspended in culture medium and ~10^6 were seeded in 75 cm^2 culture flasks. The non adherent cells were removed after 2-3 days and adherent cells were maintained by regular passaging. Cells were removed using Trypsin/EDTA for maximum of 5 min, washed and resuspended at a 1:8 split. The cell lines were confirmed to be mesothelial by the Pathology Department of Llandough Hospital based on calretinin staining, and in the Department of Oncology based on mesothelin and calretinin staining and western blotting.

2.4 Isolation of PBMC

Venous blood from healthy volunteers was obtained as described in 2.1 above. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Histopaque 1077 (Sigma) following the manufacturers instructions. Briefly; approximately 25 ml of blood was layered on top of 12 ml of Histopaque and centrifuged at 400 x g for 30 min at room temperature with the brake off. The supernatant (i.e. plasma) from this separation was frozen at -20°C in aliquots for later

analysis. Cells from the mononuclear cell interface layer were collected and diluted with an equal volume of sterile PBS and pelleted at 250 g for 10 min. Cells were then resuspend and washed twice in 10 ml of PBS at 250 g, before a final wash in RPMI + 10% FCS.

2.5 Assessment of HGF Levels

2.5.1 In Patients' Blood/Pleural Fluid

Frozen plasma from patients and healthy donors, obtained as described above (section 2.1 and plasma separated as described in section 2.5), were thawed at 4°C overnight, and HGF levels measured using R&D Systems HGF Duoset ELISA kits (R&D Systems, Minneapolis, MN, USA), following the manufactures instructions. Briefly, ELISA plates were coated overnight with HGF capture antibody, and then washed with PBS + 0.01% Tween 20. The plates were blocked with 0.1% BSA for 2 h at RT and washed again. 100 µl of sample or standard was added, in duplicates for each sample and triplicate for standards, and incubated for 2 h at RT. The wells were then washed and incubated for 1 h with biotin-conjugated HGF detection antibody; the plates were washed and incubated for 30 min with streptavidin-peroxidase; then washed and R&D detection reagent was added. The reaction was stopped after 10 min using 2N H_2SO_4. Levels of HGF were determined using a Bio-Rad 4500 plate reader reading at 570 nm and using 540 nm as reference.

2.5.2 HGF Detection in MPM Cell Line Supernatants

HGF levels in culture supernatants were determined using a commercially available ELISA kit (R&D Systems), as above Mesothelioma cell lines were seeded at 10^5 cells in 25cm^2 flasks and grown for times stated, in RPMI + 5% FBS. They were harvested and counted and the supernatant removed and frozen for later analysis. Assays were carried out as detailed above in duplicate and analysed on a Bio Rad 4500 (Bio-Rad, Hercules, CA, USA) plate reader at 450nm.

2.6 Generation of Monocyte Derived DC

DC were generated according to standard methods (Sallusto and Lanzavecchia 1994) unless otherwise stated. PBMC were resuspended in PRMI 1640 without FCS and allowed to adhere in 6 or 12 well tissue culture plates at 15 x 10^6 cells per well or 4-5 x 10^6 cells per well, respectively . After 2 h at 37°C, non adherent cells were removed by washing with PBS and cryopreserved in 20% FCS, 10% DMSO freezing mixture as described and stored in liquid nitrogen. The adherent cells were cultured in either 5 ml or 2 ml RPMI 1640 + 5% FCS (6 well and 12 well plates respectively) with recombinant human GM-CSF 500 ng/ml (Prospec-tany technogene Ltd., Rehovot, Israel) and recombinant human IL-4 500 U/ml (Gentaur, Brussels, Belgium) for 5 days. The non adherent cells were collected and the adherent cells were detached by incubating the cells with PBS for 15 min. The resulting cells were pooled, washed and counted and used as immature dendritic cells (iDC).

2.7 Generation of Macrophages

PBMC were resuspended in culture medium and allowed to adhere into 6 or 12 well tissue culture plates (as above in section 2.6). After 2 h at 37°C, non-adherent cells were removed and cryopreserved. The adherent cells were cultured in either 5 ml or 2 ml (6 or 12 well trays) RPMI 1640 + 5% FCS with 500 ng/ml recombinant human M-CSF (Peprotec, Rocky Hills NJ, USA) for 5 days. The resulting cells were detached by incubating with PBS for 15 min and the resulting cells used as macrophages.

2.8 HGF Treatment of Monocytes

The standard method of DC generation, as described above was modified by the addition of HGF or IL-10, in the following ways which are illustrated later in the Results chapters:

Co-treatment; PBMC were isolated as described previously and were resuspended in PRMI 1640 without FCS and allowed to adhere into 6 or 12 well tissue culture plates at 15 x 10^6 cells per well or 4-5 x 10^6 cells per well, respectively. After 2 h at 37°C, non adherent cells were removed by washing with PBS and cryopreserved in 20% FCS, 10% DMSO freezing mixture as described and stored in liquid nitrogen.

Adherent fractions of PBMC were treated with 30 ng/ml HGF (Peprotech, Rocky Hill, NJ), in addition to GM-CSF and IL-4 (H-iDC), as specified above (Section 2.6) for 5 days. The non adherent cells were collected and the adherent cells were detached by incubating the cells with PBS for 15 min. The resulting cells were pooled, washed and counted and used as immature HGF co-treated dendritic cells (H-iDC).

Pre-Treatment; PBMC were isolated as described previously and were resuspended in PRMI 1640 without FCS and allowed to adhere into 6 or 12 well tissue culture plates

at 15 x 10^6 cells per well or 4-5 x 10^6 cells per well respectively. After 2 h at 37°C, non adherent cells were removed by washing with PBS and cryopreserved in 20% FCS, 10% DMSO freezing mixture as described and stored in liquid nitrogen. Adherent fractions of PBMC were either treated with 30 ng/ml HGF (Peprotech, Rocky Hill, NJ) for 24 h (pH-DC), or left untreated for 24 h as controls (pC-DC) prior to the addition of GM-CSF and IL-4 in the manner specified as specified above (Section 2.6) for 5 days. The non adherent cells were collected and the adherent cells were detached by incubating the cells with PBS for 15 min. The resulting cells were pooled, washed and counted and used as immature pre-treated dendritic cells (pH-iDC or pC-iDC).

2.9 Maturation of iDC

LPS (Sigma) was used to trigger DC maturation. DC were generated as above and after 5 days in culture treated with 20 ng/ml LPS (Invitrogen Corporation, Carlsbad, CA, USA) for 48 h, to generate mature DC (mDC), mature HGF pre-treated DC (pH-mDC) or mature controls (pC-mDC). For determination of cytokine levels, cell free supernatants were collected after 2 days of culture by removing cells with centrifugation at 250 x g for 3 in.

2.10 Monoclonal Antibodies

The monoclonal antibodies (mAb) used in this study are shown in figure 2.1. The cells were analysed on a FACSCanto (Becton Dickinson & Co. San Jose, California, USA) flow cytometer using FACSDiva Software (BD).

2.11 Immunofluorescent Labelling of Cell Surface Molecules to Assay Phenotype of DC

For immuno-phenotyping, cells were washed in PBS at 4°C and incubated at 4°C on ice for 30-45 min with the following antibodies: 10 µl CD40-FITC, 5µl PD-L1-PE-Cy7, 10 µl CD83-PE, 10 µl anti-CD86 FITC-conjugated, 10 µl anti-HLA-DR PE-conjugated, 10 µl c-Met-FITC, 10 µl anti-CD80 PE-Cy5-conjugated, 5 µl anti-CCR7 PE-Cy7-conjugated, 10 µl anti-CD209 APC-conjugated, and 1.5 µl anti-CD14 APC-Cy7-conjugated or 10 µl anti-CD86 FITC-conjugated, (all antibodies are from BD Pharmingen except HLA-DR antibody (AbD Serotec, Kidlington, Oxfordshire, UK) and c-Met antibody (eBioscience, Inc., San Diego, CA, USA). Cells were washed in PBS and then analyzed using a FACSCanto flow cytometer (BD).

mAb (mouse anti-human)	Clone	Label	Source
CCR7	2H4	PE-Cy7	BD Pharmingen
CD14	MΦP9	APC	BD Pharmingen
CD209	DCN46	APC-Cy7	BD Pharmingen
CD3		APC	BD Pharmingen
CD4	SK3	R-PE	BD Pharmingen
CD40		FITC	BD Pharmingen
CD8	RPA-T8	PE-Cy5	BD Pharmingen
CD80	L307.4	PE-Cy5	BD Pharmingen
CD83		PE	BD Pharmingen
CD86	2331 (FUN-1)	FITC	BD Pharmingen
c-Met	eBioclone 97	FITC	eBioscience
HGF neutralising	24612	-	R & D Systems
HLA-DR		FITC	AbD Serotec
IFNγ	4S.B3	FITC	BD Pharmingen
IL-10		PE	BD Pharmingen
IL-10 neutralising	25209	-	R & D Systems
Mouse IgG1	11711	-	R & D Systems
Mouse IgG2b	20116	-	R & D Systems
PD-L1	MIH1	Pe-Cy7	BD Pharmingen

Figure 2.1 Table of antibodies used for analysis

2.12 ERK Phosphorylation by FACS

For measurement of ERK1/2 phosphorylation, PBMC in PBS were stimulated with HGF for 10 min in the presence or absence of an HGF-blocking antibody or its isotype control (R&D, Minneapolis, MN). Cells were then fixed and permeabilised using the BD Phosflow kit and labelled with ERK1/2p-PE (BD) or isotype control (BD), according to the manufacturer's instructions, before flow cytometry analysis. Briefly cells were fixed by addition of an equal volume of pre-warmed BD Phosflow Fix Buffer I to the cell suspension and incubating the cells at 37°C for 10-15 min. Cells were washed and permeablised by addition of 1 ml BD Phosflow Perm/Wash Buffer I for 10 min at room temperature. Cells were then washed and resuspended in BD Phosflow Perm/Wash Buffer I and stained with 5µl pERK1/2 antibody for 30 min at room temperature in the dark. Cells were washed in PBS and then analyzed using a FACSCanto flow cytometer (BD).

2.13 Detection of Cytokine Production by ELISA

ELISA kits were purchased from R&D systems for determination of IL-10, and IL-12 production. Samples from DC and T cell supernatants obtained as indicated in the results chapters were analysed following the manufacturer's instructions.

2.14 DC Migration Assay

DC migration was assessed using polycarbonate transwell plates (Greiner, Nuremberg, Germany) with 6.5 nm diameter wells and 5 µm pore size inserts. 600 µl of RPMI 1640 (Gibco), containing CCL19 (Peprotec) 200 ng/ml, 5% FBS (PAA) was added to the tissue culture plates. Control groups lacked CCL19 in medium. The upper chambers of the transwell plates were soaked in assay medium (without

CCL19) for 16 h prior to use and were inserted into each well. DC were matured using 20 ng/ml LPS for 48 h. Equal numbers of DC from each group (0.4-1 x 10^6/well) were added to the upper chambers in a final volume of 100 µl/well. The plates were incubated at 37 °C for 3 h. Medium from the lower chamber including migrated cells were collected, cell viability was assessed, and numbers of migrated cells were determined using a standard haemocytometer.

2.15 DC Phagocytosis Assay

DC phagocytic ability was assessed by measuring uptake of FITC labelled latex (Sigma) beads using FACSCanto flow cytometer (BD). DC were cultured for 5 days, then collected as described for iDC (section 2.6) washed and counted. 0.5 x 10^6 DC were cultured in medium containing 5µl of 5µm diameter FITC-latex beads (Sigma) at either 37°C or 4°C (negative control) for 30 min. Cells were washed and analysed using BD FACScanto running FACSDiva software, and the proportion of cells positive for FITC was determined.

2.16 T Cell Proliferation Assay (CFSE Dilution Method)

Mixed lymphocyte reactions (MLR) were carried out in 96 well flat bottom microtitre plates by adding DC to allogeneic non-adherent PBMC. DC were seeded at 20 x 10^3 per well in a 96 well flat bottomed tray. Non adherent PBMC from a different donor, were labelled with CFSE dye (Invitrogen) as per the manufacturer's instructions. Briefly; non adherent cells were incubated with 5 µM CFSE for 5 min at 37°C in RPMI 1640 without FCS in the dark, then washed in PBS and resuspend in RPMI 1640 + 5% FCS medium for 15 min at 37 °C before resuspending in fresh culture medium. CFSE labelled cells were added to DC at 10:1 ratio (unless otherwise stated).

Cells were collected after 7 days and analyzed using a FACScanto flow cytometer (BD)

2.17 T cell Proliferation Assays (^{3}H-Thymidine Incorporation Method)

MLR were carried out in 96 well U bottom microtitre plates by adding DC to allogeneic non-adherent PBMC. Briefly; DC were seeded at 20 x 10^3 per well in a 96 well U bottomed tray in triplicates. Non-adherent lymphocytes were added at a ratio of 10:1 or as stated of T cell:DC. ^{3}H-thymidine (Amersham, Buckinghamshire, UK) was added at 0.5 μCi/well on day 5 of culture for 16 h. The cells were harvested onto fibroglass filtermats and ^{3}H-thymidine incorporation was measured by a Wallac 1450 microbeta β-plate counter (PerkinElmer Life And Analytical Sciences, Inc. Waltham, Mass, USA).

2.18 Peptide Stimulation of T Cells by Autologous DC

Memory T cell responses to common viral peptide antigens, consisting of eleven 15-mer peptides representing EBV, HCMV and influenza-derived CD8 T cell epitopes, were measured. Briefly, 5-10 x 10^4 DC were added to 48 well trays. DC were loaded with 5 μg antigen (common viral peptide antigens as specified in figure 2.2) for 4 h prior to adding non-adherent autologous PBMC. Non adherent autologous PBMC were added at a 10:1 ratio to DC in 1ml final volume. A second stimulation was carried out 7 days later with antigen presenting cells comprised of either autologous or HLA-matched BLCL, which were co-cultured with the viral peptide mix for 4 h at 5 μg/ml and were added to T cell cultures at 1:10 ratio. After 1 h of co-incubation with

T cells, 1 µl/ml Golgi Plug® (BD) was added and the cultures were further incubated at 37°C overnight.

2.19 Intracellular Labelling for Detection of Cytokines Produced by T Cells

Cytokine production (IFNγ or IL-10) by T cells following peptide Ag-stimulation by Ag-loaded DC at a ratio of 10:1 T cell:DC as described above was measured by flow cytometry. The cells were washed with PBS, samples were fixed and permeablised using Intraprep kit (Beckamn Coulter) according to the manufacture's instructions. The cells were labelled in the presence of the permabilising agent with the following antibodies 10 µl CD8-PE-Cy5, 5 µl CD3-APC and 2 µl IFNγ-FITC for 45 min at 37°C in the dark. Cells were washed in PBS and analysed on FACSCanto flow cytometer.

HLA Class I	Score* (SYFPEITHY)	Sequence***	Antigen	Position
A1	31	IQMCTELKLSDYEGR	Flu NP	41-55
A1	35	GLLVSDGGPNLYNIR	Flu PB1	591-599
A2	30	LTKGILGFVFTLTVP	Flu M1	55-69
A2	28	IQNAGLCTLVAMLEE	EBV BMLF1	276-290
A3	30	SALILRGSVAHKSCL	Flu NP	265-273
B7	19	RKTPRVTGGGAMAGA	CMVpp65	415-429
B7	22	SQAPLPCVLWPVLPE	BZLF1	40-54
B8	32	RKCRAKFKQLLQHYR	BZLF1	187-201
B8	28	RRSQVKWRMTTLAAG	EBNA3A	154-168
B44	22	QEFFWDANDIYRIFA	CMVpp65	511-525
B44	25	QTEENLLDFVRFMGV	EBNA3c	281-290
B44, A2, A3	25	AAFEDLRVLSFIKGTK	Flu NP	336-351
B44, A1, B8	19	STLELRSRYWAIRTR	Flu NP	377-391
B44, A2,	26	SLLTEVETPIRNEW	Flu M2	1-15
A11, A2, A3	?	AVKGVGTMVMELVRMIK	Flu NP	182-198

Figure 2.2 Control Peptide Antigens Used for T cell Stimulation

Peptides representing common viral antigenic epitopes, restricted by the most frequent Caucasian HLA class I types.

*Predicts the strength of binding of peptides and the probability of being processed and presented by a given HLA allele, according to the SYFPEITHI algorithm (http://www.syfpeithi.de/Scripts/MHCServer.dll/home.htm)

** Published and/or predicted.

The peptides were selected based on (Currier, Kuta *et al.* 2002) and Influenza Sequence Database http://www.flu.lanl.gov/review/epitopes.html.

Chapter 3 - HGF in Malignant Pleural Mesothelioma (MPM)

3.1 Introduction

Elevated levels of HGF can be detected in the tumour environment and also systemically in many types of cancer. Tumour effusions represent a relatively easily available source of cells and cytokines which reflect most of the complexities of the tumour environment. Tumour effusions have been studied for their immunosuppressive effects (Chen, Ting *et al.* 2000; Sikora, Dworacki *et al.* 2004; Delong, Carroll *et al.* 2005) and a range of immunomodulatory factors have been identified such as IL-10, TGF-β, IL-6, GM-CSF and VEGF (Zou 2005). In spite of elevated levels of HGF in tumour effusions (Eagles, Warn *et al.* 1996), its immunomodulatory effect has not attracted many investigations. HGF has only recently been implicated in interactions with the immune system (Skibinski 2003).

The ongoing research project in this department studying the immunological aspects of mesothelioma provided an opportunity to study HGF in the tumour exudates which accumulate in the pleural space of majority of patients and are regularly removed as part of palliative treatment to ease breathing. DC and T cells are important in tumour elimination, as described in Chapter 1 and therefore they are possible targets for modulation by tumour-induced HGF in MPM. The aim of this chapter was to establish what levels of HGF can be found in mesothelioma patients plasma, pleural fluid and tumour cell supernatant in order to:

a) guide the development of *in vitro* work to correctly model physiological HGF concentrations present in the tumour environment

b) to see if we can establish an *in vitro* model using HGF-producing mesothelioma cell lines.

3.2 HGF in Blood and Tumour Micro-Environment of Mesothelioma Patients

Levels of plasma HGF were compared between mesothelioma patients and healthy donors. Significantly elevated levels of HGF were found in the plasma of mesothelioma patients (1980 ± 268 pg/ml, n=47, P=0.0064) compared to healthy donors (452 ± 61 pg/ml, n=12), Figure 3.1. There are three subtypes of mesothelioma. I investigated how the levels of HGF vary between the different MPM subtypes (epithelioid, mixed (biphasic) and sarcomatoid). Each group of MPM patients had elevated HGF levels (epithelioid 2409±494 pg/ml n=19, mixed 1674 ± 218 pg/ml n=11, sarcomatoid 4153±1951 pg/ml, n=4) compared to healthy donors, (Figure 3.2). There was no statistical difference in the HGF levels between the mesothelioma subtypes when compared to each other.

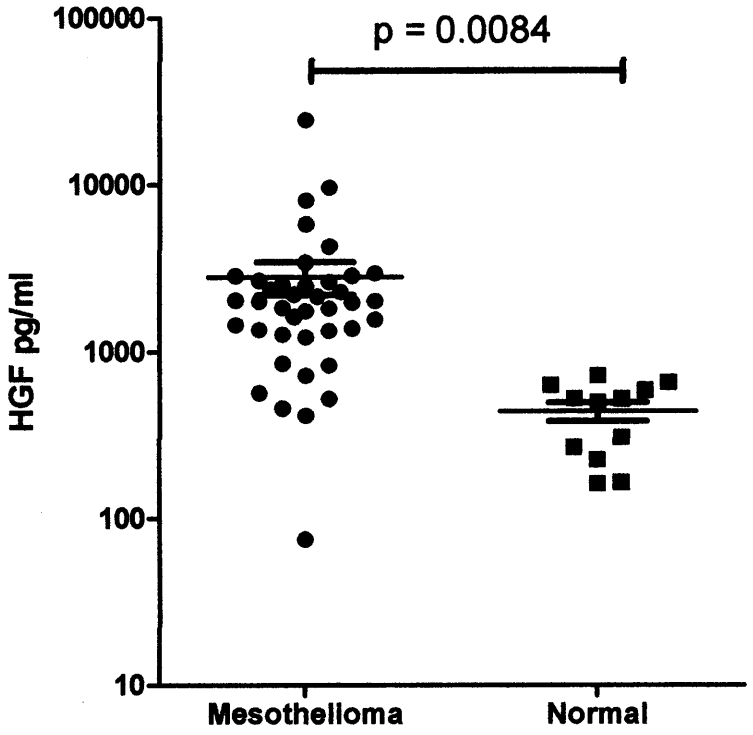


Figure 3.1 Elevated Levels of HGF in Plasma of Mesothelioma Patients Compared to Healthy Donors

HGF levels were determined from plasma, kept frozen before the assay. Mesothelioma patient HGF levels (●), were compared to healthy donors (normal) (■) HGF levels. HGF levels were determined using R&D systems HGF ELISA and expressed as pg/ml. Means of assay duplicates are plotted as individual points, the lines represent the mean HGF level for mesothelioma patients and healthy donors(normal), the bars represent the SEM. HGF levels are statistically higher (using an unpaired T test) in the plasma of mesothelioma patients (1980 ± 268 pg/ml, N=47) (P=0.0084) than in healthy donors (452 ± 61 pg/ml, N=12). The healthy and cancer samples were not age matched, however the majority of healthy donors used by the department fall within similar ages to those of mesothelioma patents, however the normal samples were skewed more towards female rather than male donors. The samples obtainable were limited by the numbers of mesothelioma patients diagnosed, due the rarety of this cancer.

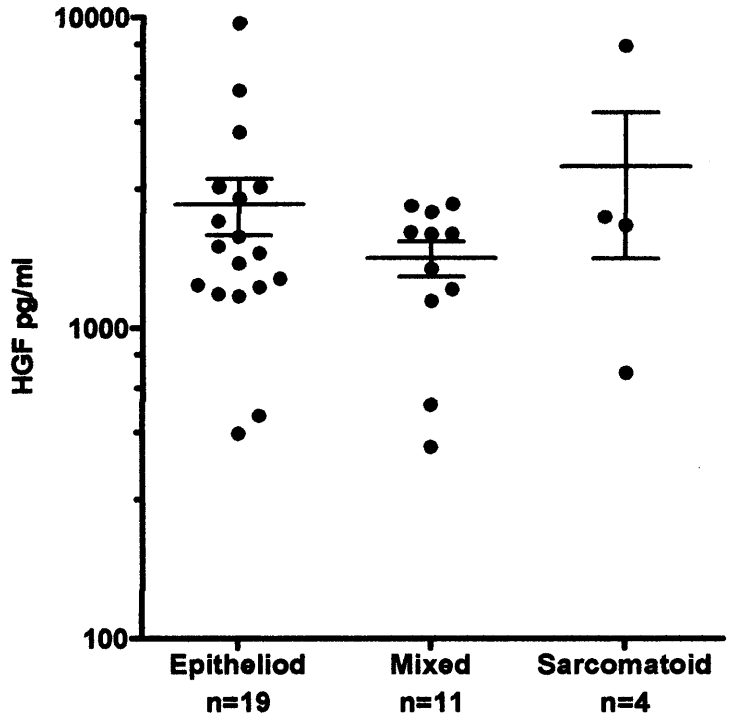


Figure 3.2 Elevated Levels of HGF in Plasma of Mesothelioma Patients of All Three Tumour Types

HGF levels were determined as in Figure 3.1. Mesothelioma patient plasma HGF levels were studied according to mesothelioma subtypes (at diagnosis). Means of assay duplicates are plotted as individual points, lines represent the mean level of HGF (2409 ± 494 pg/ml, 1674±218.9 pg/ml and 4153 ± 1951 pg/ml respectively, in epithelioid, mixed and sarcomatoid MPM) and the bars represent the SEM. There was no significant difference (using an unpaired T test) between the HGF levels in these subtypes.

3.3 High Levels of HGF in Pleural Fluid

In mesothelioma patients' tumours often produce pleural effusion which is drained as part of palliative treatment of this disease. Pleural effusions contain tumour cells, immune cells and soluble factors which are representative of the tumour environment.

HGF levels were measured in the pleural fluid in a small cohort of MPM patients. In 6 out of 7 patients both plasma and pleural fluid were available. HGF levels in tumour effusions were more than 10-fold higher than in patient's plasma ($\geq$ 7193 $\pm$ 3509 pg/ml vs. 650 $\pm$ 205 pg/ml). Two pleural fluid samples had levels of HGF above 10,000 pg/ml, the upper limit of detection; for these samples estimated values were used (estimated values of 11339 and 26789 pg/ml respectively) to determine the working concentration of HGF for experimental purposes (Figure 3.3).

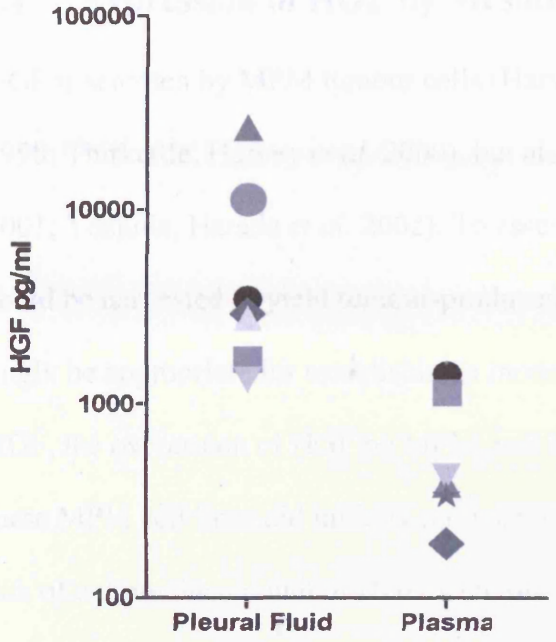


Figure 3.3 Increased Levels of HGF in Pleural Fluid Compared to Plasma in MPM

HGF levels in the pleural fluid (7193 ± 3509 pg/ml, n=7) or the plasma (650 ± 205 pg/ml, n=6) of MPM patients were measured by ELISA. The dots represent individual patients, means of duplicate samples are shown. A more than 10 fold higher level of HGF was found in the pleural fluid than in plasma.

3.4 Expression of HGF by Mesothelioma Cell Lines

HGF is secreted by MPM tumour cells (Harvey, Warn *et al.* 1998; Tolnay, Kuhnen *et al.* 1998; Thirkettle, Harvey *et al.* 2000), but also by stromal cells (Skibinski, Skibinska *et al.* 2001; Yoshida, Harada *et al.* 2002). To assess whether *in vitro* tumour cell supernatant could be harvested to yield tumour-produced HGF, and additionally whether these cell lines might be appropriate for establishing a model investigating the effect of MPM-derived HGF, the expression of HGF by MPM cell lines was examined (Figure 3.4). A few of these MPM cell lines did initially produce high levels of HGF (Figure 3.4). However, after 48h of culture the amount of HGF decreased in all cell lines (Figure 3.5). Presumably this was due to its uptake by tumour cells as shown by (Harvey *et al.* 1998; Klominek, Baskin *et al.* 1998; Harvey, Clark *et al.* 2000). Due to the low concentrations of HGF in tumour cell line supernatants, it was not feasible to establish a mesothelioma cell line-derived HGF model.

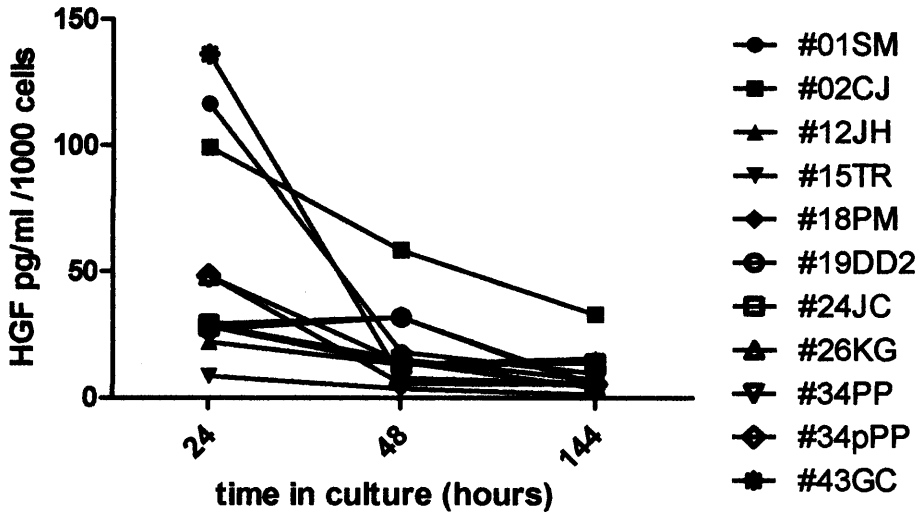


Figure 3.4 Decrease in HGF Production over Time by Mesothelioma Cells in Culture. Serial cultures of cells seeded with the same number of tumour cells were started and at the time points indicated HGF production by cells was determined analysis of supernatant HGF concentration by ELISA. At the same time tumour cells were harvested, and total cell number was determined. Levels of HGF production were calculated as the amount of HGF (pg/ml) produced by 1000 cells. Each line represents a separate MPM-line, as indicated on the right.

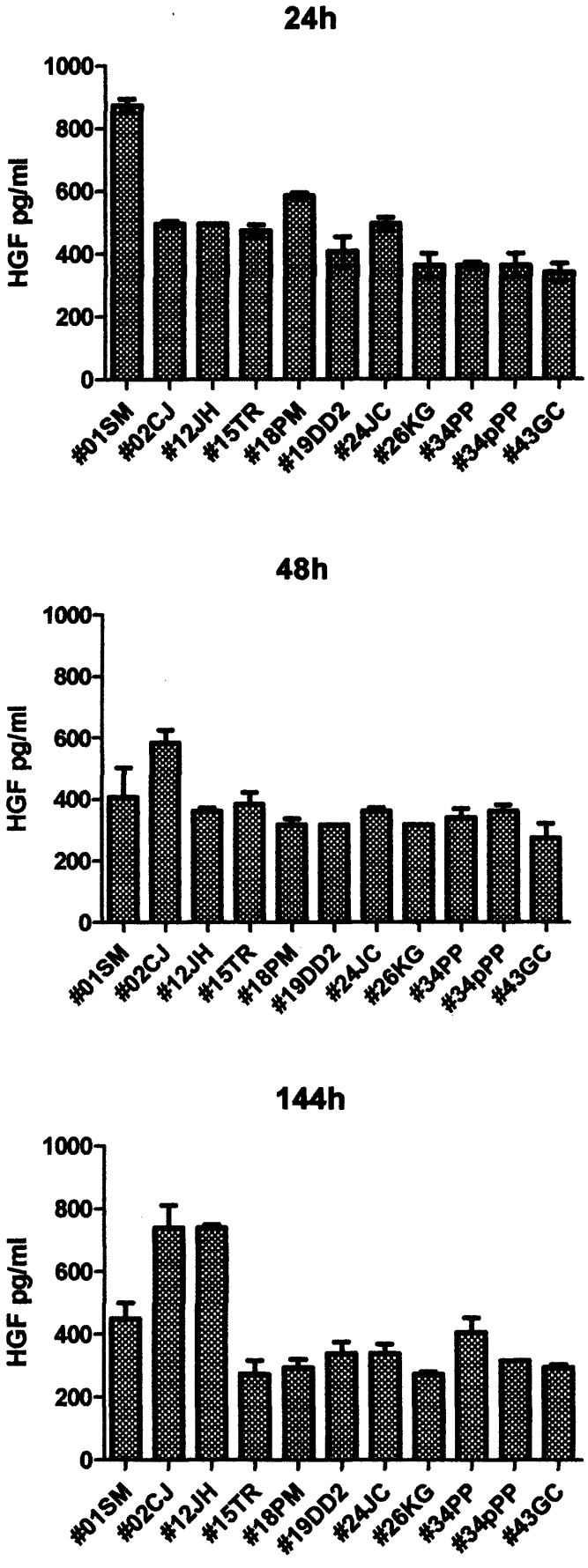


Figure 3.5 Total Levels of HGF in Mesothelioma Supernatant

Total levels of HGF were determined in the supernatant of mesothelioma cultures 24h, 48h and 144h. Briefly; wells were seeded with 10,000 Mesothelioma cells and incubated for 24, 48 or 144 hours, supernatant was then removed and HGF concentration was determined using R&D HGF ELISA kit. Each bar represents a separate MPM line.

3.5 Elevated Plasma HGF Levels Elevated Plasma HGF Levels Elevated Plasma HGF Levels

The data on HGF levels indicate that plasma HGF is elevated in all mesothelioma subtypes. Using an arbitrary cut off for high and low levels of HGF as 1000 pg/ml (~2x healthy donor levels), T cell responses (% CD8+ T cell producing IFNγ) to recall antigens were measured as described (Coleman, Clayton *et al.* 2005; Coleman, Gibbs *et al.* 2008). Results are expressed as a stimulation index (SI) (peptide-induced responses divided by control responses (T cell IFNγ production in the absence of peptides)). The T cell work was carried out in the department prior to the work for this thesis: Figure 3.6 demonstrates existing T cell data in the light of HGF levels in the plasma of these patients, determined in Figure 3.1. Mesothelioma patients with high HGF levels had slightly decreased SI values (high 4.741 ± 0.9895), but not significantly different (p=0.7522) from the low HGF group (low 5.307 ± 1.597). The results indicate that the concentration of HGF found in MPM patient plasma is not sufficient to cause systemic modulation of immune responses.

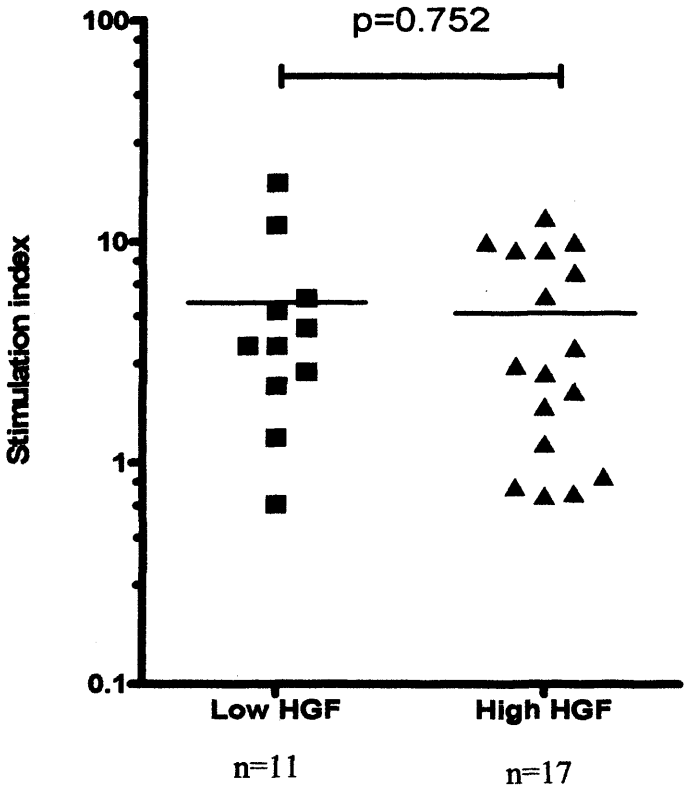


Figure 3.6 Plasma HGF Levels Do Not Affect Systemic T Cell Responses

HGF levels were determined previously using ELISA (R&D systems). Patients were split into two groups depending on HGF levels High HGF (>1000pg/ml, n=17) (■), Low HGF (0-1000 pg/ml, n=11) (▲). The stimulation index, as calculated in (Coleman *et al.* 2005; Coleman *et al.* 2008), was used as a measure of the immune response. The points indicate mean SI of duplicates; the lines are the mean SI for each group. No significant difference was observed using an unpaired T test (p=0.752).

3.6 Summary

In this chapter I set out to establish HGF levels in mesothelioma patients' plasma, pleural effusions and tumour cell supernatant. Elevated levels of HGF are found in MPM plasma compared to that in the plasma of healthy donors. This agrees with other reports showing that HGF can be elevated in the circulation of cancer patients (Eagles G, Warn A *et al.* 1996; Harvey, Warn *et al.* 1996; Taniguchi, Kitamura *et al.* 1997; Harvey *et al.* 1998; Tolnay *et al.* 1998; Harvey *et al.* 2000; Naughton, Picus *et al.* 2001; Hashem and Essam 2005; Jiang, Martin *et al.* 2005; Mukohara, Civiello *et al.* 2005; Sheen-Chen, Liu *et al.* 2005; Peruzzi and Bottaro 2006).

The average HGF level is about four times higher in MPM plasma than in healthy donors and in the range of ~1ng/ml. Similar levels are observed in the plasma of prostate and gastric cancer patients (Taniguchi et al. 1997; Naughton et al. 2001). Of greatest interest from my results is the highly elevated levels of HGF in pleural fluid, as this demonstrates that the physiological levels local to mesothelioma tumours are vastly different from that in the plasma.

HGF is routinely used at concentrations between 1-100 ng/ml *in vitro* for measuring its effects on cell migration and infiltration (Vande Woude, Jeffers *et al.* 1997; Klominek *et al.* 1998; Beilmann, Vande Woude *et al.* 2000; Harvey *et al.* 2000). In light of this and the levels found in pleural fluid levels, it was decided to use 30 ng/ml HGF as the standard dose in the experiments. This concentration maximises both any observable effects of HGF on cells, while still reflecting the physiological conditions. Additionally, as HGF binds to heparin sulphate proteoglycans and is sequestered near the cells producing it, it is likely

that levels of HGF within the tumour are higher than those in the pleural fluid. Systemically elevated HGF levels do not have a negative effect on T cell memory responses generated in vitro from PBMC of MPM patients. Systemic immuno-suppression can be observed in certain advanced cancers, e.g. ovarian cancer (Coleman et al. 2005), but this does not seem to be a feature of MPM. Local immuno-suppression in the tumour environment is a well established phenomenon, hampering anti-tumour effector function and the success of therapeutic cancer vaccines (Yang and Carbone 2004; Pinzon-Charry, Maxwell *et al.* 2005; Hegmans, Hemmes *et al.* 2006; Curiel 2007). As noted above, it was interesting to see that HGF concentrations are more than 10-fold higher in the pleural fluid than in paired plasma samples. The typical concentration of HGF in the pleural fluid is in the nanogram range, reaching >20ng/ml in several patients. Similar studies in MPM have not been carried out before, although immunomodulatory cytokines have been described in the pleural fluid of mesothelioma (Delong, Carroll *et al.* 2005; Hegmans *et al.* 2006).

Tumour cell lines established in the laboratory from the pleural fluid or tissue samples of mesothelioma patients are also studied for their ability to produce HGF. These *in vitro* HGF levels are relatively low, maybe because in mesothelioma, like in other cancers, the stromal cells are the main source of HGF (Harvey *et al.* 1996; Yoshida *et al.* 2002; Masuya, Huang *et al.* 2004). The HGF concentration does not increase with time in the culture supernatant with time, presumably due to the autocrine uptake by tumour cells, as suggested by others (Harvey *et al.* 1996; Harvey *et al.* 1998; Klominek *et al.* 1998; Harvey *et al.* 2000). Thus, although the experiments with the cell lines confirmed that HGF is produced by mesothelioma cells, it did not seem feasible that an *in vitro* model of tumour-derived HGF can be established in order to study the immunomodulatory effects of HGF.

In summary, the high levels of tumour-associated HGF found in mesothelioma patients agree with other reports that HGF levels can be elevated in cancer (Taniguchi *et al.* 1997; Naughton *et al.* 2001; Hashem and Essam 2005; Sheen-Chen *et al.* 2005), and is in agreement with the observation that HGF expression is up-regulated in mesothelioma (Harvey *et al.* 1996; Harvey *et al.* 1998; Harvey *et al.* 2000; Thirkettle *et al.* 2000; Hegmans *et al.* 2006; Jagadeeswaran, Ma *et al.* 2006). I have further shown that HGF levels are elevated in all subtypes of mesothelioma.

On examining the immune responses of these patients there is no systemic immune suppression due to increased HGF levels in plasma. However, it was determined that HGF levels in the tumour microenvironment (pleural fluid) are markedly increased (over 5 fold and possibly as much as much as 100 fold) over the levels found in patient's plasma. Therefore, the higher local levels of HGF in the tumour microenvironment may cause localised immunological effects rather than systemic ones.

Chapter 4 - The Effect of HGF on Monocyte-derived-DC (MDDC) Differentiation.

4.1 Introduction

Ovali et al. described the effects of HGF on the development of human DC from $CD34^+$ cells (Ovali, Ratip *et al.* 2000). They concluded that HGF increases DC development from $CD34^+$ bone marrow cells and, in conjuction with GM-CSF, augments the development of both DC and CD14+ cells. They suggested that further work should be done to show the expression of HGF receptors on DC and the effect of HGF on the phenotype of DC, including antigens up-regulated during maturation such as CD86 and CD80.

At the beginning of my study there was no published data on the effect of HGF on human DC development, phenotype and functions. In a review (Skibinski 2003) the immunological effects of HGF were discussed but the review did not include the possible role of HGF in affecting DC development and the knock on effect this may have on DC functions.

In an attempt to address the lack of information on the role of HGF in the development of human DC, I investigated the effects of HGF treatment prior to and during DC development. I investigated HGF effects using the widely used method of developing MDDC *in vitro* from monocytes by administration of GM-CSF and IL-4. In 2006 *Rutella et al* published their finding of accessory cells (which they termed HGF monocytes) with regulatory activity. These studies showed that HGF treated monocytes had monocyte like phenotypic features such as low expression of co-stimulatory molecules, lack of CD209

and maintenance of CD14. These monocytes produced increased levels of IL-10 and were poor activators of allogenic $CD4^+$ T cell proliferation when compared to GM-CSF and IL-4 generated DC. They also characterised the changes in gene expression of their HGF-monocytes, compared with both DC and untreated monocytes. HGF up-regulated a number genes of interest in immune responses, chemotaxis and cell adhesion, including; DAD1, IDO, ILT-3, C8XCL1, IL1B, IL18, IL1A, CXCL5, CCL2, CCR5, CD47, GPNMB, PPARD, MMP9, and IL-10 (Rutella, Bonanno *et al.* 2006). They effectively showed that these cells have different gene expression patterns to both DC and control unexposed monocytes.

Rutella *et al*'s paper does present an interesting population of monocytes with regulatory features that could be important in immune modulation. But apart from a single phenotype attempt (shown in chapter 1), measuring only CD14 and CD1a levels, their work did not address the effects of HGF on DC development. Rutella *et al* implied that IL-4 and HGF compete in the generation of DC before the HGF-specific differentiation is initiated, but no experiments addressed these questions. In the experiments presented in this chapter I investigated the effects of HGF exposure of monocytes both prior to and during GM-CSF and IL-4 (GM4) treatment. All experiments in this chapter are carried out using healthy donor monocytes.

4.2 Functional HGF-Receptor (c-Met) Expression on Monocytes

To determine whether generating MDDC is a suitable model to investigate the effects of HGF on DC differentiation, first I investigated the expression of the only known HGF receptor, c-Met, on PBMC. This was to determine the population of cells that HGF may target in peripheral blood. The levels of c-Met expression were measured on BMC either

freshly isolated or following 24 h (Figure 4.1 and 4.2) HGF or GM4 treatment or no treatment (Figure 4.2) from healthy donors. Cells in the lymphocyte region did not express the receptor, while cells in the monocytes/macrophages gate (FSc/SSc) bound c-Met antibody at a level considerably higher than the isotype control Ab (Figure 4.1). GM4 treatment reduced the level of surface expression of c-Met on monocytes after 24 h, compared to levels on fresh PBMC or 24 h untreated monocytes. HGF pre-treatment increased the level of surface c-Met expression by up to ~44% compared to levels found on freshly isolated monocytes (Figure 4.2).

The expression of c-Met and the change of expression in response to HGF indicates that monocytes express functional c-Met (Figure 4.2). To demonstrate that HGF is inducing signalling via c-Met, ERK phosphorylation, known to be part of c-Met signalling in other cell types (Choi, Park *et al.* 2004; Jagadeeswaran, Ma *et al.* 2006; Lee, Choi *et al.* 2006; Park, Nam *et al.* 2007; He, Wu *et al.* 2008) was measured in response to HGF. ERK phosphorylation was detectable after 10 min incubation with HGF. HGF stimulation increases phosphorylated ERK1/2 level above that seen in un-stimulated (resting) monocytes (Figure 4.3). This ERK1/2 phosphorylation was abolished by using HGF blocking antibody. This demonstrates that c-Met on monocytes is functional and able to transduce signals via the ERK 1/2 pathway. Based on these results I concluded that MDDC is a suitable model for studying the effect of HGF on DC development.

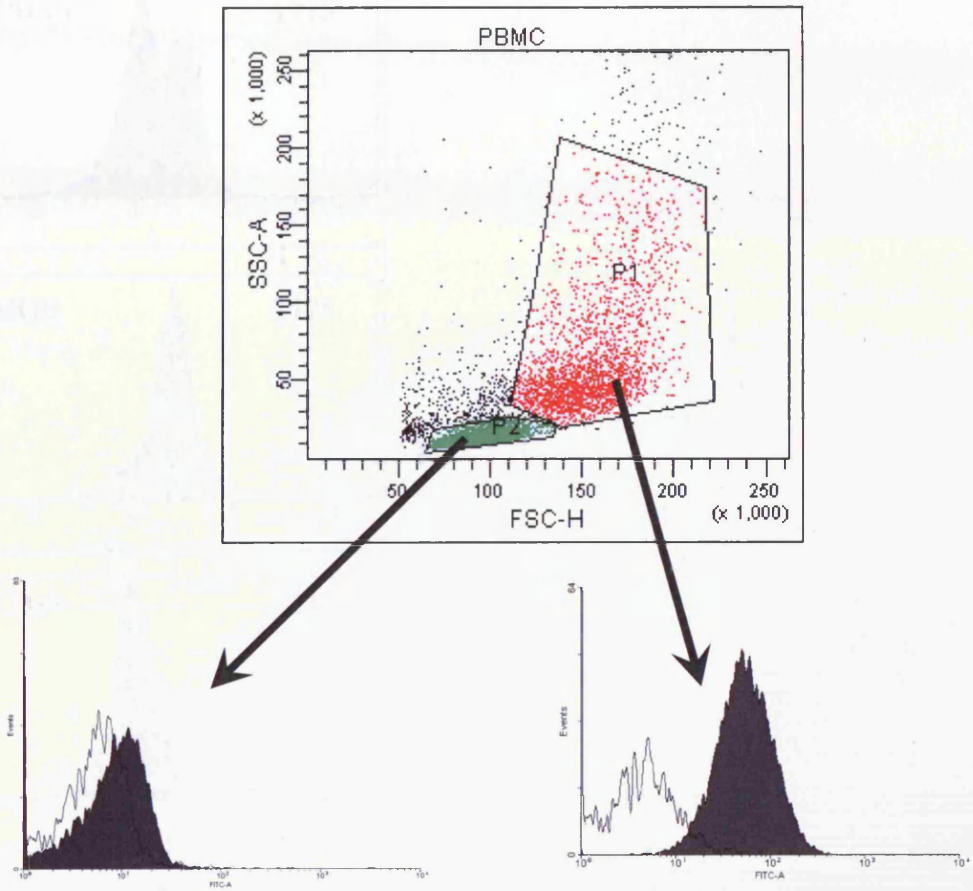


Figure 4.1 c-Met Expression on Monocytes in PBMC

FACS-analysis of PBMC. The top plot shows FSc/SSc profile of a sample of freshly isolated PBMC, P1 representing monocytes (red); P2 representing lymphocytes (green). The black filled histograms represent c-Met expression on lymphocytes (lower left hand plot) or monocytes (lower right hand plot). The black lines represent the binding of the isotype control antibody.

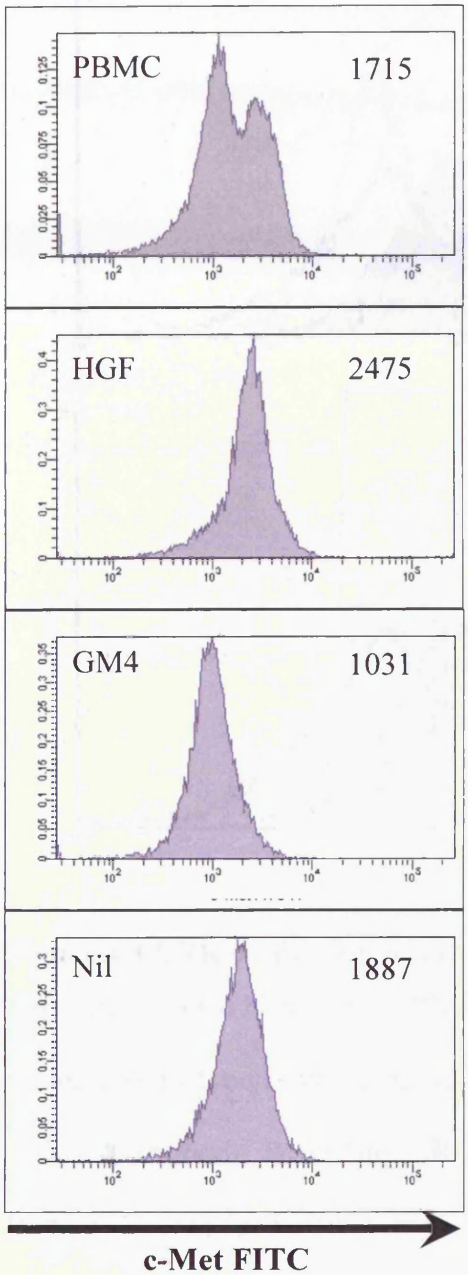


Figure 4.2 Opposite Effects of GM4 and HGF on c-Met Expression on Monocytes.

FACS-analysis of c-Met expression on the surface of monocytes of freshly isolated PBMC, or adherent monocytes cultured for 24 h in the presence of GM-CSF and IL-4 (GM4) or 30 ng/ml HGF (HGF) or without any added factors (Nil). The numbers represent MFI of c-Met expression on cells in the monocyte gate. Representative of 3 experiments.

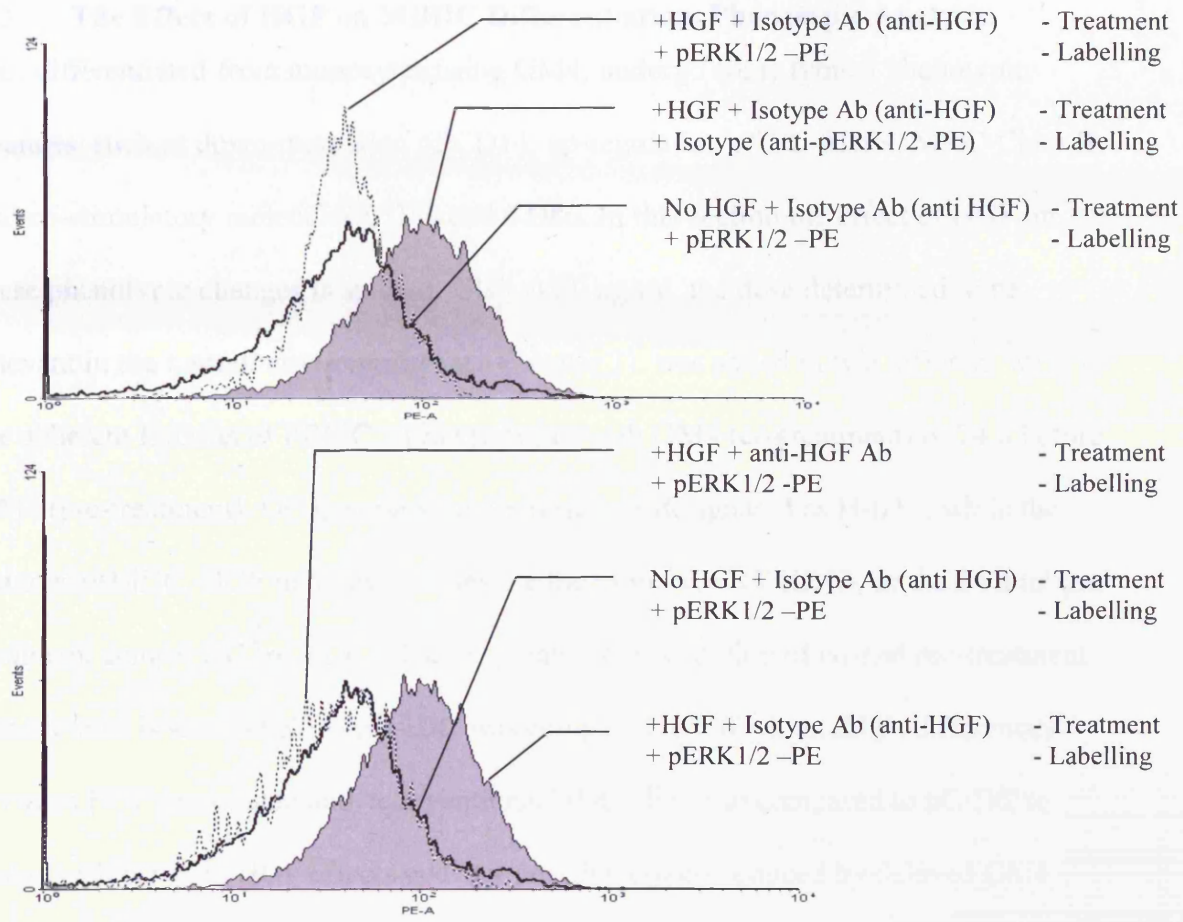


Figure 4.3 ERK1/2 Phosphorylation of Monocytes Following HGF-Treatment.

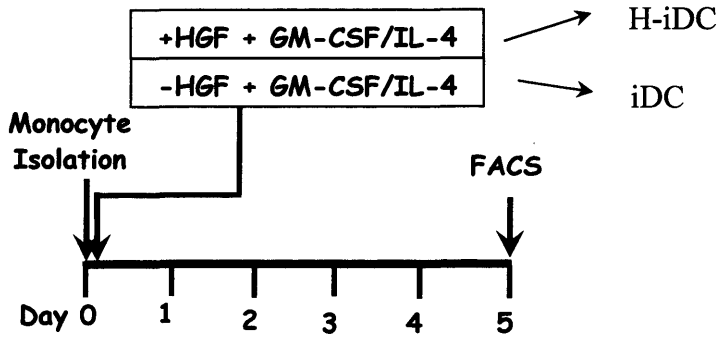
Top: Gray shaded histogram: ERK1/2p expression 10 min after HGF treatment in the presence of isotype control antibody for HGF blocking. Dotted line: isotype control of ERK1/2p antibody. Black line, ERK1/2p expression without HGF treatment.

Bottom: Gray shaded histogram and black line same as above, Dotted line: HGF treatment, with HGF-blocking antibody. Representative of 3 experiments.

4.3 The Effect of HGF on MDDC Differentiation. Phenotypic Analysis.

DC, differentiated from monocytes using GM4, undergo some typical phenotypic changes, such as down-regulation of CD14, up-regulation of DC-SIGN, MHC-Class II, and co-stimulatory molecules CD80 and CD86. In this section the effect of HGF on these phenotypic changes is studied. HGF at 30 ng/ml, the dose determined to be relevant in the tumour environment (see Chapter 3), was added in two different ways to the adherent fraction of PBMC: either together with GM4 (co-treatment) or 24 h before GM4 (pre-treatment). DC generated in the former is designated as H-iDC, while the latter as pH-iDC. HGF untreated groups are the immature DC (iDC), or the 24 h nil pre-treatment control DC (pC-iDC). The schematic representation of co-and pre-treatment experiments is shown below. pH-iDC was compared to iDC to establish differences between HGF pre-treated and "conventional" iDC, but also compared to pC-DC to distinguish between HGF effects and any possible effects induced by delayed GM4 treatment.

Co-treatment:



Pre-treatment:

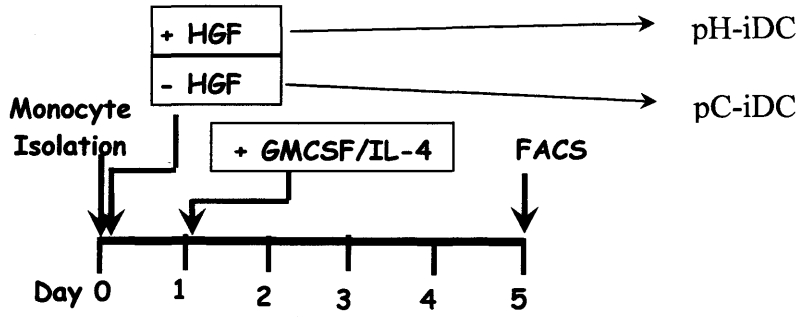


Figure 4.4 HGF Co- and Pre-Treatment Models

The figure represents the two treatment models used in this thesis in the co-treatment model HGF is added to PBMC at the same time as GM4. This si the obvious method for assessing the effects of HGF on DC as they develop from precursors

In the pre-treatment model PBMC are incubated for 24 h, prior to GM4, treatment with or with out HGF. This methods duplicates the physiological setting of cancer patients with elevated HGF levels, where DC precursors are exposed to elevated HGF levels prior to and during DC development. HGF as shown in figure 4.2 increased the level of surface c-Met expression by up to ~44% compared to levels found on freshly isolated monocytes over 24h, while GM4 treatment reduced c-Met expression. Additionally factor like VEGF have been postulated to affect development of DC by affecting precursors rather than interfering with development of DC from precursors.

4.3.1 CD14 and DC-SIGN

The expression of neither CD14 nor CD209 was significantly altered by the presence of HGF during MDDC development from monocytes. CD14 was down-regulated at the same extent on HGF-treated and untreated DC (Figure 4.5), while CD209 was up-regulated to the same level on both groups of DC (Figure 4.6). When the same experiments were carried out in the second model, applying HGF for 24 h before GM4 was added, there was a strong inhibition of CD14 down-regulation compared to that both on normal iDC and on pC-iDC (Figure 4.7). This observation suggested that exposure of monocytes to HGF before GM4, retained cells in an immature state of DC development, resembling monocytes/macrophages.

However, when the pre-treatment effect was tested on CD209 expression, it was found that CD209 levels were elevated not only compared to monocytes but also to those on iDC and pC-iDC (Figure 4.8). These differences reached statistical significance when repeated from seven different donors (Figures 4.8B). This observation suggested that HGF treated monocytes do not differentiate into normal immature DC. In order to assess if HGF pre-treatment generated $CD14^{+}CD209^{+}$ double positive cells or whether these markers were expressed on different cell subsets, two-colour analysis of DC was carried out. Increases in the proportion of double positive DC following HGF pre-treatment, and to lesser extent, without HGF but delayed DC differentiation with GM4 (Figure 4.9) were observed. While iDC contained ~5% double positive cells (Figure 4.9) after 5 days of GM4 treatment, 39.4% ± 4% (n=7) of pC-iDC and 62.5% ± 8% of pH-iDC were double positive. This confirmed that CD14 remains high and may even become up-regulated on the same cells which also express CD209 following HGF pre-treatment.

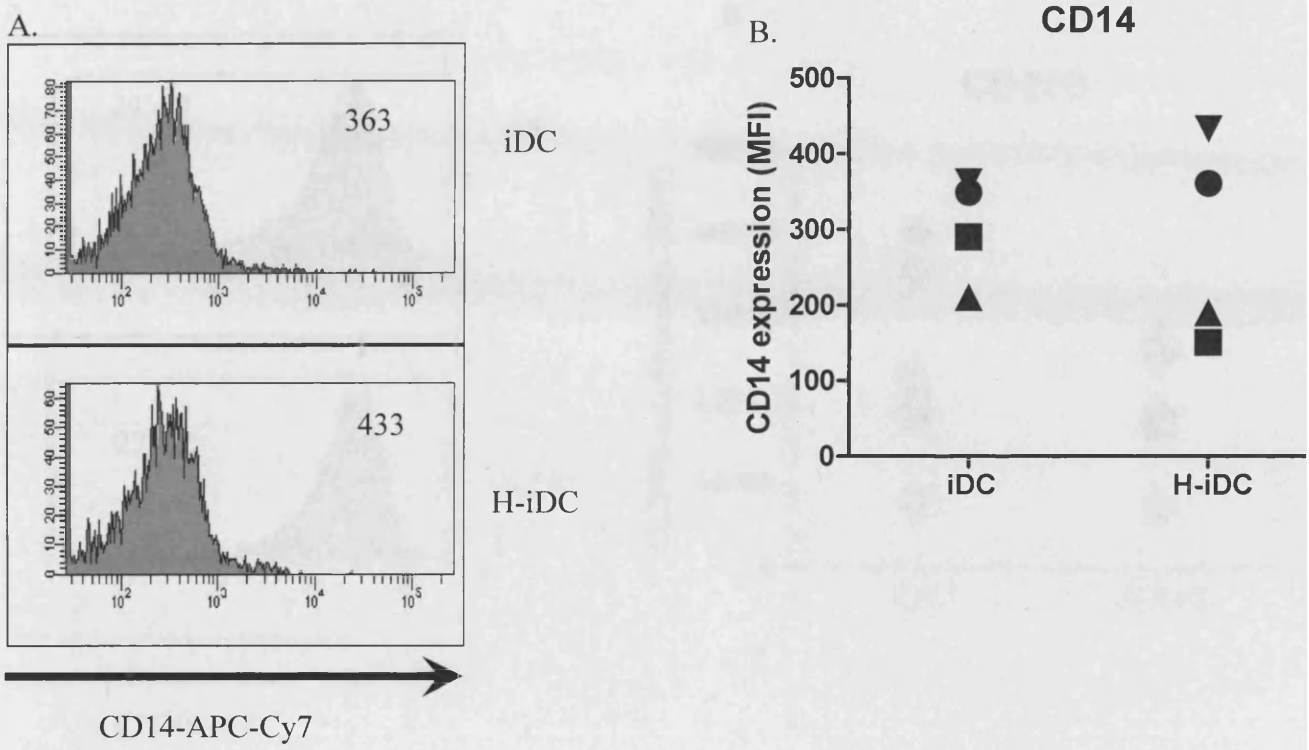


Figure 4.5 HGF Co-Treatment of Monocytes with GM4 Does Not Affect the Down-Regulation of CD14 on MDDC.

A. Expression of CD14 was measured using FACS analysis on iDC (top histogram), and on H-iDC (bottom histogram). Levels of CD14 expressed as mean fluorescence intensity (MFI) are indicated on each histogram.

B. MFI expression of CD14 on paired iDC and H-iDC samples from 4 donors connected by lines. There was no significant difference between the samples (n=4) using a paired T test $p>0.05$.

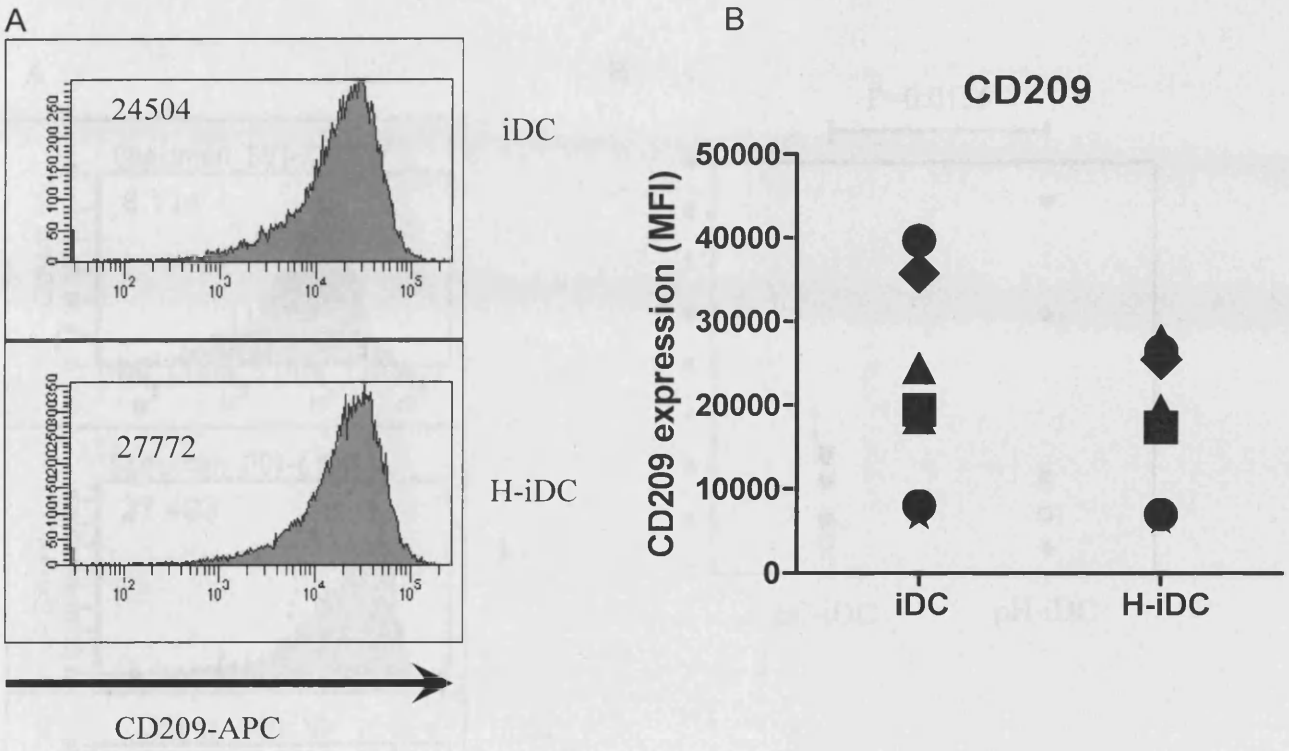


Figure 4.6 Expression of DC Marker CD209 is Not Affected by HGF Co-Treatment with GM4 of Monocytes.

A. Expression of CD209 was measured using FACS analysis on iDC (top histogram), and on H-iDC (bottom histogram). Levels of CD209 expressed as MFI are indicated on the histograms.

B. MFI of CD209 expression of paired iDC and H-iDC samples from 7 donors connected by lines, There was no significant difference between the treatment groups (n=7), using a paired T test p=0.202.

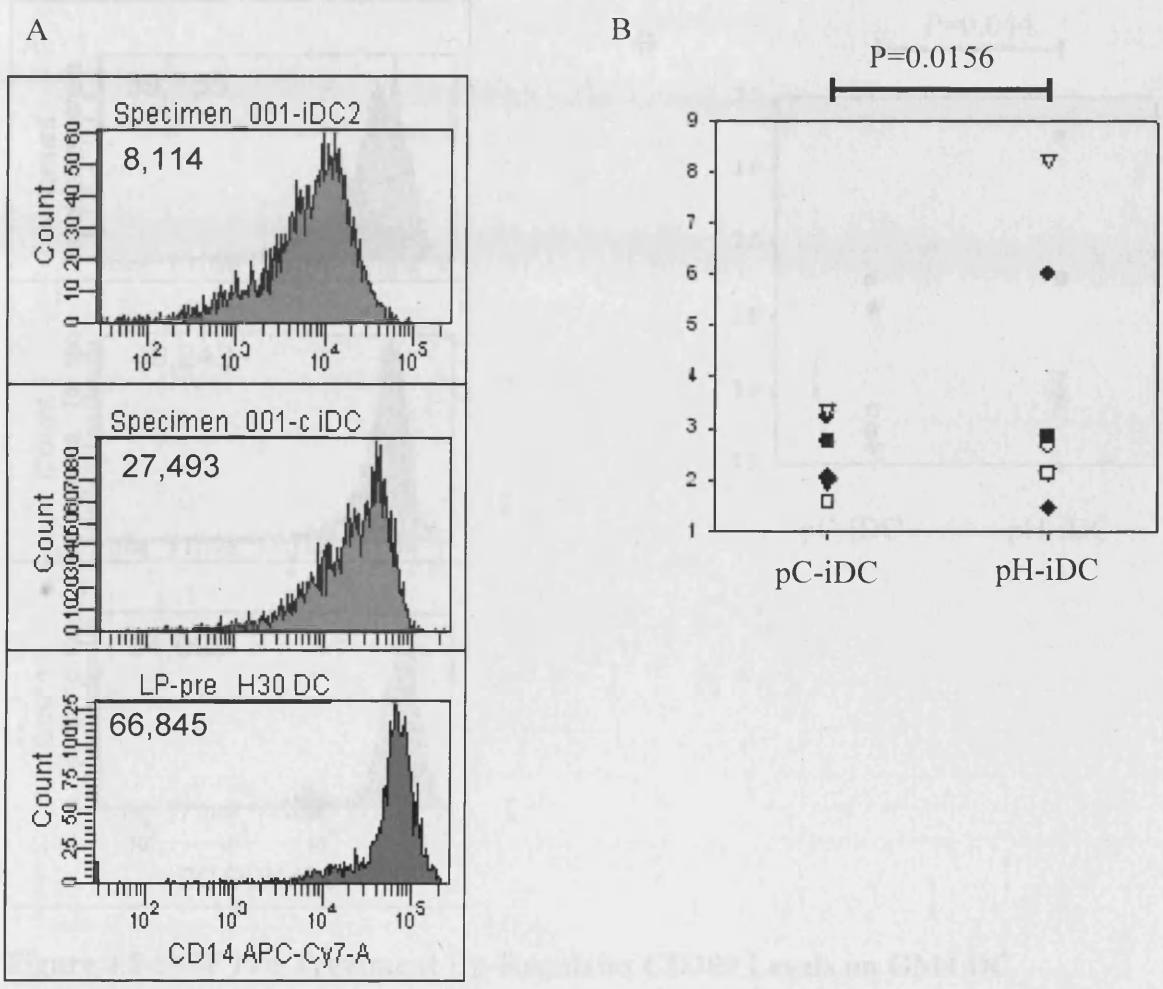


Figure 4.7 HGF Pre-Treatment Up-Regulates CD14 Levels on DC

A. Expression of CD14 was measured using FACS analysis on iDC (top histogram), control DC (pC-iDC) (middle histogram) and on HGF pre-treated DC (pH-iDC, bottom histogram). Levels of CD14 expressed as MFI are indicated on each histogram.

B. Relative expression of CD14 as a factor of expression on iDC, on paired pC-iDC and pH-iDC samples from 7 donors connected by lines. As indicated by the capped line, the difference between CD14 expression by the two groups of DC ($p=0.0156$), was significant using a paired T test. Up-regulation of CD14 on pre-treated cells was also observed in other experiments, where CD14 and CD209 were used to check development/maturation state of DC.

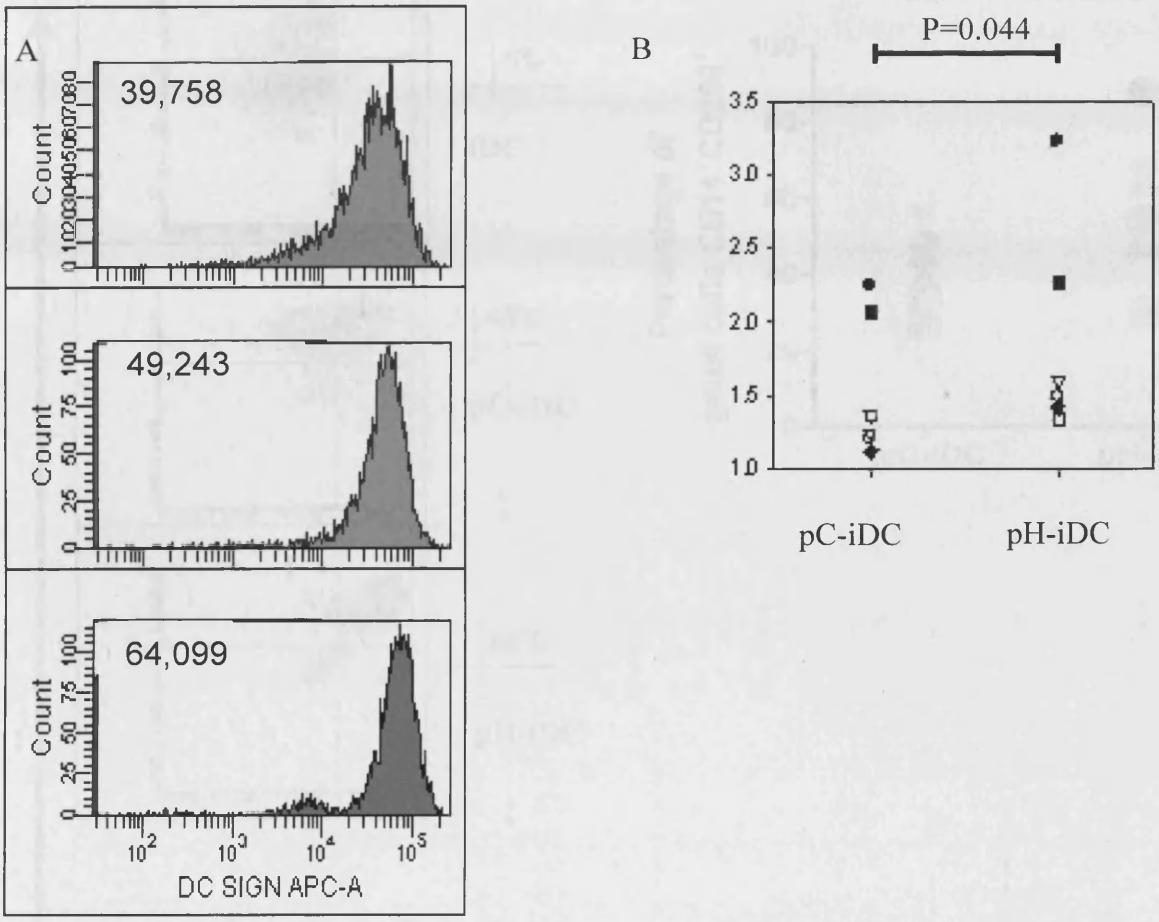


Figure 4.8 HGF Pre-Treatment Up-Regulates CD209 Levels on GM4 DC

A. Levels of CD209 were measured on day 5 iDC (top histogram), pC-iDC (middle histogram), pH-iDC (bottom histogram) are expressed as MFI and are indicated at the top of each histogram.

B. MFI of CD209 expression on paired pC-iDC and pH-iDC samples from 7 donors connected by lines. The difference between CD209 expression by control and HGF pre-treated groups was significant p=0.0156, using a paired T test, and is indicated by a capped line.Like CD14 up-regulation , increased levels of CD209 on pre-treated cells was also observed in other experiments, where CD14 and CD209 were used to check development/maturation state of DC.

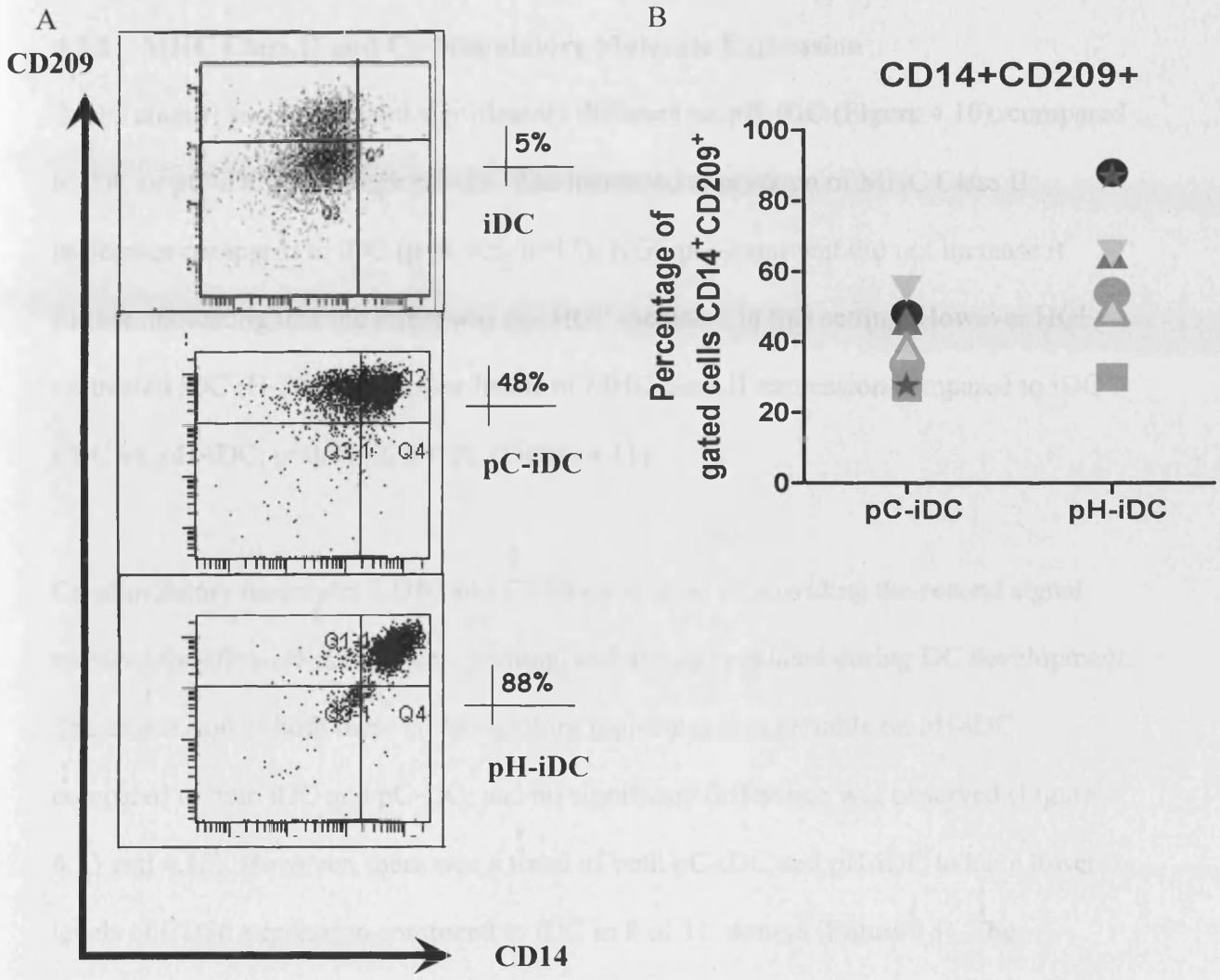


Figure 4.9 HGF Pre-Treatment Increases the Proportion of CD209+CD14+ Double Positive Cells

A. DC differentiated from monocytes (iDC), untreated monocytes (pC-iDC) after 24 h hours or from 30 ng/ml HGF pre-treated monocytes (pH-iDC) were labelled with CD14 (APC-Cy7) and CD209 (APC) specific antibodies and analysed by FACS. Percentages of $CD209^{high}CD14^{high}$ DC are indicated next to each dot-plot. A representative experiment is shown.

B. The percentages of $CD209^{+}CD14^{+}$ cells in paired pC-iDC and pH-iDC samples from 7 donors are shown, connected by lines. There was as significant difference between the frequency of double positive cells in pC-iDC and pH-iDC (P=0.0209) as indicated by the capped line.

4.3.2 MHC Class II and Co-Stimulatory Molecule Expression

MHC class II levels were not significantly different on pH-iDC (Figure 4.10), compared to iDC or pC-iDC. Although pC-iDC had increased expression of MHC Class II molecules compared to iDC ($p=0.005$, $n=15$), HGF pre-treatment did not increase it further, indicating that the effect was not HGF mediated in this setting. However HGF co-treated iDC (H-iDC) had lower levels of MHC class II expression compared to iDC (iDC vs. pH-iDC, $p=0.0042$, $n=10$), (Figure 4.11).

Co-stimulatory molecules CD80 and CD86 are critical in providing the second signal required for effective naïve T cell priming, and are up-regulated during DC development. The expression of both these co-stimulatory molecules was variable on pH-iDC compared to both iDC and pC-DC, and no significant difference was observed (Figure 4.13 and 4.15). However, there was a trend of both pC-iDC and pH-iDC to have lower levels of CD86 expression compared to iDC in 8 of 11 donors (Figure 13). The expression of CD80 expression was widely variable and no trend was observed, compared to either iDC or pC-iDC (Figure 4.15). However, in the co-treatment model H-iDC expressed significantly lower levels of CD86 compared to iDC ($p=0.0048$, $n=8$) (Figure 4.12). H-iDC also expressed significantly lower levels of CD80 compared to iDC ($p=0.0216$, $n=9$) (Figure 4.14).

4.3.3 PDL-1 Expression is Up-Regulated by HGF Pre-Treatment

Programmed death-1 (PD-1) molecule is expressed on T cells and its ligand PD-L1 is expressed on T cells and APC. The interaction between these two molecules can control T cells responses by inducing cell death or unresponsiveness of T cells as discussed in the introduction. HGF pre-treatment induced a significant up-regulation of PD-L1 on DC

(Figure 4.16) compared to DC. On pH-iDC the increase in PDL-1 on expression was 2 fold while pC-iDC the PD-L1 expression represents an intermediate level. A similar experiment in the co-treated model was not carried out.

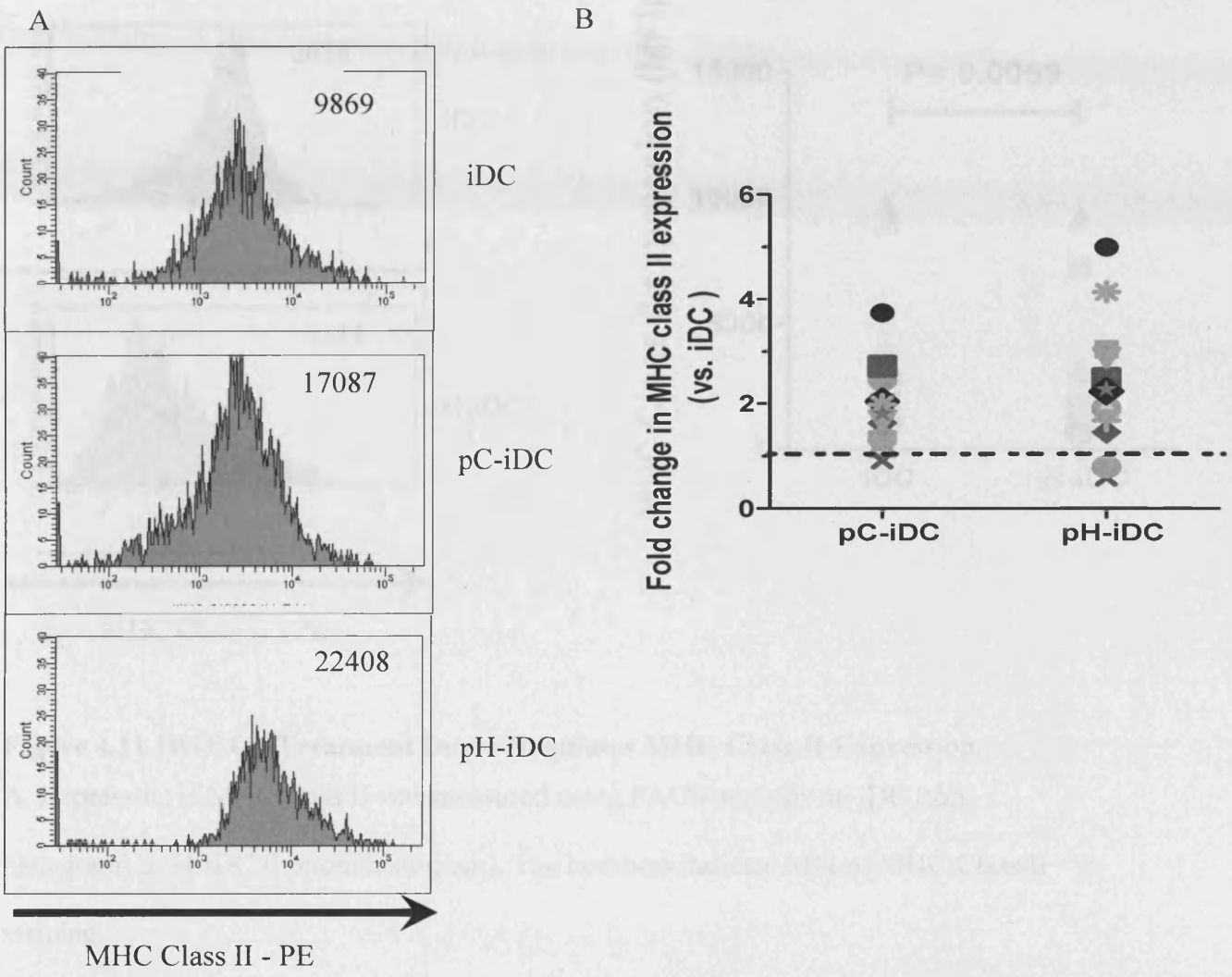


Figure 4.10 HGF pre-treated cells do not express altered levels of MHC Class II

A. The histograms show the levels of MHC Class II surface expression on iDC (top histogram), pC-iDC (middle histogram) and pH-iDC (bottom histogram). MFI are indicated at the top of each histogram.

B. The fold increases of MHC class II levels on pC-iDC and pH-iDC compared to that on iDC are shown. Each line represents an individual donor (n=15). Using a paired T test there was no statistical difference between pC-iDC and pH-iDC, ($p>0.05$ n=15). Dotted line indicates relative MHC Class II expression on iDC.

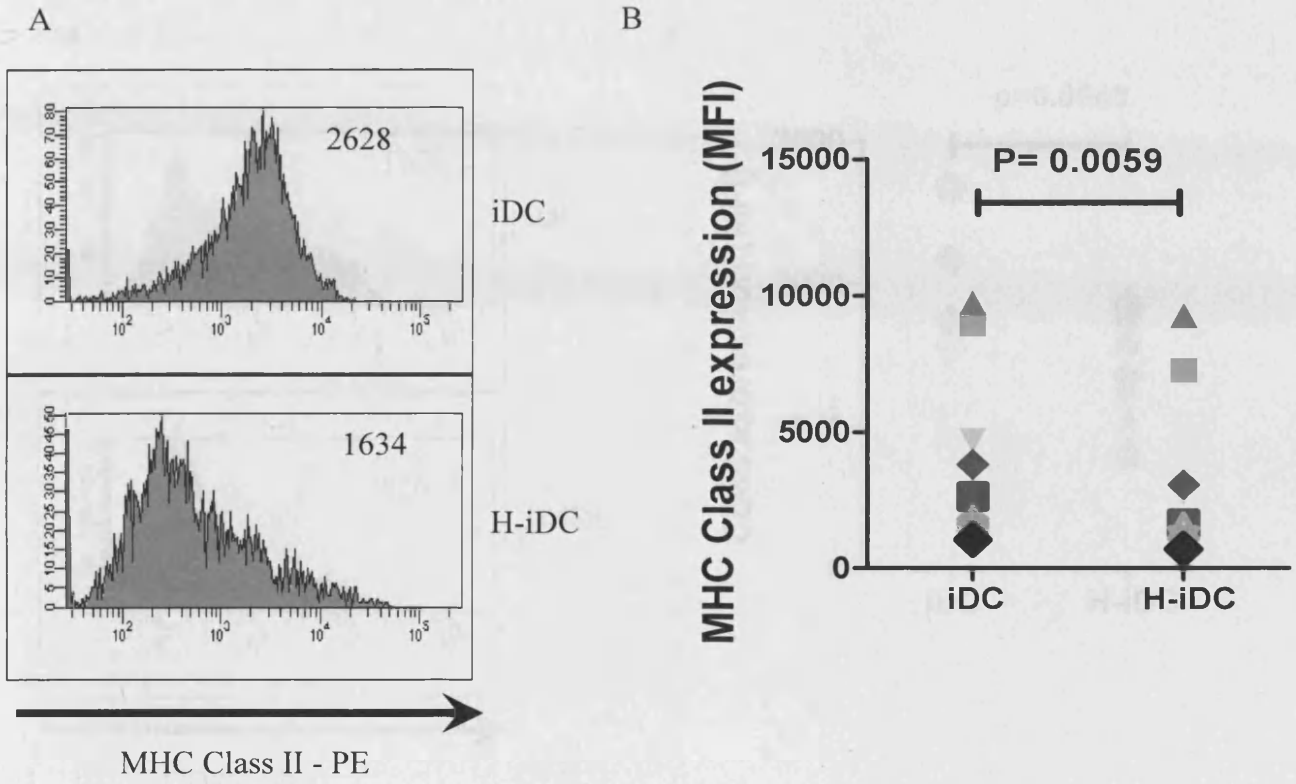


Figure 4.11 HGF Co-Treatment Down-Regulates MHC Class II Expression.

A. Expression if MHC class II was measured using FACS analysis on iDC (top histogram) or H-iDC (bottom histogram). The numbers indicate MFI of MHC Class II staining.

B. MFI of MHC Class II expression on iDC and H-iDC from 10 donors. Paired samples linked by lines. Using a paired T test, the difference between the groups was statistically different ($p=0.0059$, $n=8$).

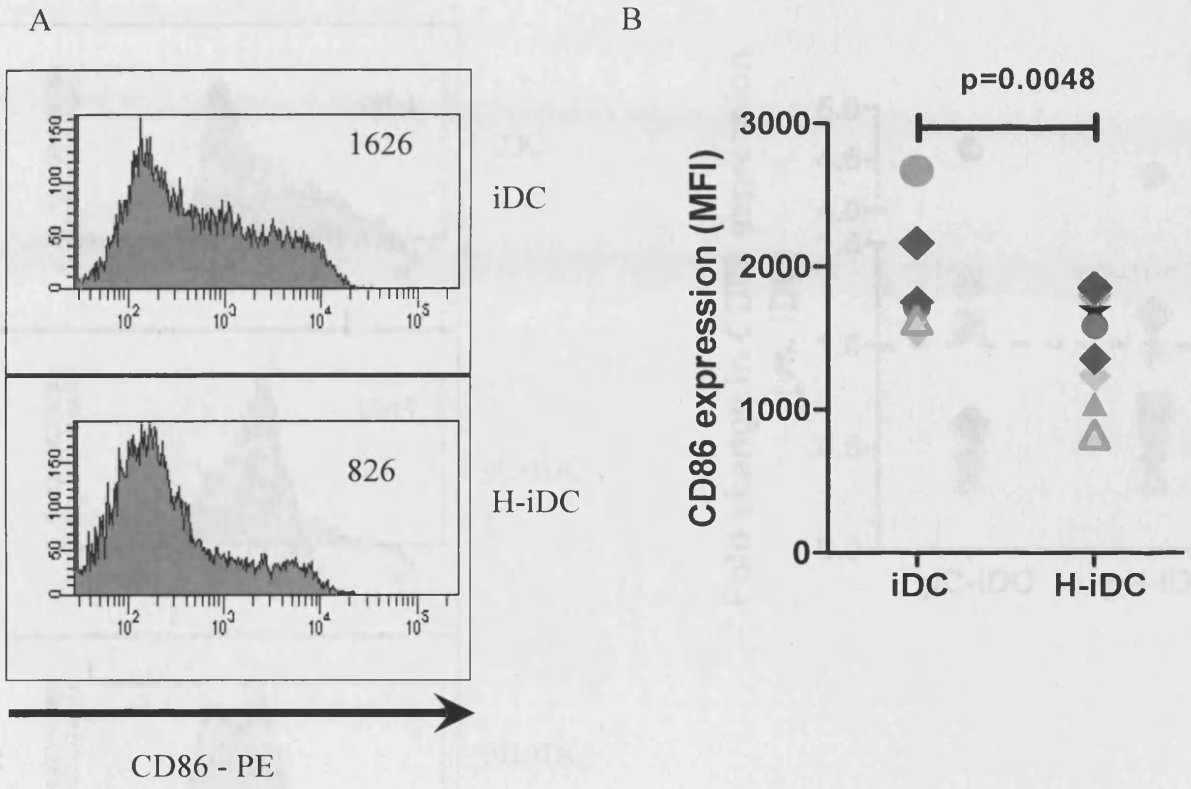


Figure 4.12 HGF Co-Treatment Down-Regulates CD86 Expression.

A. Surface expression of CD86 was measured using FACS analysis on iDC (top histogram) , and H-iDC (bottom histogram). The histograms show a representative experiment, the numbers indicate CD86 expression levels by MFI.

B. The lines show the mean expression of CD86 on iDC and H-iDC of paired samples from 8 different donors. Using a paired T test the difference between CD86 levels on iDC and H-iDC was statistically significant (p=0.0048, n=8)

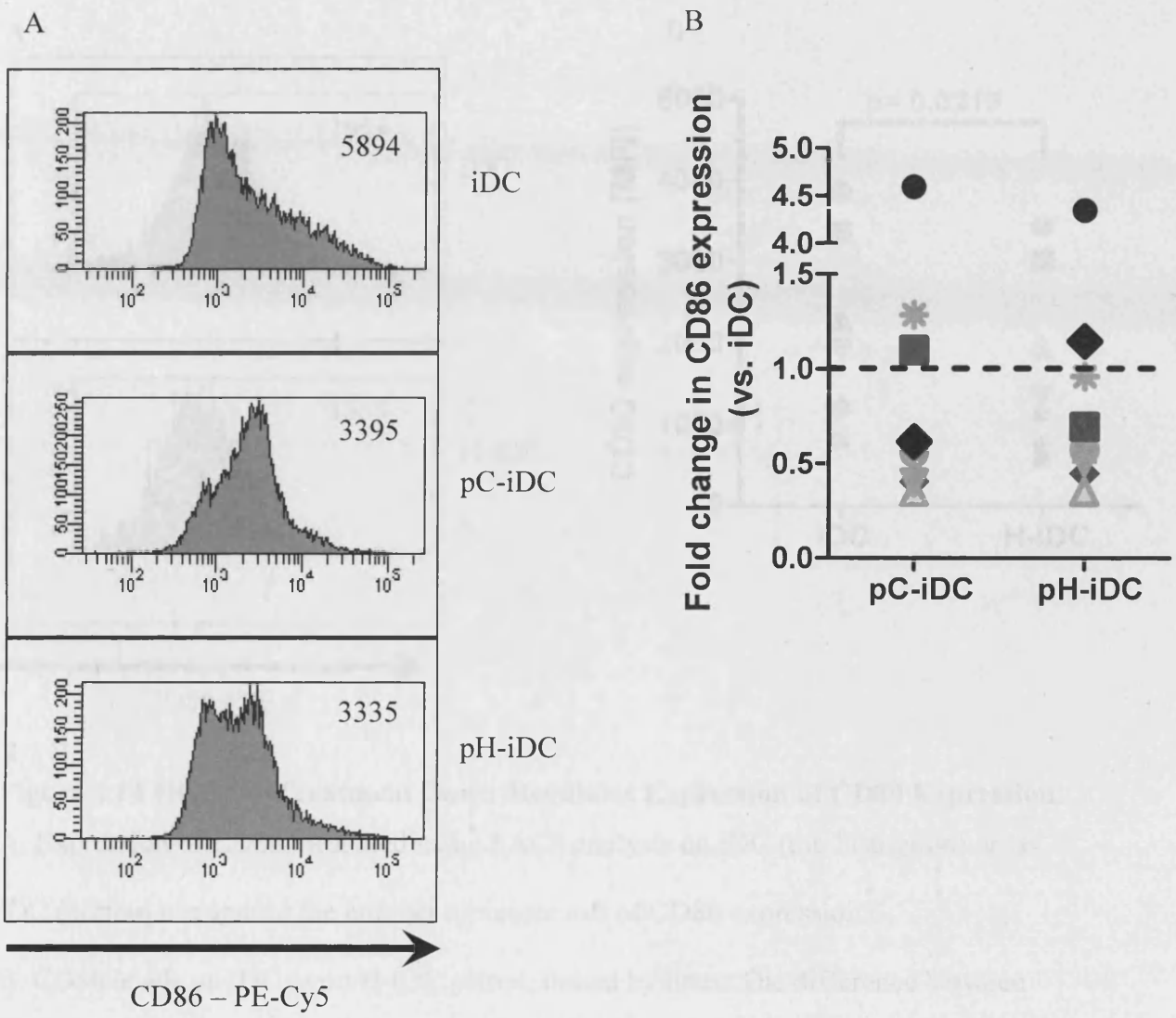


Figure 4.13 HGF Pre-Treatment Does Not Alter Expression of CD86 on immature DC

A. Surface expression of CD86 on iDC (top histogram), pC-iDC (middle histogram) and on pH-iDC (bottom histogram). The histograms show a representative experiment, the numbers indicate CD86 expression levels by MFI.

B. The fold increase of CD86 levels on pC-iDC and pH-iDC compared to iDC. Each line represents a different donor (n=11). There was no significant difference between pH-iDC compared to pC-iDC or to iDC ($p>0.05$, n=11).

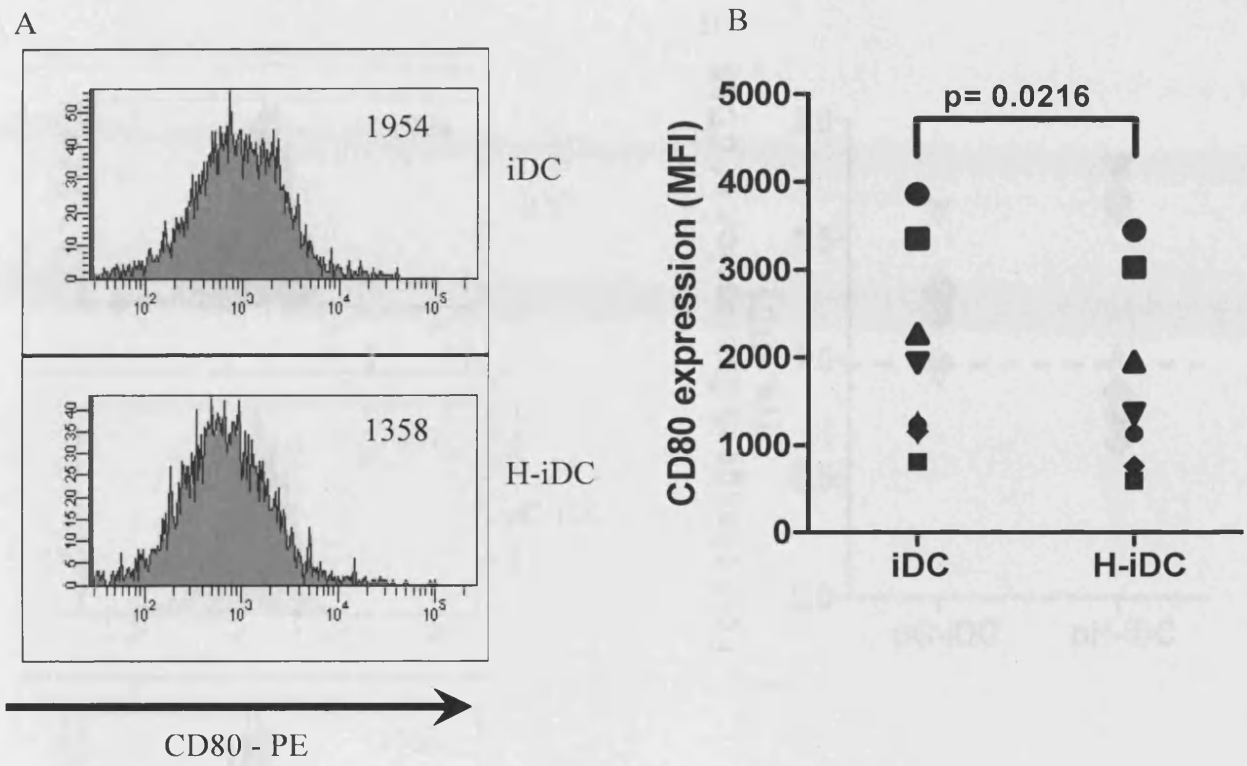


Figure 4.14 HGF Co-Treatment Down-Regulates Expression of CD80 Expression.

A. Expression of CD80 measured using FACS analysis on iDC (top histogram) or H-iDC (bottom histogram) the number represent mfi of CD80 expression.

B. CD80 levels on iDC or on H-iDC paired, linked by lines. The difference between CD80 levels on iDC and H-iDC was statistically different ($p=0.0216$, $n=9$), using a paired T test.

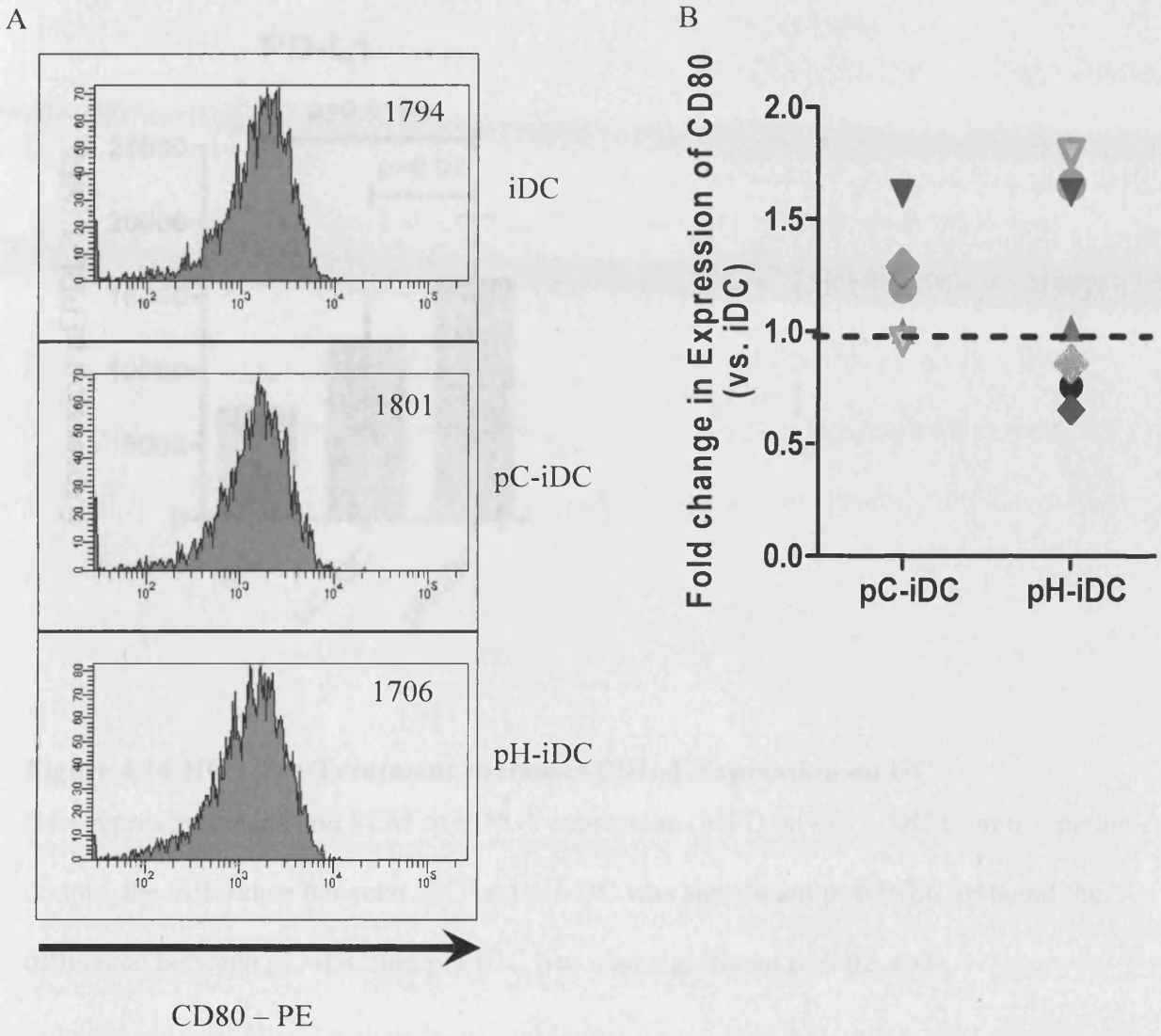


Figure 4.15 HGF Pre-Treatment Does Not Alter CD80 Expression on immature DC

A. CD80 expression was determined on iDC (top histogram), pC-iDC (middle histogram) and pH-iDC (Bottom histogram). Representative histograms showing level of CD80 expression indicated by MFI values.

B. The expression of CD80 on pC-iDC and pH-iDC compared to that on iDC. Paired samples from individual donors are linked by lines. There was no significant difference between the groups, when analysed using a paired T test, ($p>0.05$) n=6.

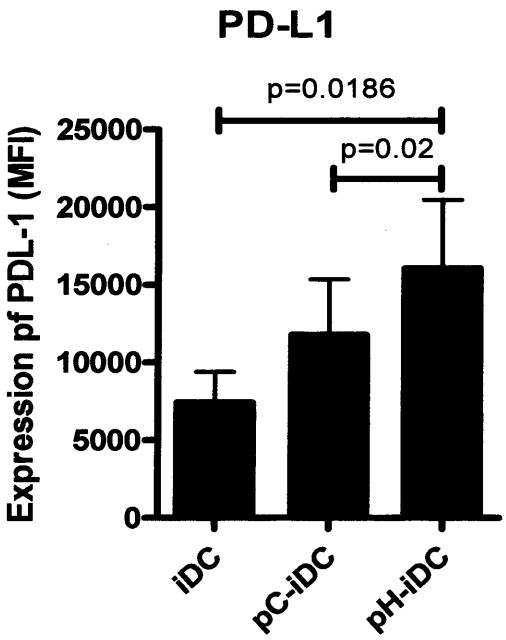


Figure 4.16 HGF Pre-Treatment Increases PDL-1 Expression on DC

Bars represent means and SEM of PDL-1 expression (MFI) on day 5 DC from 6 separate donors, the difference between iDC and pH-DC was significant p=0.0186, n=6 and the difference between pC-iDC and pH-iDC was also significant p=0.02, n=6.

4.4 Phenotypic Characterisation of HGF Pre-Treated DC.

Treatment of monocytes with HGF at the beginning of their differentiation into DC (HGF co-treatment or H-iDC) produced DC with lower expression of MHC Class II, CD80 and CD86 molecules than untreated DC. These changes are expected to have a negative effect on the T cell stimulatory efficiency of HGF-treated DC. However, HGF pre-treatment resulted in the generation of DC-like cells with a hitherto undescribed phenotype, $CD209^{+}CD14^{+}ClassII^{+}CD80^{+}CD86^{+}PD\text{-}L1^{+}$. We decided to focus on this population of DCs, so in the rest of my thesis I shall present the detailed characterisation of DC derived from HGF pre-treated monocytes.

4.4.1 Maturation

In order to confirm that HGF pre-treatment generates cells which, although expressing CD14, still behave more like DC than macrophages, pH-iDC were LPS treated and the phenotypic and functional characteristics of the resulting cells, pH-mDC were studied, according to the diagram on Figure 4.18. LPS treatment is known to activate macrophages resulting in an increase of CD14 and MHC Class II molecules expression, but the level of co-stimulatory molecules or CD83 Is not known. Their phagocytic activity also increases. LPS treatment of iDC up-regulates MHC Class II, CD80, CD86, CD40 and CD83 molecules and down-regulates CD209, CD14 molecules and phagocytic activity. The cells are labelled as mDC in the experiments

HGF pre-treatment:

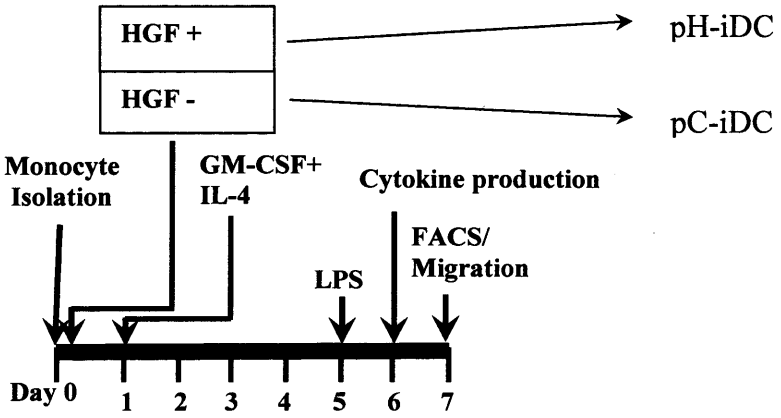


Figure 4.17 Diagram of HGF pre-treatment of matured DC

4.4.2 Up-Regulation of MHC Class II, CD209, CD14, CD40 and Co-Stimulatory Molecules on pH-DC Following LPS Treatment

When comparing mDC with pH-mDC or pC-DC, there was no significant difference between the levels of MHC Class II expression (Figure 4.18, n=5), and MHC Class II was up-regulated in all these groups compared to iDC. This demonstrates that pre-treatment with HGF does not affect the LPS mediated up-regulation of MHC Class II.

Unlike MHC Class II expression, HGF had a significant negative effect on the LPS-mediated up-regulation of CD80 and CD86 molecules (Figures 4.19 and 20). To allow easy comparison between mDC, pC-mDC and pH-mDC, the MFI values were used to calculate fold increases of expression compared to baseline expression on iDC. The inhibition of CD80 up-regulation was HGF dependent, as pC-mDC expressed higher levels of CD80 than pH-mDC. HGF pre-treatment inhibited the up-regulation of CD80 compared to both mDC ($p=0.0352$, $n=4$) and pC-mDC ($p=0.0417$, $n=3$). CD86 up-regulation was also impaired by HGF pre-treatment, compared to both mDC and pC-mDC ($p=0.0244$, $n=4$ and 0.0324, $n=5$ respectively), although it was also inhibited in the

pC-mDC group compared to that on mDC. These results suggest that HGF pre-treatment and delayed DC differentiation (induced by GM4) interact inhibiting CD86 up-regulation by LPS.

Expression of CD83 is only expected to be seen on mature DC and not on monocytes or iDC. Both control mature DC (pC-mDC) and HGF pre-treated mature DC (pH-mDC) had a slightly higher expression of CD83 than mDC but this was not significant (n=4). This indicates that HGF pre-treatment of monocytes generates DC-like cells which, like untreated DC, are able to undergo phenotypic maturation.

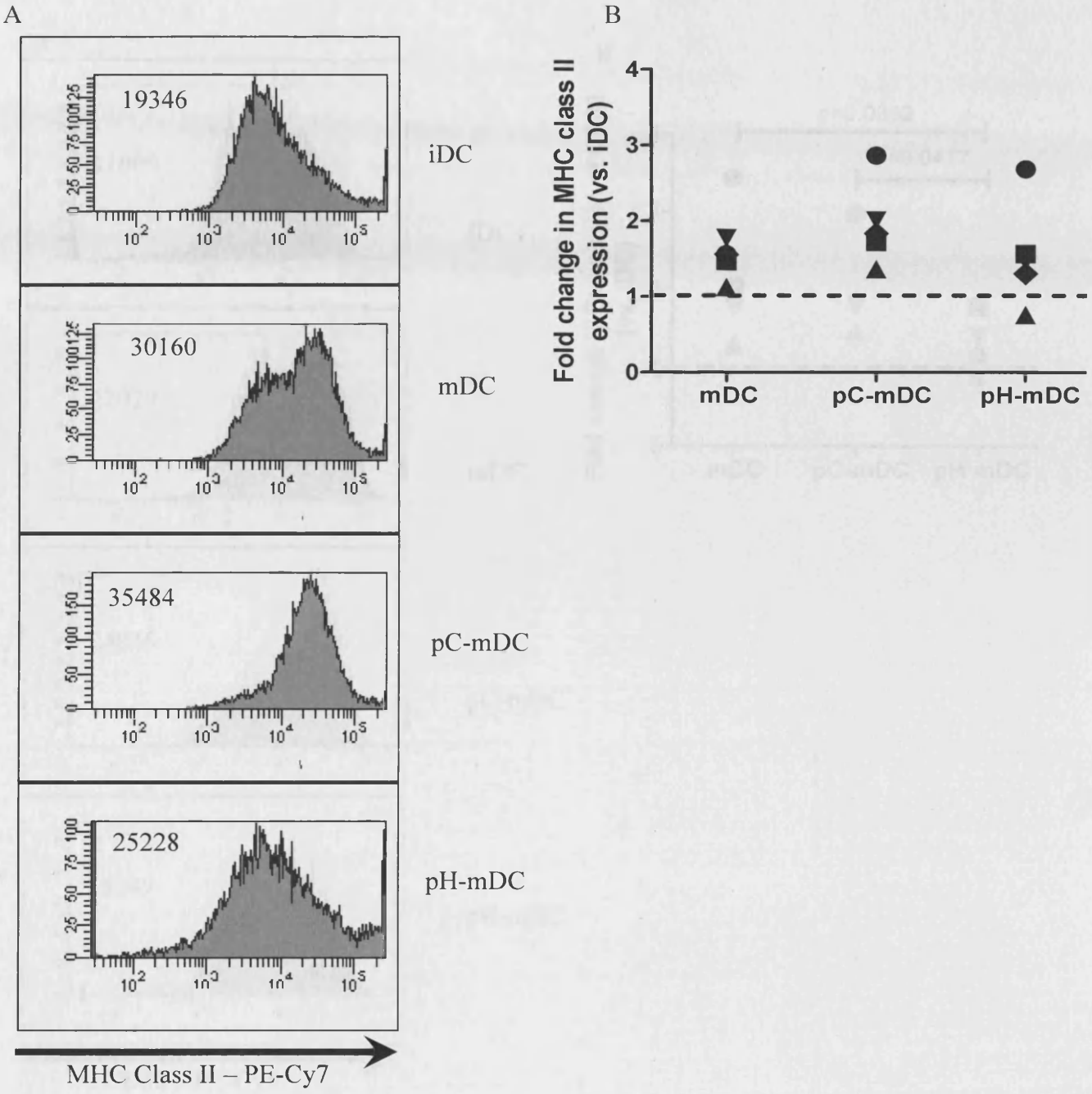


Figure 4.18 HGF Pre-Treatment Did Not Affect MHC Class II Expression on DC Following LPS Treatment.

A. Histograms show MFI of MHC Class II expression, in a representative experiment.

B. The fold increase of MHC Class II expression (MFI) compared to iDC is shown. Each line represents a different donor (n=5). Using a paired T test there was no significant difference between the groups.

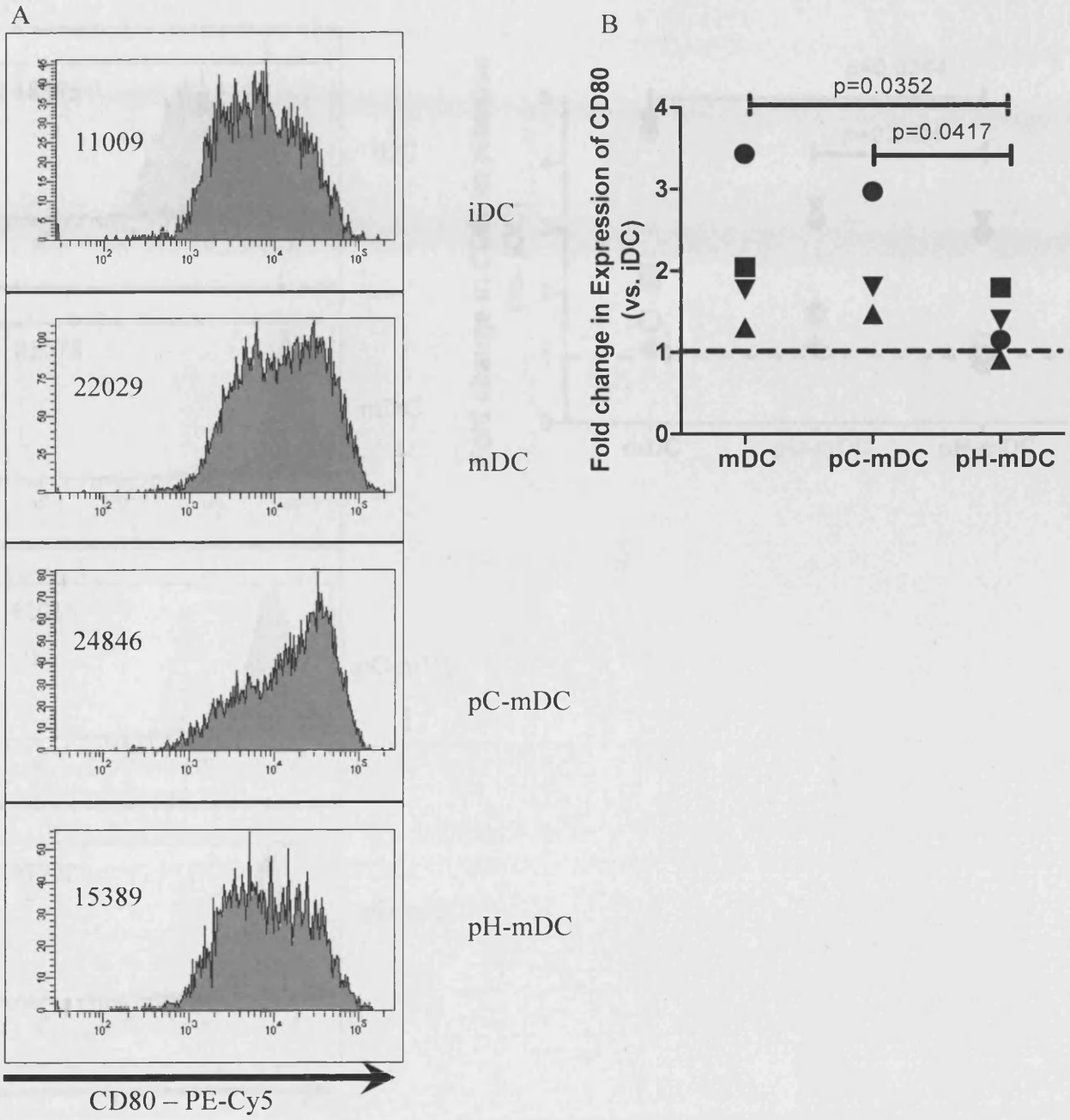


Figure 4.19 HGF Pre-Treatment Resulted in Decreased CD80 Up-Regulation Following LPS Treatment.

A. Histograms show the MFI expression of CD80 on representative samples of 4 donors, numbers represent CD80 expression.

B. The fold increase of MFI of CD80 compared to iDC. Each line represents a different donor (n=4), capped lines indicate significant differences between treatments. The significance of differences between the pairs was assessed and are indicated by capped lines using paired T tests (mDC vs. pH-mDC p=0.0352, n=4, pC-mDC vs. pH-mDC, p=0.0417, n=3).

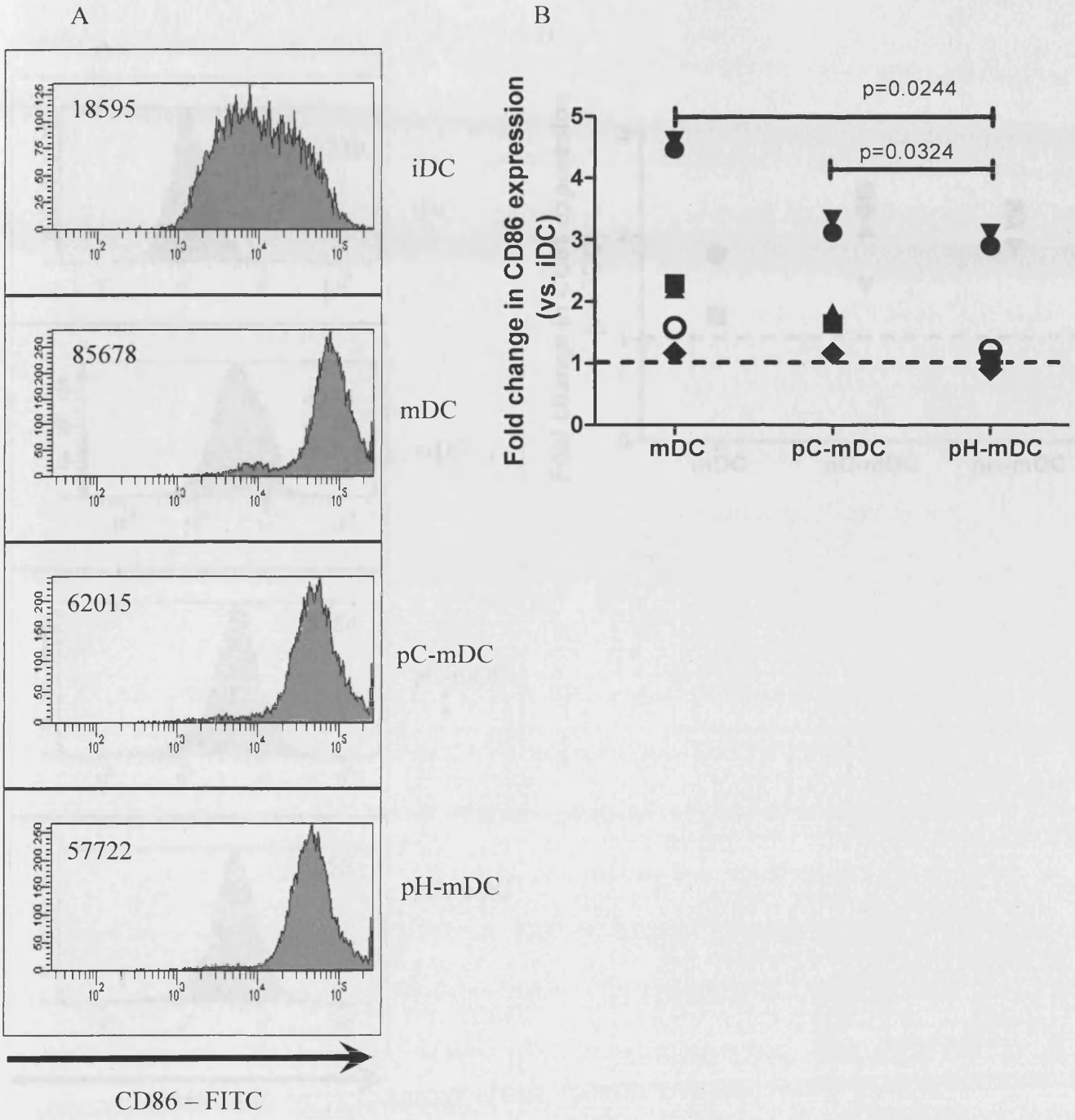


Figure 4.20 HGF Pre-Treatment Resulted in Decreased CD86 Expression Following LPS Treatment.

A. Histograms show the MFI of CD86 expression in a representative experiment of 6 donors, on groups of DC as indicated.

B. The fold increase of MFI of CD86 compared to that on iDC is shown. Each line represents a different donor (n=6), capped lines indicate significant differences between treatments, using paired T tests (mDC vs. pH-mDC p=0.0324, n=5, mDC vs. pH-mDC, p=0.0417, n=6, mDC vs. pC-mDC p=0.0472 n=5).

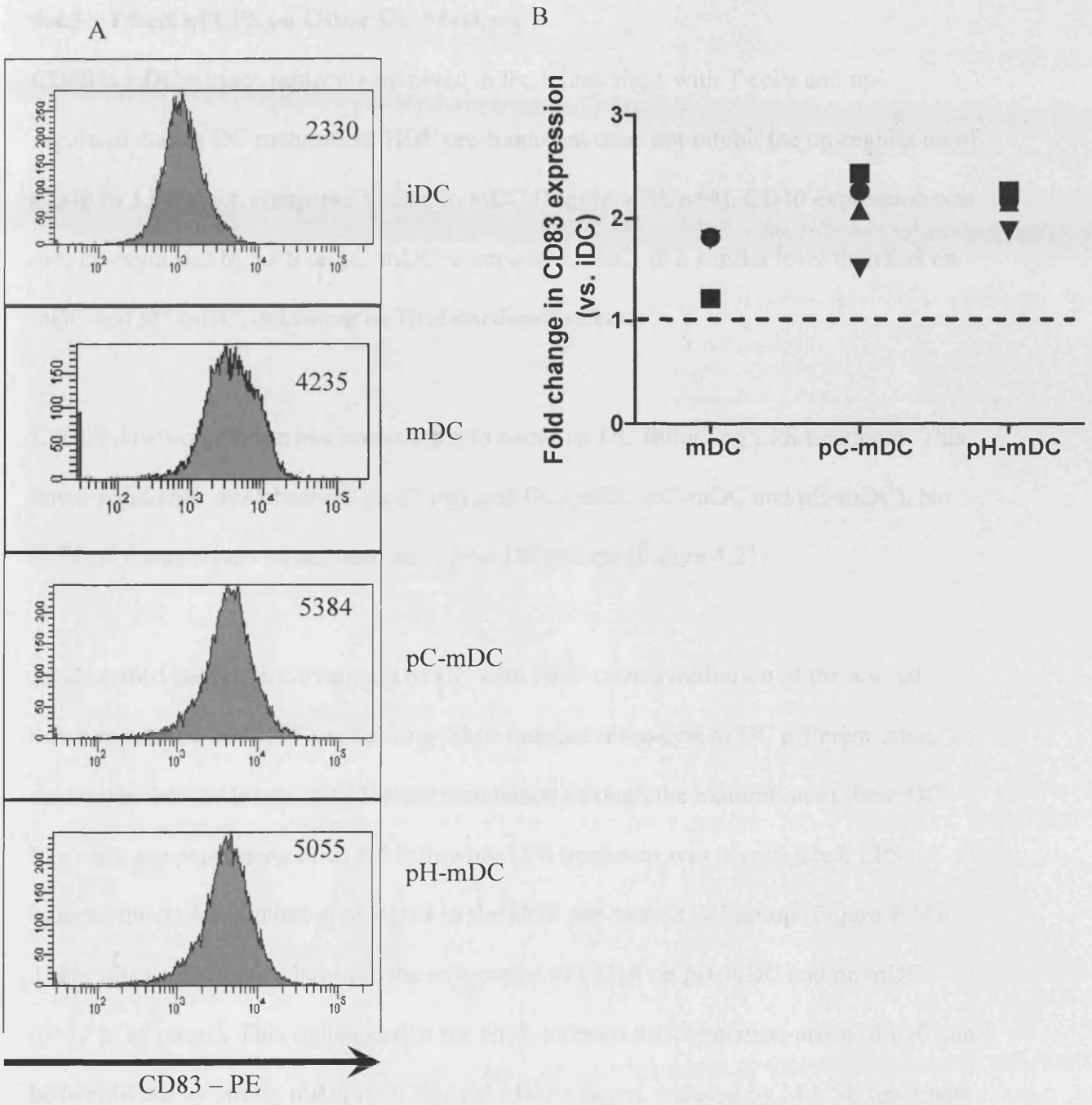


Figure 4.21 HGF Pre-Treatment Did Not Affect CD83 Expression Following LPS Treatment.

A. Histograms show the MFI of CD83 expression in a representative experiment on groups of DC as indicated.

B. Fold increase of MFI of CD83 compared to iDC is shown. Each line represents a different donor (n=4). There were no significant differences between groups using paired t tests $p>0.05$, n=4.

4.4.3 Effect of LPS on Other DC Markers

CD40 is a DC surface molecule involved in the interactions with T cells and up-regulated during DC maturation. HGF pre-treatment does not inhibit the up-regulation of CD40 by LPS when compared to that on mDC (Figure 4.22, n=4). CD40 expression was also up-regulated by LPS on pC-mDC, compared to iDC, to a similar level than that on mDC and pH-mDC, indicating no HGF-mediated effect.

CD209 down-regulation has been shown to occur on DC following LPS treatment. This down-regulation was observed on all matured DC (mDC, pC-mDC and pH-mDC). No difference was observed between any of the DC groups (Figure 4.23).

As described earlier, pre-treatment of DC with HGF causes inhibition of the normal down-regulation of CD14 seen during GM4-induced monocyte to DC differentiation. To assess whether the levels of CD14 are maintained through the maturation of these DC-like cells, the expression of CD14 following LPS treatment was investigated. LPS induced the down-regulation of CD14 in the HGF pre-treated DC group (Figure 4.24). There was no difference between the expression of CD14 on pH-mDC and on mDC (n=12 in all cases). This indicates that the HGF-induced differentiation-arrest of iDC can be overdriven by strong maturation signals. Macrophages, induced by M-CSF treatment of monocytes showed no change in the levels of CD14 when treated with LPS (n=4) (Figure 4.24).

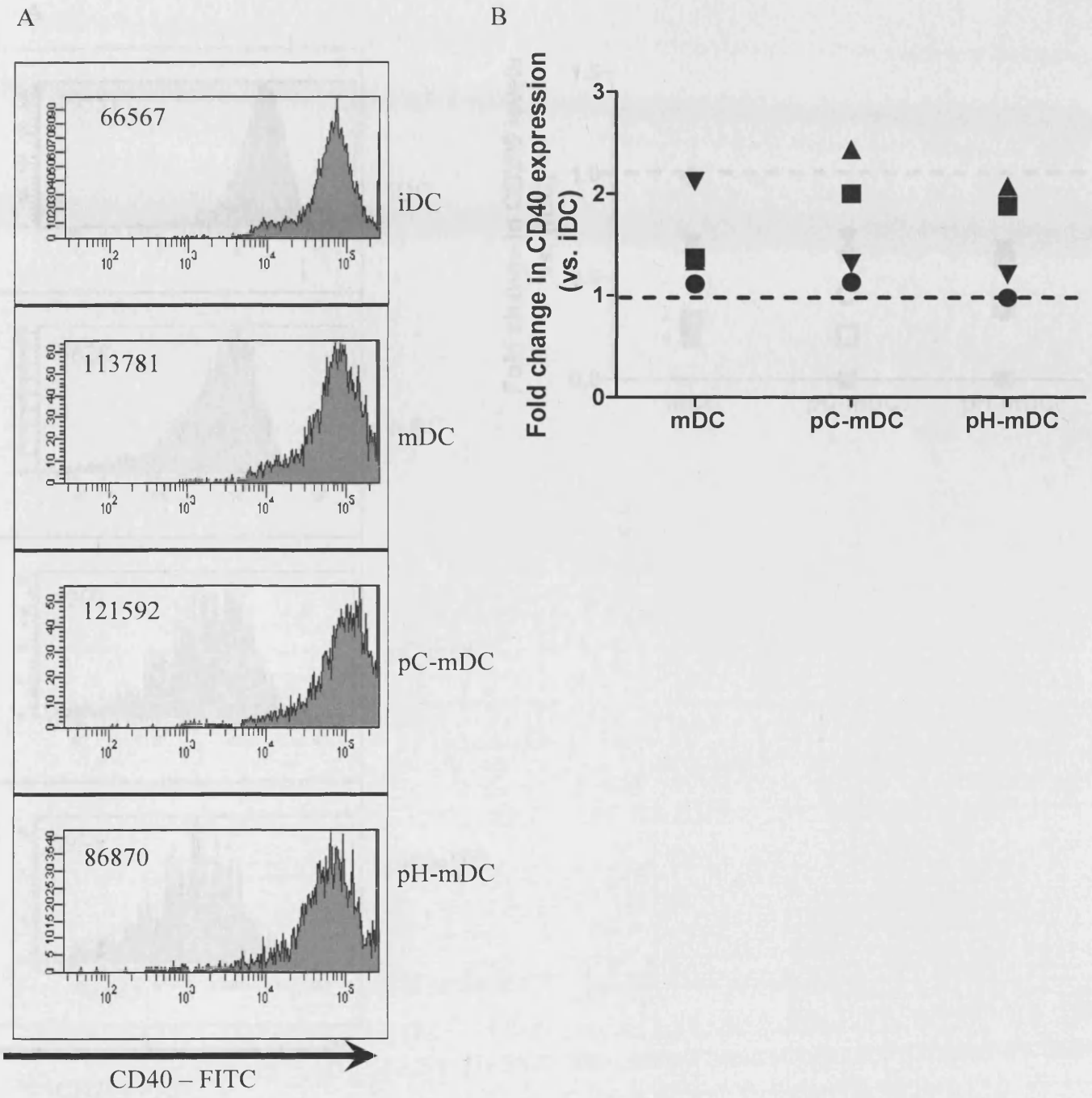


Figure 4.22 Up-Regulation of CD40 on HGF Pre-Treated DC by LPS Treatment.

A. Histograms show the MFI of CD40 expression in a representative experiment on group of DC, as indicated.

B. The increase (fold) of MFI of CD40 relative to iDC is shown. Each line represents a different donor. There was no significant difference between CD40 levels on DC in the three different groups, using a paired t tests (n=4).

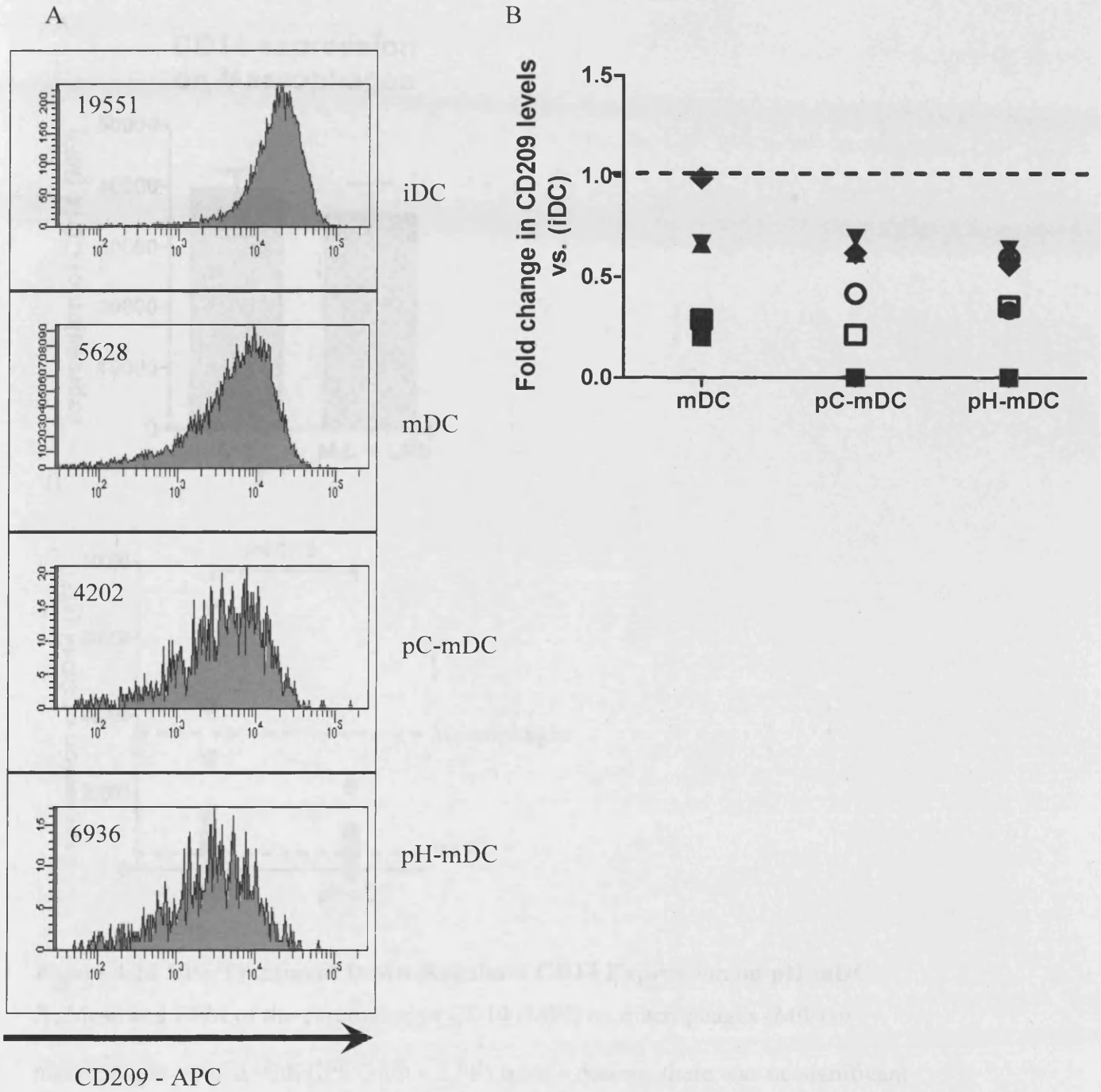
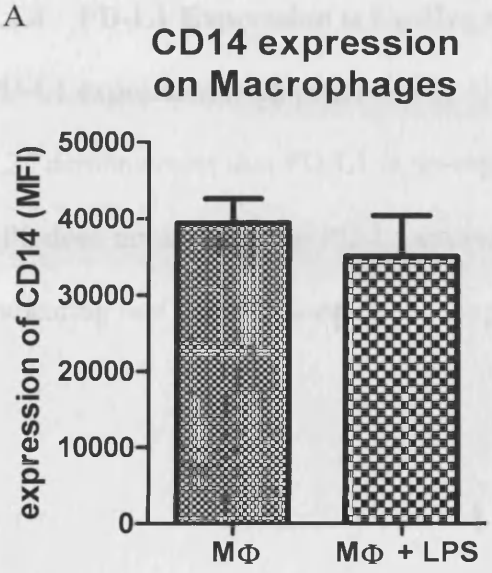


Figure 4.23 HGF Pre-Treatment Does Not Effect LPS Down-Regulation of CD209

A. Histograms show the MFI of CD209 expression in a representative experiment, on groups of DC as indicated.

B. The fold increase of MFI of CD209 relative to iDC. Each line represents a different donor (n=7). There was no significant difference between CD209 levels on DC in the three groups, using paired t tests $p>0.05$.

A



B

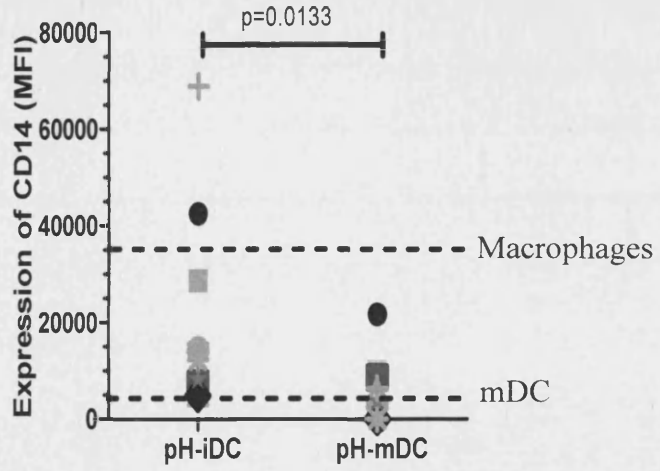


Figure 4.24 LPS Treatment Down-Regulates CD14 Expression on pH-mDC.

B

A. Mean and SEM of the expression of CD14 (MFI) on macrophages (MΦ) or macrophages treated with LPS (MΦ + LPS) from 4 donors; there was no significant difference between LPS treated or untreated samples using an unpaired t-test.

B. Paired samples of pH-iDC and pH-mDC, each line represents an individual donor (n=12). The dotted lines indicate the average levels of CD14 on macrophages (M-CSF treated monocytes for 5 days +2 days LPS treatment) and CD14 expression levels on mDC. The capped line indicates a significant difference between CD14 levels on pH-iDC vs. pH-mDC samples using a paired T-test p=0.0133, n=12.

4.4.4 PD-L1 Expression is Up-Regulated by HGF Pre-Treatment

PD-L1 expression is up-regulated by HGF pre-treatment as mentioned earlier. Figure 4.25 demonstrates that PD-L1 is up-regulated on pH-DC by LPS treatment. Interestingly LPS does not up-regulate PD-L1 expression on pC-mDC compared to that on DC, indicating that the increased PD-L1 expression is due to HGF pre-treatment.

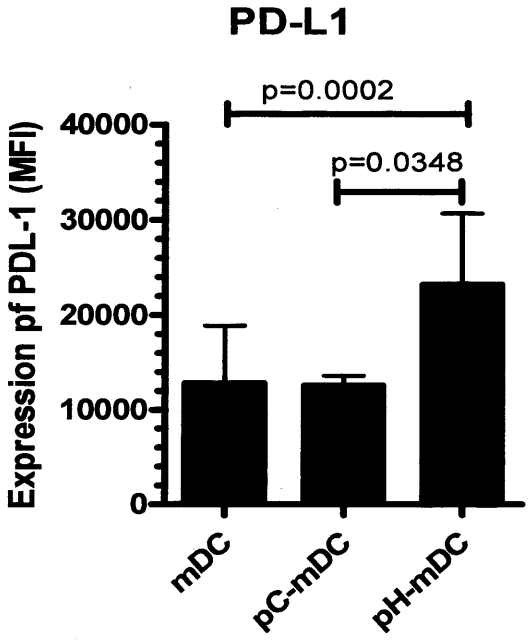


Figure 4.25 HGF Pre-Treatment Increases PDL-1 Expression on DC by LPS

Bars represent the means and SEM of PDL-1 expression (MFI) after DC have been treated with LPS for 48h, n=8. The difference between mDC and pH-mDC was significant p=0.0248 also between pC-mDC and pH-mDC p=0.0348, n=8, using paired t tests.

4.5 Functional Characterisation of HGF Pre-Treated DC

4.5.1 Phagocytosis

The uptake of antigen via the process of phagocytosis is a characteristic of immature DC. To investigate whether HGF modulates the phagocytic ability of DC, FITC conjugated latex beads were incubated with pH-iDC and control. Background levels of antigen uptake, caused by non-specific binding of beads to cell surface receptors but not being internalised by DC, was measured by incubating DC with FITC-beads at 4°C. Figure 4.26 shows that there was no difference in the level of the FITC-bead uptake by pH-iDC compared to controls. The results were reproducible in 3 separate donors.

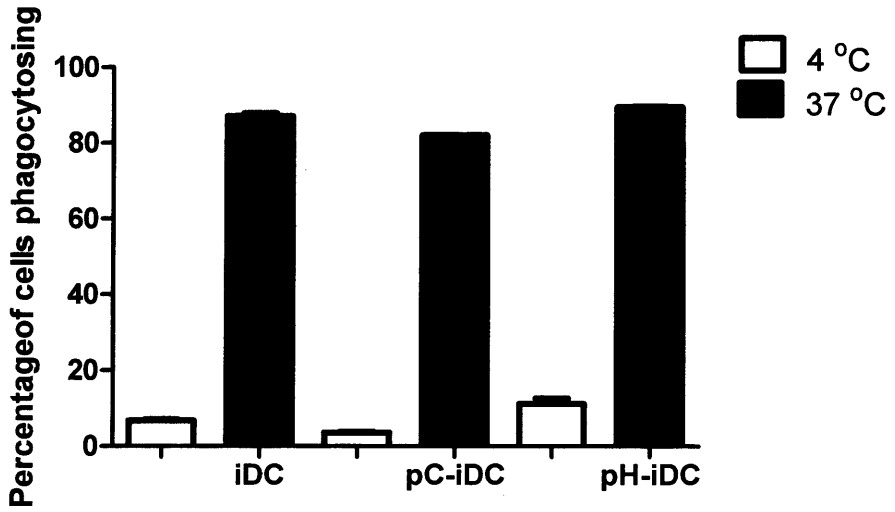


Figure 4.26 Antigen Uptake is Unaffected by HGF Pre-Treatment of DC

Uptake of FITC conjugated latex beads by iDC, pC-iDC and pH-iDC, expressed as percentage of cells that become FITC positive. The results are means of duplicate samples, the bars represent the SEM. The black bars represent FITC-latex bead uptake at 37°C. White bars are the background level of FITC-latex bead binding to DC cell surface at 4°C. Representative of 3 experiments.

4.5.2 Migratory Capacity

The migration of DC towards secondary lymphoid organs is a key function of DC. This enables DC after picking up antigen in the peripheral tissues to move to lymph nodes to interact with T and B cells. pH-mDC were assessed for their ability to migrate towards the lower chamber of a transwell migration chamber, containing CCL19, following LPS-stimulation (Figure 4.27). Results were determined as means (4 counts per well) of migrated cells as percentages of input DC numbers. As immature DC do not migrate towards CCL19, they were used as a negative control.

pH-mDC were less able to migrate towards lymphoid chemokine CCL19 when compared either to mDC or pC-mDC. 31.7% ± 7.7% pH-mDC migrated, as opposed to 62.7% ± 1.6% of mDC. This decreased migratory capacity could be due to down-regulation of the CCL19 receptor CCR7; however there was no significant change in CCR7 levels between mDC and pH-mDC (Figure 4.28). Alternatively, increased cell-cell or cell-plastic adherence may be responsible for the impaired migration; however, these possibilities were not investigated further. It is also possible that HGF affects DC by affecting altering DC responses to or production of other migratory chemokines.

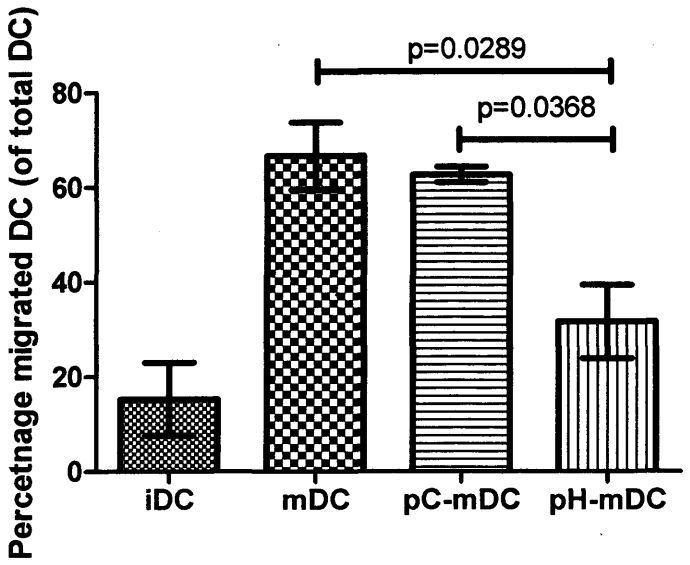


Figure 4.27 HGF Pre-Treatment Inhibits the Migration of mature DC

Percentage of DC that migrated towards CCL19. The columns represent the mean of duplicate or triplicate wells from 4 donors (each value calculated from the mean of 4 separate counts). The bars show the SEM. The capped lines indicate the significant differences between pH-mDC and mDC, p=0.0289 n=4, and pH-mDC and pc-mDC p=0.0368, n=4, using a paired t tests.

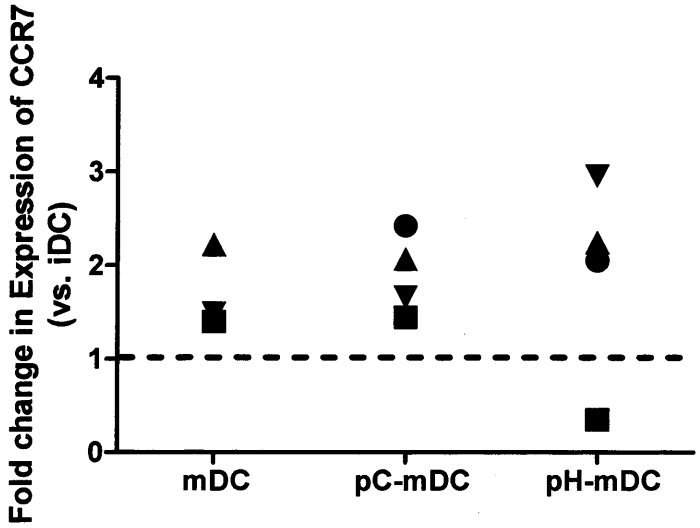


Figure 4.28 HGF Pre-Treatment Does Not Alter the Expression of CCR7, Induced by LPS

Fold increases of CCR7 expression (MFI) compared to that on iDC. Each line represents a different donor (n=4). The donors for these experiments are the same as those used in the migration experiments.

4.5.3 Cytokine Production

Il-12 was chosen as a representative T_H1 cytokine as is the most important cytokine in development of anti-tumour responses. While IL-10 was chosen as a representative T_H2 cytokine as this cytokine as been shown affect the immune responses even in the presence of IL-12, and also is the accepted as the a major cytokine involved in immune suppression. HGF has been shown to up-regulate IL-10 production in monocytes (Rutella *et al.* 2006) but the impact of GM-CSF/IL-4-treatment on HGF-induced IL-10 production has not been investigated. HGF pre-treatment resulted in elevated levels of soluble IL-10 in the supernatant of monocytes 24 h after HGF-treatment (Figure 4.29). HGF-induced IL-10 levels remained higher during 5 days of culture and following LPS-treatment, compared to that produced by iDC or pC-iDC. These results suggest that increased IL-10 production is triggered by HGF in monocytes and it is maintained and further amplified even after GM-CSF/IL-4 treatment.

During maturation, DC secrete cytokines, in particular IL-12 and IL-10, that determine T cell activation towards T_H1 or T_H2 type responses. DC differentiated using GM4 secrete cytokines that induce a strong type 1 T cell response during LPS mediated maturation, and secrete high levels of IL-12. Therefore, LPS stimulation of DC is a good *in vitro* model for studying the effect of HGF on DC cytokine release.

HGF pre-treatment did not alter LPS induced IL-12 production compared to that by either mDC or pC-mDC. However pH-mDC produced 2 fold more IL-10 compared to mDC (Figure 4.30 and 4.31), $p=0.0446$, $n=9$. HGF pre-treatment significantly increased the IL-10/IL-12 ratio, when compared to pC-mDC and mDC ($p=0.0370$ $n=6$, $p=0.0263$ $n=6$, respectively), indicating that the shift in cytokine production towards IL-10 is HGF dependent.

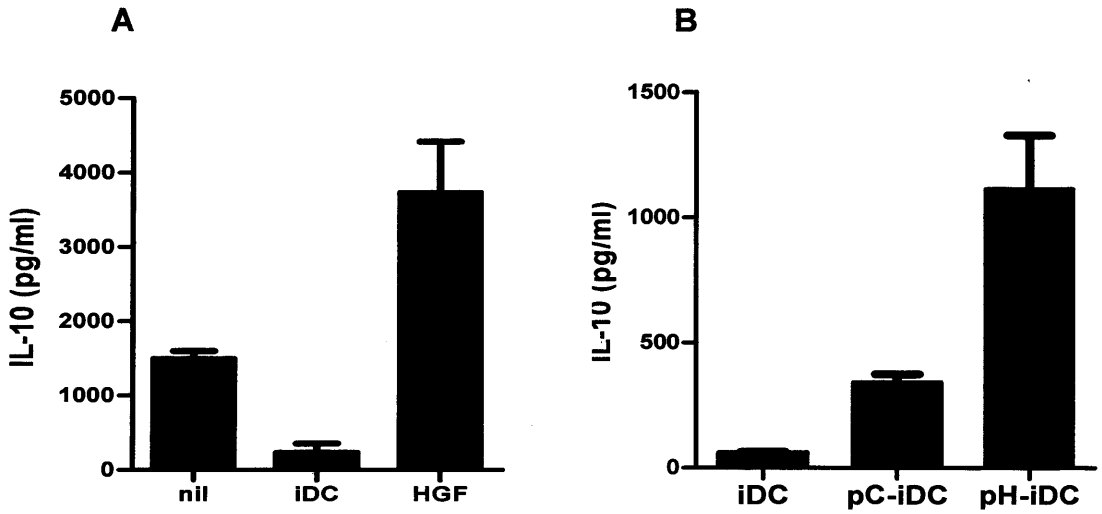


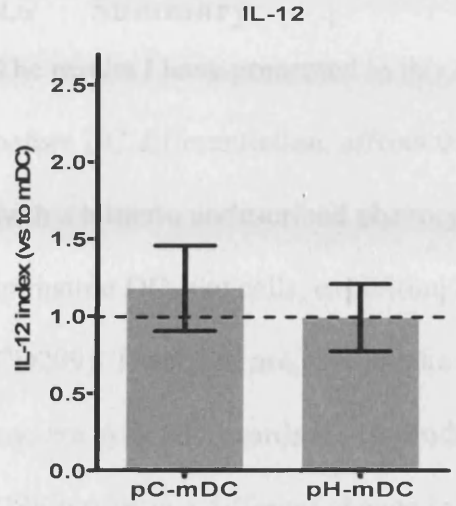
Figure 4.29 HGF Mediated Up-Regul

Persists After G

A. The levels of IL-10 produced by untr

24h of culture were determined from supernatants by ELISA. Mean and SEM of IL-10 levels (pg/ml) from 2 samples are shown.

B. IL-10 levels from day 5 DC supernatants. Mean and SEM of IL-10 levels (pg/ml) from duplicates, were determined from supernatants by ELISA.

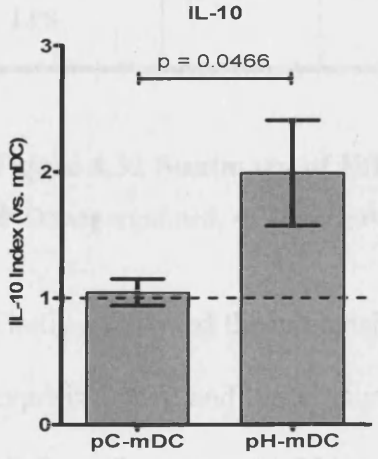


$$\text{IL-12 index} = \frac{\text{IL-12 (ng/ml) in pC-mDC}}{\text{IL-12 (ng/ml) in mDC}}$$

$$\text{IL-12 index} = \frac{\text{IL-12 (ng/ml) in pH-mDC}}{\text{IL-12 (ng/ml) in mDC}}$$

Figure 4.30 HGF Pre-Treatment Does Not Alter IL-12 Production

Levels of IL-12 in the supernatant of $1x10^6$ pC-mDC or pH-mDC 48 h after LPS stimulation were measured by ELISA and compared to that found in mDC supernatant. Dotted line indicates mDC IL-12 index. Means and SEM of ratios calculated from duplicate samples of 9 donors are shown.



$$\text{IL-10 index} = \frac{\text{IL-10 (ng/ml) in pC-mDC}}{\text{IL-10 (ng/ml) in mDC}}$$

$$\text{IL-10 index} = \frac{\text{IL-10 (ng/ml) in pH-mDC}}{\text{IL-10 (ng/ml) in mDC}}$$

Figure 4.31 HGF Pre-Treatment Causes Increased IL-10 Production

Levels of IL-10 in the supernatant of $1x10^6$ pH-mDC or pC-mDC 48 h after LPS stimulation were measured by ELISA and compared to that found in the mDC supernatant. Dotted line indicates mDC IL-10 index Means and SEM of ratios calculated from duplicate samples of 9 donors, are shown. The difference between pC-DC and pH-DC is significant as calculated by paired t test, p=0.0446, n=9.

4.6 Summary

The results I have presented in this chapter indicate that HGF, when added to monocytes before DC differentiation, affects the development of DC, by inducing a DC-like cells with a hitherto undescribed phenotype. This DC population can be described as immature DC-like cells, exhibiting markers of both monocytes and DC (CD14 and CD209). These DC are able to take up antigen, but their migratory capacity is impaired and are skewed towards IL-10 production. HGF co-treatment of monocytes with GM-CSF results in a different change in DC phenotype with MHC Class II, and co-stimulatory molecules down-regulated, but CD14 and CD209 expression is not affected, as summarised in Figure 4.32

Co-treatment	CD80↓	CD86↓	MHC Class II ↓	CD14 ns	CD209 ns	
Pre-treatment	CD80 (ns)	CD86 (ns)	MHC Class II ↑ (ns)	CD14 ↑	CD209 ↑	PD-L1 ↑
Re-treatment + LPS	CD80↓	CD86↓	MHC Class II (ns)	CD14 (ns)	CD209 (ns)	PD-L1 ↑

Figure 4.32 Summary of Effects on DC Phenotype

↓ Down-regulated, ↑ Up-regulated, (ns) – no change

Firstly I analysed the expression of HGF receptor c-Met on monocytes. Monocytes express c-Met, and lymphocytes are negative, which agrees with a previous report (Galimi, Cottone *et al.* 2001). To test that phenotypic or functional effects can be mediated by HGF, I confirmed that c-Met expressed on monocytes is functionally active by measuring ERK1/2 phosphorylation. This pathway is activated by c-Met HGF interaction in tumour cell lines of MPM and induces migration of human myeloma cells, but has not been studied in immune cells before (Jagadeeswaran, Ma *et al.* 2006; Holt, Fagerli *et al.* 2008). I also showed that an HGF blocking antibody is capable of blocking

HGF:c-Met signalling. This demonstrated that monocytes react to HGF via c-Met. I also showed that c-Met receptor is down-regulated during GM4-induced DC development. This supports the later finding that HGF-pre-treatment had more pronounced effects than co-treatment, and indicates that the timing of HGF treatment relative to the induction of DC differentiation may be important. This is physiologically relevant as levels of HGF are elevated in MPM cancer patients, both systemically and to a greater extent locally, therefore monocytes are likely to be exposed to HGF both prior to and during developing into DC.

To investigate if HGF co-applied with GM4 is sufficient to modulate DC differentiation I analysed the phenotypic development of DC. The resulting DC in this model display small but significant modulation in-particular showing down-regulation of co-stimulatory molecules CD80 and CD86 and also of MHC Class II. Markers of DC development, low CD14 and high CD209 are similar to those on untreated DC. Low levels of CD80, CD86 and MHC Class II are known to affect DC:T cell interactions, resulting in poor T cell stimulation (Ovali *et al.* 2000; Kurz, Diebold *et al.* 2002; Zou and Tam 2002; Okunishi, Dohi *et al.* 2005). This may help in explaining mouse models where HGF causes immune modulation, such as beneficial (immunosuppressive) effects in acute GVHD, autoimmune nephritis, collagen-induced arthritis, and allergic airway inflammation (Imado, Iwasaki *et al.* 2004; Ito, Kanehiro *et al.* 2005; Iwasaki, Imado *et al.* 2006; Kuroiwa, Iwasaki *et al.* 2006; Okunishi, Dohi *et al.* 2007).

HGF treatment of monocytes up-regulates c-Met expression, while GM4 down-regulates it. Thus the pre-treatment system allows HGF to interact with monocytes expressing higher levels of c-Met, for signal transduction.

Interestingly, control monocytes (pC-iDC) also exhibit a phenotype different from that of iDC. Delayed DC differentiation also generates, although at a lower frequency, DC-like cells with markers for both DC (CD209) and monocytes (CD14). GM-CSF/IL-4 treatment is a standard method of generating DC in vitro from monocytes, resembling the process which occurs in vivo during inflammatory DC development. The resulting DC is a relatively homogenous population, with a stable phenotype, which is useful for *in vitro* studies and is capable of generating sufficient DC for in vivo adoptive transfer models in vaccine studies. DC *in vivo* are however much more heterogeneous and the cytokine composition of the microenvironment during their differentiation can determine the range of subtypes. The results from pC-iDC indicate that delayed application of GM4 does not prevent DC development but allows the development of a less homogeneous DC population than that seen in GM4 iDC. The possible reason of the phenotypic bias of the pC-iDC might be the default autocrine IL-10 production, observed in the first 24 h of monocyte culture. HGF pre-treatment interacts with delayed treatment, as CD14, CD209 and PD-L1 are up-regulated further, while MHC Class II expression is generally up-regulated due to delayed GM4 application, in a HGF independent manner.

pH-iDC express markers of both DC and monocytes/macrophages, such as CD80, CD86 MHC Class II, CD209 and CD14. CD14 expression is normally used to exclude monocytes from DC populations. Using CD14 and CD209 to categorise cells I observed that $CD209^{+}CD14^{+}$ cells make the majority of DC like cells in HGF pre-treated DC, and only a small proportion of the cells express CD209 but not CD14, and therefore conforms to the phenotype of $CD209+CD14^{-}$ DC seen in iDC. The generation of $CD209^{+}$ $CD14^{+}$ cells with a tumour growth factor is interesting, as in advanced cancer

patients decreased frequencies of circulating DC ($CD14^-$), and increased numbers of immature antigen presenting cells are observed. Such DC, expressing CD14 would not be counted in "normal" lineage negative DC subsets which typically lack the expression of CD14, CD3, CD19, CD20, CD34 and CD56 molecules. DC with CD14 expression has also been observed when DC were generated in vitro from monocytes with GM-CSF and IFN-α or IL-15 (Banchereau, Pascual *et al.* 2004). It is also possible that HGF, expressed constitutively in the liver, is responsible for the presence of this unique CD14+ DC population in the liver (Cabillic, Rougier *et al.* 2006). Additionally, IL-10 and histamine are also known mediators of maintaining CD14 expression (Buelens, Verhasselt *et al.* 1997; Katoh, Soga *et al.* 2005). The data presented here adds HGF to the list of agents which inhibit CD14 down-regulation during DC differentiation.

While MHC Class II and costimulatory molecule expression was not significantly altered on pH-iDC or pC-iDC, expression of DC marker CD209 is up-regulated. CD209, DC-specific ICAM-3 grabbing non-integrin (DC-SIGN), is important in cell-cell contacts between DC and resting T cells via ICAM-3, and stabilising T cell:DC interaction in their early stages (Colmenares, Puig-Kroger *et al.* 2002; Zhou, Chen *et al.* 2006). CD209 can also mediate transmigration by interactions with ICAM-2. It is not expressed by blood DC but is expressed by MDDC and on tissue DC especially those in the skin, mucosa, liver tonsils and on some DC in spleen and lymph nodes (Geijtenbeek, Torensma *et al.* 2000; Cabillic, Rougier *et al.* 2006).

HGF interacts with delayed GM4 application, increasing the levels of CD209. Over-expression of CD209 may cause increased cellular adhesion, or accelerated trans-endothelial migration of DC with the $CD209^+$ $CD14^+$ phenotype. CD209 molecules

following cross-linking by tumour cells expressing certain tumour-associated antigens (e.g. carcinoembryonic antigen, CEA, in colorectal cancer), similar to signalling by viruses such as HIV or dengue virus may cause preferential activation of ERK1/2 and enhance IL-10 production promoting predominantly T_H2 type responses allowing tumour immune-evasion (Van Gisbergen, Aarnoudse *et al.* 2005; Caparros, Munoz *et al.* 2006; Nonaka, Izumo *et al.* 2008).

In addition to the up-regulation of CD14 and CD209, HGF pre-treatment also affects the expression of PD-L1. PD-L1 is a member of the B7 family which is expressed on human DC and monocytes and has also been described on several types of tumors (Keir, Francisco *et al.* 2007; Keir, Butte *et al.* 2008). PD-L1 can inhibit the function of PD-1 expressing T cells. Tumour factors have been shown to up-regulate PD-L1 on dendritic cells, but HGF has not been implicated in this process Here I provided evidence that HGF is a relevant factor in the induction of PD-L1 up-regulation.

The first part of this chapter indicates that HGF has a modulatory effect on DC development, the nature of which depends in the kinetics of HGF-monocyte-differentiation signal interaction.
There were two reasons why I further characterised the DC-like cells in the pre-treatment model: one is to obtain more information about their function, the other is to demonstrate whether they are more DC-like than macrophage/monocyte like.

Phenotypic maturation of DC is a process that allows DC to develop from an antigen-capturing into an antigen presenting role. It is one of the abilities of DC which underpin their "professional" status as APC in the immune system. In contrast, monocyte-derived macrophages display immune modulatory function during allo-T cell stimulation (Hoves,

Krause *et al.* 2006). They do not express CD209, nor are they able to phenotypically mature like DC, they do however retain CD14 expression. Macrophage development is also known to be inhibited by IL-4 (Canque, Camus *et al.* 1998).

I found that HGF pre-treated DC are able to phenotypically mature. LPS up-regulates the expression of MHC Class II and co-stimulatory molecules on both pH-mDC and pC-mDC. However HGF pre-treatment significantly inhibits the LPS-induced up-regulation of co-stimulatory molecules CD80 and CD86 during maturation. This is a similar effect to that observed in the HGF-co-treatment model. Although HGF pre-treatment up-regulates CD209 on immature cells, it does not interfere with CD209 down-regulation during LPS-induced maturation. CD14 expression which is also up-regulated by HGF pre-treatment, is down-regulated by LPS leading to pH-mDC expressing levels of CD14 indistinguishable from mDC or iDC. LPS also up-regulates both CD83 and CD40 on pH-mDC to levels similar to those on mDC. pH-iDC therefore undergo phenotypic maturation very similar to iDC, confirming that they are indeed DC-like. PD-L1 expression is further up-regulated during LPS-induced maturation on pH-mDC indicating the long term effect of HGF pre-treatment on the expression of this T cell inhibitory molecule.

The phenotypic data provide evidence to support the DC-like nature of cells developed from HGF pre-treated monocytes, due to their maturation characteristics. They indicate the role of HGF as an immunomodulator of DC function, via inhibiting the up-regulation of co-stimulatory molecules, and lasting up-regulation of the T cell inhibitory ligand PD-L1.

An important function of DC is the phagocytosis of antigen to allow presentation of exogenous antigen to T cells. Functional analysis of phagocytosis of immature DC indicated no effect by HGF or delayed-GM4 stimulation. Both pC-iDC and pH-iDC have similar phagocytic function to iDC, which is compared to macrophages is relatively weak (Matsuno, Ezaki *et al.* 1996). The loss of phagocytic ability of the DC groups upon LPS treatment was not studied; it may add a further line of evidence to the DC-like nature of pH-DCs.

During maturation DC express CCR7 allowing recognition of lymphoid migration signals. CCR7 recognises CCL19, the concentration gradient effect of which guides DC migration towards lymph nodes. This ability facilitates DC migration out of tissues and into draining lymph nodes where DC undergo terminal maturation and prime naïve T cells. However, pH-mDC have a significantly reduced migratory capacity towards this stimulus while expression of CCR7 is not significantly altered compared to mDC. pC-mDC are able to migrate towards CCL19, indicating that the observed effect is HGF specific. As shown in T cells, the defect may be at the signalling level of CCR7 not the surface expression level (Garcia-Zepeda, Licona-Limon *et al.* 2007). DC activated by HGF may produce CCL19 which would abolish the gradient needed for migration, or pH-DC may adhere more readily to other cells or surfaces slowing down the rate of migration.

Another functional aspect of DC is their ability to generate cytokines, and in GM4 DC these are associated with strong T_H1 type responses. pC-mDC produce levels of IL-12 and IL-10 similar to that by mDC. This agrees with both phenotypic maturation and functional aspects of these cells so far. pH-mDC produce similar levels of IL-12 as the

controls, however they produce much higher levels of IL-10 compared to pC-mDC and mDC. This alters the balance of IL-10:IL-12. IL-10 production by DC is linked to induction of regulatory function of DC and induction of Treg (Corinti, Albanesi *et al.* 2001; Zhang, Koldzic *et al.* 2004; Bellinghausen, Konig *et al.* 2006). The generation of anti-tumour T cell responses requires the presence of IL-12. However, IL-10 can induce T_H2 responses even in the presence of IL-12. (Steinbrink, Wolfl *et al.* 1997; Liu, Rich *et al.* 1998; Ria, Penna *et al.* 1998; Jonuleit, Schmitt *et al.* 2000). Therefore, the ability of HGF to increase production of T_H2 type cytokines such as IL-10 would impair the ability of these cells to induce the T_H1 response required for elimination of cancer cells.

Both the phenotypic and functional data presented in this chapter indicate that high HGF levels, like those present in the tumour environment of mesothelioma cancer patients, can affect the development of DC.

The DC-like cells induced by HGF can be characterised in their immature state as being double positive for CD209 and CD14. In their mature state they may be characterised by low co-stimulatory molecule expression and significantly increased PD-L1 expression and IL-10 production. The ability of HGF-induced DC-like cells to stimulate T cells, also the role that IL-10 may play in mediating HGF's effects merit further investigation and will be addressed in chapters 5 and 6, together with the potential physiological relevance of this cell population in MPM.

Chapter 5 - The effect of HGF pre-treatment on DC:T stimulation

5.1 Introduction

DC are uniquely able to induce primary T cell responses and efficiently restimulate memory T cells. As shown in chapter 4, HGF interferes with the development of iDC, most fundamentally by retaining CD14 expression, inhibiting migration, up-regulating PD-L1 molecule expression and increasing the ratio of IL-10:IL-12. *In vitro* induction of human T cell proliferation by HGF-treated monocytes has been shown to be impaired compared to DC (Rutella, S., Bonanno *et al.* 2006). However, there have been no studies on the effect of HGF on human DC-induced T cell responses.

The aim in this chapter was to establish whether HGF-pre-treatment affects the ability of DC to stimulate T cells. Firstly, allogenic T cell stimulation by DC was studied, a model of primary T cell responses *in vitro*. T cell proliferation was measured by using either ^{3}H-thymidine uptake assay or CFSE-dilution method; secondly, the ability of DC to induce memory responses, by measuring cytokine (IFNγ) production by T cells was studied. 15-mer peptides from common viral antigens, including published or predicted T cell epitopes, were loaded onto pH-mDC or pC-mDC and T cell recall responses were measured by cytokine flow cytometry for IL-10 and IFNγ production. IL-10 production by T cells was also measured to determine T_H1/T_H2 responses. Experiments with pH-mDC to stimulate T cells were carried out according to the diagram below (Figure 5.1)

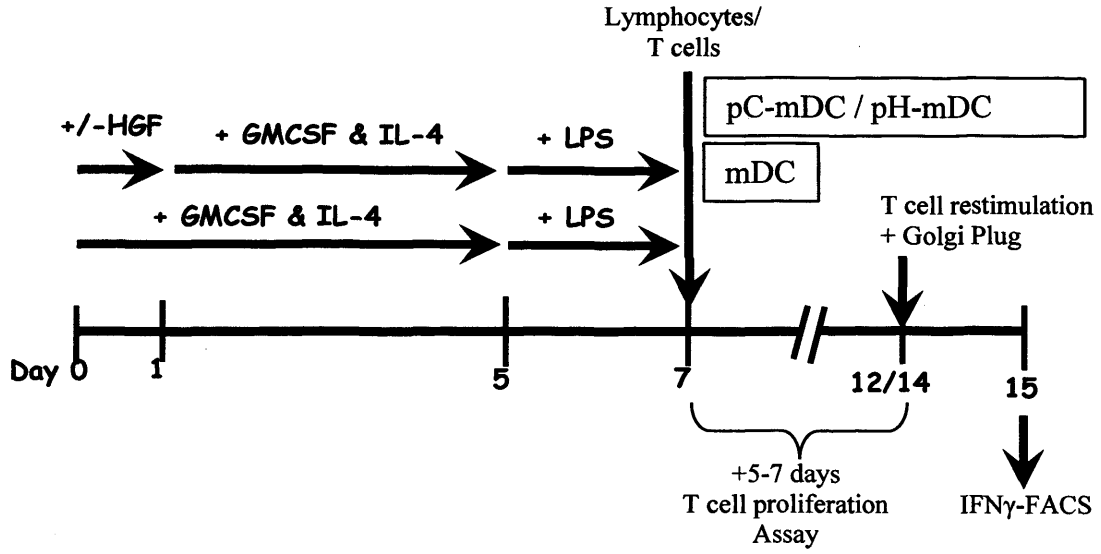


Figure 5.1 T Cell Stimulation by pH-mDC or Controls.

5.2 pH-mDC Are Less Efficient T Cell Stimulators Than Control DC

pH-mDC-induced T cell proliferation was less efficient than that induced by mDC by up to 50% (Figure 5.2A). pH-mDC, were also less efficient at stimulating T cell proliferation than pC-mDC (figure 5.2B), but the effects of delayed DC development and HGF-treatment were cumulative. It was not calculated whether pH-mDC induced fewer cycles of T cell proliferation, although it is likely as the MFI of proliferating T cells was higher in pH-mDC-compared to pC-mDC-stimulated T cells (1487± 155 pH-DC compared to 1166 ± 5 pC-DC).

T cell proliferation, measured either by ^{3}H-thymidine uptake or CFSE dilution, was lower (at all stimulator to responder ratios) with pH-mDC stimulators than with the pC-mDC stimulators (figures 5.3A and 5.3B). Inhibition varied between 8-13% using the CFSE-dilution method, while with ^{3}H-thymidine uptake inhibition of proliferation varied between 15-25% depending on DC:T ratio, T cell stimulation was not carried out in this experiment due to the lack of sufficient number of DC obtainable from an average donor. However, impairment of T cell proliferation was observed by both methods.

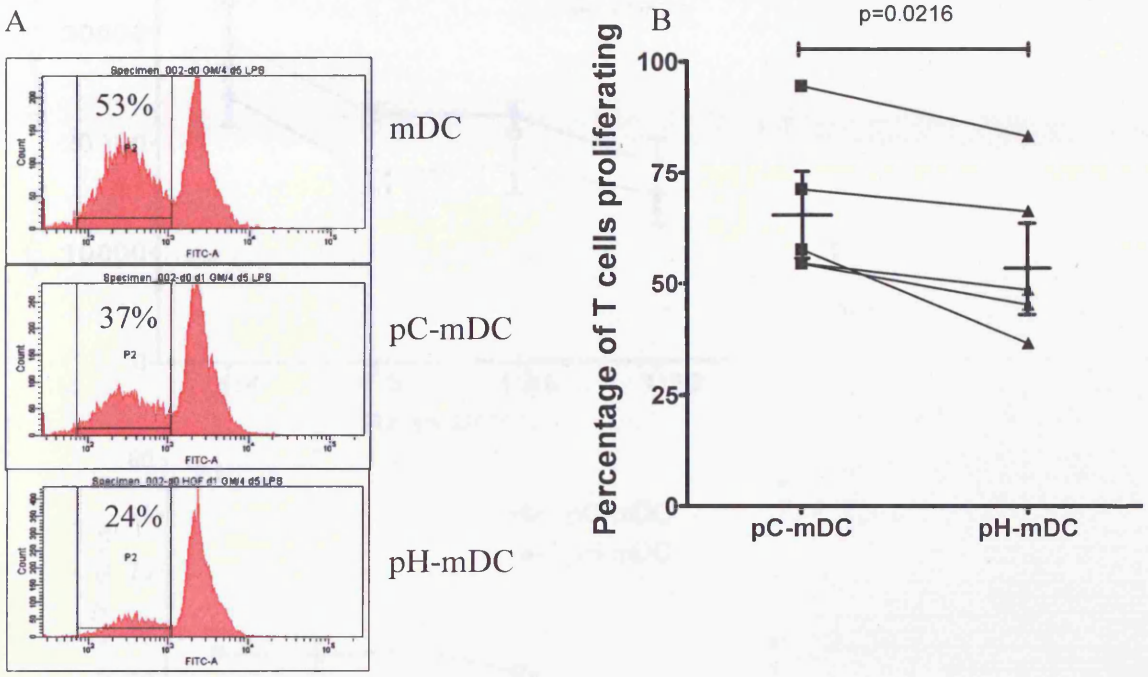


Figure 5.2 HGF Pre-Treatment Decreases the T Cell Stimulatory Capacity of DC.

A. Representative histograms show proliferation of T cells by CFSE dilution assay after 7 days, following stimulation with allogenic DC. The numbers represent the percentage of total gated $CD3^+$ T cells that proliferated (i.e. had lower CFSE intensity (MFI) than unstimulated T cells (not shown). This experiment was representative of 5 donors.

B. Proliferating T cells (based on CFSE dilution of CD3+ lymphocytes) from 5 donors following stimulation with pC-DC or pH-DC. The symbols represent means of 3 replicates per donor at a stimulator:responder ratio of 1:10. The lines and bars indicate means and SEM, while the capped line indicates a significant difference between the treatment groups using a paired T test p=0.0216, n= 5.

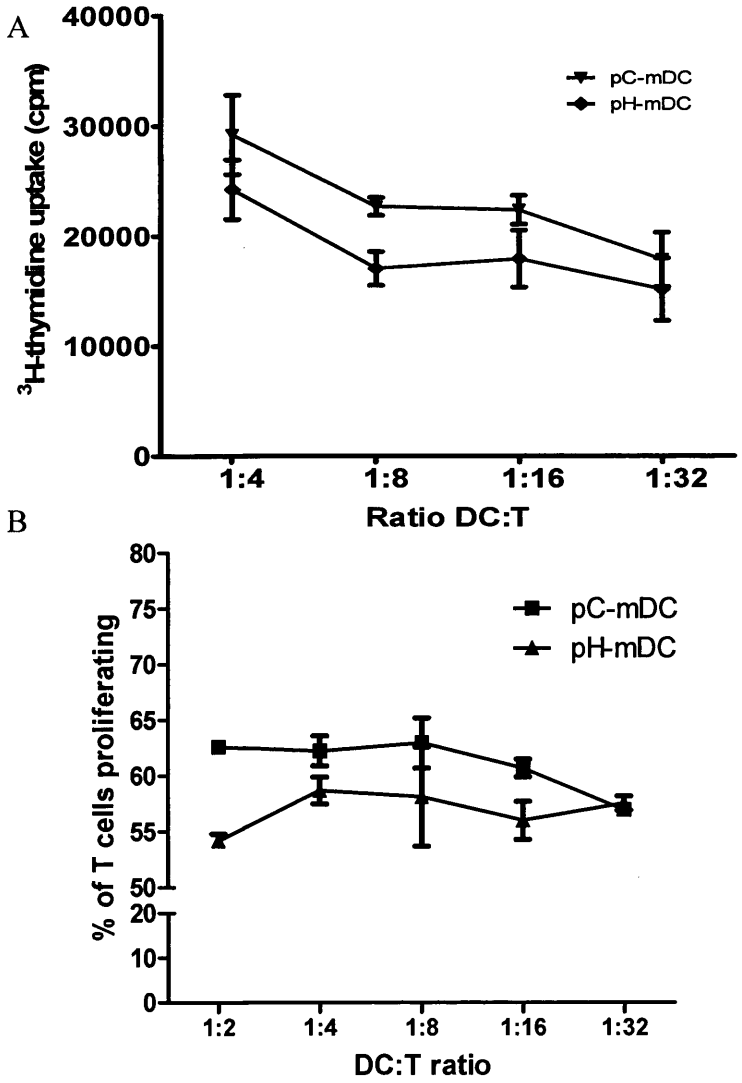


Figure 5.3 HGF Pre-Treatment Decreases the Stimulatory Capacity of mature DC

A. Percentages of CFSE-labelled proliferating T cells are shown, following stimulation with decreasing numbers of allogenic DC as indicated by the different symbols. The percentage of total T cell proliferation was calculated as the number of T cells with MFI of CFSE dye lower than that in unstimulated T cell cultures but higher than that of unlabelled cells. The symbols and bars indicate the mean and SEM of 3 replicates; the results are representative of 3 donors.B. ^{3}H-thymidine uptake by proliferating lymphocytes on day 5 following stimulation with allogenic pC-mDC or pH-mDC at decreasing DC:T ratios. The symbols and bars indicate the mean and SEM of 3 replicates. Anaylsis of proliferation by CFSE dilution method was statistically more accurate determining cell proliferation. However it was not compatible with HGF inhibitor SU11274, which due to its coloured nature interfered with CFSE measurements by flow cytometry. However determination of proliferation by ^{3}H-thymidine uptake was also able to determine differences between pC-mDC and pH-mDC although at with lower accuracy and was there for used in expermiments with SU11274.

5.3 c-Met Inhibition Partially Recovers T Cell Stimulatory Ability of pH-mDC

A c-Met specific inhibitor (SU11274, Calbiochem®, IC_{50} 20 nM) added to adherent PBMC at the same time as HGF pre-treatment began, partially restored the ability of pH-mDC to stimulate allogenic T cell proliferation (Figure 5.4). ^{3}H-thymidine uptake without SU11274 in pH-mDC stimulated cultures was 70% lower than in pC-mDC cultures, while with the addition of SU11274 the difference was only 25% (Figure 5.4). This inhibitor did not affect the T cell stimulatory capacity of pC-mDC (Figure 5.4). It was not possible to perform any FACS analysis of CFSE labelled c-Met treated DC, due to the nature of this inhibitor as it is a bright yellow/orange material in solution and interferes with FACS analysis.

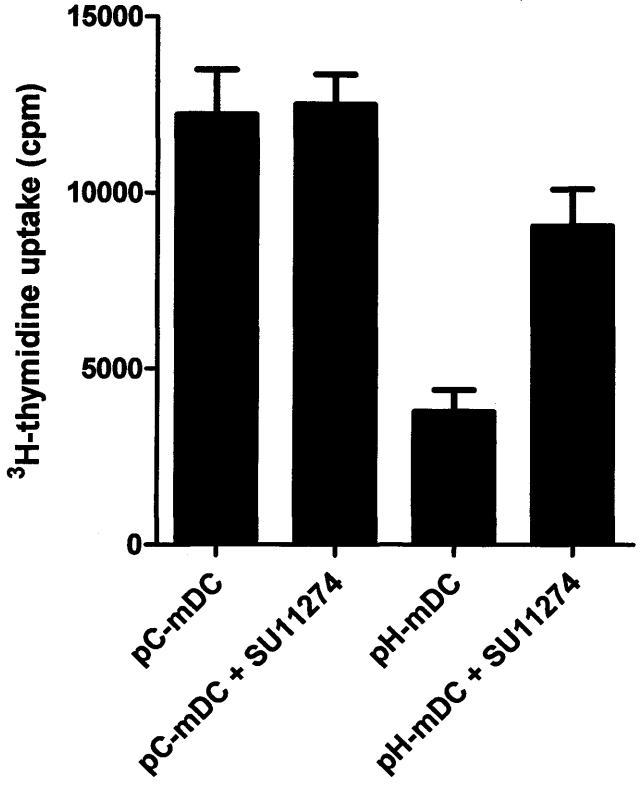


Figure 5.4 Inhibition of c-Met During HGF Pre-Treatment of Monocytes Partially Restores T Cell Stimulatory Capacity of DC.

pH-mDC or pC-mDC were cultured from day 0, in the presence or absence of SU11274 at 40 nM. The resulting cells were used as described previously to stimulate allogenic T cells at 1:10 DC:T cell ratio. ^{3}H-thymidine uptake by proliferating lymphocytes following 5 day stimulation is shown. Means and SEM of triplicates from a representative experiment of 3 is shown.

5.4 pH-mDC are Less Efficient at Generating T cell Recall Responses Than Control DC

I also investigated the ability of DC to present peptide antigens and induce memory $CD8^+$ T cell responses measured by IFNγ production. 15-mer peptides from common viral antigens, to which both $CD8^+$ and $CD4^+$ T cell recall responses have been demonstrated (Coleman, Clayton *et al.* 2005), were used. The responding cells have been characterised in the department as $CD45RA^-$, $CCR7^-$, $CD62L^-$ cells, representing the effector memory subset. The ability of DC to induce T cell responses was measured by stimulating autologous T cells with peptide-antigen loaded DC, then measuring the recall responses of T cell populations following a brief restimulation by autologous BLCL ± peptide antigen. The production of IFNγ was measured by cytokine flow cytometry.

HGF pre-treatment impaired the ability of DC to stimulate $CD8^+$ T cell recall responses, by up to 50% compared to both mDC and pC-DC ($P=0.0255$ and $p=0.0402$, respectively; $n=3$), whilst with pC-mDC compared to mDC no impairment was observed, (Figure 5.5) indicating that the effect was HGF-specific.

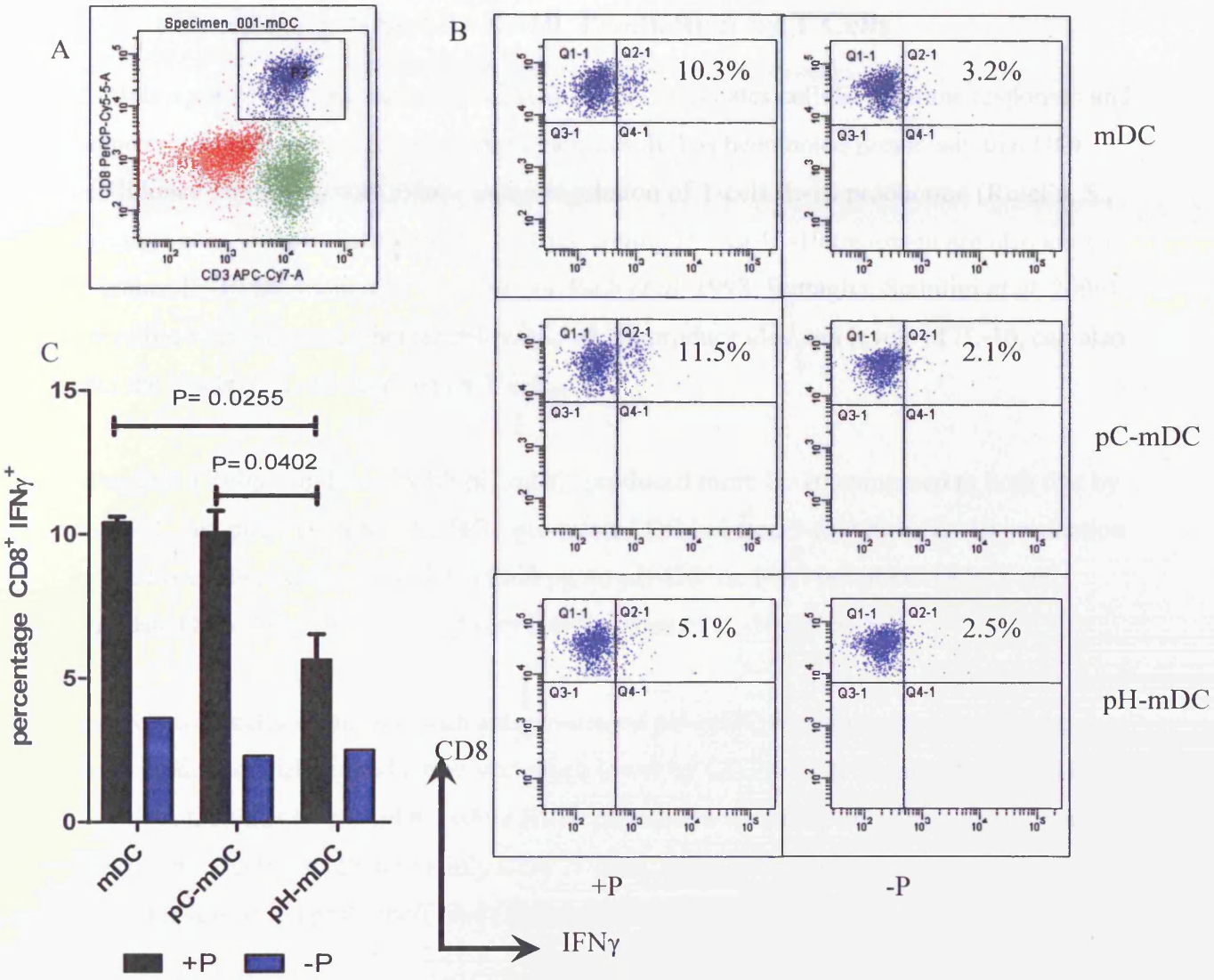


Figure 5.5 pH-mDC Stimulate Weaker CD8$^+$ Recall Responses.

A. Gating of CD3+CD8+ lymphocytes (blue) for analysis.

B. The proportion of total CD3+CD8+ T cells producing IFNγ are indicated by the numbers in the upper right quadrants. The left column: autologous CD8+ T cells stimulated with peptide antigen loaded DC. Right column: CD8+ T cell responses without peptide loading of DC. The plots are typical responses of 3 donors.

C. Summary of the percentage of total CD3+CD8+ T cells responding (triplicates). Black bars indicate mean responses (IFNγ+) of peptide stimulated T cells, error bars indicate SEM. Blue bars indicate the background T cell IFNγ responses to DC without peptide (single samples). Capped lines indicate the statistical analysis by paired t test between T cell responses induced by pH-mDC compared to pC-mDC (p=0.0402) or mDC (0=0.0255).

5.5 pH-mDC Up-Regulate IL-10 Production by T Cells

IL-10 is a potent immunosuppressive cytokine that attenuates cellular immune responses and suppresses production of inflammatory cytokines. It has been noted previously that HGF conditioned monocytes can induce an up-regulation of T-cell IL-10 production (Rutella, S., Bonanno *et al.* 2006). Additionally, IL-10-secreting DC, or IL-10 treatment are also known to induce IL-10 production by T cells (Liu, Rich *et al.* 1998; Battaglia, Stabilini *et al.* 2006). Therefore I investigated whether pH-mDC, which produce elevated levels of IL-10, can also alter the levels of IL-10 secreted by T cells.

Allogenic T cells, stimulated with pH-mDC, produced more IL-10 compared to both that by pC-mDC and mDC (Figure 5.6). HGF pre-treated DC induced 3-fold more IL-10 production by T cells compared to control DC (4360 pg/ml pH-DC vs. 1435 pg/ml pC-DC). T cells stimulated by mDC produced only very low amounts of IL-10 (57 pg/ml).

Autologous T cells stimulated with antigen-loaded pH-mDC were examined for IFNγ and IL-10 production. IFNγ production was much lower by CD3+ CD8+ T cells stimulated by pH-mDC, than that by pC-mDC, while IL-10 production remained unchanged. However, in CD3+CD8- T cells which are mainly $CD4^+$ T cells, while IFNγ production was reduced, IL-10 production was slightly increased (Figure 5.7).

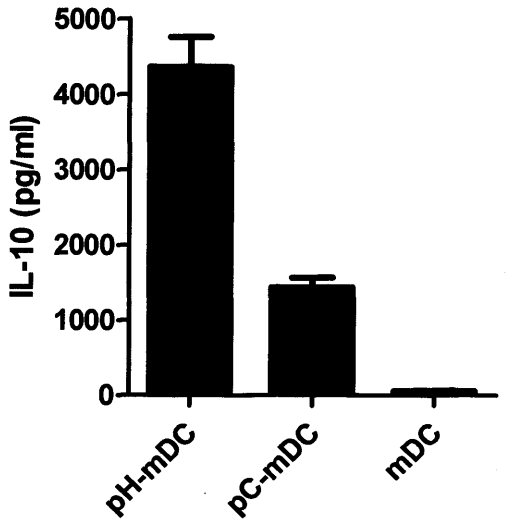


Figure 5.6 HGF Pre-Treated DC Induce Increased IL-10 Production by T Cells
The bars show the production of IL-10 (pg/ml) in 7 day cultures following allogenic T cell stimulation by irradiated pH-mDC, pC-mDC and mDC at 10:1 T:DC ratio. Means and SEM of duplicate samples are shown, measured by IL-10 ELISA (R&D systems). This is a representative of 3 donors.

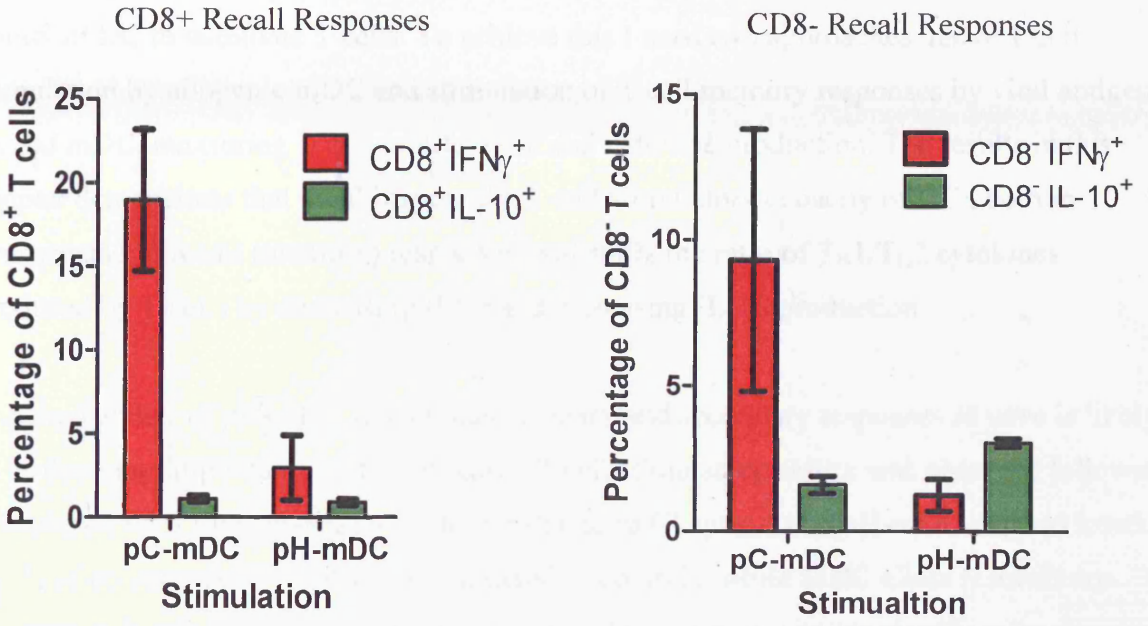


Figure 5.7 HGF Pre-Treated DC, Induce Less IFNγ and More IL-10 Production by CD8⁻ T Cells.

A. Bars represent percentage of CD8+ T cells producing IL-10 or IFNγ after stimulation with viral peptide antigen loaded DC. The bars represent means and SEM of duplicate samples.

B. Bars represent percentage of CD8- T cells producing IL-10 or IFNγ after stimulation with viral peptide antigen loaded DC. The bars represent means and SEM of duplicate samples.

are typical responses of 3 donors.

5.6 Summary

In this chapter I set out to establish whether the pre-treatment of DC with HGF affects the ability of DC to stimulate T cells. To achieve this I used two approaches, naïve T cell stimulation by allogenic mDC and stimulation of T cell memory responses by viral antigen-loaded mDC, measuring T cell proliferation and cytokine production. The results in this chapter demonstrate that HGF impairs the T cell stimulatory capacity of DC , both in primary and in recall (memory) responses, and shifts the ratio of T_H1/T_H2 cytokines produced by T cells by decreasing IFNγ and increasing IL-10 production.

Impaired ability of pH-mDC to stimulate primary and secondary responses *in vitro* is likely to be due to multiple factors. The impaired T cell stimulatory ability was observed following stimulation with LPS treated DC. I demonstrated in Chapter 4 that pH-mDC express lower levels of co-stimulatory molecules compared to controls, while MHC Class II levels are unaffected. Furthermore, IL-10 production and PD-L1 expression are significantly elevated in HGF pre-treated mature DC. It is likely that these known mechanisms of T cell suppression affect the functional behaviour of pH-mDC. Lower levels of MHC Class II and co-stimulatory molecules (Hermans, Ritchie *et al.* 1999; Almand, Clark *et al.* 2001), increased IL-10 (Allavena, Piemonti *et al.* 1998; Corinti, Albanesi *et al.* 2001; Battaglia *et al.* 2006) and increased expression of PD-L1 (Seo, Seo *et al.* 2006; Sharpe, Wherry *et al.* 2007) are all factors that can contribute to impaired T cell stimulation. The exact role of IL-10 in the HGF-mediated negative functional effect will be analysed later (Chapter 6). The functional role of PD-L1 in silencing T cell responses, by the use of PD-L1 blocking antibodies (where PD1 is up-regulated by T cell activation) (Sharpe *et al.* 2007; Wang, Han *et al.* 2007) was not confirmed due to the lack of time.

HGF's ability to dampen human T cell responses via modulation of DC function has not been observed before. However, in mice, *Okunishi et al 2005* observed the inhibition of antigen presenting function of DC and suppression of both T_H1 and T_H2 responses and a protective role in airway inflammation (Okunishi, K., Dohi *et al.* 2005). IL-10 production was not increased in their model. The same group (Okunishi, K., Dohi *et al.* 2007) found that HGF potently inhibited collagen-induced arthritis in mice via IL-10 and T_H2-mediated mechanisms. HGF-treated monocytes (^{HGF}Mo) induced weaker allo-T cell proliferation *in vitro* compared to DC (Rutella, S., Bonanno *et al.* 2006). However, as untreated monocytes

were not included as controls in the experiments, it can not be concluded from this work that HGF itself had any affect on monocyte-T cell interaction. Nevertheless, the T cell inhibitory effect was attributed to IL-10 and IDO, induced in the monocytes by HGF. Due to the lack of time I did not study the activation of IDO. Another possibility, contributing to the functional character of pH-mDC, is the up-regulation of PD-L2 (Blank and Mackensen 2007). These additional mechanisms would be interesting to study as further work, in order to completely define the functional consequences of HGF pre-treatment on DC.

Taken together, the results in this chapter provide evidence that pre-exposure of monocytes to HGF before differentiation into DC results in a long lasting and significant impairment of the ability of DC to stimulate primary and secondary T cell responses and also in a shift towards T_H2-type responses. The possible mechanisms and the relevance of these findings are explored further in the next chapter.

Chapter 6 - Possible Mechanism and Physiological Relevance of the Immuno-Modulatory Effects of HGF

6.1 Introduction

In Chapter 4, I demonstrated that both HGF-treated monocytes and DC derived from these cells produce elevated levels of IL-10. In this chapter, the impact of HGF-induced IL-10 on the phenotype and function of pH-DC is studied in order to demonstrate whether IL-10 production is the major mechanism responsible for the immunomodulatory effects of HGF. The experiments compare the similarities between IL-10- and HGF-mediated immunological effects and determine IL-10's role in the HGF-pre-treated setting by introducing an IL-10 neutralising antibody. Both DC phenotypic changes and pH-DC stimulated T cell responses are analysed this way.

In Chapter 3, elevated levels of HGF in tumour-exudate or pleural fluid of MPM patients were demonstrated. In the light of the evidence provided in chapters 4 and 5, i.e. that human recombinant HGF mediates immunomodulatory effects, it is important to determine whether tumour associated HGF is also able to deliver similar immunological effects. The pleural fluid contains tumour-cells, lymphocytes, monocytes and soluble factors and represents a relatively easily obtainable material for the study of tumour-immune interactions. The general immunosuppressive effect of pleural fluid has been demonstrated (Gottehrer, Taryle *et al.* 1991; Delong, Carroll *et al.* 2005; Hegmans, Hemmes *et al.* 2006), but the contribution of HGF to this effect has not been addressed before. For this, the cell-free filtered fraction of pleural fluid containing elevated levels of HGF was introduced during DC differentiation *in vitro* and the phenotypic and functional effects were observed in the presence of HGF-blocking and control antibodies respectively. Furthermore, the frequency of $CD209^{+}CD14^{+}$ cells, normally low in PBMC, was also studied in pleural fluid, as elevated frequency would confirm that the *in vitro* observed skewing effect on myeloid cell differentiation also exists in the tumour environment.

6.2 HGF Induces the Development $CD209^+CD14^+$ DC via IL-10

As HGF induces elevated levels of IL-10, which might then be responsible for the phenotypic and functional changes in pH-DC, first I studied whether IL-10 had the same phenotypic effects on *in vitro* DC differentiation as HGF did. Monocytes were pre-treated with either 5 ng/ml IL-10 (normal donors typically have less than 20 pg/ml IL-10 in peripheral blood serum) or 30 ng/ml HGF for 24 h before GM4 treatment. IL-10 was indeed able to generate double positive DC expressing both CD14 and CD209 molecules (Figure 6.1). In order to determine that the effect was IL-10-driven, isotype control or IL-10 neutralising antibodies, respectively, were present during DC differentiation. Both IL-10 and HGF-mediated phenotypic changes were blocked by IL-10 neutralising antibody, although only partially. Either higher concentrations of the blocking antibody may have been necessary to achieve complete blocking, or IL-10 are only partially responsible for the observed phenotypic effects.

6.3 Impaired T Cell Stimulation by pH-DC is Mediated by IL-10

IL-10 is well characterised as a powerful anti-inflammatory cytokine and its inhibitory effects on T cell proliferation and function are well established. Here, as in the previous experiment, HGF was added to monocytes in the presence of an IL-10 blocking or an isotype control antibody. On day 5, DC were collected, washed and counted, loaded with a mixture of viral peptides (see Chapter 2 and Figure 2.2) and used as antigen presenting cells to stimulate autologous memory T cell responses. Similar to results presented in Chapter 5, pH-mDC were less able to stimulate T cell responses than mDC, this time in the presence of a control isotype antibody, but when IL-10 neutralising antibody was added, both mDC stimulated and pH-mDC stimulated T cell responses were elevated to equal levels (Figure 6.2). This experiment demonstrates not only that HGF-induced IL-10 dampens antigen specific $CD8^+$ T cells to produce IFNγ but also the general immunosuppressive effects of autocrine IL-10, which acts as a default cytokine on T cell responses. Based on these blocking experiments with IL-10 it can be concluded that HGF-induced IL-10 is crucially important in the immunomodulatory effects of HGF on T cell responses.

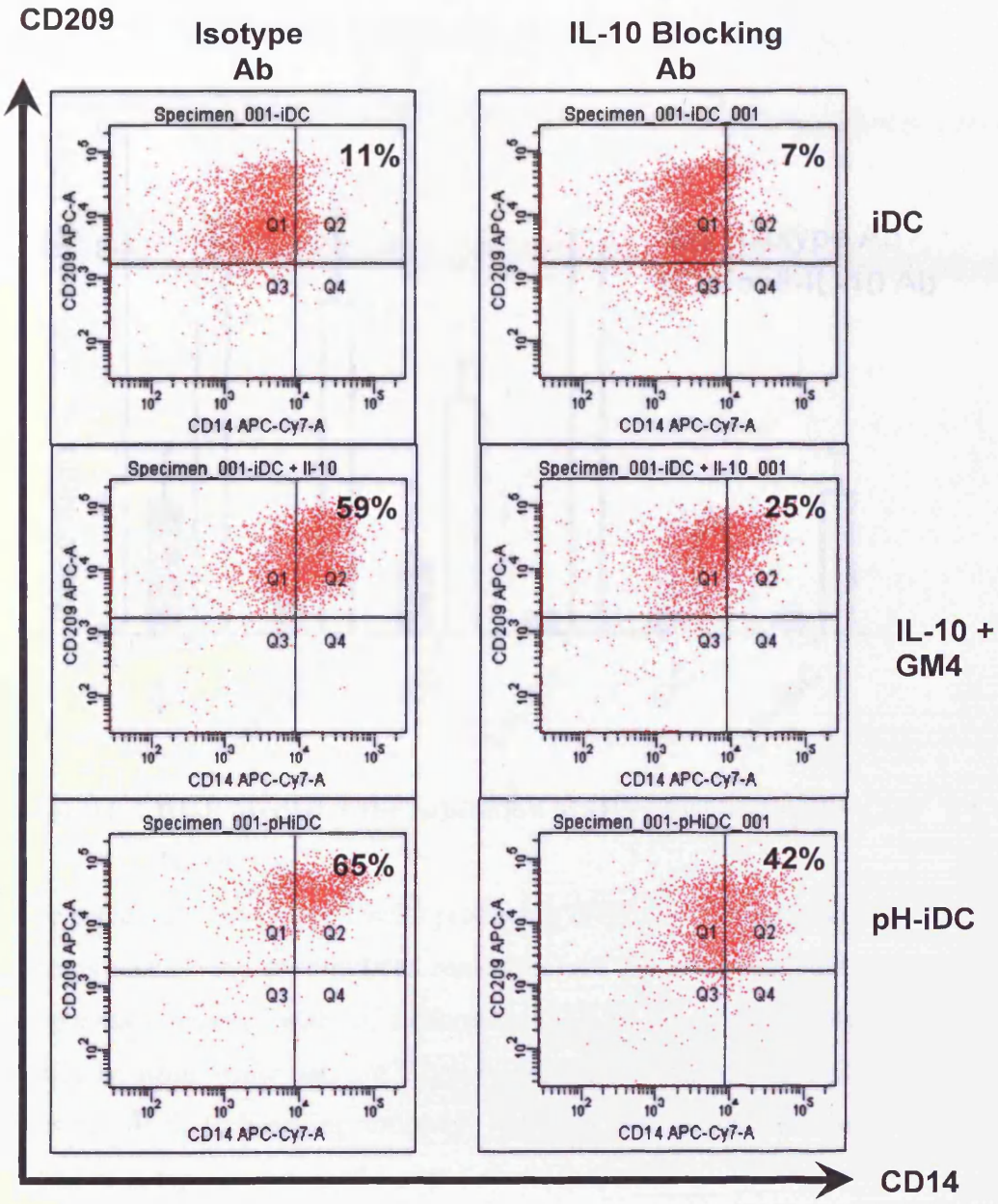


Figure 6.1 Blocking IL-10 Partially Recovers the Effect of HGF Pre-Treatment. Dot plots of the expression of CD209 and CD14 on FSc/SSc 'monocyte/DC' gated cells. The percentage of double positive ($CD209^{+}CD14^{+}$) cells is indicated on each dot plot. A representative of 3 separate experiments, however there is variability in the range of CD209 levels they there DC express, this range is also variable between donors.

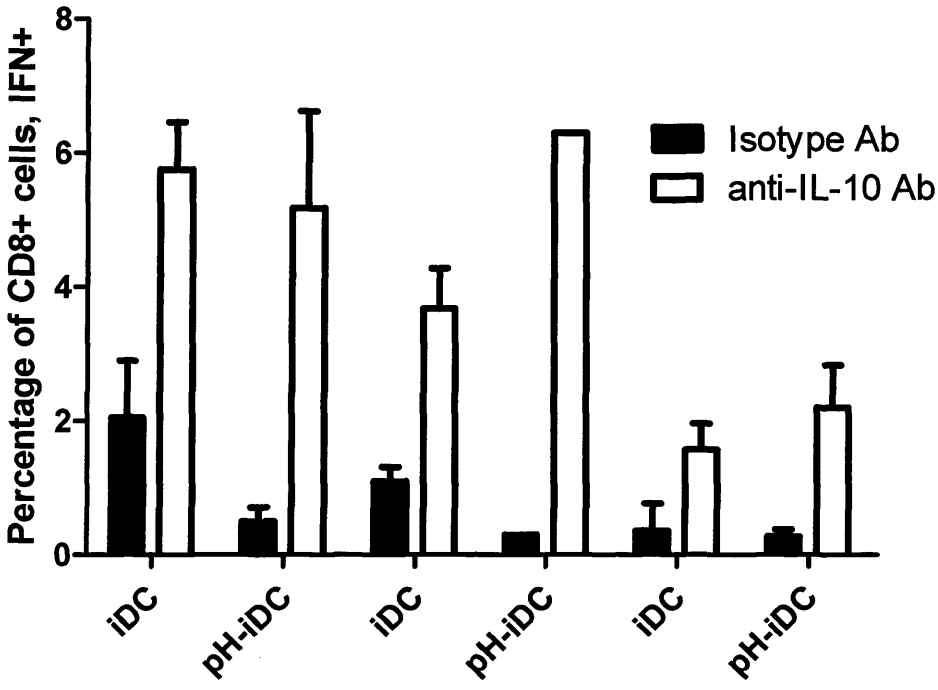


Figure 6.2 HGF Mediates the Inhibition of IFNγ Production by $CD8^+$ T Cells via IL-10

The percentages of CD8+ T cells producing IFNγ after stimulation with viral antigen (minus background unstimulated responses) are shown. Black bars represent T cell responses stimulated with DC differentiated in the presence of isotype control antibody, while white bars are T cell responses stimulated with DC differentiated in the presence of IL-10 blocking antibody. The error bars represent SEM of duplicate samples. A representative of 3 experiments is shown.

6.4 Tumour-Associated HGF Modifies DC Differentiation *in vitro*

The physiological relevance of HGF can be studied by using HGF-containing pleural fluid, representing the tumour associated environment in mesothelioma. The pleural fluid is known to contain not only well-characterised inhibitory but also immuno-stimulatory agents. therefore it is interesting to see if HGF, as part of this mixture, has a distinctly detectable effect, similar to that observed with human recombinant HGF. For this, filtered aliquots of cell free pleural fluid, containing ~30ng/ml HGF, were added at 5% concentration to normal (healthy) monocytes for 24 h before GM4 was added, either in the presence of HGF-neutralising antibody or control isotype antibody. The frequency of the $CD209^+CD14^+$ cells was analysed by flow cytometry as described. The frequency of $CD209^+CD14^+$ cells was <2% in normal iDC (Figure 6.3), while when 5% pleural fluid was added, the proportion of double positive DC increased dramatically to 81%. The effect of HGF in mediating this transmission was confirmed by employing an HGF-neutralising antibody at 10 μg/ml from the beginning of the *in vitro* culture. This specifically blocked the development of $CD209^+CD14^+$ cells (33%), as the application of a control isotype had only a slight effect on the development of $CD209^+CD14^+$ cells (63%). This experiment indicates that HGF in the pleural fluid has the ability to modify DC development.

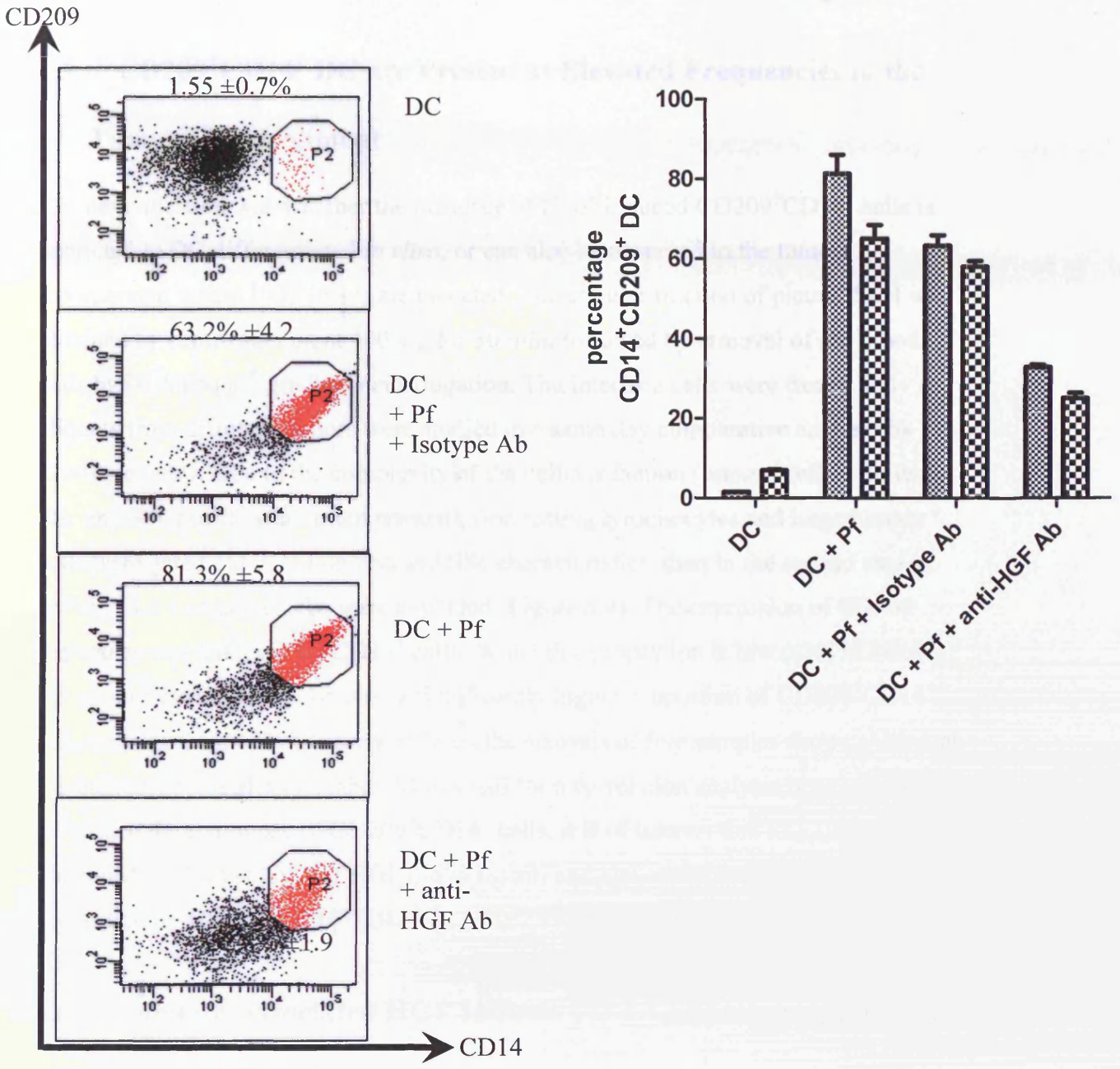


Figure 6.3 $CD209^{+}CD14^{+}$ DC Induced by MPM Pleural Fluid is Mediated by HGF

A. Dot plots of the expression of CD209 and CD14 on FSc/SSc 'monocytes/DC' gated cells. The percentage of total cells that are double positive for CD209 and CD14 are indicated on each dot plot with the mean and SEM of duplicate samples, for DC generated from Pf pre-treated monocytes with and without HGF neutralising antibody or its isotype control, as indicated. Representative of 2 separate experiments. B. Percentage CD209+CD14+ DC (FSc/SSc gated monocytes/DC). The percentage of total cells double positive for both CD209 and CD14 generated on from Pf pre-treated monocytes with and without HGF neutralising antibody of its isotype control , as indicated.

6.5 $CD209^+CD14^+$ DC are Present at Elevated Frequencies in the Tumour Environment

The next question was whether the presence of HGF-induced $CD209^+CD14^+$ cells is restricted to DC differentiated *in vitro*, or can also be observed in the tumour environment where HGF levels are elevated. The cellular fraction of pleural fluid was obtained by centrifugation at 400 x g for 30 min, followed by removal of red blood cells by Histopaque® gradient centrifugation. The interface cells were frozen and aliquots from different donors were studied in a same day comparative analysis by flow cytometry. Due to the complexity of the cellular faction (tumour cells with wide FSc and SSc profile were often present), first resting lymphocytes and large tumour cells were gated out based on FSc and SSc characteristics, then in the second step of gating, CD14 negative cells were excluded (Figure 6.4). The expression of CD209 molecules was analysed on $CD14^+$ cells. While this proportion is low (4%) in PBMC, the tumour environment contains a significantly higher proportion of $CD209^+CD14^+$ cells ranging from 10% to nearly 60%, as the analysis of five samples shows. Although the number of samples available is too small for a correlation analysis between HGF levels and the frequency of $CD209^+CD14^+$ cells, it is of interest that Pf 5 (last panel) contained the lowest level of HGF (1696 ng/ml) and also contained the lowest proportion of $CD209^+CD14^+$ (10.4%).

6.6 Tumour-Associated HGF Induces PD-L1 Expression on DC *in vitro*

In Chapter 4, an observation that HGF up-regulates the expression of PD-L1 on DC was made. The physiological significance of this finding may be interesting, as PD-L1 over-expression could also contribute to impaired T cell responses. Here, the ability of HGF-containing pleural fluid was studied on DC differentiation *in vitro*. The experiment was carried out as above, with pre-treatment using 5% Pf and HGF-neutralising or control antibody present from the beginning of the *in vitro* culture of monocyte-derived DC. Flow cytometry of CD14, CD209 and PD-L1 expression was carried out after 5 days in culture (Figure 6.5). Compared to iDC, pre-treatment of monocytes with 5% Pf resulted in the up-regulation of CD14 and CD209 molecules,

confirming the experiment shown in Figure 6.3. PD-L1 expression was also up-regulated (Figure 6.5, last column). These effects were specifically prevented by the presence of HGF-neutralizing antibodies but not the control antibody. The prevention of the PD-L1 up-regulation was partial, indicating that other components of the pleural fluid, e.g. tumour derived IL-10, may also be able to influence PD-L1 levels.

6.7 PD-L1$^+$CD14$^+$ cells are present at elevated frequencies in the tumour environment

In order to demonstrate the physiological importance of the observations of CD14 and PD-L1 expression on DC, we asked whether PD-L1 is also up-regulated on tumour-associated monocytes and DC-like cells in the pleural fluid. The cellular fractions of pleural fluid samples were isolated as described above. Flow cytometry analysis of PD-L1 expression was carried out on CD14$^+$ cells (tumour cells may also express PD-L1 which is why they were excluded by CD14$^+$ gating). The frequency of PD-L1$^+$ expressing cells was low on CD14$^+$ PBMC (6.6%). PF cells from 5 donors contained a wide range of PD-L1$^+$CD14$^+$ cells. While cells in one sample contained practically no PD-L1$^+$ cells (0.1%), the other four contained high proportions of these cells (range 8.8% - 67%; mean = 30.7 %). The absence of PD-L1$^+$ cells in one patient is interesting and would warrant further investigation of the presence of other factors which could support or counteract PD-L1 up-regulation.

These results demonstrate that PD-L1 expression can be up-regulated by pleural fluid HGF on DC *in vitro* and PD-L1$^+$CD14$^+$ cells can be found at elevated frequencies in the cellular fraction of pleural fluid. As it is not possible to prove the role of HGF in the in situ up-regulation of PD-L1, the latter observation remains indirect evidence for the physiological relevance of my observations with HGF.

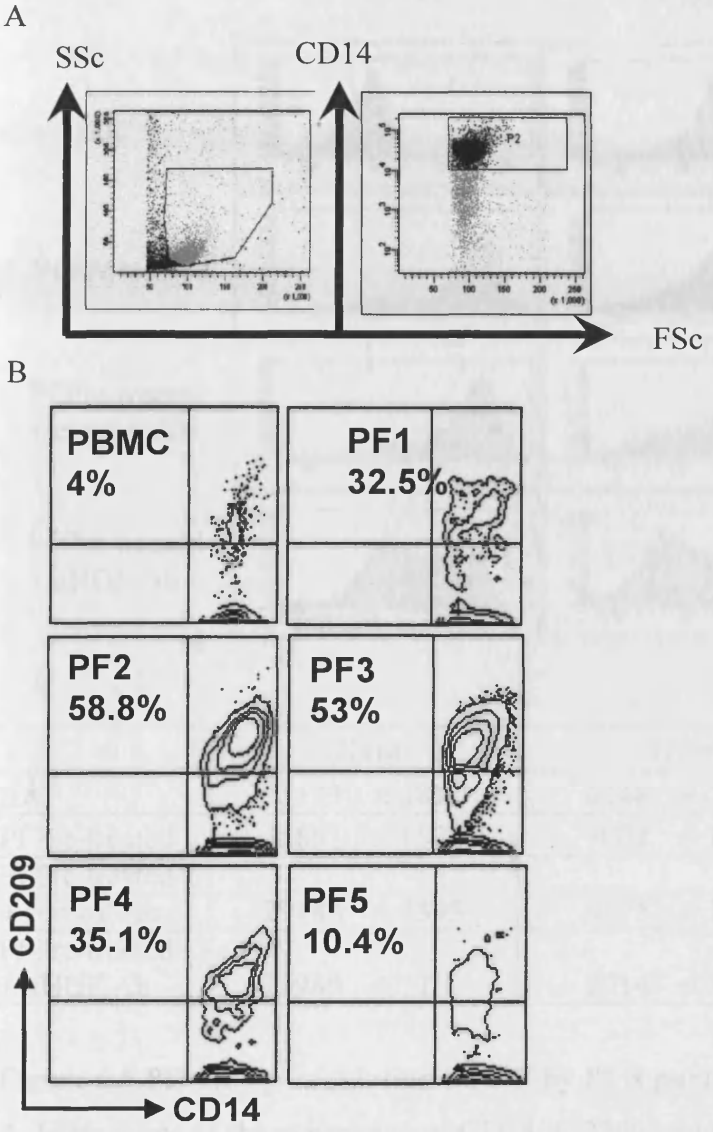
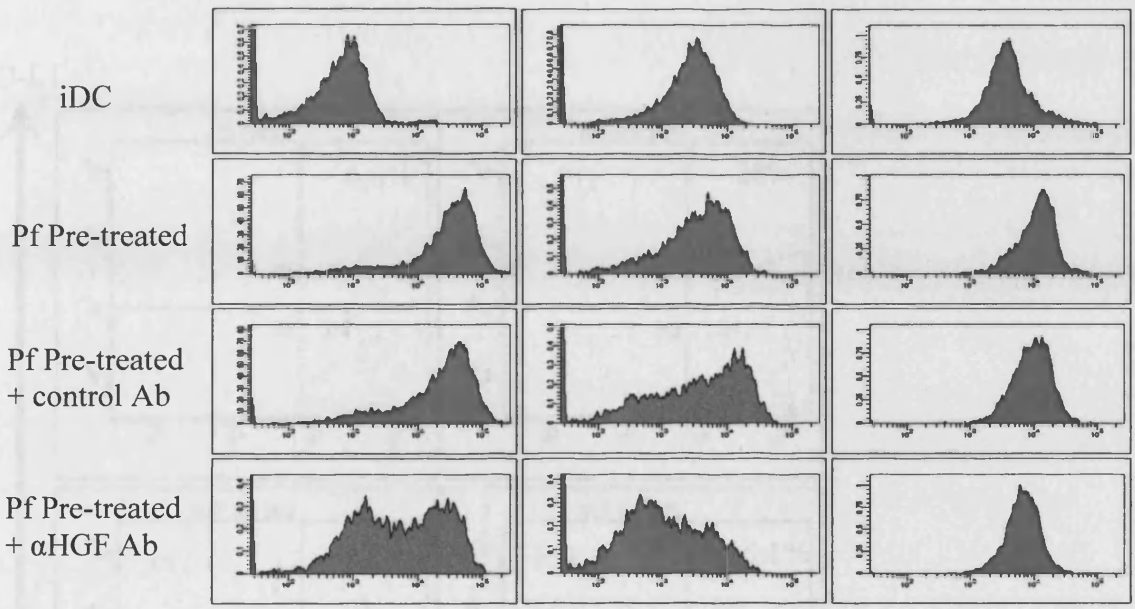


Figure 6.4 $CD209^{+}CD14^{+}$ cells can be detected in MPM pleural fluid

A. Gating of cells for CD209 analysis, first by FSc/SSc profile then by CD14 expression.

B. Dot plots of the expression of CD209 on $CD14^{+}$, FSc/SSc 'monocytes/DC' gated cells, derived from Pf (1-5) or healthy donor PBMC (first dot plot). The percentages of double positive cells ($CD209^{+}CD14^{+}$) are indicated on each dot plot.

A



B

	CD14	CD209	PD-L1
iDC	1173 ± 381	6148 ± 2783	5531 ± 489
Pf Pre-treated	55861 ± 15788	7002 ± 1916	12443 ± 657
Pf Pre-treated + control Ab	29188 ± 4395	6175 ± 1592	10123 ± 1266
Pf Pre-treated + αHGF Ab	13989 ± 77	2014 ± 145	8578 ± 641

Figure 6.5 PD-L1 up-regulation on DC by Pf is partially mediated by HGF

A. Histograms of the expression of CD14, CD209 and PD-L1 on DC pre-treated with 5% Pf for 24 h prior to adding GM4 in the absence (2nd row) or the presence of HGF neutralising antibody (4th row) or isotype control antibody (3rd row) (10ug/ml).

B. The table contains the means and SD of the MFI values of CD14, CD209 and PD-L1, from duplicates. A representative of 3 separate experiments.

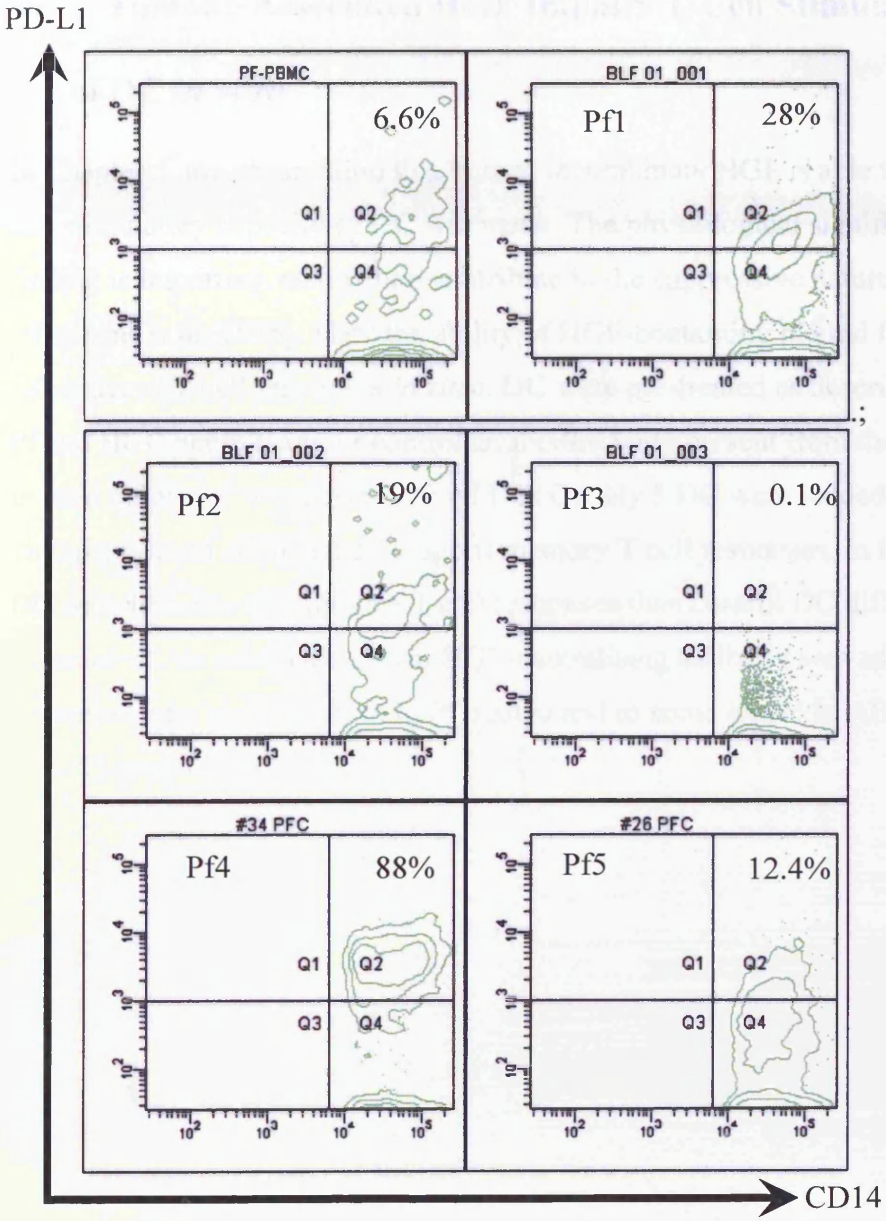


Figure 6.6 PD-L1 is expressed on CD14$^+$ cells in the pleural fluid

Dot plots of the expression of PD-L1 on CD14$^+$, FSc/SSc 'monocytes/DC' gated cells. The percentage of double positive (PD-L1$^+$CD14$^+$) cells are indicated on each dot plot, such as for healthy donor PBMC (first panel) and for Pf cells from five different individual patients (Pf 1, 2, 3, 4 and 5).

6.8 Tumour-Associated HGF Impairs T-Cell Stimulatory Function of DC *in vitro*

In Chapter 5, the observation that human recombinant HGF is able to suppress the T cell stimulatory capacity of DC was made. The physiological significance of this finding is important, as it would contribute to the suppressive nature of pleural fluid (Hegmans *et al.* 2006). Here the ability of HGF-containing pleural fluid was studied on DC-induced T cell responses *in vitro*. DC were pre-treated as described above with 5% Pf and HGF-neutralising or control antibodies were present from the beginning of the *in vitro* culture of monocyte-derived DC. On day 5 DC were loaded with a mixture of viral peptides to stimulate autologous memory T cell responses. In the presence of Pf, DC were less able to stimulate T cell responses than control DC differentiated in the presence of AB serum, but when HGF-neutralising antibody was added T cell responses were elevated both in Pf treated and to some extent in AB serum-treated cells (Figure 6.7 and 6.8).

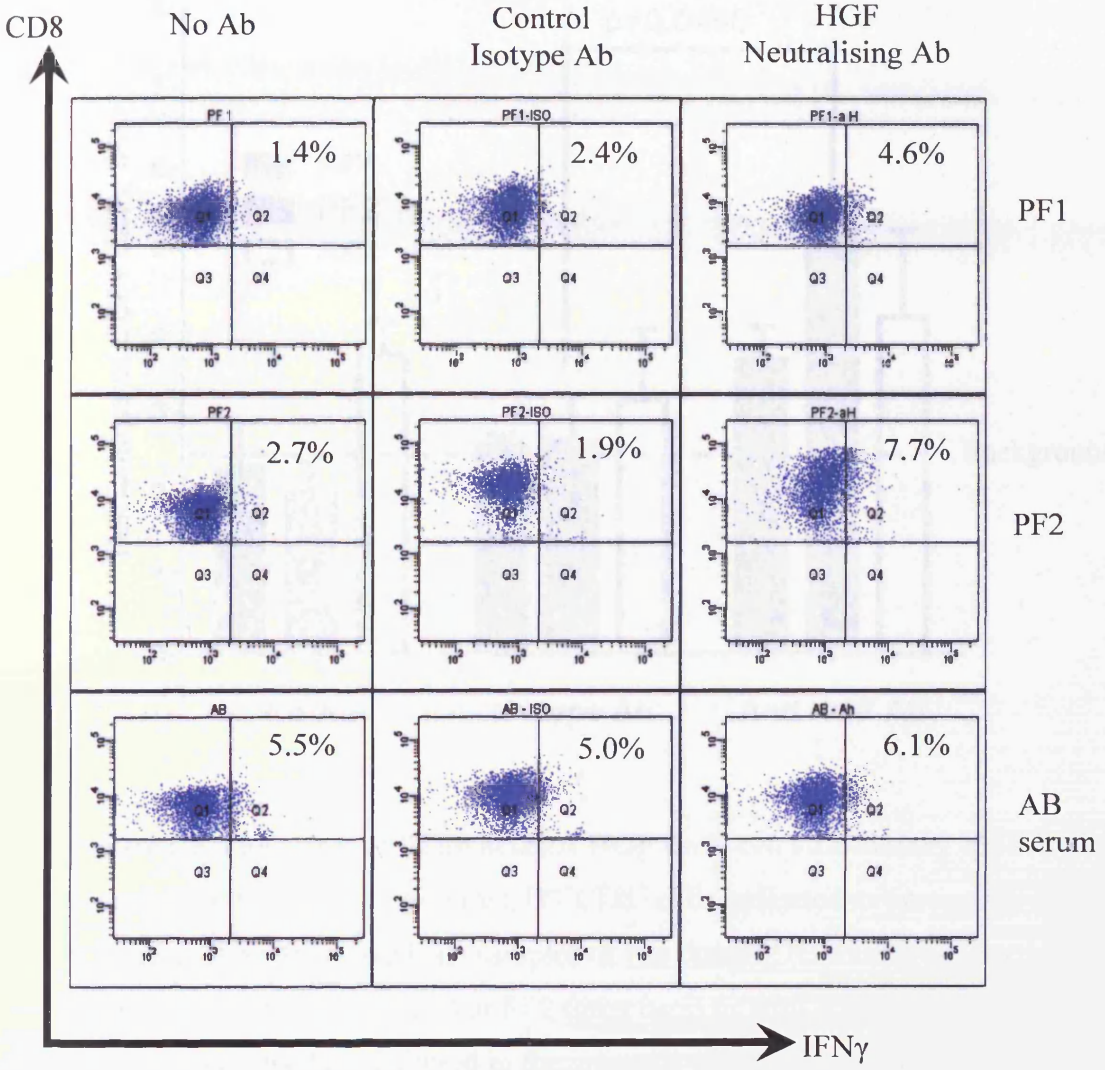


Figure 6.7 The effect of Pf-associated HGF on T cell stimulatory capacity of DC

Dot plots of IFNγ production by $CD3^{+}CD8^{+}$ cells CD8 vs. IFNγ expression are indicated by the arrows. Numbers indicate IFNγ producing cells as a proportion of total CD3+$CD8^{+}$ T cells. Healthy donor DC were pre-treated with either Pleural fluid (PF1 or PF2) or with AB serum as a control, in the presence of no Ab (left column), isotype control Ab (middle column) of HGF neutralising Ab (right hand column). Autologous T cell responses were detected against common viral antigens by intracellular cytokine staining as described in Chapter 2. Representative samples of triplicates are shown. Background responses induced by DC in the absence of peptide were 2.4 ± 0.4%.

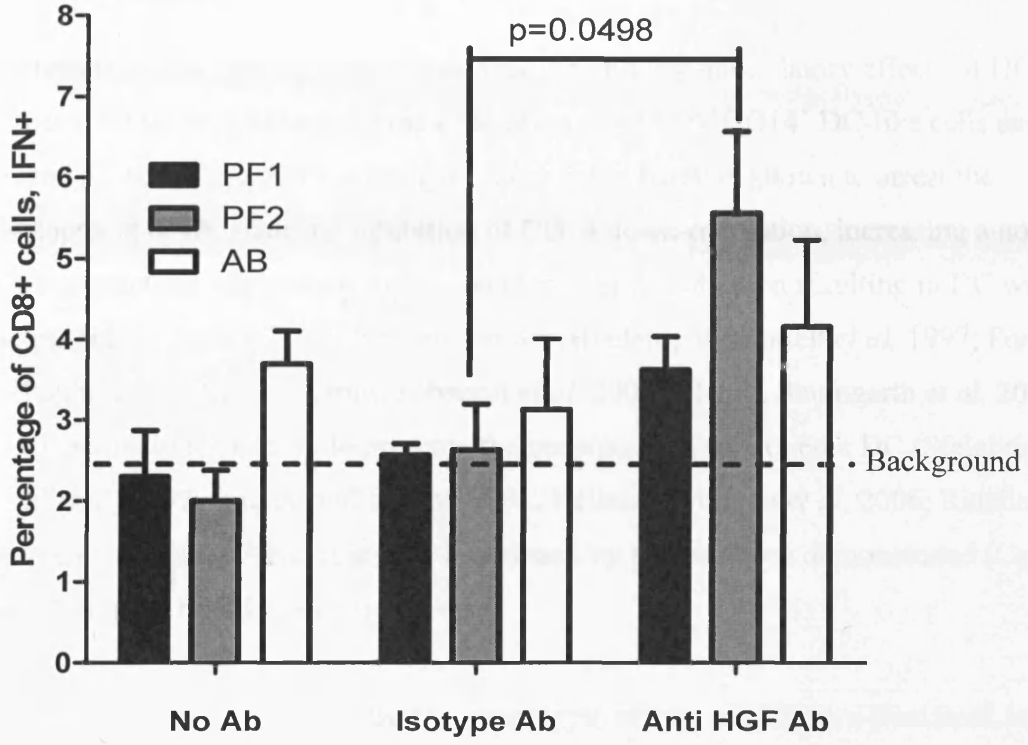


Figure 6.8 The effect of Pf associated HGF on T cell stimulatory capacity of DC
Summary of IFNγ production by $CD3^{+}CD8^{+}$ cells indicated as percentage of cells producing IFNγ from triplicate samples of one donor. DC were pre-treated with either pleural fluid, Pf 1 (black bars) or Pf 2 (grey bars) or with AB serum (white bars) as a control. The cells were cultured in the presence of no antibody (no Ab), Isotype Ab or HGF neutralising Ab (anti HGF Ab), as indicated. Background responses induced by DC in the absence of peptide were 2.4 ± 0.4% and is indicated by the dotted line. T cell responses recovered by HGF Blocking Ab were significantly higher than in the presence of the isotype control Ab (p=0.0498, n=3, PF2) using a paired t test in PF2 while the responses in PF1 were also elevated but not significant p=0.0771.

6.9 Summary

The results in this chapter demonstrate that the immuno-modulatory effects of HGF are mediated by IL-10, particularly the generation of $CD209^{+}CD14^{+}$ DC-like cells and the impaired T cell stimulatory capacity of these cells. IL-10 is known to arrest the development of DC, causing inhibition of CD14 down-regulation, increasing autocrine IL-10 production, stimulating lower levels of IL-12 production resulting in DC which are less able to induce T cell IFNγ responses (Buelens, Verhasselt *et al.* 1997; Fortsch, Rollinghoff *et al.* 2000; Corinti, Albanesi *et al.* 2001; Chang, Baumgarth *et al.* 2007). IL-10 production is known to promote the generation of tolerogenic DC (Steinbrink, Wolfl *et al.* 1997; Rutella and Lemoli 2004; Bellone, Carbone *et al.* 2006; Rutella, Danese *et al.* 2006). Autocrine IL-10 produced by DC has been demonstrated (Corinti *et al.* 2001) to affect DC functions.

Blocking IL-10 partially prevents the phenotypic effects of HGF-pre-treatment, while the ability of pH-DC to stimulate $CD8^{+}$ T cells is completely recovered by the blockade of IL-10. IL-10-treated DC exhibit similar features to HGF-pre-treated DC, such as CD14 expression, ability to stimulate T cells and IL-10 production.

The similarity between IL-10 and HGF pre-treated DC, coupled with partial restoration of phenotype with IL-10 blocking antibody indicates that HGF mediates its effects via IL-10. However, as IL-10 blocking does not completely prevent the phenotypic effects of HGF, HGF may also mediate some of its effects directly, especially as HGF:c-Met interactions signal via ERK1/2, MAPK and JNK (Boisleve, Kerdine-Romer *et al.* 2005; Jiang, Martin *et al.* 2005; Caparros, Munoz *et al.* 2006; Luft, Rodionova *et al.* 2006; Nakahara, Moroi *et al.* 2006; Qian, Jiang *et al.* 2006). ERK1/2 signalling in monocytes is involved in the induction of cytokine production such as IL-10 and TNFα, it is also involved in increased monocytes survival.

Although IL-10 seems to be the main mechanism responsible for HGF's effects on inhibiting T cell responses, other mechanisms should also be considered. As shown in Chapter 4, HGF pre-treatment up-regulates PD-L1, a B7 family member that can inhibit lymphocyte activation, by signalling through activation of PD-1 ITIM motifs in T cells (Freeman, Long *et al.* 2000). This up-regulation of PD-L1 expression may also

contribute to inhibition of T cell stimulation by HGF pre-treated DC with IFNγ or IL-10 (Yamazaki, Akiba *et al.* 2002; Brown, Dorfman *et al.* 2003; Matsumoto, Inoue *et al.* 2004). Up-regulation of PD-L1 is also seen by Pf, partially mediated by HGF, as shown by neutralisation of HGF in Pf. Other groups have shown *in vitro* that PD-L1 and PD-1 interactions can cause suppression of T cell activation and decrease of cytokine production, which can be recovered by blocking the interaction of PD-L1 and PD-1 with antibodies (Brown *et al.* 2003). Additionally PD-1 deficient knock out mice exhibit multiple autoimmune conditions (Freeman *et al.* 2000; Dong and Chen 2003; Liu *et al.* 2003). The increased expression of inhibitory factors such as PD-L1 either by recombinant HGF or by pleural fluid HGF in my pre-treatment model provides strong evidence that HGF contributes to the development of a tolerogenic DC phenotype in vitro.

My *in vitro* studies prove that HGF promotes the development of DC with immuno regulatory characteristics. To demonstrate the relevance of HGF's immuno modulatory role in cancer, I analysed cells collected from mesothelioma patients' pleural fluid. Pleural fluid contains high levels of HGF compared to that in blood from both mesothelioma patients and healthy donors. There is a high degree of up-regulation of $CD209^+$ on $CD14^+$ cells in Pf, compared to that observed on healthy donor PBMC, providing indirect evidence about the possible in situ effect of HGF.

Taken together, IL-10 mediates some of the effects of HGF, including phenotypic and functional effects (T cell stimulatory capacity). This agrees with physiological data where DC with phenotypic characteristics seen on HGF DC are found among patient pleural fluid cells. Furthermore, Pf can also induce the same effects seen by human recombinant HGF in an HGF dependent manner. However, most notably, blockade of HGF in Pf restores the ability of DC to stimulate T cell responses. This indicates that blocking of HGF in cancer apart from its direct anti-tumour effects may result in the restoration of immune responses and in particular may contribute to the restoration of antitumour $CD8^+$ IFNγ responses.

Chapter 7 - General Discussion

The study of HGF on immune responses conducted in this thesis focused on the following points:

- Development of models for analysis of the immunological effects of HGF
- Analysis of the effects of HGF on DC development
- Analysis of the effects of HGF on T cell responses (via DC)
- Identification of possible mechanisms of HGF-mediated immune modulation
- Demonstration of the physiological relevance of the effects of HGF on DC development in the tumour environment

To study HGF's effects on cellular immune responses, I first confirmed HGF's target cell population in peripheral blood. It has been previously reported that human monocytes express c-Met, the only reported HGF receptor, while mature lymphocytes do not (Chen, Defrances *et al.* 1996; Beilmann, Odenthal *et al.* 1997; Beilmann, Vande Woude *et al.* 2000; Ovali, Ratip *et al.* 2000). My experiments confirmed c-Met expression on cells within the monocyte gate of PBMC. Functional expression of c-Met has not been demonstrated on human monocytes before this. To confirm that the c-Met expressed is active on monocytes, based on available information on HGF-mediated c-Met signalling in tumour cells and in epithelial cells (Stella and Comoglio 1999; Sunitha, Shen *et al.* 1999; Derksen, Keehnen *et al.* 2002; Fan, Gao *et al.* 2005; Peruzzi and Bottaro 2006; Ramos-Nino, Blumen *et al.* 2007; Ramos-Nino, Blumen *et al.* 2008), I chose the downstream signalling molecule ERK1/2, demonstrating its phosphorylation upon c-Met engagement by HGF. The results confirmed that c-Met expressed on monocytes is functionally active, so further experiments were planned to assess the effect of HGF on the development of the most important immune cells, dendritic cells, from monocytes.

The effects of HGF were analysed using two *in vitro* models. Both these models utilised the well established method of generating monocyte-derived DC by treating monocytes with GM-CSF and IL-4 (Sallusto and Lanzavecchia 1994). In the first model, which I termed co-treatment (H-iDC), GM-CSF, IL-4 and HGF are applied together. In the second, which I called pre-treatment, HGF is added to monocytes 24 h

before GM-CSF and IL-4 are added. DC development from monocytes occurs in the skin and peripheral tissues *in situ*, although the resulting DC are phenotypically and functionally much more diverse than those generated *in vitro* (Vuckovic, Clark et al. 2002; Gabrilovich 2004; Leon, Lopez-Bravo et al. 2005; Pinzon-Charry, Maxwell et al. 2005; Rutella, Danese et al. 2006; Svane, Nikolajsen et al. 2006). The-pre-treatment approach has not been used widely *in vitro*. Monocytes for all experiments were isolated from peripheral blood samples of healthy donors by plastic adherence, because magnetic bead separation with anti-CD14 antibody coated beads lead to the down regulation of CD14, similar to that observed by others using anti-CD14 antibody (Bazil and Strominger 1991).

Generation of monocyte-derived DC obtained from the blood of healthy volunteers imposed a limit on the number of DC available for each experiment. 50 ml of blood provides, on average, 75 x 10^6 mononuclear cells, which yields about 2-3 x 10^6 DCs. This is sufficient to study 5-8 treatment groups in duplicates per experiment. Thus, it was important to establish a working concentration of HGF, which is high enough to induce an *in vitro* effect and comparable to that used by others for *in vitro* HGF work (Klominek, Baskin *et al.* 1998; Beilmann *et al.* 2000; Harvey, Clark *et al.* 2000), while also being in the range which can be found within the tumour environment. I chose 30 ng/ml HGF as the standard dose to be used in experiments in this thesis. There is considerable individual variation in the sensitivity of monocytes to HGF, and some of the experiments were carried out from 5-10 donors to establish a statistically significant effect, especially in the DC phenotypic experiments.

The effects of HGF differs on DC development in the co-treatment and in the pre-treatment models. HGF co-treatment results in DC with MHC Class II, CD80 and CD86 being down-regulated by about 25%, in a statistically significant manner, while HGF pre-treatment only affects CD86 levels. HGF co-treatment dose not have any other phenotypic effects. The effect of HGF on the co-stimulatory molecule expression of MDDC has not been tested before, as only CD14 and CD1a expression was studied and the level of these did not change (Rutella, Bonanno *et al.* 2006). The effect of MHC Class II and co-stimulatory molecule down-regulation is likely to lead to sub-optimal T cell stimulation; characterised by reduced IFNγ production by T cells, and decreased T cell proliferation due to reduced IL-2 production, (Van Gool,

Vandenberghe *et al.* 1996; Howard, Hope *et al.* 2002; Dilioglou, Cruse *et al.* 2003; Shin, Kennedy *et al.* 2003; Waeckerle-Men, Scandella *et al.* 2004; Greenwald, Freeman *et al.* 2005; Jen, Jain *et al.* 2006). Sub-optimal stimulation of T cells by impaired DC has been seen in studies with other agents including; direct blocking with neutralising antibodies (*in vitro* and *in vivo*), virus infection and also by cancer induced factors (Gajewski 1996; Saito, Yagita *et al.* 1996; Villegas, Wille *et al.* 2000; Moutaftsi, Mehl *et al.* 2002; Aalamian-Matheis, Chatta *et al.* 2007; Bae, Mitsiades *et al.* 2007).

The pre-treatment model, more interestingly, results in up-regulated CD209 expression on DC at the same or higher level than on control DC, but failure to down-regulate CD14. Cells with $CD209^{+}CD14^{+}$ phenotype can be observed in normal human skin where they are referred to as interstitial dendritic cells (Berges, Naujokat *et al.* 2005; Bechetoille, Andre *et al.* 2006). However CD209 expressing macrophages have also been reported in peripheral tissues (Granelli-Piperno, Pritsker *et al.* 2005; Rappocciolo, Jenkins *et al.* 2006; Ochoa, Loncaric *et al.* 2008). Recently $CD83^{+}CD209^{+}$ non dendritic APC, induced by IFNα from monocytes, have also been described (Gerlini, Mariotti *et al.* 2008), indicating a high level of functional plasticity of DC induced by different stimuli.

Maturation of pC-DC and pH-DC with LPS results in down-regulation of CD14 and CD209 expression and up-regulation of maturation markers, indicating that these pC-DC and pH-DC are more like DC than macrophages. pC-DC and pH-DC phagocytose antigen at a similar level to that observed in DC, and the ability of pC-DC to produce cytokines and to migrate towards lymphoid signals does not differ from DC. However, while HGF does not affect the ability of DC to phagocytose, it greatly increases IL-10 production and decreases migratory capacity of pH-DC. These findings, particularly the increased IL-10 production, indicate that pH-DC may be more similar to tolerogenic/regulatory DC (Buelens, Willems *et al.* 1995; Jonuleit, Schmitt *et al.* 2000; Steinbrink, Graulich *et al.* 2002; Enk 2005). A further finding also indicates that pH-DC resembles tolerogenic DC more than human skin interstitial DC. This finding that the up-regulation of PD-L1 expression on pH-iDC and its further up-regulation following DC maturation. PD-L1 expression is especially high on pH-DC which also express high levels of CD209 and CD14 molecules.

Thus I found that while HGF co-treatment has a relatively mild impairment of DC markers important in T cell stimulation, pre-treatment results in a population of DC-like cells with different characteristics from 'normal' DC. Some of the effects in the pre-treatment model can be considered additive, as delayed DC differentiation alone, (the control in this model (pC-DC)), is also observed to have considerable effects on DC phenotype. It is therefore important to ensure that HGF has a significant effect compared to both GM4 and delayed GM4 controls when analysing the results.

The effect of delayed DC differentiation alone may indicate that a default mechanism, protecting monocytes from becoming activated in the absence of pro-inflammatory stimuli may have a lasting effect on DC development. Further analysis of the maturation and LPS-induced cytokine expression revealed wider differences between pH-DC and pC-DC, indicating that exposure of monocytes to HGF before DC development has long lasting, HGF-specific effects which act synergistically with the effects of delayed GM4 application.

The mechanistic experiments revealed that IL-10 can be produced by monocytes without any treatment, while GM4 treatment alkmost completely stops IL-10 production. As HGF alone is also able to induce significant IL-10 production, it seems relatively easy to explain how HGF, in the pre-treatment model, is having a significant skewing effect on DC differentiation. Autocrine IL-10 production by monocytes has been observed before (Demangel, Bertolino *et al.* 2002; Chang-Rodriguez, Ecker *et al.* 2004; Raftery, Wieland *et al.* 2004; Samarasinghe, Tailor *et al.* 2006; Chang, Baumgarth *et al.* 2007). However, my findings indicate that the presence of cytokines and growth factors in the microenvironment before DC differentiation is triggered may determine the differentiation pathway of these cells. Similar effect of environmental cytokines on DC development has been suggested by (Zou and Tam 2002).

HGF impairs the ability of pre-treated DC to stimulate naïve T cell proliferation which can be recovered by inhibiting c-Met signalling. The ability of HGF to influence T cell stimulation has been reported in mouse models, particularly by preferentially enhancing T_H2-type responses and T_H2 cytokines (Kuroiwa, Kakishita

et al. 2001; Skibinski, Skibinska *et al.* 2001; Ito, Kanehiro *et al.* 2005; Okunishi, Dohi *et al.* 2007). All the major effects observed on pH-DC such as lower CD86 expression, increased IL-10 production and increased PD-L1 expression are known to affect T cell stimulation (Allavena, Piemonti *et al.* 1998; Hermans, Ritchie *et al.* 1999; Almand, Clark *et al.* 2001; Corinti, Albanesi *et al.* 2001; Battaglia, Stabilini *et al.* 2006; Seo, Seo *et al.* 2006; Sharpe, Wherry *et al.* 2007). However HGF pre-treatment does not affect IL-12 production and therefore suppression of T_H1 responses does not seem to occur by this mechanism. The up-regulation of IL-10 production, which is involved in the generation of T_H2 type responses, indicates that HGF's main effect is to skew DC development towards T_H2 type responses (Liu, Rich *et al.* 1998; Bellinghausen, Brand *et al.* 2001; Kuroiwa *et al.* 2001; Daly, Johnson *et al.* 2005; Ito *et al.* 2005; Okunishi *et al.* 2007; Chhabra, Chakraborty *et al.* 2008).

HGF also impairs the ability of DC to induce recall antigen induced cytokine production, measured by detecting IFNγ production by CD8 T cells. This effector mechanisms is also important in the efficient elimination of tumours (Rosendahl, Kristensson *et al.* 1998; Prevost-Blondel, Neuenhahn *et al.* 2000). Impaired HGF-mediated production of IFNγ has been reported in mouse models studying immune responses (Kuroiwa, Iwasaki *et al.* 2006; Okunishi *et al.* 2007). Both the reduction of CD86 and increased PD-L1 expression may contribute to this by sub-optimal activation of antigen specific T cell and also by inducing apoptosis, or anergy of activated T cells via PD-L1:PD-1 interactions. IL-10 production by CD4 T cells may also contribute to the inhibition of $CD8^+$ IFNγ production, by reducing the ability of T helper cells to contribute to CD8 T cell activation (Steinbrink, Wolfl *et al.* 1997; Steinbrink, Jonuleit *et al.* 1999; Corinti *et al.* 2001; Mcbride, Jung *et al.* 2002). The production of IL-10 by CD4 T cells also raises the possibility of pH-DC mediating immune suppressive effects by induction of Tregs. $CD4^+CD25^+Foxp3^+$ Tregs have been reported in mesothelioma pleural fluid and DC exposed to IL-10, and it maybe that HGF mediated production of IL-10 by DC contributes to the development of Treg in the pleural fluid (Delong, Carroll *et al.* 2005; Hegmans, Hemmes *et al.* 2006). Treg induction may also contribute to the inhibition of T cell activation mediated by HGF.

IL-10 also induces some of the same characteristics as pC-DC and pH-DC, such as phenotypic changes but with no effect on phagocytosis, and decreased T cell antigen

stimulation (Faulkner, Buchan *et al.* 2000; Fortsch, Rollinghoff *et al.* 2000; Mcbride *et al.* 2002). Therefore, HGF pre-treatment was compared IL-10 treatment to evaluate those effects mediated by IL-10 and those which are independent of IL-10. IL-10 induces, to some extent, development of $CD14^{+}CD209^{+}$ cells, and blocking of IL-10 in HGF treated monocytes cultures partially recovers the effects of HGF on DC phenotype. IL-10 blocking also recovers the T cell stimulatory capacity of pH-DC. Therefore, the suppression of antigen specific T cell responses by HGF is mediated by monocyte- and DC-derived IL-10.

It would be of interest to study the effects of HGF in vivo, however as both HGF and C-Met knockouts are embryonically lethal, studying the effects of HGF on immune functions in vivo would need to be accomplished by knocking out c-mET or DC-precursors, using C-Met/HGF or blacking antibodies or by using gene silencing mechanisms such as siRNA. Additionally as IL-10 production by by DC and T cells occurs it would be interesting to study the effects of HGF on DC and T cells in IL-10 knockout mice and using adoptive transfer of DC of T cells, would help to further elucidate the contribution to the immune suppression I have observed.

It would be useful using IL-10 or IL-10 receptor knock mice to investigate if HGF can suppress antigen recall responses and T cell proliferation and can $CD209^{+}CD14^{+}$ DC develop in the absence of IL-10? And does the up-regulation of CD14 or CD209 depend on IL-10 or is it independent. Additionally it would be of interest to investigate immune suppression by HGF in CD209, PD-L1 and PD-L2 knockout mice if these contribute to HGF induced DC:T cell inhibition, either by their up-regulation as in the case of PD-L1 and PD-L2 or by signalling as in the case of CD209. It would also be interesting to study the development of mesothelioma producing high levels of HGF in wild type mice and compare these to IL-10 knock-out mice. First to determine if analogs of CD14+CD209+ DC can be seen in both. Second to investigate what effects these have on the immune responses of these mice, and tumour development and progression. This could be compared to IL-10 knock out mice, mice treated with RNAi, small molecule inhibitors and blocking antibodies to inhibit c-Met or IL-10 function and to further examine the role of HGF in immune interactions.

Mesothelioma pleural fluid is high in HGF and it is capable of inducing cells with similar phenotype to those induced by human recombinant HGF, such as $CD209^{+}CD14^{+}$ cells. The development of $CD209^{+}CD14^{+}$ cells is inhibited using blocking of HGF by antibodies. Furthermore, pleural fluid from MPM, similarly to HGF is also able to up-regulate PD-L1 expression on healthy DC. Up-regulation of PD-L1 was only partially mediated by HGF. Pf induced DC also resemble tolerogenic/regulatory DC. This agrees with reports that mesothelioma pleural fluid contains cytokines and regulatory cells (Enk, Jonuleit *et al.* 1997; Mahnke, Schmitt *et al.* 2002; Gajewski, Meng *et al.* 2006; Hegmans *et al.* 2006). Analysis of cells recovered from pleural fluid revealed an unreported phenotype of cells, similar to those generated by pleural fluid, or by human recombinant HGF pre-treatment of healthy monocytes. These cells represent a significant proportion of the cells in the monocyte gate that express CD14. $CD209^{+}CD14^{+}$ cells also display increased PD-L1 expression. Most importantly the pleural fluid also inhibits the generation of T cell responses by Pf-treated DC, in a HGF-dependent manner. This demonstrates that the pleural fluid of MPM patients can mediate suppression of T cell responses induced by DC, by affecting DC development. It also demonstrates that this immuno-suppression is mediated, at least in part, by HGF which is elevated in MPM-derived Pf as it can be prevented by blocking HGF.

The role of HGF in cancer is of interest and a number of drugs aimed at c-Met are undergoing clinical trials, such as PF2341066, CE-355621 and PHA665752, small molecule inhibitors of c-Met. The small molecule RTK-inhibitor XL880 (Exelixis) is being evaluated in patients with advanced solid tumors, including papillary renal carcinoma and gastric cancer (Christensen, Zou *et al.* 2007; Zou, Li *et al.* 2007; Chandrani Chattopadhyay 2008; Tseng, Kang *et al.* 2008; Yang, Wislez *et al.* 2008). It would be of interest to see whether systemic blocking of c-Met results in any immunological effects, which may, in the light of the work presented here, be further exploited for optimising new treatments of cancer.

The main finding in this thesis is that HGF induces DC-like cells with $CD209^{+}CD14^{+}CD86/CD80^{med}MHCII^{high}IL\text{-}10^{high}PD\text{-}L1^{high}$ phenotype. These DC are able to phenotypically mature, but have reduced T cell stimulatory capacity, and generates a T_H2 bias. $CD14^{+}$ cells with similar phenotype can be found in the tumour

microenvironment (e.g. MPM pleural fluid) where high levels of HGF are present. Neutralisation of HGF (both recombinant human and Pf associated HGF) or IL-10 which is the mediator of the HGF effect can recover the ability of DC to induce T cells response and partially recovers DC phenotype.

The work presented here provides an insight into the potential immunological effects of HGF. The results describe basic observations and provide some mechanistic explanation, but they also raise further questions which I did not have time to explore. To mention a few:

- Can $CD14^{+}CD209^{+}$ DC be induced by other mechanisms and can HGF pre-treatment affect DC development using other mechanisms of developing human DC such as using $CD34^{+}$ cells or using buffy coat preparations?
- What is the effect of HGF on DC developed from monocytes by stimuli other than GM4 (e.g. high concentration GM-CSF, which is used in immunotherapy)? What other tumour factors can in this model system effect DC development?
- Do $CD209^{+}CD14^{+}$ and $CD209^{+}CD14^{-}$ mature to the same state? Are $CD209^{+}CD14^{+}$ immature DC solely inhibitory in nature and is CD14 expression, by DC, a marker of inhibitory/suppressive function? Can $CD209^{+}CD14^{+}$ DC be isolated from health donor and cancer patient perpheral blood, as well, as tumour effusions? Does $CD209^{+}CD14^{+}$ expression by Tumour infiltrating DC correlate with prognosis?
- Is IL-10 the sole mechanism by which HGF has suppressive effects on DC:T cells? Do IL-10 knockout *in vivo* models reduce of eliminate the effects of HGF on DC and T cells?
- Do pH-DC generate functionally active Treg cells? It would be interesting to investigate if HGF pre-treated DC induce Treg phenotype in T cells and whether this effect is HGF dependent and if it is, is the HGF dependence also dependent of HGF induced IL-10 production? Additionally if HGF pre-treated DC do induce Treg development can this also be observed using patient pleural fluid and do HGF inhibitors (such as inhibitor SU11274) and HGF blocking antibodies inhibit this effect of plural fluid in vitro? Also can high

HGF levels in vivo models of mesothelioma occur and do the correlate with increased levels of Tregs?

- Does high expression of CD209 on pH-DC when these DC encounter tumour cells, induce/enhance T_H2 skewing? It would be interesting to determine the exact effect of HGF on the full T_H1 and T_H2 cytokine profiles of DC and T cells using a cytokine bead array. It would also be useful to sort DC depednign on CD209/CD14 expression profiles and determine the contributions of CD209+CD14- and CD209+CD14+ DC, to IL-10 and IL-12 production by DC upon maturation and the possible skewing of T cell responses from T_H1 to T_H2. It would also be interesting to determine what contribution these two phenotypes play to stimulation or suppression of T cell proliferation.
- Does PD-L1 expression on pH-DC play a role in inhibition of T cell function? To follow up on the finding of up-regulation of PD-L1 expression separation, blocking experiments with PD-1 and PD-L1 blocking antibodies would resolve the role of PD-L1 up-regulation on T cell inhibition by pH-DC. Also experiments to characterise the phenotype of PD-L1+ cells do they express CD14? Also does HGF effect he expression PD-L2 and does this also contribute to T cell inhibition?
- It would be interesting to investigate the prognostic value of CD209+CD14+ DC in cancers and auto-immune diseases. Also can analysis of T responses, DC numbers in peripheral blood (including and excluding CD14+ DC) can be correlated with increased HGF levels in mesothelioma pleural fluid and plasma, by looking immune responses of 3-4000 mesothelioma patients, analysing their immune responsiveness and HGF levels at diagnosis and through treatment, can this be correlated with disease progression or prognosis? I would also be interested in starting a program of work investigating in other cancers (with increased HGF expression) does; T cell responses, T cell-IL-10 production, T_H1/T_H2 balance, the levels of IL-10 in the tumour microenvironment or number of circulating DC be correlated with HGF production by tumours in either microenvironment of systemically. Also I would like to investigate study both patients and animasl models does HGF act at a systemic level to suppress the immune system or is it more localised in

its affects. And are these localised effects mediated by its affects on DC development?

- I would like to further explore HGF, IL-10 histamine and other molecules that generate CD14+ DC and the functionally of DC and immune responses in cancers expressing these molecules and also other disease such as EBV, HCMV which may use this mechanism to evade immune recognition.

Taken together, HGF in MPM pleural fluid and possibly in other tumour microenvironments is likely to contribute to tumour immune escape by supporting the development of $CD209^{+}CD14^{+}CD86/CD80^{med}MHCII^{high}IL\text{-}10^{high}PD\text{-}L1^{high}$ DC-like cells, resulting in a reduced ability of tumour-exposed DC to stimulate anti-tumour T cells responses.

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