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Modification of dendritic cell phenotype and function:

Consequences for allergic asthma

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
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MODULATION OF DENDRITIC CELL PHENOTYPE AND FUNCTION: CONSEQUENCES FOR ALLERGIC ASTHMA

By Angelica Maria Cazaly

Allergic asthma is a potentially fatal disease. In developed countries the prevalence of atopic asthma has almost doubled over the past 20 years. The symptoms of allergic asthma are associated with an ongoing airway inflammation characterised by activated Th2 cells. T cell activation requires antigen-specific interaction with an antigen presenting cell (APC). Dendritic cells (DCs) are professional APCs that are able to stimulate naïve, as well as primed T cells. During antigen-specific interaction with T cells, the DC provides i) support for T cell survival, ii) surface molecules enhancing T cell activation and iii) soluble factors influencing the route of T cell differentiation. The ability of the DC to efficiently activate T cells and dictate their differentiation is strongly influenced by environmental factors. In this thesis, the hypothesis addressed is that *components within the local environment in the allergic asthmatic lung, including IgE and elevated levels of Th2 cytokines, histamine, MIP-1α and PGD_2, change a DC in a way that would perpetuate allergic inflammation.*

The initial work aimed to characterise monocyte-derived dendritic cells (Mo-DCs) from normal and asthmatic subjects to identify inherited differences with possible relevance for allergic asthma. It was found that Mo-DCs from asthmatic subjects expressed significantly lower levels of CD23 at their surface and produced more IL-6 than Mo-DCs from normal subjects.

A primary screen was then developed to address the hypothesis. The primary screen aimed to identify individual agents found at elevated levels in the asthmatic lung, referred to as allergic mediators, that significantly change the phenotype or function of Mo-DCs. Further in-depth investigation of their effects on Mo-DCs and possible relevance in allergic asthma would then be conducted. The effects of allergic mediators on Mo-DCs from both normal and asthmatic subjects were studied. The experimental approach taken showed that the Mo-DC phenotype and function was significantly changed in response to TNF-α (the positive control), IFN-γ, IL-3, IL-5 and IgE, but not in response to MIP-1α, histamine, PGD_2 and IL-13. Four areas of particular interest were identified. 1) Phenotypically mature IFN-γ treated Mo-DCs failed to significantly enhance T cell proliferation (further investigated in Chapter 5). 2) CD23 disappeared from the surface of Mo-DCs in response to TNF-α, IFN-γ and IgE (further investigated in Chapter 6). 3) IL-3 and IL-5 frequently induced a mature Mo-DC phenotype. 4) Mo-DCs from normal and asthmatic subjects responded differently to allergic mediators.

The aim of the work presented in Chapter 5 was to investigate possible mechanisms underlying the failure of phenotypically mature IFN-γ treated Mo-DCs to enhance T cell proliferation to a level expected by mature DCs. The results obtained suggested that the ability of IFN-γ treated Mo-DCs to drive T cell proliferation may be impaired due to 1) fewer viable Mo-DCs surviving during the proliferation assay, and 2) suppression of T cell proliferation by TGF-β1 present in the Mo-DC : T cell co-cultures. The reason for the reduced viability of IFN-γ treated Mo-DCs was not identified, however, was shown not to be due to Fas- or NO-mediated apoptosis.

The aim of the work presented in Chapter 6 was to identify the mechanisms by which TNF-α, IFN-γ and 100 nM IgE downregulate CD23 on the surface of Mo-DCs. By investigating the kinetics of CD23 downregulation, CD23 internalisation, CD23 gene transcription, the cellular distribution of CD23 and shedding sCD23 it was shown that TNF-α and IFN-γ reduced CD23 surface expression by increasing shedding of sCD23 and by reducing the rate of appearance of new CD23 at the surface. In contrast, 100 nM IgE reduced CD23 surface expression by rapidly clearing surface expressed CD23 by internalisation and by blocking the exposure of new CD23 at the surface. The results also show that control Mo-DCs maintain a steady-state level of surface expressed CD23 by a balanced clearance of surface CD23, involving internalisation and shedding, and appearance of new CD23 at the surface.

The work presented in this thesis supports a role for DCs in allergic asthma. This was evident as mediators of an allergic environment changed DCs from both normal and asthmatic subjects in ways that could increase activation of T cells and other cells in the asthmatic lung. Evidence also suggested that DCs from asthmatic subjects are more prone to initiate or perpetuate the chronic allergic inflammation seen in asthmatic airways.

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Quotation

"Stress does not exist. It is only coping with a situation badly"

(Nicholas Cazaly, 1961 - , CSE art, 25 yard breast stroke)

For Angelica

By Jennifer Hayashida, March 17, 2001

In the beginning, there were sandwiches: another kind of science. Butter and bananas on whole wheat, carrot marmalade and cheese, cottage cheese and tomatoes on crip bread, cucumber and bell pepper. "This" she may say, "is a great combination," biting into a sandwich and marvelling at bananas and butter melting together on the tongue.

Such were here fanciful, pre-scientific concoctions.

When we were eight, she said dandelions were a surefire way to remove mud from pants. Determined, she rubbed each of my knees with yellow blossoms until large and muddy dandelion blotches decorated my overalls. On the way home, I was even more stained and unhappy, fearful of my mother's reaction, but still confident in her knowledge. If Angelica believed in it, I did too.

Today, when we are on the phone, she tells me she has to go: the timer has rung, and it's time to check on the Petri dishes. When I come to visit, she shows me the cells she works with: she calls them "her" cells, and it is as though they are part of a well-tended garden, buds ready to bloom. I look into the microscope, the quivering image of her universe: its amorphous shapes, its vast scope, its precision.

How brave she is, to not be afraid of all the questions in that universe, to see opportunity in the unknown. How fanciful and confident she must be: what good qualities as a scientist, and what good qualities as a friend.

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Many people have been involved and are held responsible for the progress and content of the work presented in this thesis.

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All poor blood donors deserve a medal, as none of this would have been possible without them. Special thanks to recurring poor souls including Nick, Will, Mark B. and John. At the same time I am very grateful to Zoe, Kathy and Jude who provided such professional stabbing service and who understood when I had to leave the room not to "dent" the floor with my forehead!

Thanks also to all the people on Level F who have put up with me on a daily basis and who have made my days smiley, in particular Tamara and Vanessa.

What would I have done without Jude? To sufficiently thank you I would have to hijack your ears for weeks! You have doubled as computer specialist, technical support, endless source of useless facts, confidence booster, bouncy-brain, carol singer and marriage councillor. Without doubt, the best friend on this side of the Nordic sea! Thank you!

Amanda, thank you for everything! I said I wanted a challenge, you gave me one and I have thoroughly enjoyed it! But who am I going to "bake" reports with now? I'll add 2 oz of flour, 3 eggs, a pinch of salt and water, but who will stir, add more salt and say "try again!"?

Thank you Clive for never failing to give problems a new interesting angle. I also have you to thank for where I am today as you believed in me and offered me a job back in 1996. You are the kindest man I know! (Sorry Nick!)

I also want to thank my Mum who gave me the courage to surpass my abilities. ♥

Nick. How do you say run? How do you say Y's? Where is the cheek and do you remember when we used to be like that? Neah, neither do I... thanks for being catering manager, chauffeur, great entertainer, stress reliever, completely useless, absolutely gorgeous and for marrying me. I hope you agree with me when I dedicate this thesis to My Sun.

Lots of love,
♥Jelly♥

Abbreviations

1n	1nM IgE
100n	100 nM IgE
4-1BBL	4-1BB ligand
7-AAD	7 aminoactinomycin D
AD	atopic dermatitis
AMP	adenosine monophosphate
APC	antigen presenting cell
B7RP-1	B7 related protein-1
BAL	bronchoalveolar lavage
beta c	β-chain of the IL-3, IL-5 and GM-CSF receptors
BSA	bovine serum albumin
cAMP	cyclic AMP
CD40L	CD40 ligand
cDNA	compementary DNA
c-FLIP$_L$	Fas-associated death domain-like IL-1β-converting enzyme-inhibitory protein ligand
CFSE	carboxyfluorescein-diacetate succinimidylester
CLA	cutaneous lymphocyte-associated antigen
cSMAC	central supramolecular activation clusters
CMP	counts per minute
CR2	complement receptor 2
C_T	threashold cycle value
CTLA-4	cytotoxic T lymphocyte antigen 4
DAG	diacylglycerol
DC	dendritic cell
DC-CK1	dendritic cell-derived CC chemokine
DC-SIGN	DC-specific ICAM-3 grabbing nonintegrin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DMSO	dimethylsulphoxide
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbant assay
Eo	eosinophils
ER	endoplasmic reticulum
EU	endotoxin units
Fab	fragment of antibody binding
FACS	fluorescence activated cell scanner
FADD	Fas associated protein with death domain
FasL	Fas ligand
FcεRI	high affinity receptor for immunoglobulin E
FcεRII	low affinity receptor for immunoglobulin E
Fcγ	IgG Fc fragments
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
gp96	glycoprotein 96
HEL	hen egg lysozyme
HEPES	N-[2-Hydroxyethylipiperazine-N'-[2-ethanesulphonic acid]]
Hist	histamine
HLA	human leukocyte antigen
HRP	horse radish peroxidase
HsAg	heat stable antigen
Hsp	heat shock protein
ICAM	intracellular adhesion molecule
ICOS	inducible co-stimulatory
IDDM	insulin-dependent diabetes mellitus
IDEC	inflammatory dendritic epidermal cells
IFN	interferon
IFN-γR	IFN-γ receptor
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G
Ii	transmembrane invariant chain
IL	interleukin
IL-3R	IL-3 receptor
ILT	immunoglobulin-like transcript receptor
IP_3	inositol-1,4,5-triphosphate
ITAM	immunoreceptor tyrosine based activation motif
ITIM	immunoreceptor tyrosine based inhibition motif
K_d	kilo dalton
KLH	keyhole limpet hemocyanin
LCs	Langerhans' cells
LFA	lymphocyte function associated antigen
L-NAME	Nomega-Nitro-L-Arginine Methyl Ester Hydrochloride
LPS	lipopolysaccharide
MIIC	MHC class II compartment
mAb	monoclonal antibody
MCP	monocyte chemotactic protein
M-CSF	macrophage colony stimulating factor
MDC	macrophage-derived chemokine
MEM	non essential amino acids
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLR	mixed lymphocyte reaction
Mo-DCs	monocyte-derived dendritic cells
MR	mannose receptor
mRNA	messenger RNA
MV	measle virus

NK-cell	natural killer cell
NO	nitric oxide
$NO_2^{\bullet}$	excited nitrogen dioxide
NO_2^-	nitrite
NOD	non-obese diabetes mouse
NOS	NO synthase
eNOS	endothelial NOS
iNOS	inducible NOS
nNOS	neural NOS
ODN	oligonucleotides
OVA	ovalbumin
OX40L	OX40 ligand
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed death - 1
PD-L	programmed death – ligand 1
PE	phycoerythrin
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PI	propidium iodide
PI 3-K	phosphatidylinositol 3'-hydroxyl kinase
PIP_2	phosphatidylinositol-4,5-biphosphate
PKC	protein kinase C
PLCγ1	phospholipase Cγ1
PMA	phorbol myristate acetate
PS	phosphatidylserine
PSGL	P-selectin glycoprotein ligand
pSMAC	peripheral supramolecular activation clusters
PTK	protein tyrosine kinase
RANTES	regulation on activation, normal T cell expressed and secreted
rh	recombinant human
RLV	Rauscher Leukemia Virus
RNA	ribonucleic acid
RPI	relative proliferation index
RRNA	ribosomal RNA
RRX	rhodamine red X
-RT	negative reverse transcription reaction
+RT	positive reverse transcription reaction
RT-PCR	reverse transcription-polymerase chain reaction
SAC	*Staphylococcus aureus* Cowan
sCD23	soluble CD23
sCD40L	soluble CD40L
sCD95	soluble CD95
SDF	stromal derived factor
SEM	standard error of the mean
SF	synovial fluid
sFasL	soluble Fas Ligand
TARC	thymus and activation-regulated chemokines
Tc	cytotoxic T cell
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
TMB	tetramethylbenzidine free base
TNF	tumour necrosis factor
Tr	regulatory T cell
TRANCE	tumour necrosis factor-related activation-induced cytokine
TRANCE-R	TRANCE receptor
UHQ	Ultra high quality
VCAM	vascular cell adhesion molecule

Chapter One

General Introduction and Hypothesis

1.1 The immune system

The immune system is an adaptive defence system that has evolved in vertebrates to protect them from invading pathogens and harmful foreign substances[1,2].
Cells of the immune system develop from a common bone-marrow derived stem cell under the influence of specific growth factors and cytokines, a process referred to as hematopoiesis (Figure 1.1). During early hematopoiesis, the pluripotent stem cell develop into a lymphoid or myeloid stem cell. The lymphoid stem cell has the capacity to further develop into T lymphocytes, B lymphocytes, natural killer cells (NK-cells) and lymphoid dendritic cells, whereas the myeloid stem cell can form granulocytes, including eosinophils, basophils, neutrophils and mast cells, and mononuclear cells, including monocytes, macrophages and myeloid dendritic cells. Platelets and red blood cells also descend from the myeloid stem cells.

The immune system contains a non-specific and a specific component for eliminating pathogens[2]. The non-specific component, also referred to as innate immunity, involve structures and cells that do not distinguish between different pathogens. Physical and anatomical barriers as well as the complement system and phagocytic cells, e.g. macrophages, belong to this branch of the immune system. In contrast, through specific or acquired immunity, the ability has developed to specifically recognise and consequently eliminate invading pathogens or foreign harmful substances using the most efficient means. Cells such as lymphocytes and antigen presenting cells (APCs) play an important role in acquired immunity.

The immune responses can further be divided into humoral and cell-mediated responses. Whereas the humoral immune response involves elimination of the invading pathogen or substance by interaction with antigen-specific antibodies produced by activated B cells, the cell-mediated immune response leads to direct eradication of pathogens or pathogen infected cells via cells of the immune system, such as T lymphocytes and NK-cells.

Although segregated into innate versus acquired, and humoral versus cell-mediated immune responses, all components of the immune system cooperate to defend the organism. For example, products secreted by cells of the acquired immune response, e.g. cytokines and antibodies, augment the activity of the innate immune response, and at the same time phagocytes are intimately involved in the activation of acquired immunity. This makes the immune system highly complex, yet effective for protection against disease.

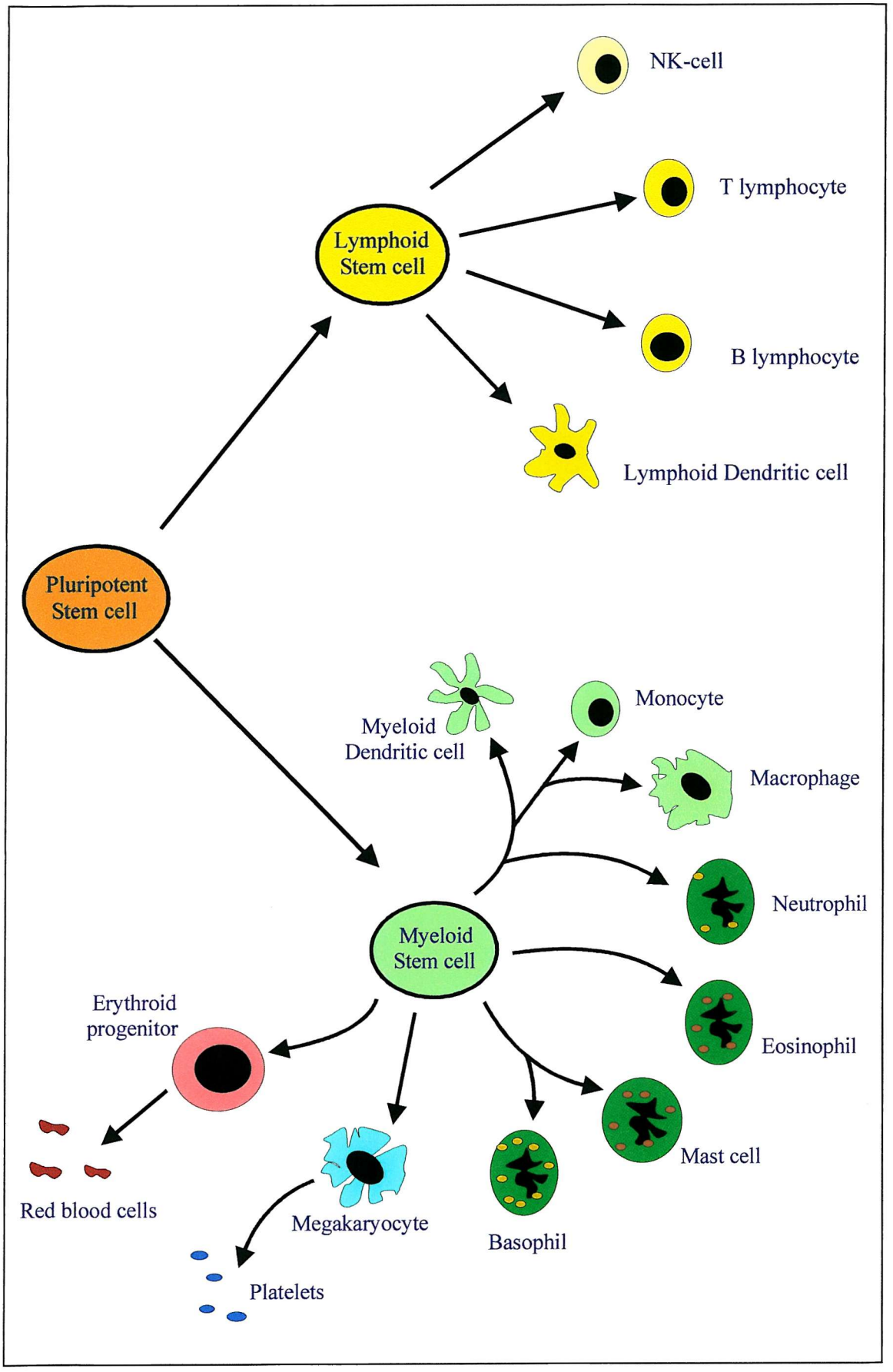

Figure 1.1 Cells of the immune system

1.2 T lymphocytes

1.2.1 Role in acquired immunity

The total human peripheral T lymphocyte pool consists of three compartments; naïve, memory and effector T cells. While the naïve T cell pool maintain an overall constant size throughout life, the memory T cell pool expands with age and the effector T cell pool expands dramatically upon activation followed by apoptosis or development into memory T cells[3].

During the primary immune response i.e. the first encounter of the immune system with an antigen, CD4 expressing ($CD4^+$) and $CD8^+$ naïve T cells may respond by initiating their development and differentiation into effector cell subsets, a process which normally is completed within 4-5 days following antigen exposure [4]. Further, activation of these antigen-specific effector cells leads to enormous proliferation, a process referred to as clonal expansion. Accumulated $CD8^+$ effector T cells acquire cytotoxic functions enabling specific destruction of infected target cells and hence participate in cell-mediated immunity. These cells are termed cytotoxic T cells (Tc) and differentiate into two known subsets, Tc1 or Tc2. The $CD4^+$ effector T cells develop the capacity to direct responses of various cell types and hence indirectly participate in both cell-mediated and humoral immunity. These cells are the helper T cells (Th) and differentiate into at least four subsets, Th0, Th1, Th2 or Th3, distinguished by their cytokine production profile.

Effector cells are also derived from the memory T cell compartment upon re-exposure to antigen during the secondary immune response. The secondary response is faster and stronger than the primary response due to: (i) more rapid development of effector cells from $CD4^+$ and $CD8^+$ memory T cells than from naïve T cells, (ii) higher frequency of antigen-specific cells as a result of clonal expansion during the primary immune response,(iii) a less strict requirement for costimulation, (iv) larger quantities of cytokines secreted and (v) activation triggered at lower antigen concentrations. The memory T cells maintain the polarised cytokine production pattern established following effector cell deviation during the primary immune response [3]. This further contributes to quick onset of the memory T cell-derived effector immune response. However, the memory effector T cell cytokine profile is not stable and may change under the influence of strong polarising cytokines, e.g. interleukin-12 (IL-12) [5].

1.2.2 T cell activation

For activation, T cells must interact with an APC resulting in an array of signalling events mediated through numerous T cell surface receptors. Following the establishment of T cell - APC contact, a process known as immunological synapse formation, the most critical signal for T cell activation is established. This is mediated through the antigen-specific T cell receptor (TCR) and the CD3 complex following binding to major histcompatibility complex (MHC) expressed on the APC, and is referred to as "signal 1". However, for complete T cell activation, amplification of the TCR-mediated signal is required. Such amplification signals, referred to as "signal 2", are provided by interaction with costimulatory as well as adhesion molecules expressed on APCs and T cells.

1.2.2.1 The immunological synapse

The immunological synapse is an area that forms at the point of interaction between a T cell and an APC that has a defined pattern of surface molecules specialised for T cell activation [6]. The plasma membrane of inactive T cells show an even distribution of these surface molecules, associated with distinct lipid structures called rafts. These rafts undergo actin-mediated polarisation to one area of the lymphocyte under the influence of chemokines, enabling synapse formation [7]. During immunological synapse formation, leukocyte function-associated antigen-1 (LFA-1) expressed on the T cell, first ligates intracellular adhesion molecule-1 (ICAM-1) on the APC, in the centre of the immature immunological synapse [6]. At this stage, referred to as junction formation, the TCR scans the APC surface for MHC-peptide complexes. During the second stage, the TCR/MHC complexes take the place of the LFA-1/ICAM-1 complexes at the centre of the mature immunological synapse. The transport of TCR/MHC to the centre and LFA-1/ICAM-1 to the periphery is also actin-mediated and has been shown to depend not only on the nature of the peptide and the ligation of LFA-1 to ICAM-1, but also on the ligation of CD28 to CD80/CD86 [6,8,9]. The last stage of immunological synapse formation is stabilisation of the TCR-MHC complexes in the central cluster, (central supramolecular activation clusters or cSMAC), and adhesion and costimulatory molecules in the outer ring surrounding this, referred to as peripheral SMAC (pSMAC)[6,9](Figure 1.2).

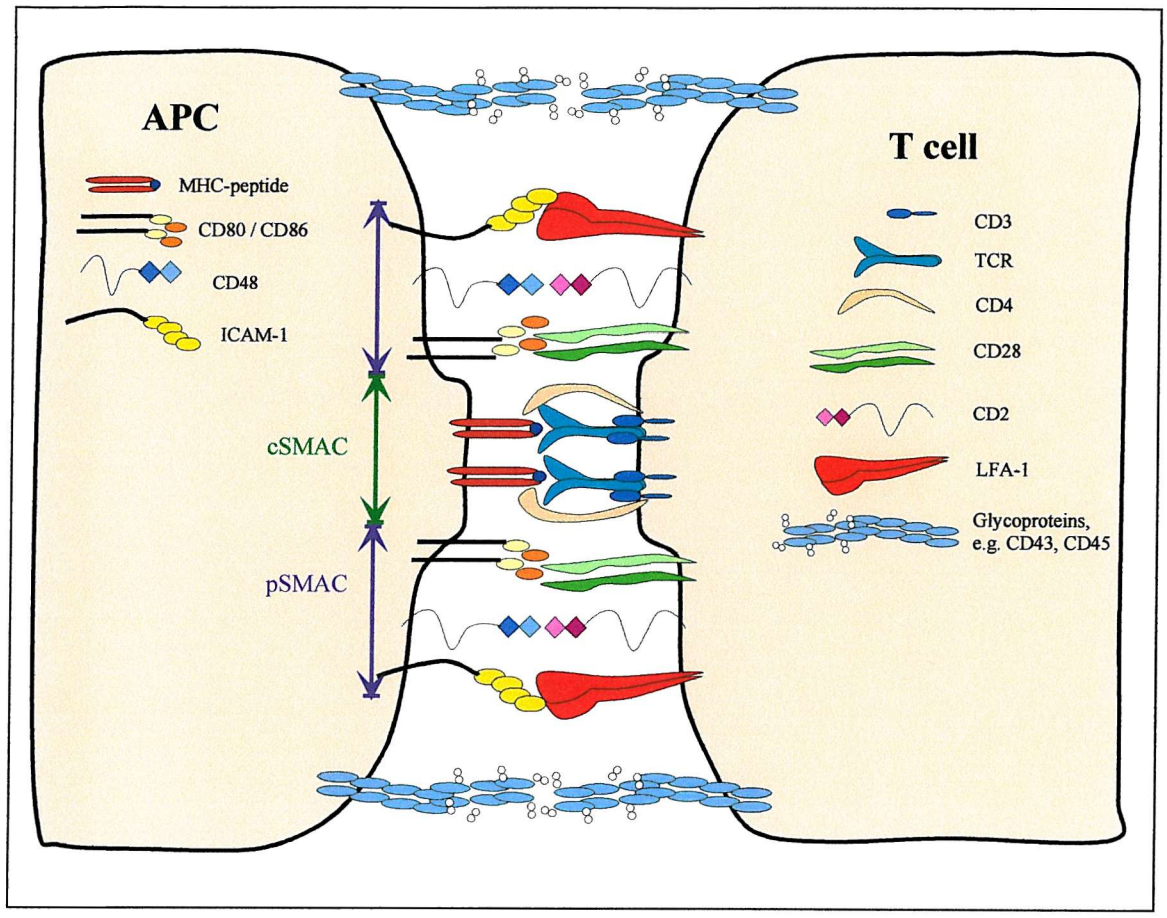

Figure 1.2 The immunological synapse

In the stabilised immunological synapse, TCR-MHC complexes are located in the centre of the synapse (cSMAC) surrounded by adhesion and costimulatory molecules (pSMAC). Bulky and charged glycoproteins are excluded from the immunological synapse.

1.2.2.2 "Signal 1": T cell receptor / CD3 complex – mediated signals

The TCR-mediated "signal 1" occurs during the early stages of immunological synapse formation as the TCR recognises the MHC-peptide complex on the APC.

The TCR is a heterodimer most commonly comprising an α and β chain but also existing as a γδ dimer. Cell surface TCR is associated with a group of polypeptides collectively known as CD3. CD3 comprises 3 single chains (α, δ, ε) and a ξξ dimer. Following ligation of the TCR/CD3-complex by MHC-peptide, the protein tyrosine kinase (PTK) lck phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 ξ-chain leading to the recruitment and activation of ZAP-70 [7,10]. In turn, ZAP-70 phosphorylates several adapter proteins leading to the recruitment of for example phospholipase Cγ 1 (PLCγ1) and phosphatidylinositol 3'-hydroxyl kinase (PI 3-K) to the TCR/CD3-complex. TCR-mediated activation ultimately leads to Ca^{2+} flux and intracellular accumulation of inositol-1,-4,-5 triphosphate (IP_3) and diacylglycerol (DAG). However, the requirement of additional signals provided by costimulatory molecules for T cell activation is evident as it has been reported that only in the additional presence of CD28-mediated signaling does the PI 3-K become fully activated [10].

1.2.2.3 "Signal 2": Costimulatory molecules in T cell activation

It has been suggested that the primary role of adhesion and costimulatory molecules may be to amplify the TCR signal by recruiting lck containing lipid rafts to the immune synapse resulting in enhanced phosphorylation of substrates[11]. Although LFA-1 and ICAM-1 and CD28 and CD80/CD86 are the only adhesion and costimulatory molecules for which effects on immunological synapse formation have been described [8,12], CD2/CD48 complexes have been identified at increased density in the immunological synapse and hence may be involved in amplifying the TCR signal (Figure 1.2). Several other adhesion and costimulatory molecules have also been suggested to be involved in T cell activation [13]. Examples of such molecules are (i) CD40 on APCs and CD40 ligand (CD40L or CD154) on T cells, (ii) 4-1BB ligand (4-1BBL) on APCs and 4-1BB (CD137) on activated T cells [13], (iii) OX40 ligand (OX40L) on APCs and OX40 (CD134) on activated T cells [13] and (iv) B7 related protein-1 (B7RP-1) on APC and inducible co-stimulatory (ICOS) molecule on activated T cells[14]. The CD80/CD86-CD28/ cytotoxic T lymphocyte antigen 4 (CTLA-4)

and the CD40-CD40L costimulatory pathway in T cell activation have been extensively investigated over the past two decades and are therefore further reviewed below.

1.2.2.3.1 Costimulation by members of the B7-family: interaction of CD80 / CD86 with CD28 / CTLA-4, and other B7 costimulatory pairs

CD28 is a transmembrane disulfide-linked homodimeric glycoprotein constitutively expressed on most $CD4^+$ T cells and on 50 % of the $CD8^+$ T cells in humans [15]. CD28 binds members of the B7-family, including CD80 and CD86 expressed on APCs[14] (Table 1.1). CD80 was the first CD28 ligand to be described[16]. Although CD80 shares many structural similarities with CD86, these receptors differ in size and only share 23 % amino acid sequence homology in humans [17]. The cytoplasmic domain of both molecules contains three potential sites for protein kinase C (PKC) phosphorylation, possibly suggesting the involvement of both CD80 and CD86 in signal transduction in the APC [15-17].

The most apparent consequence of CD28 ligation by CD80 and CD86 is T cell activation and cytokine production, in particular high levels of IL-2[13] (Table 1.1). CD28 ligation further prevents cell death through upregulation of bcl-X_L, a gene associated with protection against apoptosis [15]. Following 1-3 days of CD28 ligation, a second receptor for CD80/CD86, CTLA-4 (or CD152) is expressed on the surface of the T cell [15]. CTLA-4 indirectly downregulates the CD80/CD86-induced T cell response as CD80 and CD86 have a higher affinity for CTLA-4 than for CD28, resulting in downregulation of bcl-X_L expression and increased T cell apoptosis [15].

Recently, new members of the B7-family have been identified that bind to equally novel receptors on T cells[14] (Table 1.1). In contrast to CD28, the T cell expressed receptors for these novel B7 molecules, namely ICOS, programmed death - 1 (PD-1) and the receptor for B7-H3, are only expressed on T cells following T cell activation[18-20]. Ligation of these receptors either support or inhibit CD3-mediated T cell activation *in vitro* (Table 1.1). T cell activation is supported by signalling via ICOS, that in contrast to CD28-mediated costimulation results in production of IL-4 and IL-10[18]. Similarly, B7-H3 provides positive costimulation and preferentially promotes the production of high levels of interferon-γ (IFN-γ)[20]. On the other hand, binding of programmed death – ligand 1 (PD-L1) or PD-L2 to PD-1 have been shown to suppress T cell proliferation at optimal[21,22], but not suboptimal CD3-stimulation[23] when PD-1 signalling instead leads to T cell proliferation and IL-10

production. At suboptimal CD3-stimulation, a third PD-1 ligand, B7-DC, also supports T cell proliferation, however, this preferentially induces IFN-γ rather than IL-10 production [24].

Table 1.1 Costimulatory molecule pairs of the B7-family

APC expressed B7 molecules	T cell expressed B7 receptors	Effect on T cell activation	Effect on T cell cytokine production
CD80 / CD86	CD28	↑	IL-2
CD80 / CD86	CTLA-4	↓	N/A
B7RP-1	ICOS	↑	IL-4, IL-10
B7-H3	unknown	↑	IFN-γ
B7-DC	PD-1	↑	IFN-γ
PD-L1	PD-1	$\uparrow^{+}$ / $\downarrow^{++}$	IL-10^{+} / N/A^{++}
PD-L2	PD-1	$\uparrow^{+}$ / $\downarrow^{++}$	IL-10^{+} / N/A^{++}

+ Optimal CD3-stimulation

++ Suboptimal CD3-stimulation

1.2.2.3.2 CD40-CD40L interaction

CD40L (CD154), also referred to as gp39, is a 39 kDa type II membrane glycoprotein with extensive sequence homology with tumour necrosis factor-α (TNF-α) [25]. CD40L is predominantly expressed on activated $CD4^{+}$ T cells but also on some $CD8^{+}$ T cells [25]. CD40L binds CD40, a 50 kDa glycoprotein of the TNF-α receptor superfamily [25] expressed on APCs such as dendritic cells (DCs), B cells, monocytes and macrophages [26,27]. CD40L surface expression is regulated via CD28-mediated signals, that stabilise the CD40L mRNA resulting in quicker CD40L translation and enhanced T cell activation through increased interaction with CD40 on APCs [15,28]. T cell activation is further enhanced as CD40-mediated signaling in APCs results in increased surface expression of CD80 and CD86 [15].

At least three signaling pathways are activated following CD40L ligation. The Rac 1 and JNK/p38-K signaling pathway and the PLCγ1 / PKC signaling pathway are both activated via lck [29]. As following TCR/CD3 ligation, CD40-mediated activation of PLCγ1 / PKC leads to an increase in intracellular IP_3 and Ca^{2+} flux, further emphasising the close connection of signals mediated via TCR and costimulatory molecules. A third tyrosine-

kinase independent CD40 signaling pathway is mediated via sphingomyelinase, resulting in the production of ceramide [29]. CD40L-mediated signaling in T cells eventually leads to CD40L downregulation and internalisation, terminating all CD40-CD40L interaction [29].

1.2.3 T cell polarisation

Polarisation of $CD4^+$ Th cells was first demonstrated in mice where the Th1 and Th2 subsets were characterised by their cytokine profile [30]. Even though the cytokine profiles from specific Th subsets in mice are far more rigid than the cytokine profiles from Th subsets in humans [31], cross-production of cytokines between subsets in response to pathogens of variable origin occurs, that adds to the complexity of T helper cell deviation. However, in general the murine Th1 subset is identified as cells preferentially producing IL-2, IFN-γ, TNF-β, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 in response to viral infection or intracellular pathogens [2]. These cytokines are involved in activation of cytotoxic T cells, thus the Th1 subset is indirectly involved in cell-mediated immunity. The murine Th2 subset preferentially produces IL-3, IL-4, IL-5, IL-10 and IL-13 in response to extracellular microorganisms and parasites. Th2 cytokines are involved in humoral immunity through activation of, for example antibody producing B cells. Recently a third Th subset, designated Th3 has been identified that shows many phenotypic similarities to the Th2 subset. However, Th3 cells differ in production of high levels of transforming growth factor-β (TGF-β). TGF-β is capable of suppressing T cell proliferation, hence the alternative nomenclature for Th3 cells as the immunoregulatory Th subset (Tr1). The human Th1 and Th2 cytokine profiles are similar to those found in mice although IL-10 production has been detected in both subsets [31]. IL-10 has further been shown to induce tolerance in humans by suppressing antigen presenting functions [32,33]. Hence, IL-10 is possibly better described as an immunoregulatory cytokine belonging to the Th3/Tr1 subset, although this subset is not well defined in humans.

The cytokines present during Th cell activation strongly influence T cell differentiation. For example IL-12, released from activated macrophages and DCs, has the ability to control Th1 differentiation via the IL-12 receptor expressed on T cells [34]. On the other hand IL-4 is the most efficient promoter of Th2 cell differentiation. Once polarised, the differentiation state is further reinforced by cross-regulation as cytokines from one T cell subset inhibit the development of the opposite T cell subset[35]. Thus, IL-4 and IL-10 have

been shown to inhibit the production of IL-12 and also IFN-γ[36], and conversely IFN-γ inhibits the differentiation of the Th2 subset[37,38].

Several other factors are able to direct differentiation of Th cells into either the Th0, Th1, Th2 or Th3/Tr1 subset including the nature of the antigen, its dose and APC dependent factors [39,40].

1.2.3.1 Antigen presenting cell-dependent T cell polarisation - "signal 3"

Recent literature indicates that information on the nature of the pathogen encountered by an APC in the periphery is provided to the naïve T cells in lymphoid organs not only via the pathogen derived peptide (signal 1) in conjunction with adequate costimulatory molecules (signal 2) but also via a T cell polarising signal 3. The T cell polarising signal 3 has been suggested to be dependent on the type and origin of the APC as well as the nature of costimulation provided during Th activation and the APC cytokine profile.

1.2.3.1.1 The antigen presenting cell type and origin

The significance of the type and origin of the APC on T cell polarisation has been emphasised both in murine and human *in vitro* culture systems. In mice, B cells and macrophages were found to drive the differentiation of Th0 cells in the presence or absence of exogenous IL-2[41]. However, Th0 cells generated in the presence of IL-2 were found to produce significantly more IFN-γ when restimulated with B cells and significantly more IL-4 when restimulated with macrophages. This may suggest that during a secondary immune response, B cells and macrophages preferentially promote skewing of Th1 and Th2 cells, respectively. Further, peripheral blood DCs of the myeloid lineage ($CD11c^+$) (see section 1.3.1.1) and lymphoid lineage ($CD11c^-$) (see section 1.3.1.2) preferentially induced Th1 and Th2 differentiation, respectively[42]. Similarly, *in vitro* cultured monocyte-derived dendritic cells (Mo-DCs) and DCs derived from $CD4^+$, $CD11c^-$, $CD3^-$ plasmacytoid T cells have been shown to preferentially drive Th1 and Th2 differentiation, respectively [43]. However, although these DC lineages have their own potential to induce a predisposed Th response, they can all change their Th driving capacity under the influence of certain environmental factors[42]. $CD11c^+$ peripheral blood DCs treated with IFN-α were shown to reduce the level

of IFN-γ and increase the level of IL-10 produced by allogeneic T cells in a mixed lymphocyte reaction (MLR), indicating a change in these DCs from supporting Th1 to Tr1 development[42]. Further, mature Mo-DCs, that were shown to promote both Th1 and Th2 differentiation at high DC : T cell ratios, supported Th2 differentiation at lower DC : T cell ratios [44].

In vivo, APC are constantly influenced by surrounding cells and environmental factors as well as being present at varying numbers, and are therefore more likely able to drive several different Th responses.

1.2.3.1.2 The nature of antigen presenting cell costimulation

Costimulatory molecules may also differentially affect T cell differentiation following activation. In this respect much attention has focused on the role of CD80 and CD86 in driving Th1 or Th2 differentiation. However, no consistent trend for CD80 or CD86 driving Th1 or Th2 has been identified, as some reports suggest that usage of CD80 leads to Th1 differentiation and CD86 to Th2 differentiation[45-47], whereas others suggest the reverse[48] or even no CD80 or CD86 dependency for T cell differentiation[49,50].

Other members of the B7-family have also been suggested to promote distinct T cell differentiation based on the T cell cytokine pattern they induce *in vitro*. By inducing IL-4 and IL-10 production in T cells, B7RP-1 has been suggested to drive Th2 differentiation following binding to ICOS[14,18]. By inducing IL-10 production, PD-L1 has been suggested to drive Tr1 differentiation following binding to PD-1 [23]. Further, Th1 development was suggested as ample IFN-γ production was evident following both binding of B7-H3 to its receptor[20] and following B7-DC binding to PD-1[24].

Costimulatory molecules other than those belonging to the B7-family have also been reported to skew T cell differentiation, including 4-1BBL and OX40L. Whereas T cells stimulated via 4-1BB have been shown to produce both Th1[51] and Th2[52] cytokines depending on the culture system used, several studies indicate that T cells stimulated via OX40 preferentially produce Th2 cytokines [44,53,54].

1.2.3.1.3 Influence of pathogens on antigen presenting cell cytokine profile

Using human Mo-DCs as a model system, it has been demonstrated that the nature of the APC dependent "signal 3" is highly influenced by pathogen-derived factors, or pathogen-induced tissue responses, influencing the immature Mo-DC to adjust the level of cytokine produced, especially IL-12, following stimulation [55] (Figure 1.3).

Prostanoids have been suggested to promote Th2 differentiation as Mo-DCs derived in the presence of prostaglandin E_2 (PGE_2) show reduced IL-12 production [56]. These Mo-DCs induce naïve T cells to secrete a Th2 cytokine profile in co-culture, including high levels of IL-4 and IL-5 and low levels of IFN-γ. A similar response to PGE_2 was observed when added to maturing Mo-DCs in combination with low levels of IL-1β and TNF-α [57]. Under these circumstances, PGE_2 was shown to synergise with IL-1β and TNF-α to induce cyclic AMP (cAMP)-dependent Mo-DC maturation. It has been suggested that other mediators, either derived from or induced by pathogens, that increase the levels cAMP in APCs may have similar Th2 skewing properties. In accordance, cholera toxin was recently shown to increase the levels of cAMP in Mo-DCs, resulting in reduced IL-12p70 production following treatment with CD40L, and the preferential development of Th2 cells[58]. Another substance predicted to have similar effects on cAMP and Th2 skewing is histamine[55].

IL-10 is another cytokine that has been suggested to influence APCs to drive Th2 differentiation. It has been shown that Mo-DCs exposed to IL-10 in combination with IL-1β and TNF-α have reduced IL-12 p70 production [57]. Furthermore, IL-10 treated murine DCs have been demonstrated to induce Th2 differentiation [59]. However, IL-10 treated Mo-DCs are more commonly regarded to be involved in tolerance induction as immature Mo-DCs exposed to IL-10 show reduced capacity to stimulate $CD4^+$ T cells in an allogeneic MLR [33,57]. Furthermore, these T cells show suppressed IFN-γ and IL-2 production, indicative of T cell anergy [33].

Type I IFNs (IFN-α and IFN-β), produced in response to some viral infections, have recently been shown to increase the IL-10 production by myeloid $CD11c^+$ peripheral blood DCs[42]. These IFN-α treated DCs were further suggested to drive the development of Tr1 cells producing high IL-10 and low IFN-γ levels. Type I IFNs have also been shown to inhibit Mo-DC p40 IL-12 secretion resulting in reduced T cell IFN-γ production [60]. In contrast, type II IFN (IFN-γ) has been shown to promote the production of IL-12 p70 in $CD11c^+$ peripheral blood DCs leading to the development of Th1 cells as indicated by

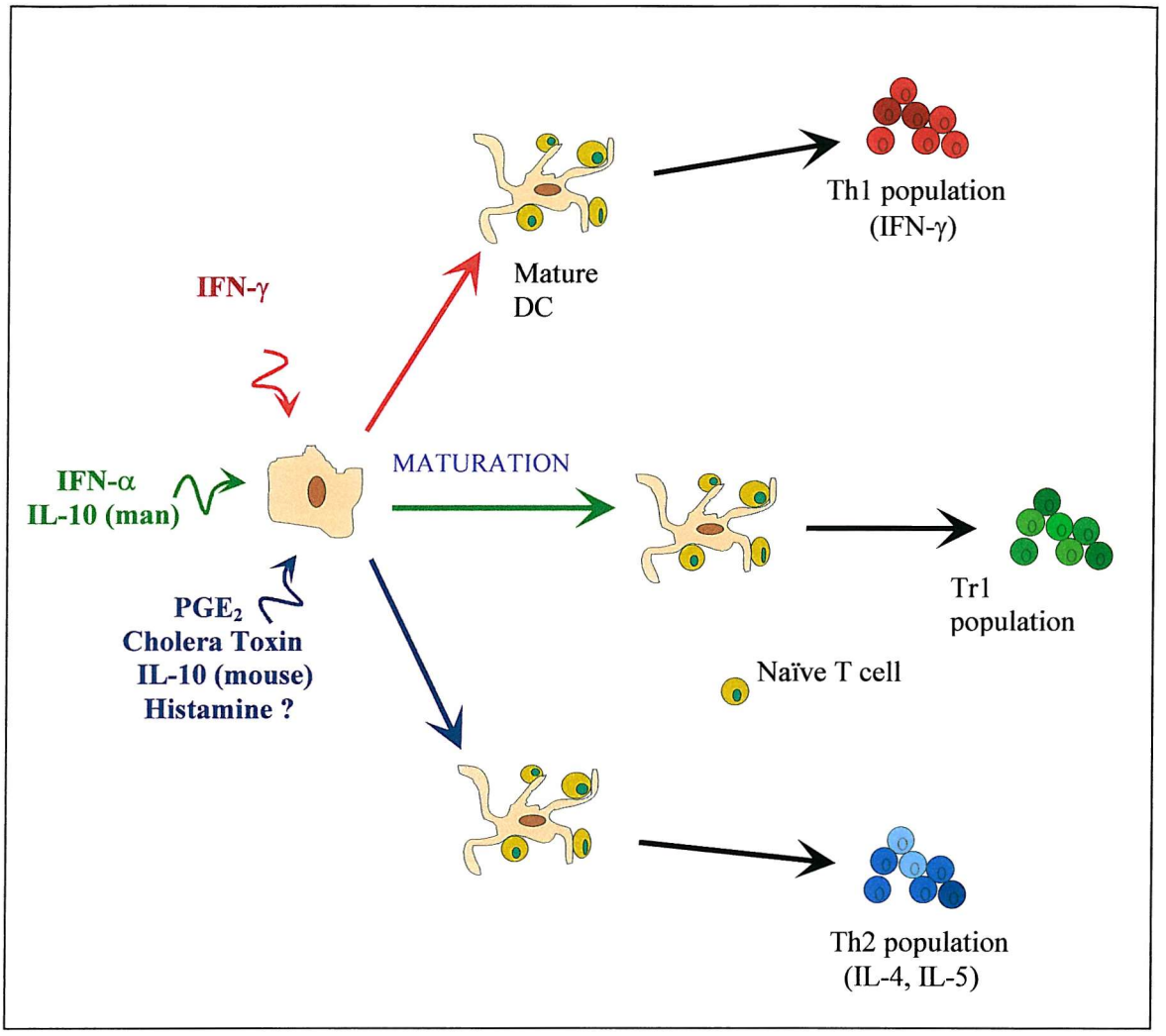

Figure 1.3 APC and pathogen-dependent shaping of signal 3

The T cell polarising signal 3 is influenced by pathogen-derived factors, or pathogen-induced tissue responses that alter the cytokine profile produced by APCs.

increased IFN-γ and decreased IL-4 and IL-10 production [42]. The presence of IFN-γ during lipopolysaccharide (LPS)- or IL-1β / TNF-α-induced maturation of Mo-DCs has also been demonstrated to enhance IL-12 production following CD40 ligation [61].

Common to IL-10, PGE_2 and IFN-γ is the inability to effect IL-12 production by terminally mature Mo-DCs [57,61]. Collectively, this suggests a mechanism by which DCs and possibly other APCs are protected from further environmental influences on the way to or in lymphoid organs. Thus, the effects of pathogen-derived and induced factors on APCs could be restricted to the periphery.

Although the current literature suggests that APCs respond to factors from or induced by pathogens primarily by altering their IL-12 production, and thus direct a cell-mediated (Th1) or humoral (Th2) immune response, other cytokines produced by APCs may contribute to T cell polarisation. For example, murine spleen DCs produce IL-4 following phagocytosis of hyphae[62]. These DCs further showed a concomitant reduction in IL-12 p70 production and were found to drive Th2 differentiation of naïve T cells. Similar effects on T cell differentiation may be predicted by Rauscher Leukemia Virus (RLV) treated bone marrow-derived murine DCs that also were shown to produce IL-4 but only low levels of IL-12 [63]. It is therefore possible that pathogen derived, or induced factors in addition regulate the levels of IL-4 produced by Mo-DCs.

1.3 The Dendritic Cell

Different types of APCs vary in their ability to activate T cells given an equal amount of antigen. This is due to differences in for example the ability to take up and process antigen, the ability to migrating to lymph nodes to present antigen, the surface density and type of costimulatory and adhesion molecules, the surface density of MHC and differential production of T cell chemoattractants for establishment of cell-cell interaction. On the basis of these criteria, DCs have been proposed to be the most efficient APC type[64].

1.3.1 Dendritic cell origin and tissue distribution

DCs are bone marrow-derived professional APCs that are widely distributed throughout lymphoid and non-lymphoid tissues as well as existing in the circulation[65].

Human and murine *in vitro* studies have provided a large body of evidence supporting the existence of two DC lineages, myeloid and lymphoid. Both DC precursors

arise from the $CD34^+$ pluripotent haematopoitic stem cells, but differ in their anatomical location, phenotype, function and the cytokines required for differentiation.

1.3.1.1 The myeloid dendritic cell lineage

Human myeloid DCs can be divided into interstitial (e.g. dermal DCs) and intraepithelial (e.g. Langerhans' cells (LCs))[66]. Whereas immature LCs are selectively localised to the epidermis, immature interstitial DCs are found in other non-lymphoid tissues [67]. Myeloid DCs are migratory cells that following activation can be found in the T cell zones of lymphoid organs[66]. Myeloid DCs are therefore thought to be involved in the induction of immune responses to antigen taken up in the peripheral tissues [68].

In the presence of GM-CSF and TNF-α, $CD34^+$ stem cells differentiate into $CD13^+$, $CD33^+$, $CD11c^+$, $CD123^-$ myeloid DCs *in vitro* [66,68]. A proportion of these myeloid DCs also express Birbeck granules, a characteristic of LCs. This may be explained by the existence of two subsets of DC precursors in peripheral blood characterised by their expression of cutaneous lymphocyte-associated antigen (CLA) [69]. In the presence of GM-CSF and TNF-α the CLA^+, $CD34^+$ and the CLA^-, $CD34^+$ subsets have been shown to differentiate into Birbeck $granule^+$ LCs and Birbeck $granule^-$ dermal DCs, respectively[69]. LCs also develop from $CD34^+$ stem cell in the presence of GM-CSF, TNF-α and TGF-β *in vitro* [65]. Myeloid DCs have been suggested to be closely related to monocytes. When cultured *in vitro* in the presence of GM-CSF and IL-4 or IL-13 monocytes have been shown to develop DC-like morphology and immature phenotype [70-73]. Monocytes also develop LC-like characteristics when cultured *in vitro* in the presence of GM-CSF, IL-4 and TGF-β[74]. Evidence suggesting that the monocyte may be a DC precursor *in vivo* has been published. It was shown that human monocytes diverge towards DCs *in vitro* following migration across the endothelium in the abluminal-to-luminal direction, a process referred to as reverse transmigration [75]. Reverse transmigration corresponds to movement of cells from the tissue to the lymphatics *in vivo*. Further, peripheral murine monocytes have been shown to migrate to lymph nodes and differentiation in to DCs *in vivo* following phagocytosis of injected microsphers [76].

Murine myeloid DCs can be divided into $CD8\alpha^-$ DCs and Birbeck $granule^+$ LCs [66]. As in man, immature murine LCs and $CD8\alpha^-$ DCs are localised in the epidermis and the marginal zones of the spleen, respectively, and move to the T cell zones of secondary lymphoid organs following activation.

1.3.1.2 The lymphoid dendritic cell lineage

Lymphoid DCs are non-migratory cells found in the thymus and spleen. It is therefore not surprising that the best characterised lymphoid DCs are the $CD8\alpha^+$, DEC-205^+, $CD11c^+$ thymic and splenic DCs of murine origin. However, evidence supporting the existence of a human lymphoid counterpart, termed plasmacytoid DCs, has been reported.

In human tonsils, the $CD4^+$, $CD11c^-$, $CD3^-$ plasmacytoid T cell has been shown to develop DC morphology and phenotype following *in vitro* culture with IL-3 and CD40L [77]. Similarly, IL-3 was shown to induce a mature DC-like phenotype and support the survival of a $CD1a^-$, $CD11c^-$, $CD123^+$ population isolated from the blood [42]. IL-3 also induced a DC-like morphology of an immunoglobulin-like transcript receptor 3^+ ($ILT3^+$), $ILT1^-$, $CD123^+$ plasmacytoid monocyte population isolated from blood [78].

Lymphoid DCs have been suggested to be involved in tolerance induction, as murine $CD8\alpha^+$ DCs isolated from the T cell areas of secondary lymphoid tissue express MHC occupied with self-peptides and induce proliferation followed by apoptosis of self-peptide specific T cell clones *in vitro* [79]. $CD8\alpha^+$ spleen DCs have also been reported to induce Fas-dependent apoptosis of $CD4^+$ T cells and to reduce proliferation of $CD8^+$ T cells by inducing suboptimal IL-2 production [80]. Further, human plasmacytoid DCs have been reported to produce high levels of IFN-α[42,78] that *in vitro* has been shown to make myeloid DCs preferentially promote the differentiation of IL-10 producing Tr1 cells [42].

1.3.2 Dendritic cell life cycle - role of the local microenvironment

The life cycle of a DC involves two functional stages, antigen capturing in the tissue and antigen presentation to T cells in the lymphoid organs, or in the tissue if inflamed. The transition between the two functional stages is characterised by DC activation or maturation (Figure 1.4). In the case of a primary immune response, it is further essential that the antigen is carried from the peripheral site to naïve T cells in regional lymphnodes, ensured by migration of the maturing DC. Both DC maturation and migration are influenced by environmental factors.

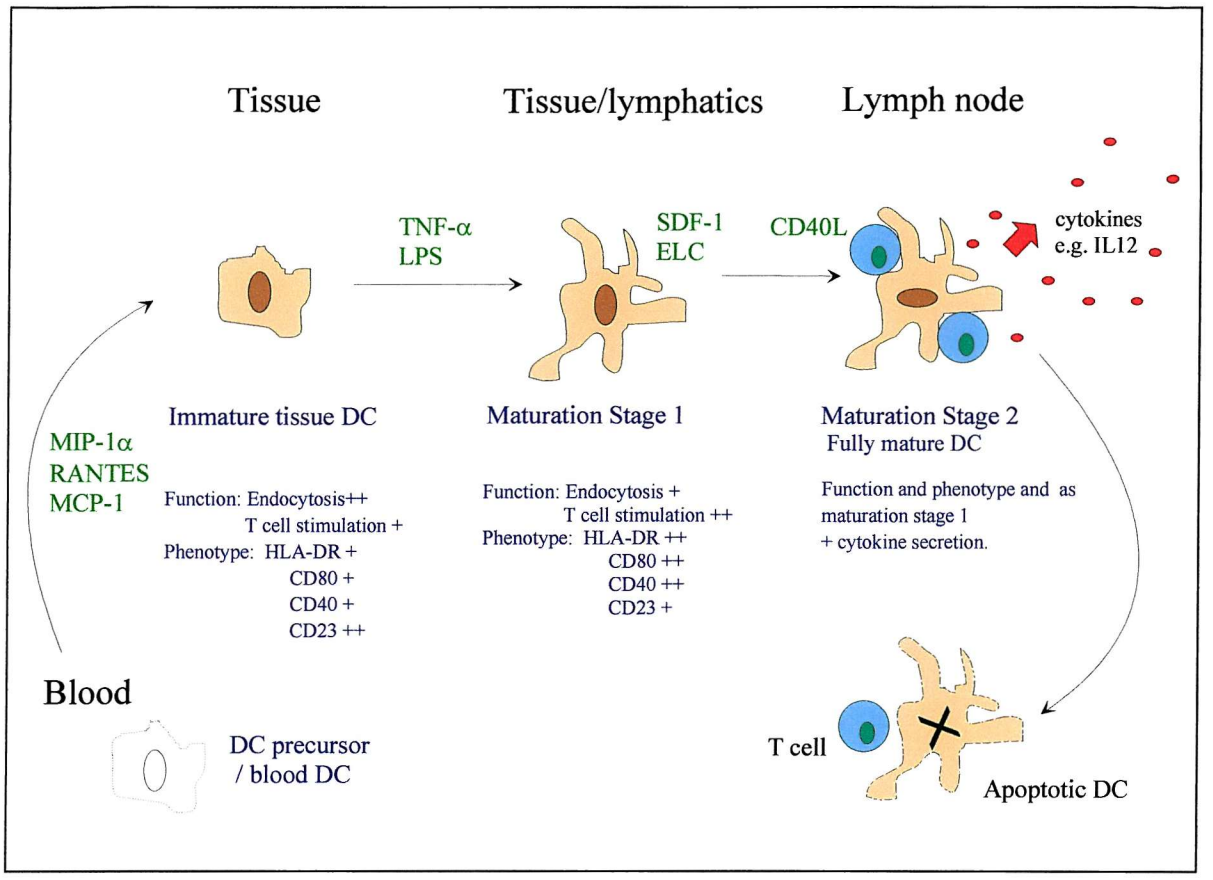

Figure 1.4 Dendritic cell life cycle

The DC life-cycle is under strong influence of environmental factors including chemokines for recruiting DC precursors from the blood to the tissue and migration from tissue to lymph nodes via lymphatics, and inflammatory mediators such as TNF-α and LPS for induction of maturation.

1.3.2.1 Immature tissue dendritic cells - phenotype and function

In the peripheral tissues DCs are situated predominantly at the epithelial interfaces with the external environment, where they constantly sample antigens by several antigen uptake mechanisms, including macropinocytosis, receptor-mediated endocytosis and phagocytosis. Accordingly, these DCs show high surface expression of molecules involved in receptor-mediated endocytosis e.g. mannose-receptor (MR)[81], CD32[82], CD64[83], FcεRI[82,84] and DEC-205[85] (the murine equivalent of the human MR). In the event of tissue inflammation, immature DCs are exposed to GM-CSF, produced by endothelium and epithelium, to inflammatory mediators such as TNF-α [72,86] and IL-1β[87], fungi [62], microbial products such as LPS[88], bacterial toxins[58] and nucleic acids[89], that can all initiate DC maturation (see 1.3.2.2). Some of these mediators also initiate immigration of blood DCs or DC progenitors from the circulation by increasing the expression of E-, and P-selectins on the surface of the endothelium lining the blood vessels, to which blood DCs expressing P-selectin glycoprotein ligand 1 (PSGL-1) can tether [90]. From here, DCs expressing CC-chemokine receptors respond to inflammatory chemokines, including macrophage inflammatory protein 1α (MIP-1α)*, monocytes chemotactic protein 1 (MCP-1) and regulated on activation of normal T cell expressed and secreted (RANTES), produced by various cells in the inflamed tissue [91]. The production of these chemokines is also regulated by inflammatory mediators [91,92].

1.3.2.2 Initiation of dendritic cell maturation and emigration by inflammatory mediators

To initiate an immune response the DC needs to be activated. DC maturation characterises DC activation and is induced by mediators that announce infection, stress or damage to cells[93]. These mediators are therefore referred to as "danger signals" and include for example the inflammatory mediators, microbial products and viruses mentioned in section 1.3.2.1. DC maturation is characterised by upregulation of cell surface MHC class II, CD80, CD86, CD40, CD54 and CD58 [81,94]. These "danger signals" also induce the

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* A new nomenclature for chemokines has recently be introduced by Zlotnik *et. al.*[297]. However, throughout the thesis all chemokines will be referred to by their old nomenclature with a footnote indicating their new name. MIP-1α (CCL3), MCP-1 (CCL2), RANTES (CCL5).

secretion of inflammatory chemokines by DCs resulting in autocrine downregulation of inflammatory chemokine receptors [91]. Concurrently, surface expression of CXCR4 and CCR7 are increased on maturing DCs, enhancing their responsiveness to the constitutive chemokines stromal derived factor 1 (SDF-1)* and MIP-3β, respectively [95-97]. Whereas SDF-1 is constitutively expressed in a broad range of tissues [98] the expression of MIP-3β is restricted to lymph nodes, thymus and appendix [99]. This suggests an important role for MIP-3β in directing the migration of maturing DCs from the inflamed tissues to the T cell-dependent regions of lymph nodes, where antigen presentation and terminal differentiation of the DC takes place.

1.3.2.3 T cell-dependent terminal differentiation of dendritic cells

The maturing and migrating DC reaches the lymph node in a semi-mature state expressing high levels of surface molecules for optimal antigen-presentation. Surface molecules involved in antigen capturing are now completely downregulated. Terminal maturation of the DC is accomplished following CD40L encounter during T cell interaction in the lymph node [100].

CD40 ligation stimulates the production of several soluble mediators by DCs, including IL-12[101], IL-8, TNF-α and MIP-1α[26]. CD40 ligation also stimulates further upregulation of surface molecules including HLA-DR, CD54, CD58, CD80 and CD86[26,102,103]. These are final adjustments that contribute to the shaping of a superior antigen presenting cell. Moreover, CD40 ligation results in upregulation of bcl-2 and bcl-X_L, two genes involved in the prevention of cell death, prolonging the interaction between the DC and the T cell [104].

1.3.2.4 Dendritic cell apoptosis

DCs have not been identified in the efferent lymphatics leaving secondary lymphoid organs suggesting that following antigen presentation to T cells DCs die within the lymph node. The route by which DCs apoptose *in vivo* is currently uncertain. Although several *in vitro* studies show that both human [105,106]and murine [107]DCs are resistant to Fas-mediated apoptosis, Fas-mediated signaling has been suggested to play a role in the induction of

* SDF-1 (CXCL12), MIP-3β (CCL19), MIP-1α (CCL3)[297]

apoptosis of murine splenic DCs incubated with keyhole limpet hemocyanin (KLH) and a KLH-specific Th1 clone[108]. Further, Fas-resistance in Mo-DCs has been shown to be reversible *in vitro* under the influence of measles virus [109]. Therefore, Fas-mediated apoptosis cannot be excluded as a mechanism for DC clearance *in vivo* where many cell types and environmental factors influence the DC.

1.3.3 Dendritic cells as professional antigen presenting cells

DCs are the only APCs able of T cell priming. Several qualities contribute to their potency as professional antigen presenting cells. Highly efficient antigen uptake, processing and presenting mechanisms ensure maximal availability of MHC-peptide complexes for TCR-ligation. The nature and level of costimulatory and adhesion molecules facilitates full T cell activation and have in addition been suggested to direct Th cell differentiation. Finally, DCs are very susceptible to changes in the pathogen-influenced microenvironment, altering the DC production of chemokines and cytokines, enabling them to make contact with naïve and primed T cells and dictate the immune response by directing T cell differentiation.

1.3.3.1 Efficient antigen uptake

Immature DCs have been shown to be 100-300 times more efficient in antigen uptake than B cells or peripheral blood mononuclear cells (PBMCs)[81]. The immature DCs efficiently capture antigen non-specifically through bulk uptake of extracellular fluid by macropinocytosis. The estimated fluid uptake via macropinocytosis is 3 000 μm^3/hour, due to very active membrane ruffling in DCs [81]. In DCs the antigens contained in macropinosomes fuse with lysosomal compartments where the antigen is concentrated as a result membrane recycling back to the cell surface and loss of water and permeable compounds through the compartment membrane. DC macropinocytosis is constitutive in contrast to other macropinocytic cells, such as macrophages and epithelial cells, where growth factors regulate non-specific antigen uptake[81].

Immature DC expresses receptors involved in receptor-mediated endocytosis, including DEC-205 (murine DCs)[85], MR[72,81] and Fc receptors such as FcγRII (CD32), FcγRI (CD64) [110] and FcεRI[84]. MR and DEC-205 belong to the C-type lectin family sharing

27 % sequence homology [85,111]. As C-type lectins, MR and DEC-205 are specialised for the uptake of carbohydrate containing particles, such as yeast [85,111]. Fcγ and Fcε receptors have the potential of capturing antigens via immunoglobulin G (IgG) and IgE, respectively, at low concentration via coated pits, vesicles and endosomes [84,112,113]. Recently, murine DCs have also been found to take up peptides associated with the heat shock protein (hsp) glycoprotein 96 (gp96) via surface expressed CD91[114]. This route of antigen uptake was found very efficient for antigen presentation via MHC class I[115].

In addition, immature DCs capture large particles and apoptotic cells by phagocytosis. Phagocytosis of apoptotic cells by DCs is predominantly mediated by αvβ5 integrin and CD36 and has been shown to support presentation of exogenous antigens to cytotoxic T cells[116], a function attributed to phagocytosing DCs but not to phagocytosing macrophages. Phagocytosis has also been shown to promote efficient antigen presentation via MHC class I in a growth-factor dependent DC line, D1[117]. D1 cells were shown to present ovalbumin (OVA) in association with MHC class I 10^6 fold more efficiently following phagocytosis of OVA-expressing bacteria as compared to uptake of soluble OVA [117]. Following phagocytosis of bacteria, D1 cells demonstrated a more mature phenotype including upregulation of MHC class I and II, CD80, CD86 and CD40. Similarly, D1 maturation was also observed following phagocytosis of high numbers of apoptotic cells [118], possibly as a direct consequence of TNF-α and IL-1β production by these phagocytic DCs.

1.3.3.2 MHC class II peptide loading and redistribution for efficient antigen presentation

Mature DCs efficiently activate naïve and primed $CD4^+$ T cells by presenting peptides from exogenous proteins in association with MHC class II. DCs differ from other APCs by requiring maturation for efficient formation and surface transport of MHC class II - peptide complexes[119]. Regulation of MHC class II expression and consequences of this for antigen presentation have been studied *in vitro* using human Mo-DCs[120,121] as well as murine bone-marrow derived DCs[122,123].

Synthesis of MHC class II is high in both murine and human immature DCs. Human immature DCs, the newly synthesised MHC class II molecule is first express on the cell surface. It is then not associated with peptide but the invariant chain (Ii) [121]. Shortly after surface exposure, the MHC-class II-Ii complexes are internalised into endosomal compartments, possibly MHC class II compartment (MIIC)-like organelles, where Ii is

degraded by the cysteine protease cathepsin S and replaced by peptide [124]. Peptide-loaded MHC class II then recycle back to the cell surface where it is present for a short period of time before it is internalised and degraded by proteases [120]. Consequently, immature human DCs only briefly expose the peptide for activation of primed T cells. In contrast, murine immature DCs retain newly synthesised MHC class II - Ii complexes in lysosomes [123]. During maturation murine DCs redistribute these MHC II - Ii complexes to peripherally located endocytic vesicles where Ii is degraded by cathepsin S and MHC class II loaded with peptide[123]. MHC class II – peptide complexes are then expressed on the surface of the mature murine DC [122]. Maturation further increases MHC class II synthesis and antigen processing and loading in both murine and human DCs, resulting in increased MHC class II -peptide surface expression[119]. In human mature DCs the half-life of the MHC class II - peptide complex on the cell surface is extended 10-fold during DC maturation due to reduced endocytosis [120]. Consequently, the peptide on murine and human DCs is presented during a prolonged period of time allowing the DC to migrate to the regional lymph nodes where it presents the peptide to naïve T cells. Moreover, DCs are capable of serial receptor triggering allowing stimulation of several TCRs by the same MHC class II - peptide complex [125].

Immature DCs further differ from other APCs by presenting empty MHC class II at their surface[126]. *In vitro*, these empty MHC class II molecules have been shown to bind peptide and to facilitate antigen-specific proliferation of T cells[126]. Immature DCs also secrete proteases capable of protein processing[127] and express functional HLA-DM molecules on their surface[128], that in endosomes assist in peptide loading. This may suggest that DCs are able to bypass MHC class II internalisation for antigen presentation.

1.3.3.3 MHC class I and cross-presentation

Mature DCs efficiently activate naïve and primed $CD8^+$ T cells by presenting peptides from endogenous proteins in association with MHC class I. However, as the only APC capable of presenting antigen to naïve T cells, DCs also initiate cytotoxic immune responses to peptides derived from exogenous proteins in association with MHC class I. This process is referred to as cross-priming[119]. Cross-priming is crucial for evoking immune responses to for example tumour cells or virally infected nonhematopoietic cells. To allow cross-priming, exogenous antigens must be internalised and presented on MHC class I, a process referred to as cross-presentation. Antigen uptake via phagocytosis[129],

macropinocytosis[130], hsp-protein[115] as well as Fc receptors[131] have all been shown to support cross-presentation in DCs. However, it is not clear where MHC class I is loaded with peptide for cross-presentation as evidence exist for peptide-loading in endosomes as well as in endoplasmic reticulum (ER)[119]. Peptide-loading is perhaps more likely to take place in the ER as this is the conventional site for association of endogenous peptide with MHC class I and since DCs show constitutive transport of antigen from endosomes to the cytoplasm[132] where endogenous antigens classically are processed by proteasomes.

1.3.3.4 Adhesion and costimulatory molecules

Although most adhesion and costimulatory molecules expressed by DCs are also expressed on other APCs, an adhesion molecule with high affinity for ICAM-3 was recently shown to be selectively expressed by human DCs *in vivo* and *in vitro*[133]. This surface molecule, called DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) was found to mediate clustering as well as proliferation of ICAM-3$^+$ resting, but not activated T cells. The selective expression of DC-SIGN to DCs may therefore contribute to making the DC the only APC capable of initiating a primary immune response. Mature DCs are also better suited for activation of naïve T cells than other APCs as they present peptide-loaded MHC in association with CD86 on their surface [134]. These clusters of MHC and CD86 may thus enhance the formation of the immunological synapse.

The density of commonly expressed costimulatory molecules, in particular CD80 and CD86, has been shown to be a critical factor in determining the efficiency of T cell activation by human APCs. It has been proposed that APCs with high levels of surface CD80 and / or CD86 are more efficient activators of T cells than APCs with low CD80 and / or CD86 density [73]. The density of adhesion molecules and MHC class II has been suggested to have negligible effects on antigen presentation in comparison to the density of CD80 and CD86. This was exemplified in macrophages that were poor APCs despite expressing high levels of adhesion molecules, and in cultured DCs that expressed higher levels of MHC class II than freshly isolated mature DCs, yet possess similar antigen presenting capacity [73].

Costimulatory molecules are not only important for activation of T cells but also for their differentiation, as already discussed (1.2.3.1.2). In this respect, by expressing a specific range or higher levels of certain costimulatory molecules, DCs may have an advantage over other APCs in shaping a specific T cell response. B7-DC is an example of such a

costimulatory molecules as it is preferentially expressed on DCs and has been found to induce high IFN-γ production in T cells following binding to PD-1 [24].

1.3.3.5 Chemokine production by dendritic cells

Chemokines are proteins with a molecular weight of between 8 - 14 kDa that via interaction with transmembrane G protein-coupled receptors are primarily involved in cell trafficking [135]. The chemokines are divided into subgroups according to the spacing of conserved cysteine motifs in the NH_2-terminus and are hence termed CC, CXC, C or CX3C chemokines[135,136]. Chemokines are also functionally categorised into constitutive chemokines, predominantly expressed in secondary lymphoid organs[137-139], and inflammatory chemokines, produced following exposure to inflammatory stimuli such as TNF-α [139].

Human DCs produce several inflammatory and constitutive chemokines of both the CC and CXC families that facilitate the attraction and interaction with T cells. Together with other APCs, DCs produce the inflammatory chemokines MIP-1α*, MIP-1β, RANTES[139] and MCP-1[140]. However, other chemokines are more specifically produced by DCs and may hence contribute to making the DC a superior APC. These include the constitutive chemokines thymus and activation-regulated chemokines (TARC) [139], dendritic cell-derived CC chemokine (DC-CK1) [141], macrophage-derived chemokines (MDC)[142] and MIP-3β[140]. Although constitutively expressed, the production of these chemokines can be further increased following DC maturation as was shown for MDC, TARC and MIP-3β following stimulation with LPS[140].

The primary function of DC-produced chemokines is to assist in establishing contact between DCs and T cells. In this role, DC-CK1 and MIP-3β have been suggested to be of particular importance for initiation of the primary immune response by bringing naïve T cells together with mature DCs[95,97,141,143]. DC-CK1 selectively attracts $CD45RA^+$ naïve $CD4^+$ and $CD8^+$ T-cells via binding to an unidentified receptor [141], whereas MIP-3β binds to CCR7 expressed on naïve T cells [144,145]. Accordingly, naïve T cells respond to MIP-3β *in vitro* [139,140,143]. Other DC-produced chemokines may in addition to establishing DC : T cell contact also favour the expansion of a specific T cell subset by selectively recruiting Th1 or Th2 cells. For example, MDC and TARC bind to CCR4 selectively expressed on Th2 cells

* MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), MCP-1 (CCL2), TARC (CCL17), DC-CK1 (CCL18), MDC (CCL22), MIP-3β (CCL19) [297]

[144,146] and have been shown to specifically attract Th2 cells *in vitro* [147,148]. Recent literature has also reported on the role of chemokines in driving the differentiation of Th1 or Th2 cells[149]. It was shown that activation of $CD4^+$ T cells in the presence of MCP-1 or MIP-1α resulted in increased incidence of IL-4 or IFN-γ producing cells, respectively[150]. Since DCs have been shown to produce both MCP-1 and MIP-1α, T cells differentiation may be directly influenced by DC produced chemokines.

1.3.3.6 Cytokines produced by dendritic cells

Cytokines strongly influences T cell differentiation. Both human and murine DCs, of various origins and maturation states, have been reported to produce numerous cytokines. Although most cytokines produced by human DCs are induced upon maturation, immature Mo-DCs can secrete low levels of IL-6[151], TNF-α [152] and IL-8[78]. The production of these cytokines by Mo-DCs is increased following stimulation with either LPS[152] or CD40L[43,153], two maturation stimuli that also induce the secretion of IL-1α, IL-1β[43], IL-10, IL-12 p70[152], IL-18[154] and IL-23[155]. Mo-DCs have also been shown to secrete low levels of IL-15 following stimulation with influenza virus[78]. In contrast, the DC-like plasmacytoid monocytes cultured in IL-3 and stimulated with the same virus, instead produced high levels of type I IFN[78]. In addition to these cytokines, human DCs have the potential to influence T cell activation and differentiation through elaboration of other cytokines that using RT-PCR have been shown to be transcribed *in vitro*. These cytokines include TGF-β1, detected in both $CD34^+$-derived DCs[32] and Mo-DCs[156], and M-CSF and GM-CSF, both detected in $CD34^+$-derived DCs relating to epidermal LCs and dermal DCs[32]. $CD34^+$-derived LCs and $CD34^+$-derived dermal DCs also express IL-13 mRNA following stimulation with phorbol myristate acetate (PMA)[32].

Most of the cytokines produced by human DCs have also been identified in murine DCs. However, to date murine DCs differ from human DCs by their production of IFN-γ[157] and IL-4[62,63], two cytokines with potentially very high impact on T cell differentiation.

In the following sections DC-expressed cytokines of particular importance for T cell differentiation are reviewed.

1.3.3.6.1 Interleukin-6

Human DCs produce high levels of IL-6 in response to inflammatory stimuli such as LPS[88,158]. In mice, DCs have been identified as the major source of IL-6 within lymph nodes during primary immune responses to cutaneous antigens [159]. In mice, IL-6 has in addition been reported to induce Th2 differentiation of naïve $CD4^+$ T cells as a result of initiation of T cell IL-4 production [160]. It is therefore possible that IL-6 derived from human DCs may in a similar manner have the potential to directe Th2 differentiation. Unlike other proinflammatory cytokines, IL-6 is not capable of inhibiting IL-4 production by primed human T cells, promoting Th2 differentiation [161].

IL-6 produced by DCs may also have autocrine functions, affecting DC antigen processing and possibly T cell development as a consequence [162]. Recent murine studies have shown that presentation of particular peptide epitopes, derived from hen egg lysozyme (HEL), by mouse DCs, was enhanced under the influence of IL-6 *in vivo* and *in vitro* [162]. As the expression of costimulatory or adhesion molecules was unaffected, enhanced peptide presentation was ascribed to differential processing of whole HEL by IL-6 treated DCs [162]. It is possible that differential peptide processing in the presence of mediators such as IL-6 could influence the pathway of Th1 - Th2 differentiation although no evidence of Th1 or Th2-directing peptides exists to date.

1.3.3.6.2 Interleukin-10

Immature Mo-DCs produce low levels of IL-10 that have been shown to suppress spontaneous maturation of Mo-DCs as well as maturation induced by LPS and CD40L *in vitro*[152]. Following LPS- or CD40L-induced maturation, Mo-DCs produce elevated levels of IL-10[152]. IL-10 production may in addition be further enhanced by treating the Mo-DCs with PGE_2 prior to the exposure to bacterial products [56].

IL-10 has been implicated in promoting both Th2 differentiation and tolerance induction of T cells. Although IL-10 has been shown to directly affect T cells, for example by inducing a state of T cell anergy [163], most effects of IL-10 on T cell activation and differentiation are induced indirectly via DCs.

DCs exposed to IL-10 have been suggested to promote Th2 differentiation as a consequence of reduced IL-12 production. Murine DCs treated with IL-10 at the time of CD40L-induced maturation showed inhibited IL-12 p70 production[164]. Similarly, purified

spleen DCs cultured in the presence of IL-10 were found to produce reduced levels of IL-12 p40[59]. When pulsed with antigen and injected into the footpads of syngeneic mice, these IL-10 treated DCs were found to drive Th2 differentiation. This was shown as purified lymph node cells from these syngeneic mice produced elevated levels of IL-4 and IL-5 and reduced levels of IFN-γ following stimulated with antigen *in vitro*[59]. In the human, immature DCs treated with IL-10 also demonstrate reduced IL-12 p70 production[57,165], however, no direct link to Th2 differentiation has yet been shown. On the other hand, in addition to having reduced IL-12 p70 production, IL-10 treated Mo-DCs demonstrate impaired maturation driven by IL-1β and TNF-α and showed reduced capacity to drive proliferation of autologous naïve T cells primed with the superantigen SEB[57]. This may suggest that treatment of human DCs with IL-10 preferentially promotes tolerance induction. In accordance with this, another study showed that immature Mo-DCs exposed to IL-10 expressed reduced levels of CD83, CD58 and CD86 and were found to induce anergy in $CD4^+$ T cells and in the influenza hemagglutinin-specific T cell clone HA1.7 [33].

With the exception of one study on human DCs [165], the effects of IL-10 on murine and human DCs were observed only when IL-10 was applied to immature or maturing, but not already mature DCs[33,57,152]. Collectively, these data may suggest that the presence of IL-10 in the periphery at the time preceding DC maturation inhibits terminal DC maturation and may convert immature DCs into Th2 skewing or tolerogenic DCs.

1.3.3.6.3 Interleukin-12

IL-12 is a heterodimeric cytokine, comprising two disulfide-linked subunits designated p35 and p40 [166]. IL-12 p35 shows sequence homologies to IL-6 and granulocyte - colony stimulating factor (G-CSF). IL-12 p35 and p40 both lack biological activity on their own. However, the 70 kDa dimer, IL-12 p70, binds to the IL-12R, expressed on activated T cells[34,166 167] resulting in the production of IFN-γ. The IL-12R consists of two subunits called IL-12Rβ1 and IL-12Rβ2 [166]. Surface expression of both IL-12Rβ1 and IL-12Rβ2 are required for high-affinity IL-12 binding. IL-12Rβ2 is suggested to be the signal transducing subunit and contains conserved tyrosine residues in its cytoplasmic portion [166]. Recent studies of both murine[34] and human[34,167,168] Th2 cells have shown a lack of surface expression of IL-12Rβ2 due to downregulation by IL-4, TGF-β2 and IL-10. This may explain the IL-12 unresponsiveness of Th2 cells.

Human DCs produce bioactive IL-12 in response to at least four stimuli: (i) following CD40 ligation [28,32,169], (ii) following exposure to pathogens including *Staphylococcus aureus* Cowan strain I and LPS [169-172], (iii) following exposure to CpG-oligonucleotides (ODN)[173], and (iv) following MHC II ligation[164]. Of these, CD40 ligation is the most efficient means of inducing IL-12 production [101,174]. The CD40-mediated IL-12 p70 production is markedly enhanced in the presence of IL-4[101]. In contrast, LPS-mediated IL-12 p70 production is inhibited by IL-4 [101]. In addition to increasing IL-12 p70 production in CD40L treated Mo-DCs, IL-4 also reduces the production of IL-12 p40, possibly further augmenting the effect of IL-12 as the inactive p40 homodimer may act as an IL-12 antagonist competing for the IL-12R[175]. Another cytokine known to greatly enhance IL-12 production in DCs is IFN-γ[170,174,176]. However, in contrast to IL-4, IFN-γ has been shown to increase both IL-12 p70 and p40 production in CD40L- as well as LPS-stimulated Mo-DCs [101].

By using a neutralising anti-IL-12 mAb, IL-12 produced by DCs has been shown to be responsible for driving the development of Th1 cells in both murine[171] and human[60,169] allogeneic DC : T cell co-cultures and in a murine antigen specific culture system[177]. In these culture systems the neutralising anti-IL-12 mAb was shown to reduce the level of IFN-γ produced by T cells. Reduced levels of IL-12 produced by Mo-DCs exposed to cholera toxin[58], glucocorticoids[178], PGE_2[56,57] or type I IFNs[60] has also been associated with reduced T cell IFN-γ production.

In addition to producing IL-12, murine DCs have been shown to respond to IL-12 resulting in the production of IFN-γ[157,179], that further may reinforce the capacity of DCs to promote Th1 differentiation.

1.3.3.6.4 Interleukin-18

IL-18 is an 18 kDa protein, previously referred to as interferon-γ-inducing factor [180]. IL-18 is constitutively produced by a range of immune cells, including DCs[154], macrophages, T cells, and B cells, as well as non-immune cells, including keratinocytes, airway epithelial cells and microglia[181]. In DCs, IL-18 resides within intracellular organelles in an inactive pro-IL-18 form that is secreted in its biologically active form following antigen-specific interaction with T cells [182]. Activation of pro-IL-18 has been suggested to result from proteolytic cleavage mediated by caspase-1[181].

IL-18 was initially believed to promote Th1 differentiation since IL-18, in synergy with IL-12, induced IFN-γ production in DCs[179], T cells, NK-cells and B cells[181]. Perhaps more importantly, in the additional presence of TCR-mediated signalling, IL-12 and IL-18 have also been shown to drive the differentiation of Th1 cells [180,183,184]. In this context, IL-18 produced by murine DCs enhanced IL-12-dependent Th1 differentiation [185]. However, in the absence of IL-12, IL-18 was recently shown to induce IL-13 and GM-CSF production in naïve T cells and to promote Th2 development in the presence of TCR-mediated signalling[181]. IL-18 has also been shown to induce the release of IL-4, IL-13 and histamine from basophils and mast cells when applied together with IL-3, possibly resulting in perpetuation of the allergic response. In conclusion, these data imply that IL-18 may drive both Th1 and Th2 differentiation depending primarily on the concurrent presence or absence of IL-12.

1.3.3.6.5 Interleukin-23

Murine and human DCs have recently been shown to produce IL-23[155]. IL-23 is a heterodimer consisting of a recently discovered subunit termed p19 that combines with the p40 subunit of IL-12. It is therefore not surprising that IL-23 shares many biological functions with IL-12 including binding of IL-12Rβ1, STAT4 activation and stimulation of T cell proliferation and IFN-γ production. Through this induction of IFN-γ production by T cells, IL-23 produced by DCs may have an important role in driving Th1 cell differentiation.

1.4 Asthma

1.4.1 Incidence and severity of asthma

Asthma is a chronic allergic disorder that has become a growing problem world-wide during the latter part of the 20th century. Over the past 20 years the incidence of asthma has almost doubled in developed countries [186] and 1 child in 4 in Great Britain is today suffering from asthma [187]. Asthma is a serious disease not only as it causes suffering and disability but it also leads to mortality [188,189].

The clinical expression of asthma is strongly influenced by genetic factors. However, for manifestation of symptoms, interaction between genetic and environmental

factors is required [190]. Although, recent changes in our life style including housing, introduction of vaccination, change in diet and increased indoor activities may partly explain the increased incidence of allergy, the increase in allergic asthma seems to be attributed to additional, as yet unknown factors.

1.4.2 Symptomatology, etiology and pathophysiology of asthma

Asthma is characterised by reversible airflow obstruction, with associated airway hyperresponsiveness, excess mucus hypersecretion, contraction of smooth muscles and damage to the epithelial lining of the airway [188]. The symptomatology is directly linked to the presence of ongoing airway inflammation. In most instances of asthma the nature of this inflammation is allergic i.e. atopic. Atopy is the genetic tendency to develop IgE against common allergens encountered in the environment. The clinical disorders that result from allergen-specific IgE mediated inflammation are called atopic diseases.

The conducting airways in the lungs of atopic asthmatic subjects are characterised by an allergic inflammatory cell infiltrate rich in activated $CD4^+$ T cells, activated mast cells and activated eosinophils. The deposition of subepithelial collagen is markedly increased as is thickening of the epithelial basement membrane and epithelial damage and disruption due to protease activity.

1.4.3 Disease mechanisms

1.4.3.1 IgE and the immediate-phase or type I hypersensitivity response

IgE was first discovered as a blood-borne agent that transfers allergen sensitivity to non-allergic subjects[191]. Since much asthma is associated with atopy it is not surprising that elevated levels of IgE have been demonstrated in the respiratory tract and in the serum of asthmatics [192,193]. In children, a relationship between total serum IgE and the prevalence of asthma has been suggested. No children with serum IgE levels < 32 IU/mL were reported to suffer from asthma whereas 36 % of children with serum IgE levels over 1 000 IU/mL were asthmatic [193].

IgE can bind to FcεRI on mast cells and basophils. Here, the IgE is available to bind specific allergen. Allergen recognition by neighbouring FcεRI molecules leads to FcεRI cross-linking, mast cell degranulation and the release of mediators such as histamine,

proteases, prostaglandins, leukotrienes and cytokines including IL-4, IL-5 and TNF-α[194]. This constitutes the IgE-mediated type I hypersensitivity response (Figure 1.5). The mediators released as a result of degranulation trigger the symptoms associated with the immediate-phase allergic response including increased vascular permeability, local oedema, itching and in the case of atopic asthma, bronchoconstriction [195,196]. Generally the immediate-phase response lasts between 20-30 minutes [197]. Cytokines released as a consequence of mast cell degranulation has been demonstrated to increase the expression of adhesion molecules on the vascular endothelium[198], especially vascular cell adhesion molecule-1 (VCAM-1), and the secretion of various chemokines by epithelial cells, including RANTES* and eotaxin [197,199]. These factors enhance the influx of inflammatory cells into the airways and hence contribute to the progression of the asthmatic response.

1.4.3.2 T helper 2 lymphocytes and the late-phase or delayed type IV hypersensitivity response

The asthmatic late-phase response arises around 4-6 hours following exposure to allergen, and is accompanied by symptoms such as bronchospasm, bronchial hyperreactivity and nasal congestion [197]. Infiltrating pro-inflammatory cells, notably eosinophils and T cells, and resident non-hematopoetic cells including epithelial cells and airway smooth muscle, release cytokines when activated that evoke and maintain the late-phase response [200] (Figure 1.5).

In situ hybridisation studies suggest that the activated $CD4^+$ T cells in asthmatic airways preferentially produce the cytokines IL-3[201,202], IL-4[201-203], IL-5[201-203], IL-9[204], IL-13[205] and GM-CSF[201,202]. On the basis of this cytokine profile, these activated T cells have been suggested to belong to the Th2 subset. However, other inflammatory cells such as activated mast cells and eosinophils have also been shown capable of producing IL-4[203,206], IL-5[203,206,207] and IL-9[204], and thereby contribute to the pathophysiology of the allergic disease.

The Th2 cytokines mentioned above have been found to cluster together on chromosome 5q[208]. Despite some inconsistency between different populations, strong linkage between the atopic, asthmatic phenotype and the Th2 cytokine gene cluster has been reported[208]. The role of these Th2 cytokines in the pathophysiology of human asthma has further been suggested by their activity *in vitro*. IL-3 supports the development and

* RANTES (CCL5), eotaxin (CCL11) [297].

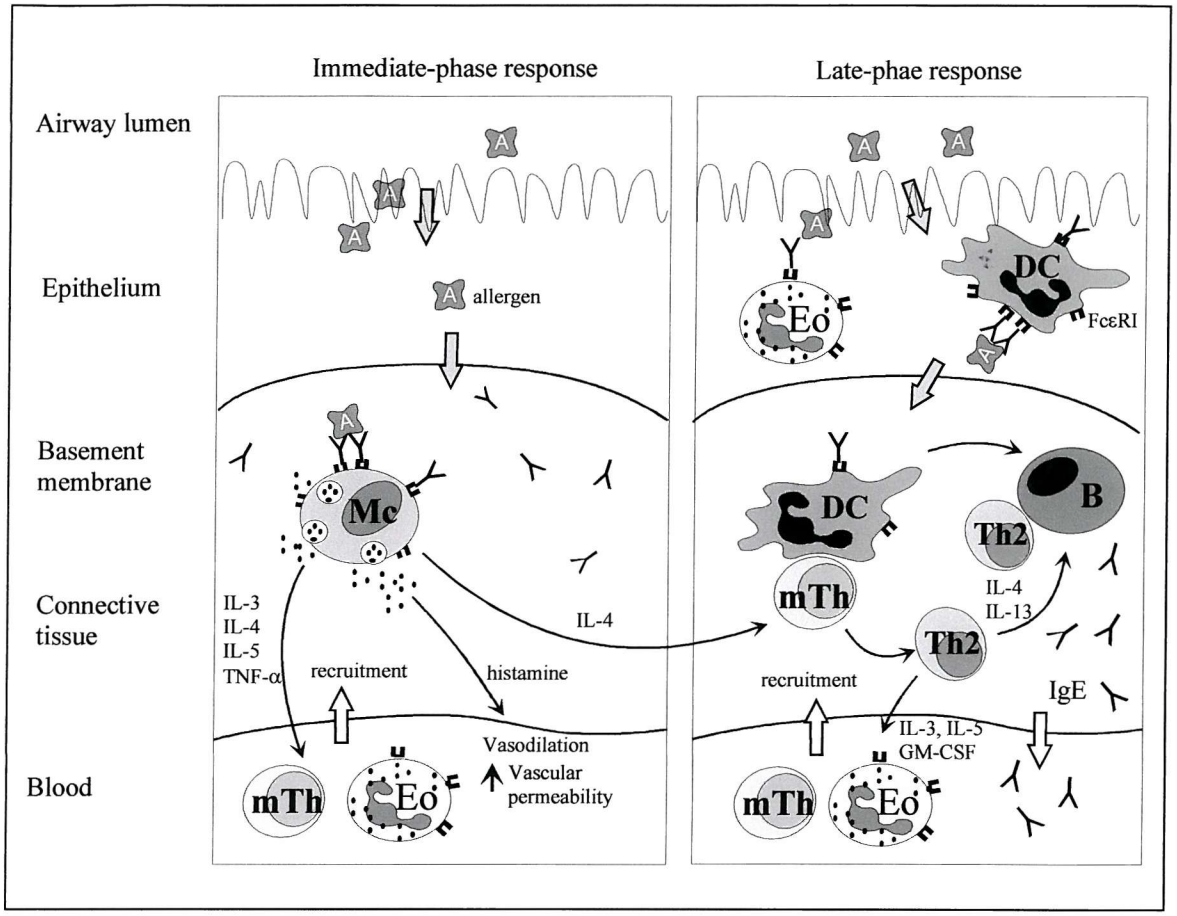


Figure 1.5 The immediate- and late-phase allergic response in the conductive airways

In the immediate-phase response, allergen cross-links IgE bound to FcεRI on the mast cell (Mc), causing degranulation. Mediators released as a consequence of degranulation lead to recruitment of inflammatory cells such as memory $CD4^+$ T cells (mTh) and eosinophils (Eo) from the blood. During the late-phase response, APCs, typically dendritic cells (DC), activate Th2 cells leading to the release of Th2 cytokines that further augment recruitment and activation of eosinophils and B cells. Adapted from[234].

histamine release by mast cell [209,210] and basophil [211-213] and is also involved in the regulation of CD23 expression and IgE secretion in B cells [214]. IL-5 controls the proliferation of eosinophils, and together with IL-3 and GM-CSF enhances the activation, differentiation and migration of eosinophils into the lung epithelium [197,215-218]. Eosinophils in turn perpetuate the allergic inflammation through damaging the epithelial barrier, resulting in increased airway hyperresponsiveness and susceptibility to environmental antigens, hence contributing to the chronicity of atopic asthma [219]. IL-4 is a potent promoter of Th2 differentiation and may initiate the development of allergen-specific Th2 cells in atopic asthmatic airways. Through the use of a common receptor component, IL-4 shares many biological functions with IL-13[220], including initiation of the production of allergen-specific IgE in B cells [221,222], eosinophil infiltration and the induction of mucous hypersecretion [219]. However, IL-13 also has functions that are independent of IL-4, including the induction of airway hyperresponsiveness[223] collagen deposition and the production of eotaxin[224]. IL-9 has been shown to potentiate IL-4-induced IgE production by B cells[225] and to support the growth and proliferation of mast cells[226,227].

The significance of these Th2 cytokines for the asthmatic response has also been emphasised in *in vivo* studies using animal models of asthma [215-217,222,224,228-231]. For example, eosinophilia, lung damage, and airway hyperresponsiveness were abolished following aeroallergen challenge of IL-5-deficient mice [216] or BALB/c mice in the presence of anti-IL-5 antibody [217]. However, during a recent clinical trial it was evident that IL-5 may not play such an important role in the onset of the late asthmatic response in humans, as a blocking IL-5 mAb failed to affect airway hyperresponsiveness to histamine[232]. The role of IL-13 was demonstrated using IL-13 transgenic mice in which this cytokine was shown to promote the development of airway hyperresponsiveness and collagen deposition independently of IL-4[224]. Similarly, by neutralising IL-13 with an IL-13 receptor fusion protein in allergen challenged mice, airway hyperresponsiveness was abolished [223]. IL-13 has therefore been suggested to have a prominent role in airway hyperresponsiveness in mice and possibly also humans.

In the ongoing pulmonary allergic response the segregation of the function of IgE and Th2 cytokines into the immediate- and late-phase response respectively, is not absolute. Although the activation of allergen-specific Th2 cells is sufficient to provoke increased airway responsiveness in a late allergic inflammatory response, the simultaneous presence of IgE results in a more severe pulmonary response. This was recently observed in a study where allergen and allergen-specific murine Th2 cells were transferred into the lungs of naïve mice. High levels of IL-4 and IL-5 were found in the bronchoalveolar lavage (BAL),

along with a modest increase in the numbers of eosinophils and mucous-secreting goblet cells [233]. However, allergen-specific serum IgE was not detected. In contrast, mice sensitised and challenged with the same allergen showed higher levels of pulmonary eosinophilia, mucous goblet cells, airway responsiveness and the presence of allergen specific serum IgE despite lower levels of BAL IL-4 and IL-5 [233].

1.5 Lung dendritic cells and allergic asthma

1.5.1 Initial priming of allergen-specific T cells - role for lung dendritic cells

Environmental antigens normally induce tolerance or provoke a weak Th1 response in adults including production of low levels of IFN-γ [235]. However, in some individuals the same antigen may act as an allergen, inducing a Th2 response characteristic of atopic disease. A major challenge in allergy research is understanding how this abnormal, inappropriate response is induced.

Allergen-specific lymphoproliferation has been observed for cord blood T cells *in vitro*, irrespective of whether atopic disease developed later in childhood [235]. Thus, T cell priming to common food and aeroallergens has been proposed to occur *in utero* [236]. The neonatal immune system offers an environment that strongly promotes Th2 immunity [237]. Priming of naïve T cells to become allergen-specific under these conditions is therefore likely to produce Th2 memory. However, even though all infants are born with Th2-skewed immunity most infants will develop a normal Th1 response to antigens, including allergens, indicating that the adult response is shaped postnatally as the developing neonatal immune system is exposed to higher levels of antigens. This process of allergen-specific T cell selection is thought to be dependent on APCs, in particular DCs that are believed to be the only APC capable of T cell priming.

In the lung, DCs form a network within the airway epithelium. Interestingly, in mice these intraepithelial DCs only develop postnatally. Thus, during the preweaning period the intraepithelial DC network is poorly established and the DCs undeveloped in terms of surface expression of MHC class II, APC function and responsiveness to cytokines, including IFN-γ and GM-CSF [238,239]. As a possible reflection of incompletely developed antigen presenting functions, DCs purified from central lymphoid organs in newborn mice have been found deficient in their ability to drive Th1 differentiation of naïve T cells, instead leading to production of a Th2-cytokine profile [240]. A similar process may occur in human fetal lung, although at an earlier stage during the perinatal period. Delayed postnatal

maturation of intraepithelial DC immune functions, possibly as a result of genetic predisposition, have been suggested to greatly increase the risk of keeping a Th2 response to allergens [235], emphasising the possible role of lung DCs in the development of allergic asthma.

1.5.2 The location and dynamics of lung dendritic cells

Most studies on lung DCs have been performed using animal models, predominantly the rat and mouse in which DCs are identified according to their dendritic morphology and strong immunoreactivity for MHC class II (Ia)[241,242]. In the rat as well as man, lung DCs are most prominent in the epithelium of the conducting airways (Figure 1.5) [241,242]. DCs have also been identified in lung parenchyma[242,243], as the only resident antigen presenting cell within non-inflamed rat alveolar septa[244] and at low numbers in human BAL[244,245].

The density of intraepithlial DCs in the conducting airways of the rat varies according to the extent of antigen exposure. For example, larger airways, exposed to high levels of antigens, contain approximately 600-800 DCs per mm^2 of epithelium, whereas smaller airways exposed to less antigen, contain approximately 75 DCs per mm^2 of epithelium [242,246]. During inflammation the density of intraepithelial DCs increases as a result of elevated levels of chemokines recruiting MHC class II-expressing DC precursors to the airway epithelium [247](Lambrecht 2001). MIP-3α may be one of these chemokines as lung DCs have been shown to express the MIP-3α receptor CCR6 [248], and MIP-3α has been shown to attract $CCR6^+$ DCs derived from $CD34^+$ progenitor cells *in vitro* [249,250] Although experimental studies of the dynamics of DC populations cannot easily be performed in human lung, the number of intraepithelial DCs has been reported to be increased in human asthmatic lung [251-254].

Shortly after encounter with inflammatory stimuli, maturing lung DCs migrate to the regional lymph nodes where antigenic peptides are presented to naive T cells [247]. The turnover of intraepithelial DCs in the lung has been estimated to a half-life of close to 2 days in the rat [255]. Only DC populations in the gut epithelium have a comparably rapid population turnover [256], indicating a requirement for fast DC responses at mucosal surfaces exposed to heavy antigenic load [246,247,255]. However, even in a resting state, the whole lung DC population is renewed in less than three days, reflecting a generally heavy trafficking between the airway mucosa and the regional lymph nodes [234,257].

1.5.3 The function of lung dendritic cells

The intraepithelial DC network is strategically placed for capturing airborne antigens. Furthermore, the immature phenotype of these cells facilitate antigen uptake, as demonstrated for DCs isolated from rat lung that are capable of MR-mediated endocytosis of FITC-conjugated dextran [258]. Following maturation in response to GM-CSF these rat DCs demonstrate increased levels of surface expressed MHC class II and CD86 and reduced levels of IL-10 mRNA [258], that may enhance antigen presentation to naïve T cells in the lymph nodes.

The ability of pulmonary DCs to induce antigen sensitisation was demonstrated using DCs pulsed with OVA *in vitro* that were then injected into the trachea of naïve mice and rats[259]. The DCs were shown to migrate to the regional lymph nodes where they induced a primary response to OVA, evident by the presence of proliferating OVA-specific T cells. Moreover, following challenge with aerosolised OVA these animals developed eosinophilia and goblet cell hyperplasia, characteristic of asthma.

In animal models DCs have also been suggested to drive secondary immune responses. By treating OVA-sensitised mice, transgenic for the thymidine kinase gene with ganciclovir it was possible to selectively deplete DCs but not B cells, T cells or marcophages[260]. Following OVA challenge these DC-depleted animals failed to develop eosinophilia and goblet cell hyperplasia with a concurrent reduction in the level of airway IL-4 and IL-5 and OVA-specific IgE. It has been suggested that DCs play an important role in antigen presentation in the lung due to an unexpectedly high dependency on costimulation by lung memory T cells. Purified mouse lung DCs have been shown to express common costimulatory and adhesion molecules including CD80, CD86 and CD54 [261]. In the asthmatic lung it is possible that the prominent antigen presenting role of DCs is further enhanced due to the expression of the high affinity IgE receptor, FcεRI, which may mediate selective and efficient capture of allergen via allergen-specific IgE.

1.5.4 FcεRI$^+$ dendritic cells in atopic diseases

1.5.4.1 FcεRI expression by dendritic cells in asthma and other atopic diseases

In a recent study, it was found that Mo-DCs derived from allergic asthmatic patients display a different phenotype relative to Mo-DCs from normal subjects, including increased expression of FcεRI as well as HLA-DR and CD11b [262]. These DCs demonstrated an enhanced ability to stimulate allergen-specific T cell proliferation. Increased expression of the FcεRI α-chain has also been demonstrated on human lung DCs in asthmatic patients [251,252,263]. Surface expression of FcεRI is also dramatically increased on LCs from patients with atopic dermatitis (AD) when compared with nonatopic individuals[264].

Increased FcεRI surface expression on DCs in atopic disease is possibly due to elevated levels of IL-4 and IgE in the allergic environment, as these cytokines have been shown to regulate FcεRI expression by mast cells[265-267] and DCs[268] *in vitro*. However, it cannot be excluded that the increase in FcεRI$^+$ DCs in the inflamed asthmatic lung may be a result of recruitment of FcεRI expressing DCs from the circulation [234,251].

1.5.4.2 The function of FcεRI on dendritic cells

FcεRI$^+$ effector cells, such as mast cells and basophils, bind monomeric IgE, some of which may be allergen-specific. Subsequent binding of multivalent allergen induces FcεRI cross-linking resulting in degranulation and release of inflammatory mediators. However, on FcεRI$^+$ APCs carrying IgE, cross-linking results in internalisation of the receptor and its bound IgE-allergen complexes [84,124]. This process of FcεRI-mediated allergen uptake is commonly known as allergen focusing. Recently it was found that allergen focusing in DCs targets allergen-derived peptides to MIIC-like organelles where peptide loading onto newly synthesised MHC class II molecules involves cathepsin S [124]. It has been suggested that peptide loading via cathepsin S-dependent MIIC compartments may be a particularly effective route for antigen presentation [124]. In accordance, antigen presentation was found 100 –1000 fold more efficient when the antigen was targeted to FcεRI via antigen-specific IgE[269]. Furthermore, DCs were found around 10 times more efficient at FcεRI and IgE-mediated antigen presentation than monocytes[270].

Through allergen-specific IgE, allergen focusing also provides efficient uptake of allergens at very low concentrations. Allergen focusing further favours capture of allergens to which an individual already has been sensitised, consequently perpetuating the allergic response [84,234,242,251]. Accordingly, FcεRI$^+$ peripheral blood DCs have been shown to stimulate allergen-specific secondary T cell responses in a FcεRI/IgE-dependent manner [270].

Together these efficient uptake- and processing mechanisms may result in a reduced threshold for induction of allergen-specific T cell responses in atopic individuals' [271 269]. However, it has yet to be determined whether FcεRI-mediated signaling, following ligation and / or cross-linking and internalisation of FcεRI, alters the DC phenotype and function. Support for this comes from reports on FcεRI$^+$ monocytes that show increased expression of CD54 in the presence of IgE, F(ab)$_2$ or Fab fragments of the anti-FcεRIα mAb 15.1 [272] and reduced apoptosis following stimulation with monomeric IgE[273]. Similar FcεRI-mediated changes in DCs may potentially result in a DC optimally suited for the induction of allergen-specific T cell responses.

1.6 Hypothesis and aims

Since the identification of DCs in 1973 [274], the importance of the DC in the initiation and maintenance of the immune response has been emphasised. The complex lifecycle of the DC, with different functions being spatially and temporally separated, is attributed to the plasticity of the DC phenotype. To a large extent, DC phenotype is influenced by environmental factors that have been shown to be responsible for shaping parts of the APC-dependent T cell polarising signal 3. **The hypothesis for this work was that components within the local microenvironment in the atopic asthmatic lung, including elevated levels of IgE and Th2 cytokines, bring about phenotypic and functional changes in DCs. These changes in turn modify the outcome of the DC - T cell interaction, resulting in initiation or perpetuation of the chronic allergic inflammation seen in asthmatic airways.**

To address this hypothesis the aim of the work presented here was to evaluate the effects of mediators found at elevated levels in the asthmatic lung, collectively referred to as allergic mediators, on 1) DC phenotype and 2) DC function in stimulating T lymphocytes and driving their differentiation. Dendritic cells derived *in vitro* from monocytes (Mo-DCs) were used as a model system, and the response of Mo-DCs from, non-atopic, non-asthmatic individuals and atopic, asthmatic individuals was compared.

A primary screen was developed (Chapter 4) to evaluate the effects of individual allergic mediators on Mo-DC phenotype and function using four assays: 1) flow cytometric analysis of surface expression of CD23, CD40, CD80, CD83, CD86 and HLA-DR by Mo-DCs (section 4.2), 2) flow cytometric analysis of receptor-mediated endocytosis by Mo-DCs (section 4.2), 3) evaluation of IL-6 and IL-10 production by Mo-DCs (section 4.3), and 4) evaluation of Mo-DC-induced proliferation of allogeneic T cells ($CD45RA^+$) (section 4.4). All four assays were designed to identify allergic mediators that result in a statistically significant or marked change in Mo-DC phenotype and / or function, for further in-depth investigation of their effects on Mo-DCs and possible relevance in allergic asthma.

The experimental approach taken identified four areas of interest, two of which were further investigated. One of the areas that were further investigated was the effects of IFN-γ on Mo-DCs. It was found that IFN-γ induced Mo-DC maturation, yet failed to enhance T cell proliferation to a level expected for mature Mo-DCs. The work presented in Chapter 5 therefore aimed to investigate the mechanisms underlying this disparity between the phenotype and function of IFN-γ treated Mo-DCs. The other area that was further investigated was the disappearance of CD23 in response to treatment. It was found that CD23 disappeared from the surface of Mo-DCs in response to TNF-α, IFN-γ and 100 nM IgE. As these mediators differ in their ability to physically interact with CD23, the work presented in Chapter 6 aimed to determine the role of possible mechanisms involved in clearing surface expressed CD23 in response to TNF-α, IFN-γ and 100 nM IgE.

Chapter Two

General materials and methods

2.1 Materials

2.1.1 Standard laboratory chemicals

Chemicals for general laboratory use were purchased from either Sigma Chemical Company Ltd. (Poole, UK), Fisher Scientific U.K. (Loughborough, UK) or LAB3 Ltd. (Northampton,UK). All chemicals were of analytical or molecular biology grade.

2.1.2 Buffers and solutions

Solutions were either prepared in sterile pyrogen-free water (Pharmacy, Southampton General Hospital, Southampton, UK) or in Ultra high quality (UHQ) reverse osmosis water purified through an ElgaStat UHQ-PS system. All buffers used for the preparation of cells were filter-sterilised through 0.22 μm filter units (Corning Costar Ltd., High Wycombe, UK). Sterile phosphate buffered saline (PBS), for cell preparation, was prepared from 10 × PBS stock, without Ca^{2+}, Mg^{2+} (Life Technologies Ltd., Pairsley, UK) that following 1:10 dilution in pyrogen free water consisted of 2.67 mM KCl, 1.47 mM KH_2PO_4, 138 mM NaCl and 8.1 mM $Na_2HPO_4 - 7H_2O$, pH 7.3. This was used to prepare the buffers described in Table 2.1. For all other applications, PBS was prepared from tablets (OXOID, Basingstoke, UK) using UHQ water according to the suppliers instructions, giving a final composition of 3.0 mM KCl, 1.0 mM KH_2PO_4, 160 mM NaCl and 8.0 mM Na_2HPO_4, pH 7.3. FACS buffer is a commonly used buffer prepared from this PBS, consisting of PBS, 0.1% sodium azide (w/v), 1% bovine serum albumin (BSA) fraction V (w/v) (Sigma).

Table 2.1. PBS based solutions for cell preparation.

Buffer	Composition
Elutriation buffer	PBS, 1% BSA fraction V (w/v), 270 nM EDTA
MACS buffer	PBS, 0.5% BSA fraction V (w/v), 2 mM EDTA
Wash buffer	PBS, 1% foetal calf serum (FCS), 1 mM EDTA

2.1.3 Cell culture media

RPMI-medium 1640 with and without phenol red, penicillin / streptomycin and L-glutamine were purchased from Life Technologies. Pooled human AB serum (Sigma) and defined FCS (Pierce and Warriner, Chester, UK) was heat inactivated at 56 °C for 30 minutes, put on ice and when cold, filter-sterilised through 0.43 μm filter units (Corning Costar). Aliquots of penicillin / streptomycin, L-glutamine, AB serum and FCS were stored at -20°C.

2.1.4 Cytokines and cell culture additives

Recombinant human (rh)GM-CSF, rhIL-4, rhIFN-γ, rhIL-3, rhIL-5, rhIL-10, rhIL-13, rhMIP-1α and rhTNF-α was obtained from R&D Systems Europe Ltd. (Abingdon, UK). Azide free, chimaeric human IgE was obtained from Serotec Ltd. (Oxford, UK) and dihydrochloride histamine and prostaglandin D_2 (PGD_2) from Sigma.

Stock solutions of all cytokines, rhMIP-1α, PGD_2 and histamine were prepared by dissolving the lyophilised powder in sterile PBS containing 1 % low endotoxin BSA, fraction V (Sigma), then aliquoted and frozen. The chimaeric human IgE supplied in PBS and the stock solutions of IFN-γ, IL-3, IL-5 and IL-13 were screened for endotoxin contamination using a limulus amebocyte lysate assay (2.2.1.1). Consequently the chimaeric human IgE was treated for endotoxin contamination (2.2.1.2), then aliquoted and frozen.

2.1.5 General consumable

Standard tissue culture plasticware, including flat-bottomed 6, 12, 24, 48 and 96 well tissue culture plates, FACS tubes and 15 and 50 mL Falcon tubes from Becton Dickinson (Oxford, UK), were chosen for low endotoxin levels. 0.22 μm Millex-GV filter units were purchased from Millipore (Watford, UK), 30 mL and 7 mL Universal tubes from Western Laboratories (Aldershot, UK) and V-bottomed 96 well plates from Life Technologies.

2.1.6 Antibodies

Monoclonal antibodies used for flow cytometric analysis of cell surface markers are listed in Table 2.2. The antibodies were unconjugated or directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or rhodamine red X (RRX) and stored at 4 °C.

2.1.7 Subjects

Venous blood was collected from mild asthmatic subjects or normal, healthy volunteers. Mild atopic asthmatic subjects, receiving treatment with inhaled β_2-agonists only, were selected on the basis of a history of asthmatic episodes and positive skin-prick tests for common aeroallergens, i.e. wheal response $\geq$ 3 mm in diameter. Healthy subjects on no regular medication were selected on the basis of negative skin-prick tests to common aeroallergens and no symptoms of allergic disease. All subjects were non-smokers. All subjects gave informed consent and the study was approved by the Southampton and S. W. Hants Joint Research Ethics Committee, approval number 038/98.

Table 2.2. Antibodies for flow cytometric analysis

Antibody specificity (clone)	Isotype	Conjugation	Supplier
FcεRI-α (15.1)	IgG1	FITC	J-P. Kinet, (Boston, USA)
CD1a (NA1/34)	IgG1	FITC	Dako (Ely, UK)
CD4 (C4120F)	IgG1	FITC	Prof. M. Glennie, (Tenovus, Southampton, UK)
CD5 (BL1A)	IgG2a	FITC	Coulter/Immunotech (Beckman, High Wycombe, UK)
CD5 (L17F12)	IgG2a	FITC	Becton Dickinson
CD8 (24.13.4)	IgG1	FITC	Prof. M. Glennie
CD14 (2D-15C/ FMC-32)	IgG1	FITC	Chemicon (Harrow, UK)
CD14 (UCHM1)	IgG2a	PE	Serotec
CD14 (MϕPg)	IgG2b	PE	Becton Dickinson
CD16 (B73.1)	IgG1	PE	Becton Dickinson
CD19 (J4.119)	IgG1	FITC	Immunotech
CD20 (B9E9)	IgG2a	FITC	Coulter/Immunotech
CD20 (L27)	IgG1	PE	Becton Dickinson
CD23 (BU38)	IgG1	unconjugated	The Binding Site (Birmingham, UK)
CD23 (EBVCS-5)	IgG1	PE	Becton Dickinson
CD40 (MAB89)	IgG1	PE	Immunotech
CD45RA (L48)	IgG1	FITC	Becton Dickinson
CD45RA (ALB11) CD45RO	IgG1	PE	Immunotech
(UCHL-1)	IgG1	PE	Becton Dickinson
CD54 (LB-2)	IgG2b	PE	Becton Dickinson
CD56 (MY31)	IgG1	PE	Becton Dickinson
CD80 (L307.4)	IgG1	PE	Becton Dickinson
CD83 (HP15a)	IgG2b	PE	Coulter
CD86 (IT2.2)	IgG2b	PE	PharMingen (Becton Dickinson)
CD95 (ANC95.1/5E2)	IgG1	FITC	Alexis Corporation, Nottingham, UK
CD95 (LOB3/11)	IgG1	unconj.	Prof. M. Glennie
Goat anti Mouse	IgG1	PE	Serotec
HLA-DR (L243)	IgG2a	PE	Becton Dickinson
Isotype control	IgGγ1	FITC	Becton Dickinson
Isotype control	IgGγ1	PE	Becton Dickinson
Isotype control	IgG2a	FITC	Becton Dickinson
Isotype control	IgG2b	PE	Becton Dickinson

2.2 Methods

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

2.2.1 Endotoxin detection and treatment of cell culture additives

2.2.1.1 Limulus amebocyte lysate assay for detection of endotoxin

Detection and semi-quantitation of endotoxin in the stock solutions of chimaeric human IgE, rhIFN-γ, rhIL-3, rhIL-5 and rhIL-13 were performed using an E-Toxate multiple test vial kit (Sigma) according to the suppliers instruction. In brief, an E-Toxate® working solution was prepared by adding endotoxin-free water (supplied with the kit) to the lyophilised E-Toxate® using a pyrogen-free graduated plastic pipette. The E-Toxate® was dissolved by gentle swirling, then chilled on ice. The lyophilised endotoxin standard was dissolved in endotoxin-free water by vigorous shaking for 2 minutes followed by 30 seconds shaking at 10 minutes intervals over a 30 minute period. A standard curve ranging from 400 endotoxin units (EU)/ mL to 0.015 EU/mL was prepared in baked borosilicate tubes (Sigma) by serially diluting the endotoxin standard provided in endotoxin-free water. Dilution of the sample was made in endotoxin-free water. 0.1 mL sample, diluted standard or endotoxin-free water (negative control) was added to the baked tubes. An equal volume of E-Toxate® working solution was added to the sample, diluted standard or endotoxin-free water, then gently mixed and covered with Parafilm. Following 1 hour incubation at 37°C, the tubes were gently inverted to detect gelation. A positive test was defined as formation of a hard gel, whereas all other gel consistencies were considered negative. The level of endotoxin in the sample was calculated by multiplying the in verse of the highest dilution of sample found positive (e.g. 1/64) by the lowest dilution of standard found positive (e.g. 0.06 EU/mL). Under the above conditions the endotoxin level would equal 3.8 EU/mL according to the following equation. (2.3 EU = 1 ng endotoxin.)

$$\text{Endotoxin (EU/mL)} = (1/(1/64)) \times 0.06 = 3.8 \text{ EU/mL}$$

2.2.1.2 *Removal of endotoxin contamination*

Endotoxin contamination was removed from stock solutions using an END-X B15 endotoxin removal affinity resin kit (ACC-UK, Liverpool, UK). The samples were incubated in the supplied microcentrifuge tubes containing affinity resin for 16 hours at 4 °C under rotation. The affinity resin was separated from the sample by centrifugation at 1 200 g for 2 minutes at 4 °C. The endotoxin-free supernatant was transferred to the supplied endotoxin-free microcentrifuge tube.

2.2.2 Cell purification

2.2.2.1 *Purification of leukocytes from human peripheral blood*

100 - 150 mL peripheral blood was collected into 10 mL Vacutainer containing K_3 EDTA (Becton Dickinson). 10 mL Lymphoprep (Life Technologies) was pipetted into 30 mL sterile Universal tubes and overlayed with an equal volume of peripheral blood followed by centrifugation at 895 g, 25 minutes, 20 °C, without braking. The resulting interface between the upper plasma layer and the underlying Lymphoprep, which comprised blood mononuclear cells, was collected and transferred into 50 mL Falcon tubes and washed once in sterile wash buffer (Table 2.1) at 895 g, 10 minutes, 20 °C. The pellets were pooled into one 50 mL Falcon tube and further washed at decreasing speeds of 500 g and 270 g, each for 10 minutes at 4 °C. The pellet was resuspended in 1 mL wash buffer, viable cells counted based on exclusion of 0.04 % (w/v) trypan blue in PBS, and the concentration of the cell suspension adjusted according to the requirements of the following procedures.

2.2.2.2 *Enrichment of monocytes and T cells from mononuclear cells by counter-current elutriation*

Separation of PBMCs was performed using a J2-21 centrifuge and JE-6B counter-current elutriation rotor from Beckman.

The rotor was assembled and connected to the centrifuge according to the manufactures instructions. The elutriation buffer (Table 2.1) was prepared and kept on ice. The rotor chambers and connecting tubing were sterilised in 6 % (v/v) H_2O_2 in UHQ water for 30 minutes followed by extensive rinsing with sterile PBS. The ice-cold elutriation

buffer was run through the system at a starting flow rate of 18 mL / minute, while the centrifuge was cooled to 4 °C. Air bubbles were removed from the chambers as the rotor reached the starting speed of 3130 rpm. The mononuclear cells suspended in 4 mL elutriation buffer in a sterile 5 mL syringe were loaded into the elutriation chamber over a period of 10 minutes (Table 2.3). The T cell enriched fractions 1-3 and the monocyte enriched fractions 7-10 were collected in 50 mL Falcon tubes on ice.

Table 2.3. Elutriation protocol

Cell fraction	Time (min)	Time interval	Speed (rpm)	Flow rate (mL/min)
Cell loading	0	10	3130	18
1	10	2.5	2930	21
2	12.5	2.5	2930	21
3	15	2.5	2790	20
4	17.5	2.5	2630	19
5	20	2.5	2580	18
6	22.5	2.5	2580	18
7	25	2.5	2230	20
8	27.5	2.5	2100	20
9	30	2.5	2100	20
10	32.5	2.5	1950	20

2.2.2.3 Enrichment of monocytes from human mononuclear cells by discontinuous Percoll gradients

The original Percoll gradient protocol, (Sallusto *et. al* [72]) gave unsatisfactory separation of monocytes from human PBMC populations. The density of the gradients and the volumes of the most dense layers have been adjusted to those described in Table 2.4 to give improved monocyte separation.

The osmolarity of the Percoll (Amersham Pharmacia Biotec UK Ltd., Little Chatfont, UK) was adjusted by adding one part × 10 PBS, without $Ca^{2+}$and Mg^{2+}, to 9 parts Percoll. The Percoll was adjusted to the required density (Table 2.4) by dilution with RPMI 1640 containing 10 % FCS. For alternate layers the Percoll was diluted with RPMI 1640 with phenol red / 10 % FCS and RPMI-medium 1640 without phenol red / 10 % FCS.

Table 2.4. Percoll gradients

Percoll gradient	Approximate density (g/mL)	PBS adjusted Percoll (mL)	RPMI / 10 % FCS (mL)	Volume loaded / Percoll gradient (mL)
26.5 %	1.035	1.325	3.675	2
37 %	1.046	2.590	4.410	3
43 %	1.055	3.870	5.130	4
53 %	1.067	4.770	4.230	4

Each layer was carefully prepared in 2 × 15 mL Falcon tubes starting with the 53 % layer. Approximately 5.0×10^7 cells in 1 mL wash buffer was loaded onto each Percoll gradient followed by centrifugation at 895 g, 30 minutes, 20 °C, without braking. The monocyte-enriched fraction between the 43 % and the 53 % interface was collected and washed in wash buffer at 895 g, 10 minutes, 20 °C, followed by × 2 washes in cold wash buffer at 440 g for 7 minutes at 4°C. The pellet was resuspended in 1 mL wash buffer, a cell count performed using trypan blue exclusion, and the concentration of the cell suspension adjusted.

2.2.2.4 Immunomagnetic separation of cells using MACS

Cells obtained either by counter-current elutriation or density sedimentation were further purified by depleting contaminating cells using magnetically conjugated mAbs (Microbeads) and associated materials from Miltenyi Biotec (Bisley, UK), unless otherwise stated.

2.2.2.4.1 Indirect immunomagnetic depletion

The monocytes were suspended in cold MACS buffer (Table 2.1) containing 10 % human AB serum (80 µL / 1.0×10^7 cells) to block non-specific binding of immunoglobulins to Fcγ receptors. Following 15 minutes on ice the cells were labelled with mAb RFB9 against human CD19 (50 µg / 1.0×10^8 cells) (In house) and mAb OKT3 against human CD3 (50 µg / 1.0×10^8 cells) (In house) followed by 30 minutes incubation on ice. The cells were washed twice in cold MACS buffer at 440 g for 5 minutes at 4 °C, then resuspended in cold MACS buffer (80 µL / 1.0×10^7 cells) and magnetically-labelled $F(ab)_2$ fragments of GAM MicroBeads (20 µL / 1.0×10^7 cells). After 15 minutes

incubation in the refrigerator, the cells were resuspended in 15 mL MACS buffer then pelleted by centrifugation at 440 g for 5 minutes at 4 °C. The cells were resuspended in 500 µL cold MACS buffer and was loaded onto a Midi MACS VS^+ column that had been pre-washed with 3 mL cold MACS buffer and placed in a MidiMACs magnet on a MACS MultiStand. Unlabelled cells were eluted in 4 × 3 mL of cold MACS buffer. The cells was washed once in RPMI as above, and a cell count performed using trypan blue exclusion.

2.2.2.4.2 Direct immunomagnetic depletion

Non-specific binding of immunoglobulins to Fcγ receptors on enriched T cells or monocytes was blocked as described (2.2.2.4.1). For monocyte purification, cells were labelled with anti-human CD19 Microbeads and anti-human CD3 Microbeads by adding 20 µL of each bead / 1.0×10^7 cells to the cell suspension. For enrichment of $CD45RA^+$ T cell, anti-human CD19 Microbeads and anti-human $CD45RO^+$ Microbeads were used at 20 µL of each bead / 1.0×10^7 cells. In both cases, following 20 minutes incubation in the refrigerator, the cells were resuspended in 15 mL MACS buffer then pelleted by centrifugation at 440 g for 5 minutes at 4 °C. The cells resuspended in 500 µL cold MACS buffer were separated when passed through a MACS VS^+ column as described (2.2.2.4.1).

2.2.3 Monocyte-derived dendritic cell cultures

Mo-DCs were prepared by a modification of the methods of Sallusto *et. al.*[72]. Up to 95 % pure monocytes from human peripheral blood were obtained by a combination of leukocyte purification using Lymphoprep (2.2.2.1) followed by enrichment of monocytes by Percoll gradients (2.2.2.3) or counter-current elutriation (2.2.2.2) and finally, immunomagnetic depletion of $CD3^+$ T cells and $CD19^+$ B cells (2.2.2.4.1 or 2.2.2.4.2).

2.2.3.1 Culture conditions for conversion of human monocytes to dendritic cells

The purified monocytes were cultured at 1×10^6 cells/mL in sterile, flat-bottomed, 6-well or 12-well tissue culture plates. Unsatisfactory cell viability and poor conversion of monocytes to dendritic cells was experienced using published media, that included non essential amino acids (MEM) and 1 mM sodium pyruvate. The following approach gave

optimal result. Cells were cultured in sterile, phenol red-free RPMI 1640, containing 10 % heat inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 30 µM 2-mercaptoethanol (complete RPMI) at 37 °C and 5 % CO_2. The complete RPMI was supplemented with 1000 U/mL rhIL-4 and 35 ng/mL rhGM-CSF, a reduced amount of GM-CSF compared to the 50 ng/mL used in the original published protocol. Every other day the cultures were supplied with 1 mL fresh complete RPMI. Depending on the condition of the cultures, 1 mL fresh complete RPMI was either added to the culture or used to replace 1 mL of old culture medium. The fresh complete RPMI was supplemented with additional rhGM-CSF and rhIL-4 according to the following equation;

rhGM-CSF = (Final volume per well (mL) × 35 ng/mL) / 3

rhIL4 = (Final volume per well (mL) × 1000 U/mL) / 3

Mo-DCs were cultured for 8 days before harvest and subsequent analysis. During the last 48 hours the Mo-DCs were generally cultured in the additional presence of one of several mediators.

2.2.3.2 Preparation of monocyte-derived dendritic cells for flow cytometric analysis of CD23 internalisation

2.2.3.2.1 CD23 labelling of monocyte-derived dendritic cells

FITC conjugated anti-CD23 mAb (clone BU38) (kindly FITC-conjugated by Maurine Power, Tenovus, UK) diluted to 213 µg/mL in PBS was aliquoted and stored at -40°C.

Six to eight day old Mo-DCs were gently harvested into a 15 mL Falcon tube to minimise the mechanical activation, then pelleted at 500 g for 7 minutes at 4 °C. The supernatant was aspirated and approximately 1.0×10^6 Mo-DCs gently resuspended in 200 µL cold FITC conjugated BU38 (20 µg/mL) in PBS or FITC conjugated IgG1 isotype control diluted to the same concentration in PBS. Following 30 minutes incubation on ice in the dark the Mo-DCs were washed twice in cold PBS at 500 g for 4 minutes at 4°C then resuspended to 5.0×10^5 Mo-DCs /mL in cold complete RPMI supplemented with 1 000 U/mL rhIL-4 and 35 ng/mL rhGM-CSF.

2.2.3.2.2 Culture of CD23 labelled monocyte-derived dendritic cells

5.0×10^5 BU38-labelled Mo-DCs in complete RPMI containing rhGM-CSF and rhIL-4 were plated into the wells of flat bottomed 24-well plates, to which 75 ng/mL rhTNF-α, 1.9 nM rhIFN-γ or 100nM chimaeric human IgE was added. Following 6, 12 or 24 hours incubation at 37 °C and 5 % CO_2, the 8 day old Mo-DCs were harvested then resuspended in PBS and kept on ice in the dark.

2.2.3.2.3 Removal of surface expressed CD23 using trypsin

A proportion of each Mo-DC condition was resuspended in 200 µL of 0.25 % trypsin (Life Technologies Ltd) to remove cell surface CD23. Following 10 minutes incubation at 37 °C, the Mo-DCs were washed twice in FACS buffer (2.1.2) containing 10 % FCS at 500 g for 4 minutes at 4°C.

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

Mo-DCs, grown for 8 days in the presence or absence of additional stimuli for the last 48 hours, were harvested and used to investigate (1) their ability to drive proliferation of allogeneic T cells, (2) their fate in co-culture with allogeneic T cells, (3) the phenotype of the responding T cell population, and (4) the cytokine content in the resulting co-culture supernatant.

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

Allogeneic T cells, enriched for CD45RA expression (2.2.2.4.2), were suspended in RPMI 1640, 10 % human AB serum, 2 mM L-glutatamine, 100 U/mL penicillin and 100 µg/mL streptomycin (RPMI + AB serum) and plated out at 1.0×10^5 cells / 100 µL / well in 96-well flat-bottomed plates. Eight day old Mo-DCs were irradiated with 25 gray from a caesium source to prevent any spurious proliferation of possible contaminating T cells in the Mo-DC culture. Mo-DCs were diluted in RPMI + AB serum and added to T cells resulting

in 300, 1 000, 3 000 and 10 000 Mo-DCs per well (in triplicates). Control wells were also prepared containing T cells alone or DCs alone.

The cells were incubated at 37 °C, 5% CO_2 for 5 days before 0.5 μCi tritiated [H^3] thymidine (Amersham Pharmacia Biotech) was as added to each well. Following 6 hours incubation at 37 °C, 5% CO_2, thymidine incorporation was stopped by freezing the plates. The plates were stored at -40 °C pending harvest. Using a 96-well plate cell harvester (Tomtec) the cells from each thawed plate were harvested onto separate glass fibre filters (Wallac, Milton Keynes, UK). The filters were air-dried and placed in plastic pockets containing 2 mL scintillant (Betaplate scint, Wallac). T cell proliferation was quantified by measuring H^3-thymidine incorporation as counts per minute (cpm) in a 1450 microbeta liquid scintillant counter (Wallac). Alternatively, each area on the air-dried filter corresponding to one well on the 96-well plate, was punched out and placed in a polyethylene vial (Canberra Packard, Pangbourne, UK) containing 2 mL scintillant, capped and counted in a 2500 TR liquid scintillation analyser (Packard).

2.2.4.2 Co-cultures of CFSE labelled monocyte-derived dendritic cells and unlabelled T cells

A stock solution of 5 mM carboxyfluorescein-diacetate succinimidylester (CFSE) (Sigma) was prepared by dissolving the lyophilised powder in DMSO. The stock solution was further diluted to a working concentration of 2.5 μM in RPMI 1640, then filter sterilised. Following 8 days in culture the Mo-DCs were harvested and 6.0×10^5 Mo-DCs were resuspended in 200 μL of 2.5 μM CFSE. Following 10 minutes incubation at 37 °C the Mo-DCs were washes twice in RPMI containing 10 % FCS at 500 g for 4 minutes at 4°C. Co-cultures of 3.5×10^4 CFSE-labelled Mo-DCs and 3.5×10^5 allogeneic T cells were plated into the wells of flat bottomed 96-well plates in 200 μL RPMI + AB serum. Over a period of 5 days, the number of viable CFSE-labelled Mo-DCs and unlabelled T cells in these co-cultures was estimated by counting the total number of viable cells using trypan blue exclusion of dead cells followed by an estimation of the proportion CFSE-labelled to unlabelled cells by flow cytometry (2.2.8).

2.2.4.3 Monocyte-derived dendritic cell : T cell co-cultures for investigation of phenotype, apoptosis and cytokine production

Co-cultures of Mo-DCs and allogeneic T cells were prepared to investigate the phenotype of the T cell population following culture with Mo-DCs. Mo-DC : T cell co-cultures were also prepared to investigate the apoptotic state of all cells and the cytokine production by the cells in these co-cultures.

Eight day old Mo-DCs were harvested, irradiated with 25 gray, washed twice in RPMI, then resuspended in RPMI + AB serum. In the wells of a 24-well plate, 2.0×10^5 Mo-DCs were combined with 2.0×10^6 allogeneic T cells enriched for CD45RA expression, in a total volume of 1 mL RPMI + AB serum. Following 5 days incubation at 37 °C, 5% CO_2, the cells were harvested and immunostained to identify surface molecules (2.2.5) or apoptotic cells (2.2.7) by flow cytometry, and the supernatants collected for analysis of cytokine content by enzyme linked immunosorbant assay (ELISA)(2.2.9).

2.2.5 Phenotypic analysis of T cells, monocytes and monocyte-derived dendritic cells by two colour flow cytometry

2.2.5.1 Pre-blocking cells prior to immunostaining

Cultured cells were harvested into 15 mL Falcon tubes and centrifuged at 500 g for 7 minutes at 4°C. The supernatant was aspirated and the cells resuspended in 50 µL FACS buffer (2.1.2) containing 10 % human AB serum (FACS blocking solution) per immunostaining combination. The cells were divided between the required number of wells of a V-bottomed 96 well plate, and incubated for 15-30 minutes on ice, followed by centrifugation at 500 g for 4 minutes at 4°C and removal of the blocking solution.

2.2.5.2 Immunostaining for flow cytometry

Cells were labelled (i) directly, with antibodies conjugated to FITC or to PE (Table 2.2) or (ii) indirectly with biotinylated antibodies followed by fluorochrome conjugated streptavidin. All antibodies were used at optimal concentrations for flow cytometry as determined by titration. Antibodies were re-titrated at 6 month intervals to confirm optimal dilutions.

2.2.5.2.1 Direct immunostaining for flow cytometry

The pre-blocked cells were stained for 30 minutes on ice with FITC- and /or PE-conjugated antibodies optimally diluted in FACS blocking solution. In parallel, cells were also labelled with the appropriate fluorochrome conjugated isotype control antibodies. The cells were washed 3 times in 200 µL FACS buffer at 500 g for 4 minutes at 4°C. The cells were resuspended in 200 µl FACS buffer (2.1.2.1), transferred to FACS tubes, and placed on ice in the dark pending analysis.

2.2.5.2.2 Indirect immunostaining for flow cytometry

For biotinylated primary antibodies the pre-blocked cells were incubated for 30 minutes on ice with antibody optimally diluted in 50 µL of blocking solution. The cells were washed 3 times in 200 µL FACS buffer at 500 g for 4 minutes at 4°C. The cell pellets were retained in the V-bottomed 96 wells and incubated with 50 µL of blocking buffer containing FITC or PE conjugated streptavidin for 30 minutes on ice. In parallel, cells were labelled with the appropriate primary biotinylated isotype control followed by fluorochrome conjugated streptavidin. The cells were washed 3 times as above and following the final wash were resuspended in 200 µL FACS buffer, transferred to FACS tubes, and placed on ice in the dark pending analysis.

2.2.5.3 Paraformaldehyde fixation of labelled cells

Under circumstance where immunostained cells had to be stored for 6-16 hour before flow cytometric analysis, paraformaldehyde fixation was performed in order to retain the fluorescence.

Following the final wash after immunostaining for flow cytometry, the cells, in the V-bottomed 96-Microwell plate, were resuspended in 100 µL FACS buffer and transferred to FACS tubes. 100 µL 4 % paraformaldehyde (w/v) in PBS was added to the FACS tubes and the cells were stored at 4 °C in the dark pending analysis.

2.2.5.4 Flow cytometric analysis of surface molecules

Fluorescently labelled cell suspensions were passed through a Fluorescence-Activated Cell Scanner (FACScan) (Becton Dickinson), and cell populations were analysed using Lysis II or CELLQuest software (both Beckton Dickinson). 7 aminoactinomycin D (7-AAD, Sigma) was added to unfixed cells to a final concentration of 0.5 μg/mL and was used to set a live cell gate in FL3. Unlabelled cells were used to set the sensitivity of the fluorescence detectors to appropriate levels, such that on a histogram the peak of cells lay in the first decade of a four-log scale. Singly-stained cells labelled with CD1a FITC (for Mo-DCs), CD14 FITC (for enriched monocytes) or CD5 FITC (for enriched T cells) were used to compensate the fluorescence signal in FL1. Cells stained with HLA-DR PE (for Mo-DCs and enriched monocytes) or CD3 PE (for enriched T cells) were used to compensate the fluorescence signal in FL2. This was done using the standard compensation circuits on the instrument. Enriched monocytes and enriched T cells were typically detected at forward scatter and side scatter settings of 1.0 (E-0) and log 2.10, respectively. Mo-DCs were typically detected at forward scatter and side scatter settings of 8.5 (E-1) and log 2.10, respectively. Cell debris was eliminated using a forward scatter threshold.

T cells were identified as cells showing strong labelling for CD3 or CD5, monocytes were identified as cells showing strong labelling for CD14 whereas Mo-DCs were identified as cells showing no labelling for CD14 but strong labelling for CD1a.

Using Lysis II software or CELLQuest, the percentage labelled cells in a cell population was obtained by setting a histogram marker at ≤ 1 % of the appropriate isotype control, typically on the first decade of a four log scale. The geometric mean fluorescence intensity (MFI) was obtained for the entire ungated cell population in a histogram.

2.2.6 Flow cytometric analysis of FITC-dextran uptake

2.2.6.1 Receptor mediated endocytosis of FITC-dextran

FITC-dextran, 38 260 kDa (Sigma), was suspended in PBS to 10 mg/mL. Small particles were removed by centrifugation at 13 000 rpm for 10 minutes in a MSE MicroCentaur centrifuge (Jepson Bolton Laboratory Equipment) and clear liquid transferred to new vessels.

Receptor-mediated endocytosis of FITC-dextran was performed on 1.0×10^5 cells from each DC condition at 37°C for 1 hour in a final concentration of 0.25 mg/mL FITC-dextran in complete RPMI. In parallel 1.0×10^5 cells from each DC condition was incubated in 0.25 mg/mL FITC-dextran on ice (4 °C) for 1 hour as a control for surface bound FITC-dextran. The cells were stored at 4 °C in the dark pending analysis by flow cytometry.

2.2.6.2 Flow cytometric analysis of FITC-dextran uptake

Mo-DCs incubated in FITC-dextran supplemented medium were passed through a FACScan suspended in FACS buffer (2.1.2). Mo-DCs were typically detected at forward scatter and side scatter settings of 8.5 (E-1) and log 2.10, respectively. Cell debris was eliminated using a forward scatter threshold. Mo-DCs incubated at 4 °C were used to set the sensitivity of the fluorescence detectors to appropriate levels, such that on a histogram the peak of cells lay in the first decade of a four log scale. 7-AAD was used to distinguish live from dead cells as described in 2.2.5.4, although dead cells were not excluded during flow cytrometric analysis due to difficulties in compensating the strong signal in FL1. Dead cells were later excluded using Lysis II software. 10 000 events for each preparation were collected.

2.2.7 Identification of apoptotic cells by flow cytometry

2.2.7.1 Indirect immunostaining of apoptotic cells for flow cytometry

Apoptotic cells were identified by their ability to bind annexin V. Biotinylated annexin V and × 10 annexin V binding buffer was obtained from PharMingen.

Cultured cells were harvested into 15 mL Falcon tubes and centrifuged at 500 g for 7 minutes at 4°C. The supernatant was aspirated and each culture condition were resuspended in 400 µL cold PBS and divided into 4 wells of a V-bottomed 96 well plate. Following two washes in cold PBS at 500 g for 7 minutes at 4°C the cells in two of the four wells were resuspended in biotin-conjugated annexin V diluted 1/17 in × 1 annexin V binding buffer. The remaining cells were resuspended in × 1 annexin V binding buffer alone. Following 15 minutes incubation at room temperature in the dark, the cells were washed twice in × 1 annexin V binding buffer at 500 g for 4 minutes at 18°C. The cells in

the two wells stained with annexin V and the cells in one of the unstained wells were resuspended in FITC-conjugated streptavidin (Serotec) diluted 1/20 in × 1 annexin V binding buffer. The remaining cells were resuspended in × 1 annexin V binding buffer alone. Following 15 minutes incubation at room temperature in the dark the cells were washed twice in × 1 annexin V binding buffer at 500 g for 4 minutes at 18°C, resuspended in 200 µL FACS buffer, transferred to FACS tubes, and placed on ice in the dark pending analysis. Apoptotic cells were analysed within two hours using a FACScan cytometer (2.2.9.6).

2.2.7.2 Flow cytometric identification of apoptotic cells

Mo-DCs were typically detected at forward scatter and side scatter settings of 8.5 (E-1) and log 2.10, respectively. Mo-DC : T cell co-cultures were typically detected at forward scatter and side scatter settings of 1.0 (E-0) and log 2.10, respectively. Cell debris was eliminated using a forward scatter threshold. Unlabeled cells were used to set the sensitivity of the fluorescence detectors to appropriate levels, such that on a histogram the peak of cells lay within the first decade of a four log scale. To these unlabeled cells, approximately 1µg/mL propidium iodide (PI, Sigma) was added to distinguish live from dead cells in FL2, although dead cells were not excluded during flow cytrometric analysis. These PI-labelled cells were used to compensate the fluorescence signal in FL2. Cells stained with biotinylated annexin V and FITC-conjugated streptavidin were used to compensate the fluorescence signal in FL1. No more than 10 minutes prior to flow cytometric analysis, PI was added to cells stained with biotinylated annexin V and FITC conjugated streptavidin. Early apoptotic cells were identified as cells showing labelling for annexin-V but impermeable to PI. Late apoptotic cells were identified as cells showing labelling for annexin-V and permeable to PI. Live cells were identified as cells showing no labelling for annexin-V and impermeable to PI (See Figure 5.4). 5 000 events for each preparation were collected.

2.2.8 Flow cytometric analysis of CFSE labelled cells

Mo-DC : T cell co-cultures containing CFSE stained Mo-DCs were passed through a FACScan suspended in FACS buffer. CFSE labelled cells were identified as cells fluorescing strongly in FL1 and were used to compensate the fluorescence signal in FL1.

Dead cells were identified using PI (2.2.7.2) and were excluded from the events being collected. 5 000 live events for each preparation were collected.

2.2.9 Basic method for sandwich ELISA

Commercial kits were used to detect IL-6 (R&D Systems), IL-10 (PharMingen), TGF-β1 (R&D Systems), sCD95 (IDS, Tyne and Wear, UK and sCD23 (Biosource Europe S.A, Nivelles, Belgium). ELISAs were performed at room temperature according to the supplier's instructions using the solutions supplied with the kit or prepared in house prior to use. In brief, Multi-sorp ELISA plates (Life Technologies) were coated with optimally diluted capture mAb (see Table 2.5) overnight, unless pre-coated plates were supplied. Non-specific binding of protein to the plates was blocked for 1-2 hours, unless pre-blocked plates were supplied. After thorough washing of the plates, a standard curve of the appropriate cytokine or protein supplied with the kits (except rhIL-10 from R&D Systems) was added along with the same volume of optimally diluted test supernatant. Samples for measurement of TGF-β1 content were activated prior to use by adding 1 volume of 1 M HCl to 5 volumes of sample for 10 minutes followed by addition of 1 volume of 1.2 M NaOH/0.5 M HEPES. Depending on the individual protocol, the cytokine or protein was captured by the coating mAb over a period of 1 –3 hours in the presence or absence of optimally diluted biotinylated detection mAb (Table 2.5). In cases where the cytokine or protein was captured in the absence of detection mAb the plates were thoroughly washed before the detection mAb was added for 1-2 hours. All ELISAs used streptavidin-conjugated horseradish peroxidase (HRP) (TCS Biologicals Ltd., Buckingham, UK) unless supplied with the kit) with ready made tetramethylbenzidine free base (TMB) substrate (TCS, or supplied with the kit) to detect the biotinylated detection mAb. Following thorough washing, the plates were then incubated with the streptavidin-conjugated HRP, diluted 1:8 000, for 30 minutes - 1 hour followed by thorough washing and the addition of TMB substrate. The colour reaction was typically stopped following 15 - 20 minutes incubation in the dark and the plates were read at 450 nm on a SpectraMAX 340 PC plate reader (Molecular Devices, Wokingham, UK).

Table 2.5. Working concentrations of ELISA coating and detection mAbs.

Kit	Coating mAb concentration	Detection mAb concentration
IL-6 DuoSet ELISA	3.0 μg/mL	0.2 μg/mL
IL-10 (paired mAbs)	2.0 μg/mL (clone JES3-9D7)	1.0 μg/mL (clone JES3-12G8)
TGF-β1 DuoSet ELISA	2.0 μg/mL	0.3 μg/mL
sCD95	pre-coated, pre-blocked	ready to use
sCD23	pre-coated. pre-blocked	ready to use anti-sCD23-HRP

2.2.10 Nitric oxide analysis

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

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

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

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

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

A calibration curve was constructed before any samples were analysed by stepwise addition of increasing concentrations of $NaNO_2$ prepared in PBS. The linear fit correlates

the emission (mV) generated by the chemiluminescent reaction of NO with O_3, with the corresponding NO concentration. The slope of the curve was then used to calculate the concentration of NO measured in the cell culture samples. The sensitivity for measurement of NO and its reaction products in liquid samples is approximately 1 pmol.

2.2.11 TaqMan reverse-transcription polymerase chain reaction

2.2.11.1 Purification of total RNA

Total RNA was purified using QIAShredder columns, RNeasy Mini Kit (including columns, 1.5 mL and 2.0 mL collection tubes, RNase Free water, RLT buffer, RW1 buffer, RPE buffer) and RNase-Free DNase Set Kit (including RNase Free DNase I, Buffer RDD) purchased from QIAGEN Ltd. (Crawley, UK). RNA purification was carried out according to the supplier's instructions at room temperature using sterile aerosol resistant tips (Life Technologies). With the exception of cell harvest, all centrifugations were performed in a MSE MicroCentaur centrifuge.

Cultured Mo-DCs were harvested into 15 mL Falcon tubes and spun at 500 × g for 7 minutes at 4°C. The supernatant was aspirated and the pellet, containing up to 5.0×10^6 cells, was resuspended in 350 µL RLT buffer, containing 0.145 M 2-mercaptoethanol, followed by thorough mixing. The cell lysate was loaded onto a QIAShredder column placed in a collection tube and spun at 13 000 rpm for 2 minutes. 70 % ethanol was prepared by diluting 7 parts absolute ethanol (LAB3) with 3 parts DNase/RNase free water (Life Technologies). One volume 70 % ethanol, approximately 350 µL, was added to the homogenised cell lysate in the collection tube and was mixed by repeated pipetting. The ethanol : lysate mix was loaded onto a RNeasy mini spin column placed in a new collection tube and spun at 8 000 × g for 15 seconds. The flow-through was discarded. The spin column was washed once, reusing the collection tube, by adding 350 µL RW1 buffer followed by centrifugation at 8 000 × g for 15 seconds. The flow-through was discarded and the collection tube reused. 70 µL RDD buffer was added to 10 µL DNase I (2.7 U/µL) and without further handling added to the spin columns followed by 15 minutes incubation. DNA degradation was stopped by adding 350 µL RW1 buffer followed by centrifugation at 8 000 × g for 15 seconds. The flow-through was discarded. The spin column was placed in a new 2 mL collection tube and washed by adding 500 µL RPE buffer, containing 4 volumes of absolute ethanol, followed by centrifugation at 8 000 × g for 15 seconds. The flow-

through was discarded and the collection tube reused. The spin columns were washed once more with 500 µL working RPE buffer and spun at 13 000 for 2 minutes. The spin column was placed in a new collection tube and the total RNA was eluted by adding 30 µL RNase free water followed by centrifugation at 8 000 × g for 1 minute. The eluted RNA was stored on ice pending measurement of RNA content.

2.2.11.2 Measurement of RNA concentration

The concentration of purified total RNA was measured spectrophotometrically using a GeneQuant (Amersham Pharmacia Biotec) and a 5 mm pathlength cuvette holding 5 µL of recoverable sample. The absorbance at 260 nm and 280 nm was measured using RNase and DNase free water as a reference. The absorbance value obtained at 260 nm (A_{260}) was multiplied by 2 to adjust for the use of the 5 mm pathlength cuvette. The RNA content could then be calculated as 1 OD at 260 nm corresponds to 40 µg/mL RNA. An A_{260} / A_{280} ratio between 1.8 – 2.1 indicate high RNA purity. The sample was recovered from the cuvette and stored at -40 °C.

2.2.11.3 Random hexamer primer reverse transcription

Reverse transcription of the total RNA was performed using a SUPERSCRIPT™II RNase H⁻ Reverse Transcription kit (Life Technologies) including SUPERSCRIPT II, 5 × First-Strand Buffer and DTT, and random hexamer primers (Promega, Southampton, UK). Reaction mixes were set up in 0.2 mL thin-walled tubes (Advanced Biotechnologies Ltd.). For each RNA sample one positive reverse transcription reaction (+RT) containing reverse transcriptase enzyme, and one negative reverse transcription reaction (-RT) without enzyme was set up. For each +RT reaction, a maximum of 1 µg RNA in a total volume of 10 µL RNase and DNase free water was added to 1 µL random hexamer primers (50 ng/mL) and incubated at 72 °C for 10 minutes. The +RT reaction mixtures were kept on ice while 4 µL first strand buffer, 2 µL of 0.1 M DTT, 1 µL of 12.5 mM dNTPs (Bioline Ltd., London, UK) and 1 µL of 20 U RNase inhibitor (Amersham Pharmacia Biotech) was added to each tube followed by incubations at 25 °C for 10 minutes and 42 °C for 2 minutes. The +RT reaction mixtures were kept on ice while 1 µL SuperScript II was added to each tube. The RNA was reverse transcribed at 42 °C for 50 minutes in a GeneAmp 9600 thermal cycle

(PE Biosystems, Warrington, UK). The samples were then heated to 94 °C for 3 minutes to inactivate the SuperScript II. The resulting cDNA was diluted to a final volume of 60 µL with UHQ water and stored at - 80 °C. –RT reaction mixtures were prepared and treated as the +RT reaction mixtures with the exception of SuperScript II being replaced by RNase and DNase free water.

2.2.11.4 TaqMan multiplex polymerase chain reaction

Relative quantification of human CD23 normalised to 18S ribosomal RNA (rRNA) was performed using pre-developed TaqMan assay reagents for human CD23, including 20 × primers, probes and 2 ×TaqMan Universal PCR Master Mix, and 20 × 18S rRNA primer and probe purchased from PE Biosystems (Warrignnton, UK). The probes for CD23 and 18S rRNA carry different fluorescent dyes (FAM and VIC respectively) and so both targets can be in the same tube, referred to as multiplex TaqMan polymerase chain reaction (multiplex TaqMan PCR).

Duplicate reaction mixes for each cDNA preparation were set up in 0.2 mL MicroAmp optical tubes (PE Biosystems). 10 µL of the cDNA (2.2.11.1 - 2.2.11.3) was added to 12.5 µL 2 × TaqMan Universal PCR Master Mix, 1.25 µL 20 × CD23 primer and probe and 1.25 µL 20 × 18S rRNA primer and probe. The samples were placed in an ABI PRISM 7700 sequence detection system (PE Biosystems) where amplification of the target cDNAs was recorded over 40 cycles of amplification.

After amplification, the threshold cycle value (C_T) was determined for each cDNA preparation. This was determined as the cycle number at which the exponential amplification curve crossed the threshold line (Figure 6.6). In order to accurately set the threshold, the baseline was first set such that the amplification curve growth on a linear scale begins at a cycle number greater than the highest baseline cycle number. The threshold value was then set above the background within the exponential phase of the logarithmic scale amplification plot. For each cDNA preparation the C_T value for CD23 was then normalised to the C_T value for the endogenous internal standard 18S rRNA by subtracting the 18S rRNA C_T from the CD23 C_T. The average ΔC_T for the duplicate cDNA preparations was further used in the equations below to calculate the relative quantification of treated Mo-DC cDNA preparations compared to untreated Mo-DC cDNA preparations. A relative quantification value of 4.0 indicates four times as much CD23 cDNA in the treated samples compared to the untreated sample.

Equation 1) $\Delta\Delta C_T$ = average ΔC_T (treated preparation) - average ΔC_T (untreated preparation)

Equation 2) Relative quantification = $2^{-\Delta\Delta CT}$

2.2.12 Confocal Microscopy

2.2.12.1 Immunostaining for confocal microscopy

Immunostaining for confocal microscopy was performed at room temperature. Cultured Mo-DCs were harvested into 15 mL Falcon tubes and spun at 500 × g for 7 minutes at 18°C. The supernatant was aspirated and the cells resuspended to approximately 2.0×10^6 cells/mL in RPMI. Around 1.0×10^5 cells in 100 µL were spun at 500 rpm for 5 minutes onto polylysine-coated microscope slides (LAB3) using a Cytospin 2 centrifuge (Shandon). The cells on the air-dried slides were fixed in 4 % paraformaldehyde (w/v in PBS) for 5 minutes followed by extensive washing in PBS. Free amino groups were blocked using PBS containing 50 mM NH_4Cl for 5 minutes followed by extensive washing with PBS. The cells were permeabilised in FACS buffer (2.1.2) containing 0.1 % saponin (w/v) and 10 % human AB serum (permeabilisation buffer). Following 15 minutes, the equilibrated cells were stained for 30 minutes with 20 µg/mL unconjugated anti-CD23 (clone BU38) (The Binding Site) diluted permeabilisation buffer. In parallel, cells were labelled with 20 µg/mL unconjugated isotype control antibody. The cells were washed extensively in FACS buffer containing 0.1 % saponin before the cells were stained for 30 minutes with RRX-conjugated GAM (IgG1) (Jackson ImmunoResearch Labs. Inc., West Grove, PA, USA) optimally diluted in permeabilisation buffer. The cells were washed extensively in FACS buffer containing 0.1 % saponin before a small amount of Moviol (Agar Scientific, Stansted, UK) was placed on the stained cells and covered with a circular cover slip. The slides were stored at 8°C in the dark pending analysis by confocal microscopy.

2.2.12.2 Confocal microscopy

Images were acquired using a SP2 Confocal Lacer Scanning Microscope (Leica, Milton Keynes, UK) equipped with argon, blue helium neon and red helium neon lasers with a spectral head. In all cases Mo-DCs were viewed using × 40 and × 63 oil-immersion objectives. A series of confocal sections were taken at 0.5 μm intervals.

2.2.13 Statistical analysis

Non-parametric statistical analysis was performed using SPSS version 10.0 for Windows (Microsoft Systems Inc., Northampton, MA, USA). The Wilcoxon test was used where possible for comparing two related samples within a subject group. The Mann Whitney *U* test was used where possible to compare two independent samples between the asthmatic and normal subject group. A p value of less than 0.05 was considered statistically significant

Chapter Three

Characterisation of monocyte-derived dendritic cells from normal and asthmatic subjects

Summary

Over the past decade genetic studies have revealed direct links between the disease phenotype of atopic asthma and candidate genes. Some of these candidate genes may impact on dendritic cell functions. Dendritic cells from atopic asthmatic subjects may hence be inherently predisposed to promote an atopic asthmatic disease phenotype. To identify hereditary differences between Mo-DCs from normal and asthmatic subjects, work presented in this chapter aimed to characterise Mo-DCs with regard to their surface molecule expression and cytokine production.

By using a combination of leukocyte purification using Lymphoprep followed by counter-current elutriation and immunomagnetic depletion of $CD3^+$ T cells and $CD19^+$ B cells, a $\approx$ 70 % pure monocyte population was obtained. After 8 days culture in medium supplemented with rhIL-4 and rhGM-CSF complete conversion of monocytes to Mo-DCs was achieved, yielding on average 84% $CD1a^+$ Mo-DCs. No difference in monocyte purity on day 0 or CD1a yield following 8 days in culture was noted between normal and asthmatic subjects. For the first time, a statistically significantly smaller proportion of $CD23^+$ Mo-DCs was identified in Mo-DC populations derived from asthmatic subjects compared to those from normal subject. None of the other surface molecules studied showed any significant difference in their expression on Mo-DCs from normal and asthmatic subjects. Further, increased IL-6 production in Mo-DCs from asthmatic subjects was observed but failed to reach significance. These results indicate that Mo-DCs from asthmatic subjects are different to Mo-DCs from normal subjects and may be more prone to initiate or perpetuate allergic asthmatic inflammation.

3.1 Introduction

The link between genetic factors and the clinical expression of atopic asthma has been investigated during the past decade. Although the complex nature of atopic asthma including the influence of environmental factors, difficulties in disease definition and ethnic ascertainment has made it hard to clearly identify any direct links between disease phenotype and candidate genes, linkage to regions on chromosome 5q, 6p, 11q13 and 16p have been reported [208]. Candidate genes on chromosome 5q include those encoding the Th2 cytokines IL-3, IL-4, IL-5, IL-13, GM-CSF and the b_2-adrenergic receptor. On chromosome 6p the human leukocyte antigen (HLA) gene complex has been identified, that contains genes with linkage to a phenotype of elevated levels of allergen specific IgE. Mapping of chromosome 11q13 identified the FcεRI beta chain (FcεRI-β) as a candidate gene with links to atopy. Finally, chromosome 16p has been associated with aeroallergen sensitisation linked to a candidate gene coding for the IL-4 receptor α subunit, IL-4RA.

Some of the above candidate genes may impact on dendritic cell function. Genes directly involved in DC functions include those associated with HLA, that are implicated in antigen presentation, and those encoding IL-4 and IL-13, that DCs have been shown to produce [32,63]. The IL-4RA gene may also directly impact on DC function as IL-4 has been shown to drive the differentiation of myeloid DCs *in vitro*[72]. Further, as DCs have been shown to respond to IL-3[80], IL-4[72], IL-13[70] and GM-CFS[72], genes associated with these Th2 cytokines may indirectly affect DC functions. Although no clear genetic linkage have been demonstrated for the reduced level of IL-10 observed in asthmatic subjects[275], this may also impact on DC functions as DCs both produce[32] and respond to IL-10[33]. Dendritic cells from atopic asthmatic subjects may hence be inherently predisposed to promote an atopic asthmatic disease phenotype.

The work presented in this chapter aimed to characterise untreated Mo-DCs from normal and asthmatic subjects. Characterisation has been performed with regard to surface molecule expression and cytokine production.

3.2 Materials and methods

3.2.1 Subjects

23 non-atopic, non-asthmatic normal subjects (11 female, 12 male) and 11 atopic, asthmatic subjects (5 female, 6 male), defined as in section 2.1.7, took part in this study.

3.2.2 Immunostaining for flow cytometry

Mo-DCs were blocked (2.2.5.1) prior to immunostaining with mAbs against CD1a (FITC), CD14 (PE), FcεRI-α (15.1-FITC), CD23 (PE), CD40 (PE), CD80 (PE), CD83 (PE), CD86 (PE) and HLA-DR (PE) (Table 2.2, 2.2.5.2.1). In parallel, cells were also labelled with the appropriate fluorochrome conjugated isotype control antibodies, IgG2a FITC, IgG1γ PE and IgG2b FITC.

During flow cytometric analysis dead cells were identified using 7-AAD and were excluded from the collected events (2.2.5.4). A total of 5 000 events were acquired for each sample.

3.2.3 Monocyte-derived dendritic cell cytokine production

On day 7 of culture, 500 μL of supernatant was removed from the Mo-DC cultures and stored at -40°C pending analysis of cytokine content by ELISA. Following another 24 hours in culture the Mo-DCs were harvested, counted and the total volume of supernatant measured.

The IL-6 and IL-10 content in Mo-DC supernatants was measured by ELISAs according to the supplier's instructions (2.2.9). All supernatants were measured in duplicates. Supernatants for measuring IL-6 content were diluted 1:20 to fall with in the detection range of the standard curve (9.4 pg/mL - 600 pg/mL). Supernatants for measuring IL-10 were used without dilution to fall within the detection range of 15.6 pg/mL - 2 ng/mL. Both ELISAs used streptavidin-conjugated HRP with TMB substrate to detect biotinylated mAb. The plates were subsequently read at 450 nm on an ELISA plate reader.

The concentration of cytokine in the supernatants was calculated by relating the mean absorbance value of the duplicates to the standard curve. These values were adjusted to account for the sample dilution and were expressed as pg / 5.0×10^5 Mo-DCs.

3.2.4 Statistical analysis

Using SPSS 10.0 for Windows, the non-parametric Mann Whitney *U* test was used where possible to compare responses between the asthmatic and normal subject groups. A *p* value of less than 0.05 was considered statistically significant.

3.3 Results

3.3.1 Conversion of monocytes to monocyte-derived dendritic cells

On average 70.9% ± 3.0 pure monocytes were obtained by a combination of leukocyte purification using Lymphoprep (2.2.2.1) followed by enrichment of monocytes by counter-current elutriation (2.2.2.2) and immunomagnetic depletion of $CD3^+$ T cells and $CD19^+$ B cells (2.2.2.4.2). After 8 days culture in medium supplemented with rhIL-4 and rhGM-CSF a population consisting of, on average 84.4% ± 3.2 $CD1a^+$ Mo-DCs and 2.5% ± 0.9 $CD14^+$ cells was obtained (n = 15). No difference in monocyte purity on day 0 or CD1a yield following 8 days in culture was noted between normal and asthmatic subjects (data not shown). Figure 3.1 shows a representative example of the purity of enriched monocytes from a normal subject on day 0 and the resulting $CD1a^+$ Mo-DCs on day 8.

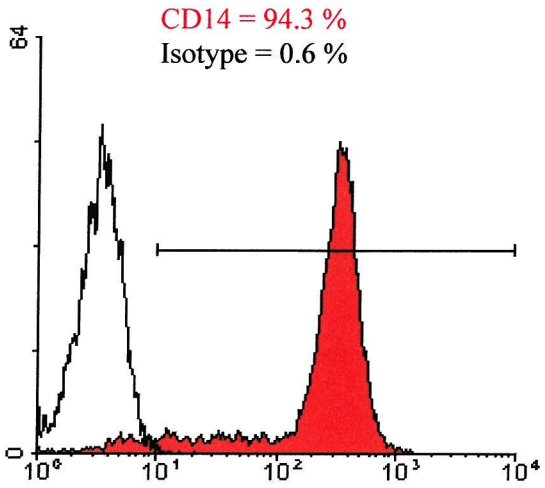
3.3.2 Characterisation of monocyte-derived dendritic cells from normal and asthmatic subjects

3.3.2.1 Surface molecule expression

Table 3.1 and Figure 3.2 show surface molecule expression on untreated Mo-DCs from normal and asthmatic subjects.

Using the Mann Whitney statistical test for non-parametric independent samples, the proportion of Mo-DCs expressing $CD23^+$ was significantly smaller in the Mo-DC population derived from asthmatic subjects (32.6 ± 6.1 % $CD23^+$ cells, n =11, $p < 0.05$) compared to those from normal subjects (48.6 ± 5.2 % $CD23^+$ cells, n =16). The level of expression of CD23 also appeared lower in Mo-DCs from asthmatic subjects (MFI = 8.5 ± 1.5, n =11) compared to Mo-DCs from normal subjects (MFI = 12.6 ± 2.0, n =16), however failed to reach statistical significance (data not shown). None of the other surface molecules studied showed any significant difference in their expression on Mo-DCs from normal and asthmatic subjects.

a) Day 0



b) Day 8

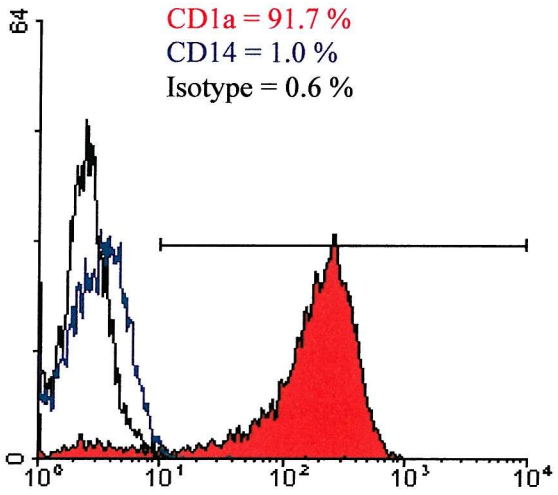


Figure 3.1 Conversion of monocytes to Mo-DCs

High purity of monocytes ($CD14^+$ cells) at start of culture on day 0 was obtained by a method using Lymphoprep, counter-current elutriation and immunomagnetic depetion of $CD3^+$ and $CD19^+$ cells as shown in a). Following 8 days of culture in medium supplemented with rhIL-4 and rhGM-CSF the cell population expressed high levels of CD1a and low levels of CD14 as shown in b).

Table 3.1 Surface molecule expression on untreated Mo-DCs from normal and asthmatic subjects

Mo-DC expressed molecule	Normal subjects, average with standard error	n =	Asthmatic subjects, average with standard error	n =
FcεRI	19.2 ± 6.6[+]	9	14.9 ± 5.5	4
CD23	48.6 ± 5.2	16	32.6 ± 6.1 *	11
CD80	39.5 ± 4.1	14	37.0 ± 3.4	10
CD83	9.8 ± 2.9	2	10.2 ± 1.5	7
CD86	9.4 ± 2.2	16	7.6 ± 1.1	8
CD40	50.7 ± 6.7	12	46.2 ± 4.6	8
HLA-DR	38.2 ± 5.0	18	38.2 ± 4.9	8

[+] Calculations based on percentage positive cells are presented in blue and calculations based on MFI are presented in red.

* = $p < 0.05$ compared to Mo-DCs

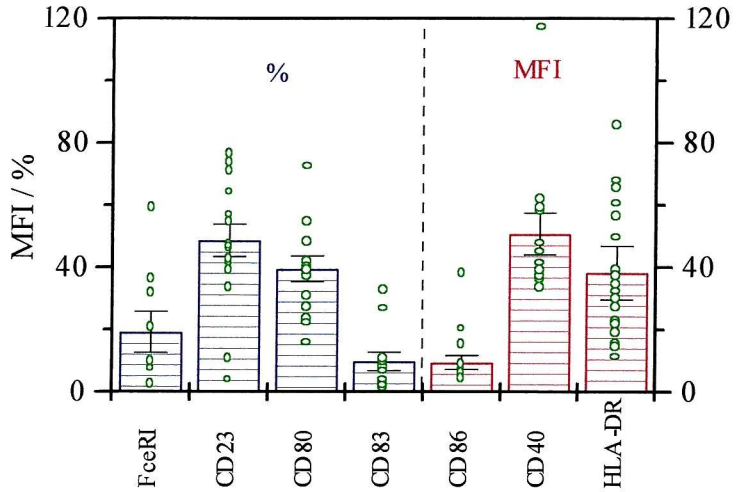
3.3.2.2 Cytokine production

Figure 3.3 and Table 3.2 show IL-6 and IL-10 production by 7 day old untreated Mo-DCs from normal and asthmatic subjects. Although the sample number is too low for statistical analysis, it appears that Mo-DCs from asthmatic subjects produce more IL-6 than Mo-DCs from normal subjects. Although not as pronounced, a similar trend of higher IL-10 production by Mo-DCs from asthmatic subjects was observed.

Table 3.2 IL-6 and IL-10 by untreated control Mo-DCs from normal and asthmatic subjects

Cytokine	Normal subjects, (pg / 5.0 × 10^5 Mo-DCs)	n =	Asthmatic subjects, (pg / 5.0 × 10^5 Mo-DCs)	n =
IL-6	5674 ± 1583	4	10009 ± 2157	3
IL-10	67 ± 16	9	107 ± 36	4

a) Normal subjects



b) Asthmatic subjects

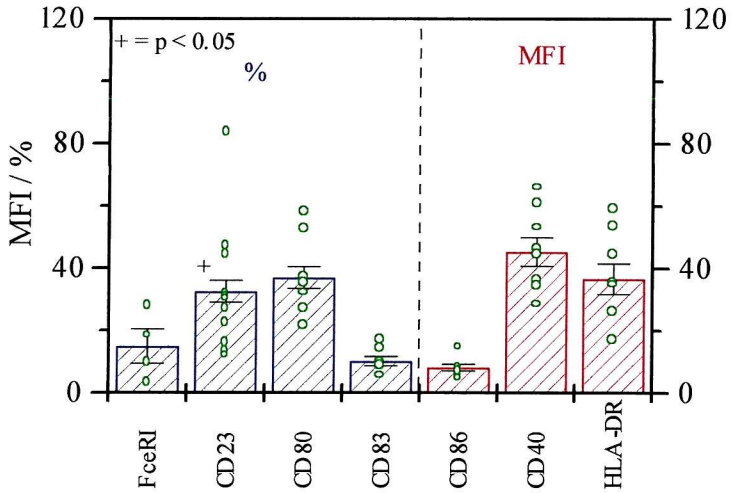
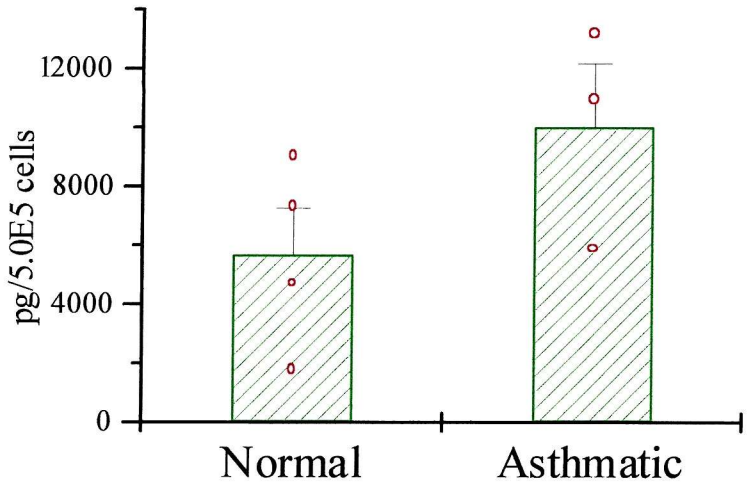


Figure 3.2 Surface molecule expression on untreated Mo-DCs

Mo-DCs from a) normal and b) asthmatic subjects were cultured in medium supplemented with rhIL-4 and rhGM-CSF for 8 days, then harvested, stained for surface expression of FcεRI, CD23, CD40, CD80, CD83, CD86 and HLA-DR and analysed by two-colour flow cytometry. Each individual value is presented as an open green circle and the mean of all values for each surface molecule is shown as a column with error bars representing the standard error of the mean (SEM). The percentage of cells positive for a given molecule is presented in blue, MFI is presented in red. For each molecule, the number of subjects studied is as indicated in Table 3.1.

+ = $p < 0.05$ compared to Mo-DCs from normal subjects.

a) IL-6 production



b) IL-10 production

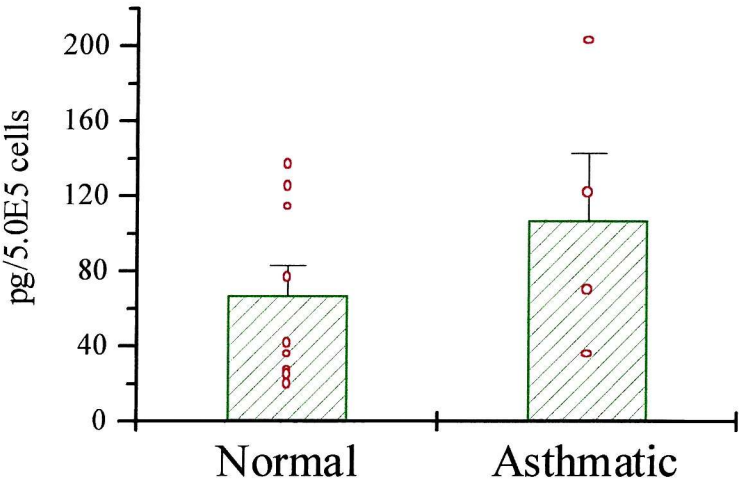


Figure 3.3 IL-6 and IL-10 production by untreated Mo-DCs

Levels of a) IL-6 and b) IL-10 were measured by ELISA in supernatants from 7 day old untreated Mo-DCs from normal and asthmatic subjects. Values were calculated as amount of cytokine (pg) expressed by 5.0×10^5 Mo-DCs. Each individual value is presented as an open red circle and the mean of all values for each subject group is shown as a column with error bars (SEM). The number of subjects studied is as indicated in Table 3.2.

3.4 Discussion

3.4.1 Conversion of monocytes to monocyte-derived dendritic cells

The current protocol for purification of monocytes using Lymphoprep, counter-current elutriation and immunomagnetic depletion yields on average 70.9% ± 3.0 pure monocytes. This procedure has several advantages over other monocyte purification protocols such as Percoll gradient centrifugation or cell adhesion, including minimal cell loss, complete depletion of platelets and availability of T cell fractions for parallel experiments. Further, in contrast to protocols that purify monocytes by adhesion, using the Lymphoprep-elutriation-MACS protocol the resulting enriched monocyte population is in suspension allowing flow cytometric analysis. However, there are also some disadvantages using this protocol including the long procedure taking between 5 - 6 hours, which may cause some cell death. It would also be preferable to further increase the purity of the final enriched monocytes. The major remaining contaminants are NK-cells and a few T cells (data not shown).

Using 1 000 U/mL rhIL-4 and 35 ng/mL rhGM-CSF, the monocyte conversion to Mo-DCs occurred over a period of 6 days (data not shown), after which the Mo-DC population could be exposed to additional stimuli. Without additional stimuli, this protocol yielded on average 84.4% ± 3.2 $CD1a^+$ Mo-DCs following 8 days in culture. The period for conversion may be decreased to 5 days using increased concentrations of rhGM-CSF (50 ng/mL) (data not shown).

3.4.2 Surface molecule expression

Although phenotypic and functional differences of untreated Mo-DCs from normal and asthmatic subjects have recently been published by van den Heuvel *et. al.*[262], the significantly lower percentage $CD23^+$ Mo-DCs observed here in the asthmatic Mo-DC population is novel. Surface expression of CD23 has been shown to be regulated by IL-4 in B cells[276,277], DCs[278] and monocytes [276,277,279]. Since IL-4 is present in these Mo-DC cultures, it is possible that the difference in CD23 surface expression on Mo-DCs from normal and asthmatic subjects could be due to differences in IL-4 related signalling pathways specific to CD23. Mo-DCs from asthmatic subjects may hence be genetically predisposed to express lower levels of CD23 at their surface. However, IL-4 has also been shown to regulate the release of soluble CD23 (sCD23) from the surface of B cells[276]. Mo-

DCs from asthmatic subjects may therefore also be predisposed to release a higher proportion of the surface expressed CD23 as sCD23. A DCs that produce elevated levels of sCD23 may sequentially perpetuate allergic disease as sCD23 have been shown to promote IgE production in B cells[280-282]. It is therefore interesting to note that the serum levels of sCD23 is significantly increased in patients with allergic disorders [283,284]. It should also be noted that not only genetic polymorphisms may shape the phenotype and function of Mo-DCs from normal and asthmatic subjects, but also the systemic environment in these two subject groups affecting the bone marrow and the circulating monocytes differently.

No differences in expression of FcεRI, CD40, CD80, CD83, CD86 or HLA-DR were found between Mo-DCs from normal and asthmatic subjects. Similarly, no difference in FcεRI surface expression was reported comparing monocytes[272] or peripheral blood DCs[285] from non-atopic and atopic asthmatic subjects. However, significantly higher FcεRI surface expression on Mo-DCs from asthmatic subjects was reported by van den Heuvel *et. al.*[262], that also showed significantly higher HLA-DR and CD11b expression on these Mo-DCs. Although in the present experiments expression of CD11b was not analysed, the discrepancy in the findings relating to the other markers between the two studies may be explained by differences in data analysis. van den Heuvel *et. al.*, base all comparisons on percentage positive cells. In contrast, for surface molecules expressed by the majority of the cells within a population we favour analysis of mean fluorescence intensity. For Mo-DCs such surface molecules typically include CD40, HLA-DR and CD54 that are expressed by over 95 % of the $CD1a^+$ population. Further, van den Heuvel *et. al.*, found on average 50 % $CD1a^+$ cells and 74 % $HLA\text{-}DR^+$ cells suggesting the presence of a contaminating $HLA\text{-}DR^+$ population or incomplete conversion of monocytes to Mo-DCs as they also report the presence of on average 20 % $CD14^+$ cells, possibly monocytes that further are known to express HLA-DR. The lack of correlation in HLA-DR, FcεRI and CD23 surface expression between these two studies of untreated Mo-DCs may hence be due to differences in developmental stages of the Mo-DCs used, contaminating cell populations as well as differences in data analysis.

3.4.3 Cytokine production

Although the sample number is too low for statistical analysis, a trend for increased IL-6 and to a lesser extent, increased IL-10 production by Mo-DCs from asthmatic subjects has been noted. As already mentioned in Chapter 1, IL-6 has been reported to induce IL-4 production in murine T cells[160] and IL-10 has been shown to suppress IL-12 production in

APCs[57]. Hence, this may suggest that Mo-DCs from asthmatic subjects are more likely to drive Th2 differentiation of naïve T cells than Mo-DCs from normal subjects, and may consequently be predisposed to initiate or perpetuate the allergic asthmatic inflammation.

At the number of subjects investigated, no correlation between the quantity of cytokines produced and the level of CD23 expressed was noted that would further support the existence of a DC phenotype predisposed for perpetuation of allergic disease.

3.4.4 Conclusions

Mo-DCs from asthmatic subjects are different to Mo-DCs from normal subjects as they express significantly less CD23 on their surface and produce slightly more IL-6 and to a lesser extent IL-10.

Chapter Four

A primary screen of the effects of allergic mediators on the phenotype and function of dendritic cells from normal and asthmatic subjects

Summary

Dendritic cell phenotype and function is strongly influenced by environmental factors. The aim of the work presented in this chapter was to develop and apply a primary screen to address the hypothesis that mediators of the allergic asthmatic environment bring about phenotypic and functional changes in DCs resulting in the initiation or perpetuation of the chronic allergic inflammation seen in asthmatic airways. The primary screen aimed to identify individual allergic mediators resulting in a statistically significant or marked change in Mo-DC phenotype or function, for further in-depth investigation of their effects on Mo-DCs and possible relevance in allergic asthma.

The primary screen consisted of four assays that are presented in section 4.2 –4.4. These assays evaluated the effects of allergic mediators on Mo-DC 1) phenotype (Section 4.2), 2) receptor-mediated endocytosis(Section 4.2), 3) cytokine production (Section 4.3), and 4) ability to drive proliferation of allogeneic $CD45RA^+$ T cells (Section 4.4). Mo-DCs were derived *in vitro* from normal and asthmatic subjects on which the effects of MIP-1α, histamine, PGD_2, IgE, IL-3, IL-5 and IL-13 were studied. In addition, cells were exposed to TNF-α as a positive control for Mo-DC maturation. IFN-γ was included as a typical Th1 cytokine.

Using this experimental approach, significant or marked changes in Mo-DCs phenotype and function were observed in response to TNF-α, IFN-γ, IL-3, IL-5 and IgE. In contrast, only slight effects were observed in response to MIP-1α, histamine, PGD_2 and IL-13. This experimental approach also identified four areas of particular interest. 1) Phenotypically mature IFN-γ exposed Mo-DCs failed to significantly enhance allogeneic T cell proliferation. 2) CD23 disappears from the surface of Mo-DCs in response to TNF-α, IFN-γ and IgE. 3) IL-3 and IL-5 frequently induced a mature Mo-DCs phenotype. 4) The response of normal and asthmatic Mo-DCs to allergic mediators was different. The inability of IFN-γ treated Mo-DCs to significantly enhance T cell proliferation and the mechanisms underlying the downregulation of CD23 in response to TNF-α, IFN-γ and IgE are further investigated in Chapter 5 and 6, respectively.

Section 4.1

Introduction

4.1.1 Aim

Dendritic cell phenotype and function is strongly influenced by the environment in which the cells are found. It is not only their state of maturation and migration that is dictated by environmental factors but also their ability to activate T cells and driving their differentiation.

As discussed in Chapter 1 (1.4) the lung of atopic asthmatic subjects is characterised by the presence of allergic inflammation. Inflammatory cells are recruited into the lung in response to chemokines, for example MIP-1α* that is produced at elevated levels by both structural cells and inflammatory cells within the asthmatic airways[286]. Amongst the cells recruited are mast cells and basophils that in atopic asthmatic subjects carry higher levels of FcεRI with bound IgE due to the elevated levels of IgE present in these subjects[193]. Cross-linking of the FcεRI by allergen and allergen-specific IgE in these cells leads to degranulation and the release of mediators including histamine[212,287] and prostaglandin D_2 (PGD_2) [196,288], also found at elevated levels in the asthmatic lung[289,290]. Finally, primarily due to activation of Th2 cells in the asthmatic airways elevated levels of Th2 cytokines, including IL-3[291], IL-4[292], IL-5[293] and IL-13[205], are also in the asthmatic lung.

The aim of the work presented in this chapter was to develop and apply a primary screen to address the hypothesis that mediators of the allergic asthmatic environment bring about phenotypic and functional changes in Mo-DCs from normal subjects resulting in the initiation or perpetuation of the chronic allergic inflammation seen in asthmatic airways. However, the aim was also to apply the primary screen to Mo-DCs from asthmatic subjects to establish whether these Mo-DCs are predisposed to perpetuate the chronic allergic inflammation when encountering an allergic environment.

4.1.2 The mediators studied in the primary screen

Evidence for elevated levels of MIP-1α[286,294], histamine[289,290,295], PGD_2[290,295], IgE[192,193], IL-3[202,291], IL-5[292] and IL-13[205,296] has been reported in the asthmatic lung. These mediators were therefore chosen for this study. Although also found at elevated levels in the asthmatic lung[206], TNF-α was chosen as a positive control since its effects on Mo-DC phenotype and function are well documented. IFN-γ was included as a typical Th1 cytokine for comparison with the Th2 cytokines IL-3, IL-5 and IL-13. Elevated levels of IL-4 [292]and GM-CSF [291,293]has also been demonstrated in asthmatic airways. However, the effects of

* MIP-1α (CCL3) [297].

these two agents could not be independently studied here as the current methodology for deriving Mo-DCs relies on the presence of GM-CSF and IL-4 throughout the culture period.

In the following section (4.1.2.1 - 4.1.2.8), known effects of MIP-1α, histamine, PGD_2, IgE, IL-3, IL-5, IL-13 and IFN-γ on DCs are reviewed. As the number of Mo-DCs obtained from each donor was limited and the range of mediators investigated extensive, each mediator could only be screened at one concentration. Reasons for the concentrations used are therefore also given in the following sections.

4.1.2.1 Macrophage inflammatory protein 1α

MIP-1α is a 7.5 kDa CC-chemokine [R&D data sheet] that bind both the receptors CCR1 and CCR5[297]. MIP-1α has the potential to attract B cells, DCs and T cells to the asthmatic lung as these cells are known to respond to MIP-1α *in vitro* [96 298]. Mo-DCs have been shown to express the receptors CCR1 and CCR5 [140,299,300]. *In vitro*, 10 nM MIP-1α is sufficient to initiate DC migration (Dr C.G. Jackson, AstraZeneca, personal communication). The same concentration of MIP-1α was chosen for this study.

4.1.2.2 Histamine

Histamine is an amine with a broad range of biological effects. Through interaction with histamine-H_1-, H_2-, H_3- and H_4-receptors, histamine is involved in such diverse functions as digestion, neurotransmission and inflammation [301,302]. There are no reports of histamine receptors on DCs, however, H_1- and H_2-receptors have been demonstrated on human monocytes where histamine ligation to H_2-receptors results in reduced IL-12 and TNF-α production [303 304 305]. The production of IL-12 p70 by monocytes in response to *Staphylococcus aureus* Cowan strain I (SAC) was completely inhibited by 0.1 nM histamine [306]. This concentration of histamine was therefore selected for the present study.

4.1.2.3 Prostaglandin D_2

PGD_2 is a prostanoid that binds to the DP-receptor with a K_d of 13 nM [307]. PGD_2 is the most abundantly produced prostanoid in human mast cells [196 288]. PGD_2 production from arachidonic acid is catalysed by cyclo-oxygenase and follows a route of several unstable

intermediates [196]. In the extracellular environment PGD_2 is quickly metabolised to 9α, 11β-PGF_2 that in the lung has been shown to have similar biological effects to PGD_2 including induction of bronchoconstriction [196,295]. Overlapping affinity of PGD_2 for other prostaglandin receptors, including the PGE_2 receptor EP2, that may potentially affect DC phenotype and function, necessitated the use of a low PGD_2 concentration of 5 nM in this study [307,308].

4.1.2.4 Immunoglobulin E

IgE comprises two identical light chains, each of 25 kDa, and two identical heavy ε chains, each of 72.5 kDa (Figure 4.1.1). Each ε heavy chain has 5 domains, a variable domain (V_H) and 4 constant domains (Cε1, Cε2, Cε3 and Cε4). Whereas IgG, IgA and IgD all have a hinge region between their C_H1 and C_H2 domains, IgE lacks this region that is replaced by an additional constant domain, Cε2. IgE adopts a bent conformation that is retained following IgE binding through Cε3 to the low affinity IgE receptor CD23 (FcεRII) [112]. IgE also binds to its high affinity receptor, FcεRI, through residues in its Cε2 domain. FcεRI, but not CD23, has been shown to be expressed by peripheral blood DCs [270,285]. In contrast, inflammatory dendritic epidermal cells (IDEC) in lesional skin of atopic eczema [309] and Mo-DCs express both FcεRI and CD23. Apart from being involved in allergen focusing in DCs[84,124], FcεRI-mediated signaling has also been shown to prevent monocytes apoptosis and to induce the production of TNF-α , GM-CSF, IL-12 and IL-1β [273], further justifying investigation of the effects of IgE binding on Mo-DCs. In this study, two concentrations of IgE were used. 1 nM IgE was used to target FcεRI, a concentration equal to 10 times the K_d for the receptor (K_d 10^{-9} to 10^{-10} M). Due to the high cost of human chimaeric IgE, targeting of FcεRI and CD23 was attempted using 100 nM IgE a concentration equal to the K_d of CD23 (K_d 10^{-6} to 10^{-7} M).

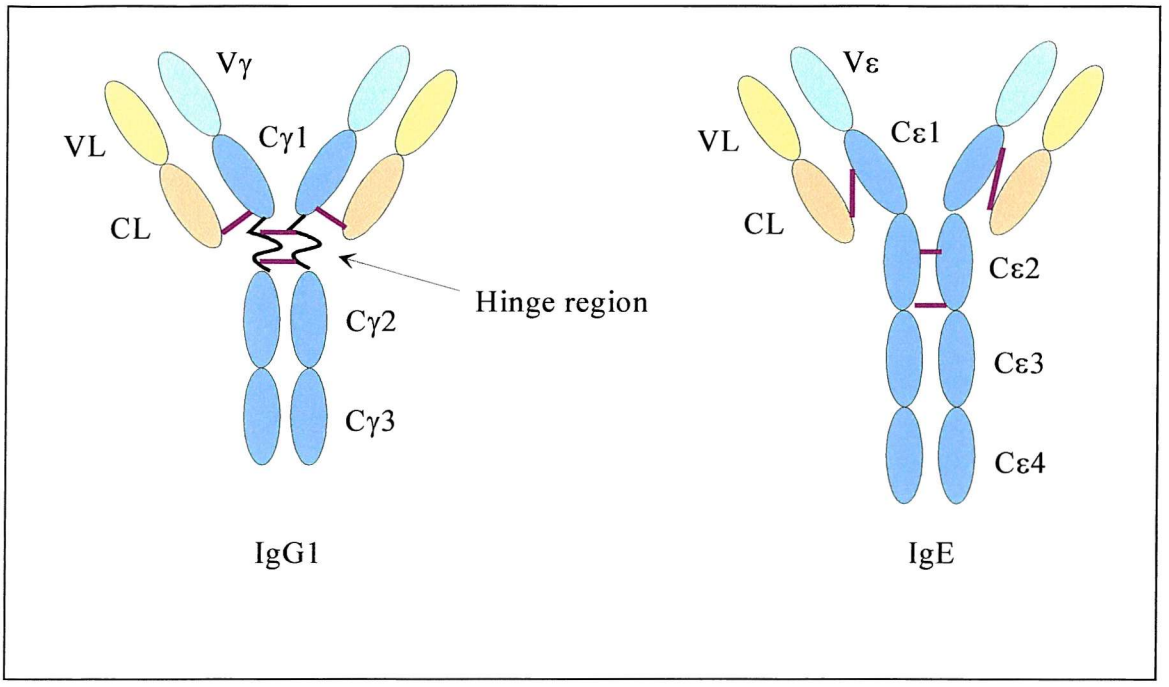


Figure 4.1.1 Structural comparison of human IgG1 and IgE

IgG and IgE consist of two identical light chains (in yellow) and two identical heavy chains (in blue) held together by disulphide bonds (purple lines). Each light and heavy chain have a variable region indicated as light yellow or light blue-green for the light and heavy chain respectively. IgE differs from IgG by an additional heavy chain domain that replaces the hinge region seen in IgG, hence Cγ3 is homologous to Cε4.

4.1.2.5 Interleukin-3

IL-3 is a 17.5 kDa Th2 cytokine [R&D data sheet]. IL-3 binds to the IL-3 receptor (IL-3R) with a K_d of 140 pM [310]. The IL-3R is a high affinity complex that like the IL-5 and the GM-CSF receptors comprises one cytokine-specific α-chain and one shared β-chain (beta c)[311]. IL-3 has been shown to be involved in the development of lymphoid DCs *in vitro*[42]. Whereas blood DCs have been shown to express both the IL-3R α- and β-chain[42,311], Mo-DCs have only been shown to express the α-chain at low levels[78]. In this study 1.4 nM IL-3 was used to target the IL-3R on Mo-DCs, a concentration equivalent to 10 times the K_d for the IL-3R.

4.1.2.6 Interleukin-5

IL-5 is a 33 kDa Th2 cytokine [R&D data sheet]. IL-5 binds to the IL-5 receptor (IL-5R) with a K_d of 150 pM [310]. The IL-5R has so far not been identified on DCs. In this study 1.5 nM IL-5 was used to target potential IL-5R on Mo-DCs, a concentration equivalent to 10 times the K_d for IL-5R.

4.1.2.7 Interleukin-13

IL-13 is a 12 kDa cytokine that binds the IL-13R with a K_d of 30 pM [220]. IL-13 has the ability to substitute for IL-4 in the generation of dendritic cells from both monocytes and $CD34^+$ progenitors *in vitro* [312 313]. One of the IL-13-binding proteins, IL-13Rα1, that associate with the IL-4Rα chain to make up the IL-13R [220], has recently been identified in Mo-DCs by RT-PCR [J. W. Holloway and A. Cazaly, data not shown]. In this study, 10 nM IL-13 was used for evaluation of the effects on Mo-DCs.

4.1.2.8 Interferon-γ

IFN-γ is a 50 kDa Th1 cytokine that binds to the IFN-γ receptor (IFN-γR) with a K_d of 0.1 to 1 nM [314]. Increased levels of IFN-γ have been reported to be produced by BAL cells from atopic asthmatic subjects when stimulated with PMA compared to BAL cells

from normal subjects [315]. However, IFN-γ has been shown to inhibit rather than promote airway hyperresponsiveness and eosinophilia in animal models of asthma [229,316]. DCs were therefore exposed to IFN-γ in light of the potential effects of increased IFN-γ detected in human asthmatic BAL cells but primarily for evaluation of the effects of a typical Th1 cytokine. Mo-DCs have been shown to express the IFN-γR (CD119)[176]. Following exposure to 500 U/mL IFN-γ, Mo-DCs show impaired ability to activate allogeneic T cells [317]. In this study, 1.9 nM (9 500 U/mL) IFN-γ, equivalent to × 2 - × 20 K_d of the IFN-γR, was used to evaluate the effects off IFN-γ on Mo-DCs.

4.1.3 Experimental approach

The primary screen was designed to identify individual allergic mediators significantly changing the Mo-DC phenotype or function, for further in-depth investigation of their effects on Mo-DCs and possible relevance in allergic asthma. In determining which mediators to select for further in-dept investigation, non-parametric statistical analysis was performed where possible. However, allergic mediators resulting in a clear, although not statistically significant change, were also considered for further investigation. For this purpose an arbitrary significance limit was applied that has been taken to be a ≥ 50 % (±) change compared to untreated control cells even when the standard error of the mean has been taken into account. This arbitrary significance limit is referred to as marked change.

All experiments presented in this chapter were performed on Mo-DCs derived from normal and asthmatic subjects as described in Chapter 2. On day 6, Mo-DCs were exposed to one of the following mediators, collectively referred to as allergic mediators; 10 nM MIP-1α, 0.1 nM histamine, 5 nM PGD_2, 1 nM human chimaeric IgE, 100 nM human chimaeric IgE, 1.4 nM rhIL-3, 1.5 nM rhIL-5, 10 nM rhIL-13 and 1.9 nM rhIFN-γ. In addition, cells were exposed to 75 ng/mL (1.5 nM) rhTNF-α as a positive control for DC maturation. Control Mo-DCs were not exposed to any of the mediators mentioned above and were used to determine baseline values for all assays in the screen.

After 48 hours exposure to the mediators i.e. on day 8, each DC treatment was harvested separately, washed twice in sterile PBS, 1% BSA and resuspended in complete RPMI awaiting analysis. The primary screen incorporated four main assays as illustrated in Figure 4.1.2. These assays included 1) flow cytometric analysis of Mo-DC phenotype 2) flow cytometric analysis of Mo-DC receptor-mediated endocytosis, 3) measurement of Mo-DC produced cytokines by ELISA, and 4) evaluation of Mo-DC-induced proliferation of allogeneic T cells ($CD45RA^+$) measured by H^3-thymidine uptake. Attempts to develop an

assay for evaluation of Mo-DC driven allogeneic T cell differentiation was also made (indicated as 5 in Figure 4.1.2). Due to limitation in number of Mo-DCs recovered following 8 days in culture, each subject was rarely exposed to all 10 mediators and all four assays were rarely performed for each mediator.

The results in this chapter are presented in sections. Section 4.2 presents the effects of allergic mediators on Mo-DC phenotype and receptor-mediated endocytosis as determined by flow cytometry. Section 4.3 presents the effects of allergic mediators on Mo-DC produced cytokines as determined by ELISA. Section 4.4 presents effects of allergic mediators on Mo-DC and the consequences for T cell function. The effects of allergic mediators on Mo-DCs from normal and asthmatic subjects are presented in parallel.

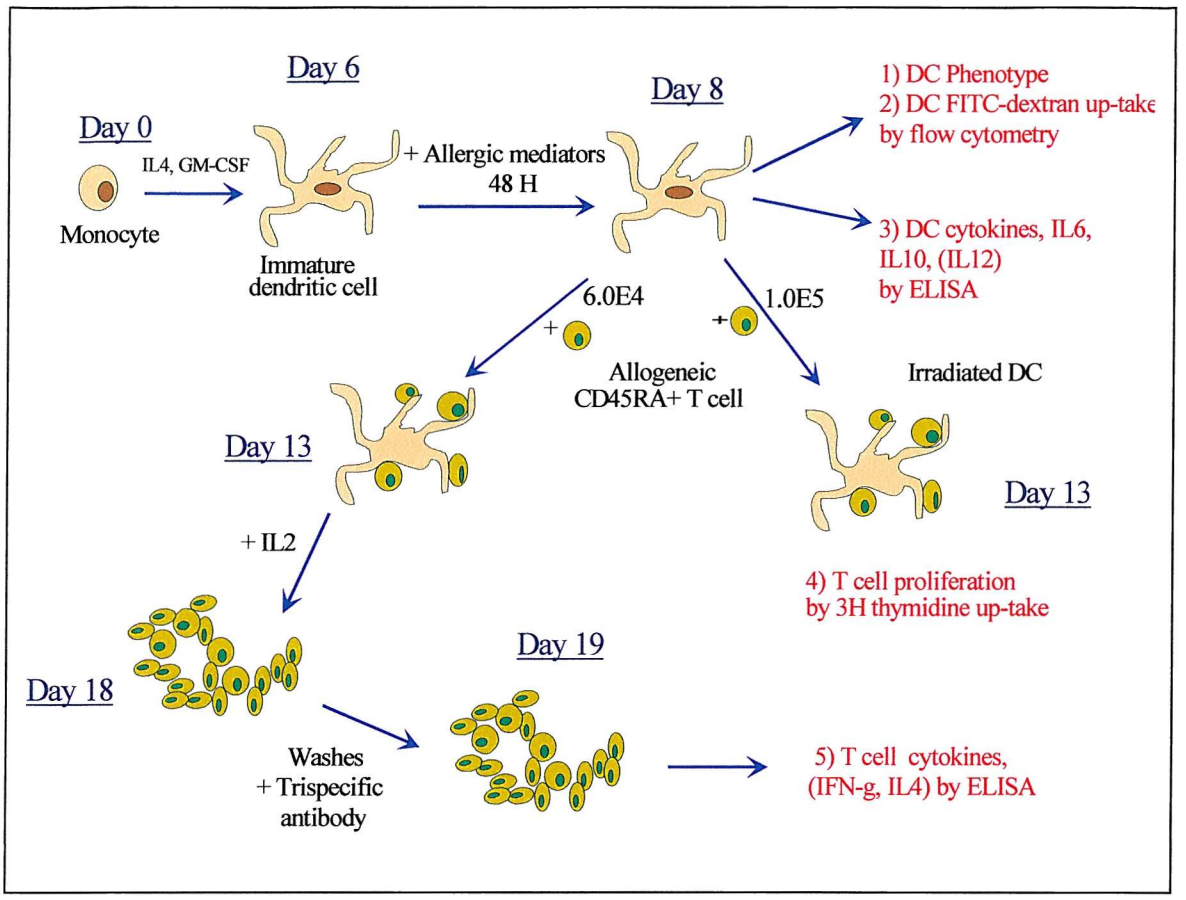


Figure 4.1.2 Schematic diagram of the primary screen

Schematic diagram showing the design of the primary screen of the effects of allergic mediators on Mo-DC phenotype and function.

Section 4.2

Effects of allergic mediators on the phenotype and receptor-mediated endocytosis of monocyte-derived dendritic cells from normal and asthmatic subjects

4.2.1 Material and methods

4.2.1.1 Subjects

24 non-atopic, non-asthmatic normal subjects (12 female, 12 male) and 10 atopic, asthmatic subjects (5 female, 5 male), defined as in section 2.1.7, took part in this study.

4.2.1.2 Effects of allergic mediators on monocyte-derived dendritic cell phenotype

To assess the effects of allergic mediators on the phenotype of Mo-DCs from normal and asthmatic subjects, 6 day old Mo-DCs were exposed to allergic mediators for a further 48 hours then harvested and stained for surface expression of CD1a, CD14, CD23, CD40, CD80, CD83, CD86 and HLA-DR as described (2.2.5.2.1).

During flow cytometric analysis dead cells were identified using 7-AAD and were excluded from the events collected (2.2.5.4). A total of 5 000 live events were acquired.

The level of surface expressed CD1a, CD14, CD23, CD80 and CD83 was assessed by analysing the percentage of positively labelled cells on a gated cell population, whereas the level of surface expressed CD40, CD86 and HLA-DR was assessed by analysing the geometric MFI of the whole cell population. The resulting percentage labelled cells or MFI value for a specific Mo-DC treatment was used to calculating the percentage change relative to untreated control Mo-DCs, according to the following equation;

((Sample value* / Control value) -1) × 100

* = percentage positive cells or MFI

4.2.1.3 Receptor mediated endocytosis of FITC-dextran

To assess the effects of allergic mediators on receptor-mediated endocytosis of FITC-dextran, 6 day old Mo-DCs from normal and asthmatic subjects were exposed to allergic mediators for a further 48 hours, then harvested and incubated in 200 µL complete RPMI supplemented with FITC-dextran for 1 hour at 37°C or 4°C (2.2.6.1). During flow cytometric analysis of FITC-dextran uptake, dead cells were identified using 7-AAD, but

were not excluded from the collected events due to difficulties in compensating the strong signal in FL1. Dead cells were later excluded using Lysis II software. A total of 10 000 events were acquired.

The level of FITC-dextran uptake was assessed by analysing the geometric MFI. Before comparison between different Mo-DC treatments was made, the MFI at 4°C was deducted from the MFI at 37°C. The resulting MFI value was used for calculating the percentage change relative to untreated control Mo-DCs according to the equation in section 4.2.1.2.

4.2.1.4 Statistical analysis

Using SPSS 10.0 for Windows, the non-parametric Wilcoxon test was used where possible to compare responses within a subject group. The non-parametric Mann Whitney *U* test was used where possible to compare responses between the asthmatic and normal subject groups. A p value of less than 0.05 was considered statistically significant.

4.2.2 Results

4.2.2.1 Effects of allergic mediators on monocyte-derived dendritic cell phenotype

Throughout this chapter, surface expression of CD23, CD80 and CD83 was analysed as the percentage of positively labelled cells on a gated cell population, whereas the surface expression of CD40, CD86 and HLA-DR was analysed by the geometric MFI of the whole cell population. The geometric MFI was the favoured analysis method for HLA-DR and CD40 as a majority of the DC population expression these two markers. However, surface expression of CD23, CD80, CD83 and CD86 could have been presented by either analysis method as only a proportion of the DC population expressed these markers. To limit the amount of data shown in this chapter, the analysis method demonstrating the most pronounced effect induced by the positive control TNF-α was chosen.

4.2.2.1.1 Effects of allergic mediators on monocyte-derived dendritic cells from normal subjects

Mo-DCs grown for 8 days in the presence of IL-4 and GM-CSF, without additional treatment, showed an immature phenotype (Figure 4.2.1) typically indicated by both low intensity (within the 1st log decade) and low percentage of cells expressing the DC maturation marker CD83 and the costimulatory molecule CD86. In contrast, all cells expressed CD40 and HLA-DR, although the latter was present at moderate levels within the 2nd log decade. Immature Mo-DCs further typically express low to moderate levels of the low affinity IgE receptor CD23 and the costimulatory molecule CD80.

The most striking changes to this immature phenotype occurred following 48 hours exposure to either TNF-α or IFN-γ (Figure 4.2.1, Figure 4.2.2 and Table 4.2.1). Both cytokines induced a more mature phenotype, indicated by a statistically significant increase in the number of cells expressing CD83 and CD80 and in the levels of expression of CD40, CD86 and HLA-DR. Further, the frequency of CD23^{+} Mo-DCs was significantly decreased by TNF-α or IFN-γ treatment.

The Th2 cytokines IL-3 and IL-5 also showed a tendency to induce Mo-DC maturation (Figure 4.2.2 and Table 4.2.1), although these effects were less pronounced than those seen in response to TNF-α and IFN-γ. Mo-DCs exposed to IL-3 showed a statistically significant increase in expression of CD80, CD83, CD86 and HLA-DR. The levels of CD40

were also increased above the arbitrary significance limit of 50 %, defining a marked increase, when treated with IL-3. Treatment with IL-3 also resulted in a significant decrease in CD23 expression compared to untreated control cells. IL-5 significantly increased surface expression of CD80 and CD86 and induced a marked increase in the expression of HLA-DR.

Treatment with 100 nM IgE, but not 1 nM IgE, resulted in a significant decrease in the number of cells expressing CD23 (Figure 4.2.2a and Table 4.2.1). 1 nM or 100 nM IgE did not markedly affect any other markers investigated.

The remaining mediators, MIP-1α, histamine, PGD_2 and IL-13 failed to change the expression of any surface molecule investigated significantly or by over 50 % relative to control untreated Mo-DCs.

4.2.2.1.2 Effects of allergic mediators on monocyte-derived dendritic cells from asthmatic subjects compared to monocyte-derived dendritic cells from normal subjects

Although fewer asthmatic than normal subjects were studied it was found that as for normal Mo-DCs, asthmatic Mo-DCs were also affected most by treatment with TNF-α or IFN-γ (Figure 4.2.2 and Table 4.2.1). Further, like in normal Mo-DCs, TNF-α and IFN-γ induced a mature Mo-DC phenotype with significantly increased expression of CD40, CD80 and CD86. Both TNF-α and IFN-γ induced a marked increase in expression of CD83 and a marked decrease in expression of CD23. However, treatment with TNF-α results in significantly increased expression of HLA-DR whereas IFN-γ only induced a marked increase in the expression of the same marker in asthmatic Mo-DCs. As shown, TNF-α and IFN-γ generally induced a similar phenotype in Mo-DCs from normal and asthmatic subjects, however, it appears that that these cytokines increased the surface expression of CD40 and CD83 to a higher extent in normal Mo-DCs than asthmatic Mo-DCs (Figure 4.2.2b and Table 4.2.1). Following statistical analysis it was found that IFN-γ induced significantly higher expression of CD83 in normal than asthmatic Mo-DCs (data not shown).

Like for normal Mo-DCs, the Th2 cytokine IL-3 was shown to induce maturation of asthmatic Mo-DCs (Figure 4.2.2 and Table 4.2.1), although the IL-3 induced effects were less pronounced than those seen in response to TNF-α and IFN-γ. IL-3 was shown to significantly increase expression of CD80 and HLA-DR, however did not alter the expression of CD23, CD83 or CD86 as was seen in normal Mo-DCs. In the case of CD23

expression, the difference in response to IL-3 between normal and asthmatic subjects was found statistically significant (Figure 4.2.2a). Normal and asthmatic Mo-DCs also responded differently to other Th2 cytokines. For example, in contrast to normal Mo-DCs, no significant or marked phenotypic changes were observed in response to IL-5 in Mo-DCs from asthmatic subjects. Mo-DCs from asthmatic subjects also increased the expression of CD80 above the arbitrary significance limit of 50 % in response to IL-13, a change not seen in Mo-DCs from normal subjects.

Whilst only 100 nM IgE on Mo-DCs from normal subjects induced a significant decrease in CD23 expression, both 1 nM and 100 nM IgE resulted in a significant increase in expression of CD80 and CD86 on Mo-DCs from asthmatic subjects (Figure 4.2.2a and Table 4.2.1). In the case of 1 nM IgE, this difference between normal and asthmatic Mo-DCs was found statistically significant (Figure 4.2.2a).

The remaining mediators, MIP-1α, histamine and PGD_2, failed to change the expression of any surface molecule investigated significantly or by over 50 % relative to control untreated Mo-DCs.

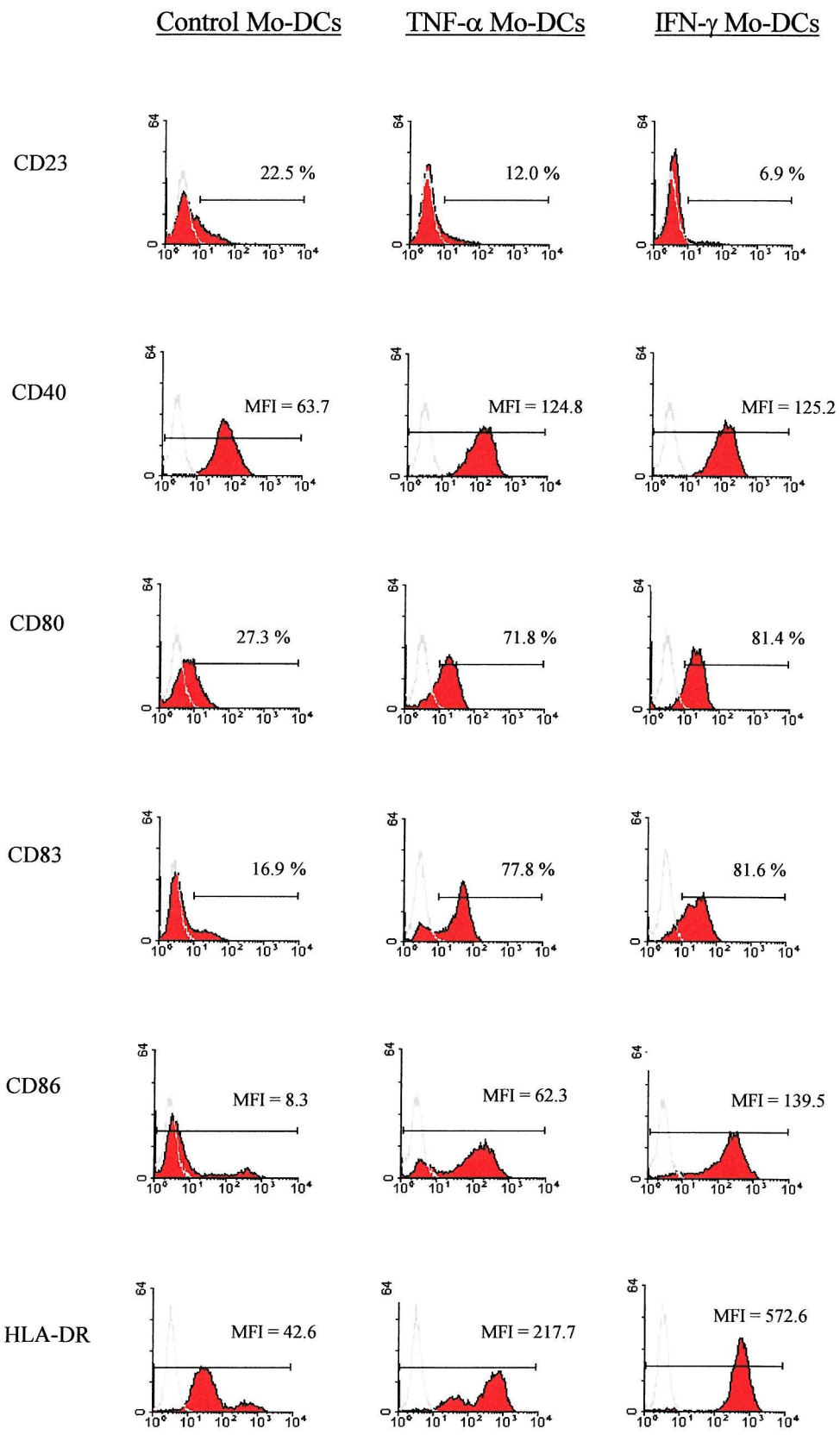


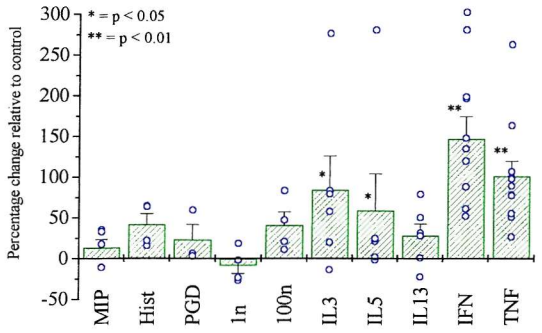
Figure 4.2.1 Surface molecule expression on Mo-DCs ± TNF-α or IFN-γ

The figure shows the effects of 75 ng/mL (1.5 nM) TNF-α and 1.9 nM IFN-γ on CD23, CD40, CD80, CD83, CD86 and HLA-DR expressed by Mo-DC. The data is derived from one representative normal donor.

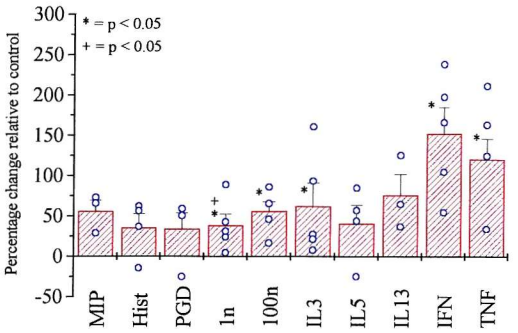
Normal subjects

Asthmatic subjects

CD80

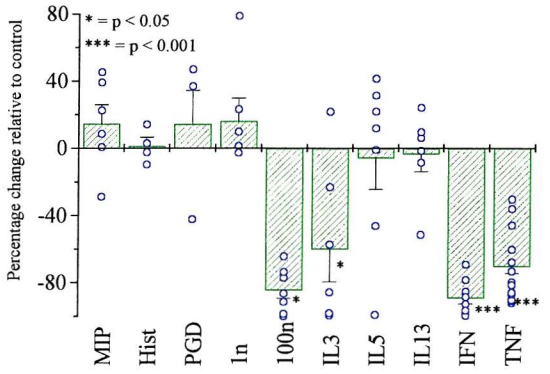


n = MIP-1α (5), Histamine (4), PGD2 (3), IgE 1nM (4), IgE 100nM (4), IL-3 (6), IL-5 (6), IL-13 (6), IFN-γ (11), TNF-α (13).

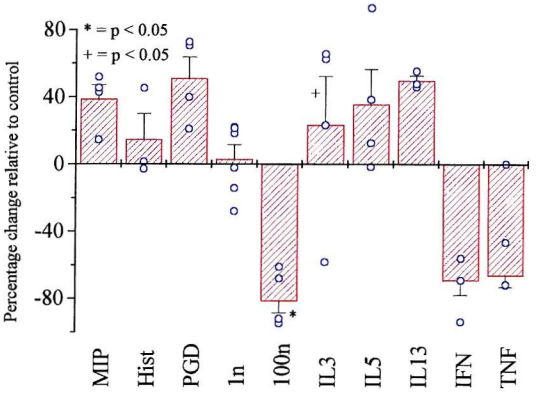


n = MIP-1α (3), Histamine (4), PGD2 (4), IgE 1nM (5), IgE 100nM (5), IL-3 (5), IL-5 (4), IL-13 (3), IFN-γ (5), TNF-α (6).

CD23



n = MIP-1α (6), Histamine (4), PGD2 (4), IgE 1nM (6), IgE 100nM (7), IL-3 (6), IL-5 (7), IL-13 (6), IFN-γ (15), TNF-α (19).



n = MIP-1α (4), Histamine (3), PGD2 (4), IgE 1nM (6), IgE 100nM (5), IL-3 (4), IL-5 (4), IL-13 (3), IFN-γ (4), TNF-α (4).

Figure 4.2.2 Effects of allergic mediators on surface molecule expression by Mo-DCs

a) Surface expression of CD80 and CD23 on normal and asthmatic Mo-DCs

Six day old Mo-DCs from normal and asthmatic subjects were exposed to allergic mediators for 48 hours then harvested and stained for surface expression of a) CD80 and CD23, and b) HLA-DR, CD40 and CD86. In a) the effects of allergic mediators are presented as percentage change relative to control based on the percentage of cells positively stained for each marker and in b) based on the MFI of the whole population. Each individual value (percentage change) is presented as an open blue circle and the mean of all values for each surface molecule is shown as a column with error bars (SEM).

The following abbreviations are used; 75 ng/mL TNF-α (TNF), 1nM IgE (1n), 100 nM IgE (100n), 0.1 nM histamine (Hist), 10 nM MIP-1α (MIP), 1.4 nM IL-3 (IL3), 1.5 nM IL-5 (IL5), 10 nM IL-13 (IL13), 1.9 nM IFN-γ (IFN), 5 nM PGD_2 (PGD),

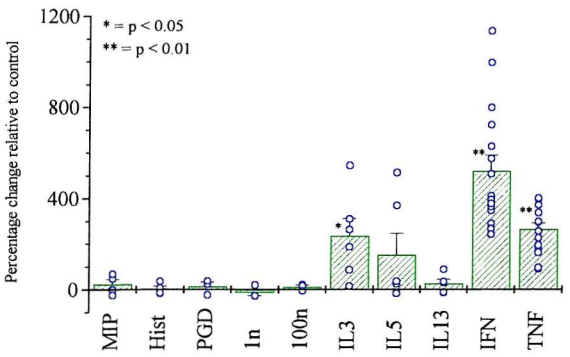
* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ compared to control untreated Mo-DCs.

+ = $p < 0.05$ compared to the same treatment in normal Mo-DCs.

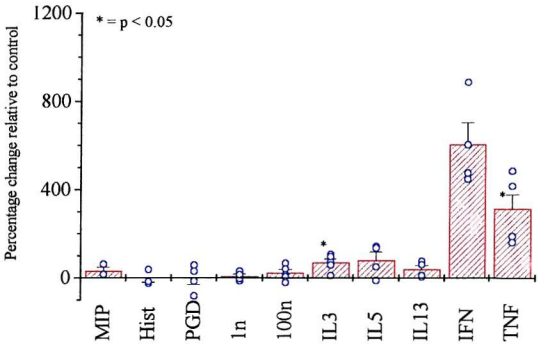
Normal subjects

Asthmatic subjects

HLA-DR

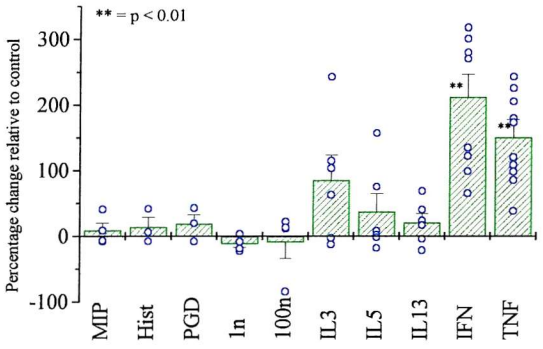


n = MIP-1α (4), Histamine (4), PGD2 (3), IgE 1nM (3), IgE 100nM (3), IL-3 (6), IL-5 (6), IL-13 (5), IFN-γ (16), TNF-α (19).

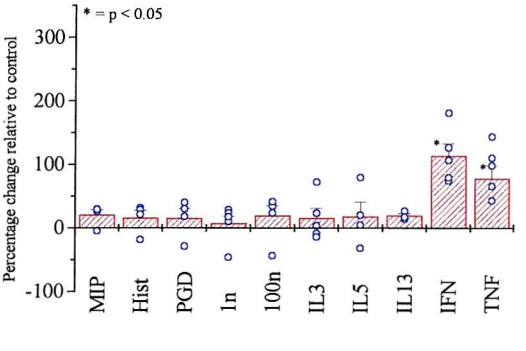


n = MIP-1α (3), Histamine(3), PGD2 (4), IgE 1nM (4), IgE 100nM (5), IL-3 (5), IL-5 (4), IL-13 (4), IFN-γ (4), TNF-α (5).

CD40

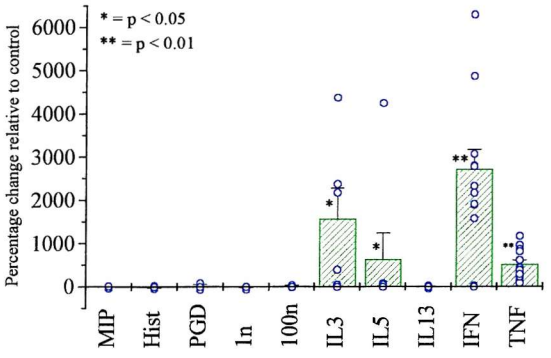


n = MIP-1α (4), Histamine (3), PGD2 (3), IgE 1nM (4), IgE 100nM (4), IL-3 (6), IL-5 (6), IL-13 (6), IFN-γ (9), TNF-α (13).

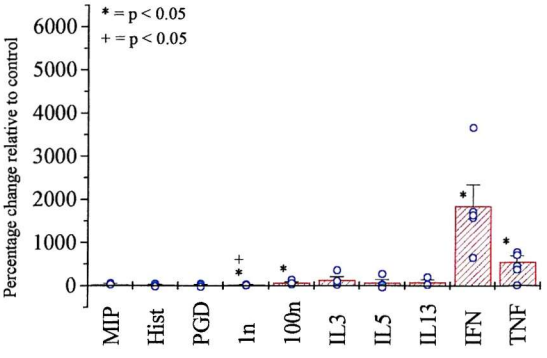


n = MIP-1α (4), Histamine (4), PGD2 (4), IgE 1nM (6), IgE 100nM (5), IL-3 (5), IL-5 (4), IL-13 (4), IFN-γ (5), TNF-α (6).

CD86



n = MIP-1α (5), Histamine (3), PGD2 (3), IgE 1nM (4), IgE 100nM (3), IL-3 (6), IL-5 (7), IL-13 (6), IFN-γ (12), TNF-α (14).



n = MIP-1α (3), Histamine (4), PGD2 (4), IgE 1nM (5), IgE 100nM (5), IL-3 (4), IL-5 (4), IL-13 (3), IFN-γ (5), TNF-α (6).

b) Surface expression on HLA-DR, CD40 and CD86 on normal and asthmatic Mo-DCs

Table 4.2.1 Summary of statistically significant and marked effects of allergic mediators on surface molecules expression by Mo-DC

Surface molecule	Normal Mo-DCs	Asthmatic Mo-DCs
CD23	↓[+] IFN-γ (-89.1% ± 3.4, n = 15)[+++] ***	↓ IFN-γ (-69.2 % ± 8.7, n = 4)
	↓ TNF-α (-70.3% ± 4.2, n = 19) ***	↓ TNF-α (-66.3 % ± 6.6, n = 4)
	↓ 100 nM IgE (-84.4% ± 5.1, n = 7) *	↓ 100 nM IgE (-81.5% ± 7.0, n = 5) *
	↓ IL-3 (-59.9 % ± 19.7, n = 6) *	
CD40	↑[++] IFN-γ (212.3 % ± 34.6, n = 9) **	↑ IFN-γ (113.6 % ± 19.3, n = 5) *
	↑ TNF-α (150.3 % ± 27.4, n = 13) **	↑ TNF-α (77.6 % ± 20.3, n = 6) *
	↑ IL-3 (85.3 % ± 38.2, n = 6)	
CD80	↑ IFN-γ (146.8 % ± 27.2, n = 11) **	↑ IFN-γ (152.3 % ± 32.7, n = 5) *
	↑ TNF-α (101.0 % ± 18.2, n = 13) **	↑ TNF-α (120.5 % ± 25.2, n = 6) *
	↑ IL-3 (84.1 % ± 41.4, n = 6) *	↑ IL-3 (62.5 % ± 28.7, n = 5) *
	↑ IL-5 (58.9 % ± 44.7, n = 6) *	↑ IL-13 (76.1 % ± 26.1, n = 3)
		↑ 1 nM IgE (38.7 % ± 14.1, n = 5) *
		↑ 100 nM IgE (56.3 % ± 11.7, n = 5) *
CD83	↑ IFN-γ (2059.4 % ± 571.8, n = 10) **	↑ IFN-γ (504.2 % ± 91.8, n = 4)
	↑ TNF-α (1506.4 % ± 391.1, n = 13) **	↑ TNF-α (499.5 % ± 57.7, n = 4)
	↑ IL-3 (1540.3 % ± 909.4, n = 6) *	
	↑ IL-5 (949.0 % ± 786.2, n = 7) *	
CD86	↑ IFN-γ (2713.3 % ± 460.4, n = 12) **	↑ IFN-γ (1845.2 %± 493.6, n = 5) *
	↑ TNF-α (515.3 % ± 91.6, n = 14) **	↑ TNF-α (544.6 % ± 141.5, n = 6) *
	↑ IL-3 (1570.3 % ± 706.1, n = 6) *	↑ 1 nM IgE (17.8 % ± 6.0, n = 5) *
	↑ IL-5 (630.6 % ± 603.7, n = 7) *	↑ 100 nM IgE (66.7 % ± 18.8, n = 5) *
HLA-DR	↑ IFN-γ (520.1% ± 67.6, n = 16) **	↑ IFN-γ (603.7 % ± 100.3, n = 4)
	↑ TNF-α (264.3 % ± 27.2, n = 19) **	↑ TNF-α (314.4 % ± 63.0, n = 5) *
	↑ IL-3 (234.8 % ± 76.3, n = 6) *	↑ IL3 (69.0 % ± 16.7, n = 5) *
	↑ IL-5 (152.2 % ± 93.7, n = 6)	

[+] ↓ = decreased surface expression.

[++] ↑ = increased surface expression..

[+++] Percentage change relative to control, standard error (SEM) and n value are presented in brackets.

* = $p < 0.05$ compared to control untreated Mo-DCs.

** = $p < 0.01$ compared to control untreated Mo-DCs.

*** = $p < 0.001$ compared to control untreated Mo-DCs.

4.2.2.2 Effects of allergic mediators on receptor mediated endocytosis of FITC-dextran by monocyte-derived dendritic cells

Virtually all immature control Mo-DCs have endocytosed moderate levels of FITC-dextran following 1 hour incubation in RPMI supplemented with FITC-dextran at 37°C, but not at 4°C (Figure 4.2.3a). The low level of FITC-dextran observed following incubation at 4°C indicated the level of non-specific binding or non-specific uptake of FITC-dextran by these cells. In contrast, only a proportion of the TNF-α treated Mo-DCs take up FITC-dextran following incubation at 37°C (Figure 4.2.3b) and these further show reduced level of endocytosed FITC-dextran, indicating Mo-DC maturation in response to TNF-α.

In accordance with the effects of allergic mediators on Mo-DC phenotype, the most striking effects observed on receptor-mediated endocytosis were those provoked by TNF-α and IFN-γ (Figure 4.2.4). In both normal and asthmatic Mo-DCs, treatment with TNF-α resulted in a significant decrease in receptor-mediated endocytosis of FITC-dextran, whereas IFN-γ induced a marked decrease in FITC-dextran uptake (Figure 4.2.4 and Table 4.2.2), both observations consistent with Mo-DC maturation.

The remaining mediators, MIP-1α, histamine, PGD_2, IgE, IL-3, IL-5 and IL-13, failed to change receptor-mediated endocytosis significantly or by over 50 % relative to control untreated Mo-DCs.

Table 4.2.2 Summary of statistically significant and marked effects of allergic mediators on receptor-mediated endocytosis by Mo-DC

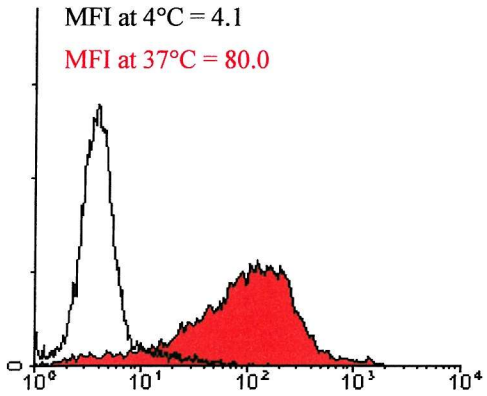
Mediator	Normal Mo-DCs[+]	Asthmatic Mo-DCs
TNF-α	↓[++] -75.3 % ± 7.6, n = 5 *	↓ -64.8 % ± 6.7, n = 5 *
IFN-γ	↓ -81.4 % ± 3.0, n = 3	↓ -60.1 % ± 8.3, n = 3

[+] Percentage change relative to control, standard error (SEM) and n value are presented.

[++] ↓ = decreased receptor-mediated endocytosis of FITC-dextran.

* = $p < 0.05$ compared to control untreated Mo-DCs

a) Control Mo-DCs



b) TNF-α Mo-DCs

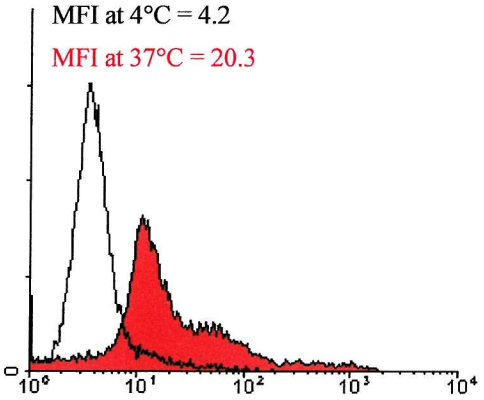
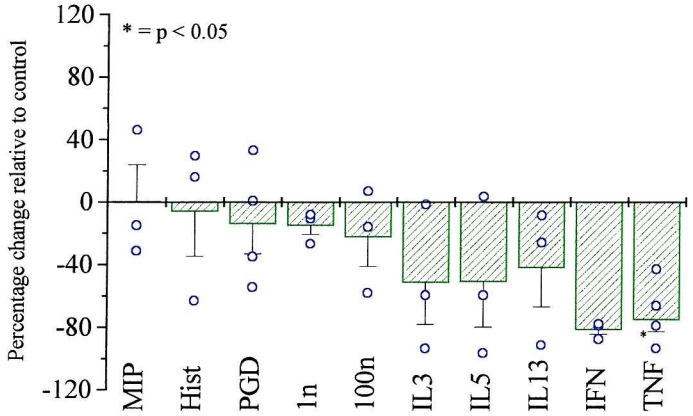


Figure 4.2.3 Flow cytometric analysis of receptor-mediated endocytosis of FITC-dextran by Mo-DCs

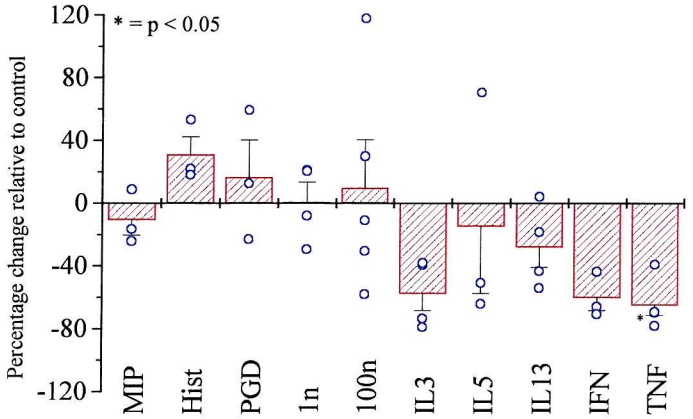
Mo-DCs were cultured for 8 days without (control) or with 75 ng/mL TNF-α for the last 48 hours. The cells were harvested and incubated in RPMI supplemented with FITC-dextran for 1 hour at 37°C or 4°C followed by flow cytometric analysis. Receptor-mediated endocytosis of FITC-dextran at 4°C (black) and 37°C (red) by a) control and b) TNF-α exposed Mo-DCs are illustrated above.

a) Normal subjects



n = MIP-1α (3), Histamine (3), PGD_2 (4), IgE 1nM (3), IgE 100nM (3), IL-3 (3), IL-5 (3), IL-13 (3), IFN-γ (3), TNF-α (6).

b) Asthmatic subjects



n = MIP-1α (3), Histamine (3), PGD_2 (3), IgE 1nM (4), IgE 100nM (5), IL-3 (4), IL-5 (3), IL-13 (4), IFN-γ (3), TNF-α (5).

Figure 4.2.4 Effects of allergic mediators on receptor-mediated endocytosis by Mo-DCs

Mo-DCs from a) normal and b) asthmatic subjects were exposed to allergic mediators for 48 hours then harvested and incubated in RPMI supplemented with FITC-dextran for 1 hour at 37°C and 4°C followed by flow cytometric analysis. Each individual value (percentage change) is presented as an open blue circle and the mean of all values for each mediator is shown as a column with error bars (SEM). The abbreviations used are listed in Figure 4.2.2. * = $p < 0.05$ compared to control untreated Mo-DCs.

4.2.3 Discussion

4.2.3.1 Effects of allergic mediators on monocyte-derived dendritic cell phenotype

While screening the effects of allergic mediators on Mo-DC phenotype it was evident that treatment with TNF-α, IFN-γ, the Th2 cytokines or IgE resulted in significant or marked changes in surface molecule expression by Mo-DCs, whereas MIP-1α, histamine and PGD_2 had insignificant effects. The phenotypic changes induced by TNF-α and IFN-γ, the Th2 cytokines and IgE are summarised and discussed below.

4.2.3.1.1 Effects of TNF-α and IFN-γ on normal and asthmatic monocyte-derived dendritic cells

The most pronounced effects on Mo-DC phenotype were those provoked by TNF-α and IFN-γ. These cytokines consistently resulted in altered phenotype on both Mo-DCs from normal and asthmatic subjects. The changes observed by TNF-α and IFN-γ were further consistent with Mo-DC maturation, showing downregulation of CD23 involved in antigen capturing, upregulation of CD40, CD80, CD86 and HLA-DR involved in antigen presentation to T cells, and upregulation of the known DC maturation marker CD83. Occasionally, TNF-α and IFN-γ appeared to alter surface molecule expression to a higher extent in Mo-DCs from normal than asthmatic subjects (Table 4.2.1). This may result from differences in level of expression of the TNF-α and IFN-γ receptor on Mo-DCs from normal and asthmatic subjects or differences in the signalling pathway leading from these receptors. Although genomic polymorphisms of TNF-α[318,319] and IFN-γ[320] related genes have been associated with asthma, no such links have yet been identified for the their receptors or associated signalling pathways.

4.2.3.1.2 Effects of the Th2 cytokines on normal and asthmatic monocyte-derived dendritic cells

The Th2 cytokines IL-3 and IL-5 also induce a mature Mo-DC phenotype although not to the same extent as TNF-α and IFN-γ. Of IL-3 and IL-5, IL-3 appeared to be the most potent initiator of Mo-DC maturation, inducing significantly increased expression of CD80,

CD83, CD86 and HLA-DR and significantly decreased surface expression of CD23 in Mo-DCs from normal subjects. Increased surface expression of CD83, CD80, CD40, HLA-DR and HLA-DQ was also reported on lymphoid CD11c$^-$ peripheral blood DCs treated with IL-3 [42]. The effects of IL-3 are consistent with evidence for the expression of the IL-3 specific α subunit of the IL-3R on both Mo-DCs[78] and peripheral blood DCs [42]. In contrast, the IL-5 specific α subunit of the IL-5R has not been detected on peripheral blood DCs and no reports exist on the presence or absence of this subunit on Mo-DCs. Nonetheless, normal Mo-DCs were shown to respond to IL-5 by significantly increasing the expression of CD80, CD83 and CD86. As Mo-DCs and peripheral blood DCs differ in surface expression of several other surface molecules, it is possible that Mo-DCs indeed express the IL-5Rα subunit, although this has yet to be determined.

It appears that IL-3, and indeed the other Th2 cytokines studied (IL-5 and IL-13), affect Mo-DC phenotype differently according to the subject group studied. In normal Mo-DCs, IL-3 and IL-5 significantly changed the expression of a range of markers as already mentioned above. However, in asthmatic Mo-DCs, IL-3 only significantly increased the expression of CD80 and HLA-DR and IL-5 did not affect surface expression of any marker investigated to any significant extent. In contrast to normal Mo-DCs, asthmatic Mo-DCs also responded to IL-13 by inducing a marked increase in the expression of CD80. This difference in responsiveness to the Th2 cytokines may reflect differences in level of expression of the cytokine receptors expressed by normal and asthmatic Mo-DCs or differences in the signalling pathway leading from these receptors. To date, no such differences between DCs from normal and asthmatic subjects have been reported. However, increased expression of the IL-5Rα on eosinophils has been reported in atopic asthmatic subjects[321].

4.2.3.1.3 Effects of IgE on normal and asthmatic monocyte-derived dendritic cells

IgE also altered the phenotype of Mo-DCs demonstrated by a significant reduction in expression of CD23 following treatment with 100 nM IgE in both normal and asthmatic Mo-DCs. Further, IgE at 1 nM and 100 nM significantly increased the expression of CD80 and CD86 in asthmatic, but not normal Mo-DCs (Figure 4.2.2 and Table 4.2.1). The increase in CD80 and CD86 expression may indicate maturation of asthmatic Mo-DCs as a result of ligation and signalling via IgE receptors. As similar responses were observed following treatment with 1 nM and 100 nM IgE, these IgE-mediated effects are more likely mediated via the high-affinity IgE receptor, FcεRI rather than the low-affinity IgE receptor,

CD23. The difference in response to IgE in Mo-DCs from normal and asthmatic subjects may result from differences in FcεRI-mediated signalling in these Mo-DCs. In contrast, it is not likely that the decrease in CD23 expression is due to induction of Mo-DC maturation, in particular since no other indications of DC maturation was observed in normal Mo-DCs. It is possible that surface expression of CD23 is reduced as a consequence of internalisation following binding of IgE to CD23, as seen in B cells [322], monocytes and macrophages [278]. The route by which CD23 disappears from the cell surface is further investigated in Chapter 6.

4.2.3.2 Effects of allergic mediators on monocyte-derived dendritic cell receptor mediated endocytosis of FITC-dextran

Of all mediators tested only TNF-α and IFN-γ significantly or markedly changed receptor-mediated endocytosis in Mo-DCs from both normal and asthmatic Mo-DCs. The reduced level of receptor-mediated endocytosis observed in response to TNF-α or IFN-γ is consistent with a mature Mo-DC phenotype as antigen uptake is a characteristic of immature Mo-DCs. TNF-α and IFN-γ-induced Mo-DC maturation was further confirmed by studying their effects on Mo-DC phenotype.

4.2.3.3 Conclusion of the effects of allergic mediators on monocyte-derived dendritic cell phenotype and receptor mediated endocytosis

The effects of allergic mediators on Mo-DCs phenotype and receptor-mediated endocytosis were investigated using both statistical analysis and an arbitrary significance limit. The results presented in this section show that;

- Mo-DCs from normal subjects responded to TNF-α, IFN-γ, IL-3, IL-5 and 100 nM IgE but not MIP-1α, histamine, PGD_2, 1nM IgE or IL-13.
- Mo-DCs from asthmatic subjects responded to TNF-α, IFN-γ, IL-3, IL-13, 1nM IgE and 100 nM IgE but not MIP-1α, histamine, PGD_2, or IL-5.
- Mo-DCs from normal and asthmatic subjects responded differently to allergic mediators. Mo-DCs from normal subjects were found to respond to a higher extent to treatment with;
    1. TNF-α and IFN-γ, that increased the expression of CD40 and CD83*.

2. IL-3, that increased the expression of CD80 and CD86 and decreased the expression of CD23*.
3. IL-5, that increased the expression of CD80, CD86 and HLA-DR.

- Mo-DCs from asthmatic subjects were found to respond to a higher extent to treatment with;
    1. IL-13, that increased the expression of CD80.
    2. 1 nM and 100 nM IgE, that increased the expression of CD80*, and CD86*.

(* = Statistically significant difference comparing the response of Mo-DCs from normal and asthmatic subjects).

Section 4.3

Effects of allergic mediators on cytokine production by monocyte-derived dendritic cells from normal and asthmatic subjects

4.3.1 Materials and methods

4.3.1.1 Subjects

9 non-atopic, non-asthmatic normal subjects (6 female, 3 male) and 4 atopic, asthmatic subjects (2 female, 2 male), defined as in section 2.1.7, took part in this study.

4.3.1.2 Cytokine production by monocyte-derived dendritic cells

To assess the effects of allergic mediators on IL-6 and IL-10 production by Mo-DCs from normal and asthmatic subjects, 6 day old Mo-DCs were exposed to allergic mediators for a further 24 hours where after 500 μL supernatant was removed from the cell cultures and stored at -40°C pending analysis of cytokine content by ELISA. Following another 24 hours in culture, the 8 day old Mo-DCs were harvested and counted for use in other assays and the total volume of supernatant measured.

The IL-6 and IL-10 content in Mo-DC supernatants was measured by ELISAs as previously described (2.2.9). The cytokine content has been presented as pg / 5.0×10^5 Mo-DCs by adjusting the supernatant concentration to the estimated volume of medium on day 7 and the final number of cells present on day 8.

4.3.2 Results

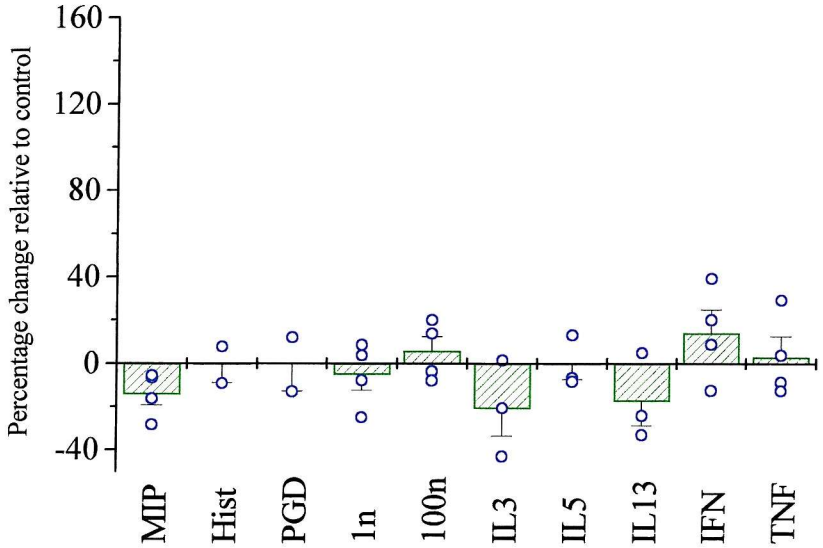
4.3.2.1 IL-6 production by monocyte-derived dendritic cells

Due to the low number of subjects studied in this assay, statistical analysis was not possible. The arbitrary significance limit, defined in 4.1.3 and referred to as marked changes was therefore used. The results in Figure 4.3.1 are presented as percentage change in IL-6 production relative to control Mo-DCs. It was evident that the IL-6 production was markedly increased following treatment with IFN-γ (105.1 % ± 19.7, n = 3) in asthmatic, but not normal Mo-DCs (Figure 4.3.1). In Chapter 3 it was shown that untreated control Mo-DCs from asthmatic subjects produce on average 10.0 ng IL-6 per 5.0×10^5 cells (Figure 3.3.2.2). The increase in IL-6 production observed following exposure to IFN-γ corresponded to an average of 20.7 ng IL-6 per 5.0×10^5 Mo-DCs (data not shown). The remaining mediators, MIP-1α, histamine, PGD_2, IL-3, IL-5, IL-13 and TNF-α, failed to change the production of IL-6 by over 50 % relative to control Mo-DCs in both normal and asthmatic Mo-DCs.

4.3.2.2 IL-10 production by monocyte-derived dendritic cells

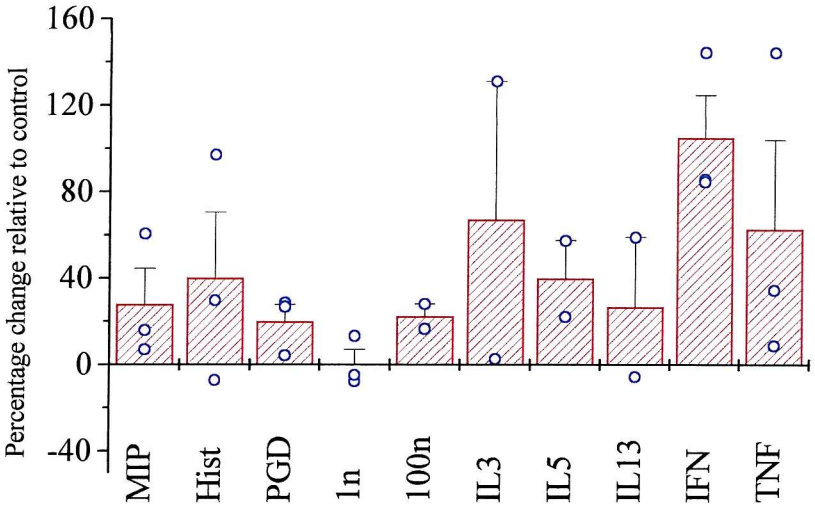
Using non-parametric statistical analysis and the arbitrary significance limit of ± 50 % change, it was shown that none of the mediators investigated significantly or markedly changed the level of IL-10 produced by either normal or asthmatic Mo-DCs (Figure 4.3.2).

a) Normals



n = MIP-1α (4), Histamine (2), PGD_2 (2), IgE 1nM (4), IgE 100nM (4), IL-3 (3), IL-5 (3), IL-13 (3), IFN-γ (4), TNF-α (4).

b) Asthmatics

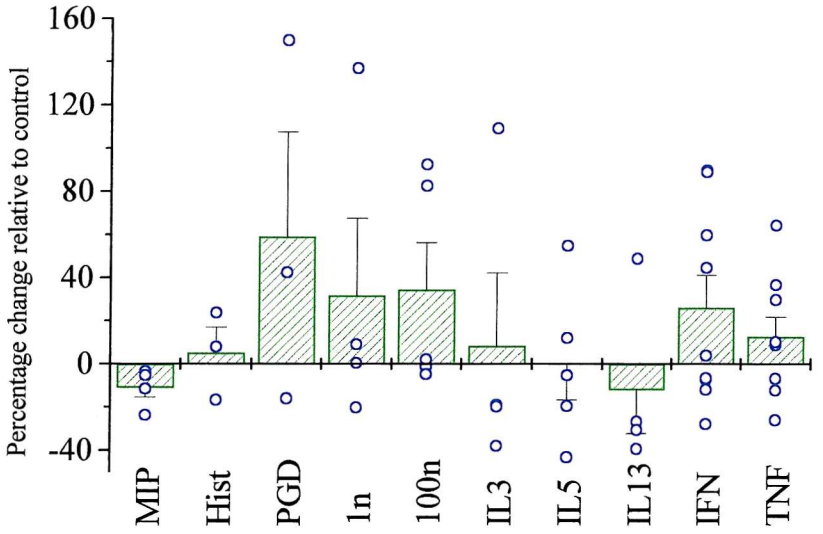


n = MIP-1α (3), Histamine (3), PGD2 (3), IgE 1nM (3), IgE 100nM (2), IL-3 (2), IL-5 (2), IL-13 (2), IFN-γ (3), TNF-α (3).

Figure 4.3.1 IL-6 production by Mo-DCs from normal and asthmatic subjects

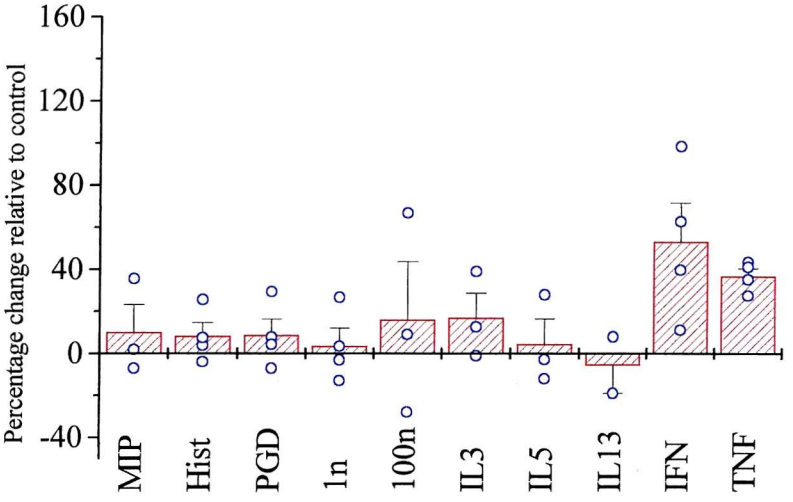
6 day old Mo-DCs from normal and asthmatic subjects were exposed to allergic mediators for a further 24 hours, followed by collection of a small volume of supernatant for measurement of IL-6 content by ELISA. The effects of allergic mediators are presented as percentage change relative to control. Each individual value (percentage change) is presented as an open blue circle and the mean of all values is shown as a column with error bars (SEM). The abbreviations used are listed in Figure 4.2.2.

a) Normals



n = MIP-1α (4), Histamine (3), PGD_2 (3), IgE 1nM (4), IgE 100nM (5), IL-3 (4), IL-5 (5), IL-13 (4), IFN-γ (9), TNF-α (9).

b) Asthmatics



n = MIP-1α (3), Histamine (4), PGD_2 (4), IgE 1nM (4), IgE 100nM (3), IL-3 (3), IL-5 (3), IL-13 (2), IFN-γ (4), TNF-α (4).

Figure 4.3.2 IL-10 production by Mo-DCs from normal and asthmatic subjects

6 day old Mo-DCs from normal and asthmatic subjects were exposed to allergic mediators for a further 24 hours, followed by collection of a small volume of supernatant for measurement of IL-10 content by ELISA. The effects of allergic mediators are presented as percentage change relative to control. Each individual value (percentage change) is presented as an open blue circle and the mean of all values is shown as a column with error bars (SEM). The abbreviations used are listed in Figure 4.2.2.

4.3.3 Discussion

4.3.3.1 Interleukin-6 and interleukin-10

This part of the primary screen of the effects of allergic mediators on Mo-DCs was designed to identify allergic mediators resulting in a statistically significant change or marked change (± 50 % change, defined in 4.1.3) in cytokine production relative to control untreated Mo-DCs. At 24 hours following exposure to allergic mediators there was no significant change in the production of either IL-6 or IL-10 by Mo-DCs from normal or asthmatic subjects. However, Mo-DCs from asthmatic subjects appeared to be slightly more responsive to IFN-γ that induced a marked increase in IL-6 production. It could be argued that an IFN-γ stimulated Mo-DC producing increased levels of IL-6 may be better suited to drive Th2 differentiation. However, since the production of IL-1β was also increased by around 50 % in response to IFN-γ (69.6 % ± 38.2, n = 12 normal subjects, data not shown) the increase in IL-6 production is more likely due to general activation of Mo-DCs in response to IFN-γ. Further, relatively large quantities of IL-6 were detected in the supernatants of untreated Mo-DCs from normal (5.7 ng/ 5.0×10^5 cells) and asthmatic (10.0 ng/ 5.0×10^5 cells) subjects in Chapter 3 (Table 3.2). It seems unlikely that a cytokine constitutively produced in such quantities and that further is not affected by treatment to any significant extent, would have any crucial role in regulating T cell differentiation. IL-10 is, on the other hand, produced at much lower quantities, on average 67 pg / 5.0×10^5 cells by Mo-DCs from normal subjects and 108 pg/ 5.0×10^5 cells by Mo-DCs from asthmatic subjects. The seemingly more controlled production of IL-10 may therefore suggest that IL-10 has a higher impact on T cell proliferation and differentiation when directly focused on proximal T cells in the microenvironment. However, as already mentioned, none of the mediators tested in this study altered the IL-10 production by Mo-DCs to any significantly extent.

As demonstrated in Figure 4.3.1 and 4.3.2, the differences in effect between allergic mediators on cytokine production at 24 hours are in general very small. This may be due to consumption and / or degradation of IL-6 and IL-10 in the culture supernatants. In support of this, the IL-6 and IL-10 concentration in supernatants retrieved 48 hours following exposure to allergic mediators were generally lower than that at 24 hours (data not shown). The difference in effects of allergic mediators at 48 hours was in addition even smaller than at 24 hours. It is also possible that Mo-DCs stimulated with different mediators have different kinetics for regulation of IL-6 and IL-10 production. Some cultures may hence

have reached maximal cytokine production whereas other cultures are still ascending when the supernatants are collected 24 hours post initial exposure to mediator. This would consequently make interpretation of any mediator-specific differences complicated. A study of the kinetics of IL-6 and IL-10 production over the first 24 hours would be essential for the identification of the time point best suited for comparison of effects of allergic mediators. However, for the purpose of this study, priority was given to other areas of interest.

With on average 84 % pure Mo-DCs obtained on day 8 (3.3.1), Mo-DCs are the most likely source of IL-6 and IL-10 detected in these cultures. However, on average 14 % of the total cell population in these *in vitro* Mo-DCs cultures are contaminating cell. Although these cells do not express common surface molecules for T cell, B cell or NK-cells (date not shown), they may be a potential source for the IL-6 and IL-10 detected in the supernatants.

4.3.3.2 Interleukin-12

IL-12 is probably the most powerful cytokine driving Th1 differentiation. It is known that DCs make IL-12 following maturation induced by CD40 ligation or LPS stimulation (1.3.3.5.3). In addition, IL-12 production induced by CD40 ligation has been reported to be greatly enhanced in the presence of exogenous IFN-γ [170] or IL-4[101].

As the effects of allergic mediators on Mo-DC phenotype and function is relatively unknown, the primary screen aimed to identify changes in IL-12 p70 production by Mo-DCs brought on by the allergic mediators without introducing any additional maturation stimuli or exogenous IFN-γ or additional IL-4. However, none of the allergic mediators was able to induce IL-12 p70 production as determined using two separate IL-12 p70 human ELISA kits (TCS and IDS) (data not shown). The induction of IL-12 p70 was therefore attempted in the additional presence of commercial soluble CD40L (sCD40) and enhancer, cross-linking sCD40L (Ancell). No effect on IL-12 p70 production by Mo-DCs was observed (data not shown).

Since finishing the primary screen, it has become possible to detect IL-12 p70 and p40 in the supernatants of Mo-DC exposed to CD40L transfected CHO cells. IL-12 p40 has also been detected in Mo-DCs treated with CD40L transfected CHO cells by intracellular cytokine staining for flow cytometry and mRNA for IL-12 p40 and IL-12 p35 have been detected following stimulation with sCD40L by TaqMan PCR.

4.3.3.3 Conclusion of the effects of allergic mediators on the cytokine production of monocyte-derived dendritic cells

The effects of allergic mediators on the production of IL-6 and IL-10 by Mo-DCs were investigated using both statistical analysis and an arbitrary significance limit. The results presented in this section show that;

- None of the mediators studied significantly or markedly changed the production of IL-6 or IL-10 by Mo-DCs from normal subjects.
- Only IFN-γ markedly increased the production of IL-6 by Mo-DCs from asthmatic subjects, whereas none of the mediators studied changed the production of IL-10 in these Mo-DCs.

Section 4.4

Effects of allergic mediators on monocyte-derived dendritic cells from normal and asthmatic subjects: consequences for T cell function

4.4.1 Materials and methods

4.4.1.1 Subjects

15 non-atopic, non-asthmatic normal subjects (7 female, 8 male) and 5 atopic, asthmatic subjects (2 female, 3 male), defined as in section 2.1.7, took part in this study.

4.4.1.2 Monocyte-derived dendritic cells for T cell proliferation and differentiation assays

To assess the effects of Mo-DCs treated with allergic mediators on the proliferation of driven allogeneic, $CD45RA^+$, T cells, 6 day old Mo-DCs from normal and asthmatic subjects were exposed to allergic mediators for a further 48 hours, then harvested and washed twice in sterile PBS, 1% BSA. Mo-DCs suspended in complete RPMI were irradiated with 25 gray, then washed once and kept on ice resuspended in complete RPMI.

4.4.1.3 Allogeneic, $CD45RA^+$ T cells for T cell proliferation assays

Allogeneic T cells were obtained by purification of leucocytes from human peripheral blood followed by counter-current elutriation (2.2.2.1 and 2.2.2.2) and enrichment of $CD45RA^+$ cells by immunomagnetic depletion of CD19 and CD45RO expressing cells (2.2.2.4.2).

4.4.1.4 Allogeneic $CD45RA^+$ T cell proliferation assay

T cell proliferation assays were set-up in 96-well plates as described in 2.2.4.1. In brief, 1.0×10^5 T cells were co-cultured with serially diluted irradiated Mo-DCs in triplicates for 5 days, then pulsed with 0.5 μCi H^3-thymidine for a further 6 hours before thymidine incorporation was stopped by freezing the plates. T cell proliferation was quantified by measuring H^3-thymidine incorporation as counts per minute (CPM) following harvest of the thawed plates onto glass fibre filters that were counted in a plate reader.

Differences in the T cell proliferation driven by treated Mo-DCs has been expressed as relative proliferation index (RPI). This shows the magnitude of the difference in T cell

proliferation between untreated control Mo-DCs and allergic mediator treated Mo-DCs, and was calculated according to the following equation;

Relative proliferation index = (CPM allergic mediator - CPM control) / CPM control

The CPM values at the Mo-DC dilution giving the greatest difference in CPM between control Mo-DCs and Mo-DCs treated with allergic mediator were chosen for calculation of the relative proliferation index for each allergic mediator. For example, in Figure 4.4.2 the difference in CPM between TNF-α exposed Mo-DCs and control Mo-DCs was greatest at 3 000 Mo-DCs / well (indicated with blue arrows). In this example, these CPM values were chosen for calculations of the relative proliferation index.

4.4.1.5 Statistical analysis

Using SPSS 10.0 for Windows, the non-parametric Wilcoxon test was used where possible to compare responses within a subject group. A p value of less than 0.05 was considered statistically significant.

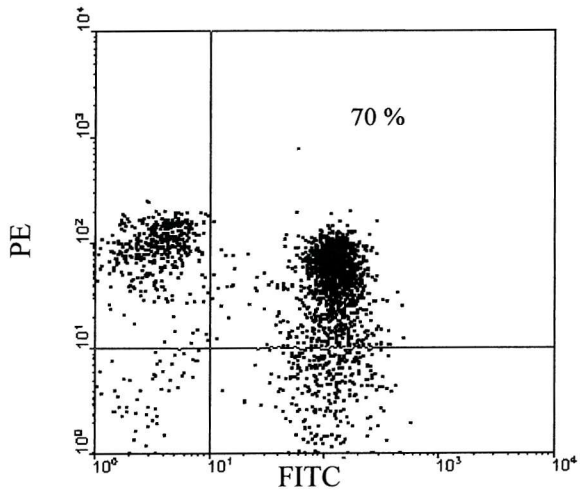
4.4.2 Results

4.4.2.1 Enrichment of allogeneic CD45RA$^+$ T cells

Using a protocol combining purification of leukocytes from human peripheral blood, counter-current elutriation and immunomagnetic depletion of CD19$^+$ and CD45RO$^+$ cells, a cell population consisting of on average 76.0 % ± 3.3 (n = 8) CD5$^+$ T cells was obtained. Of these CD5$^+$ T cells 55.4 % ± 6.4 (n = 8) were shown to be CD45RA$^+$.

A representative example of an enriched CD45RA$^+$ T cell population is illustrated in Figure 4.4.1a. Further, 66.0 % ± 1.0 (n = 3) of the T cell population identified by anti-CD3, was shown to express CD4 (Figure 4.4.1b).

a) CD45RA PE v.s. CD5 FITC



b) CD3$^+$ T cell population

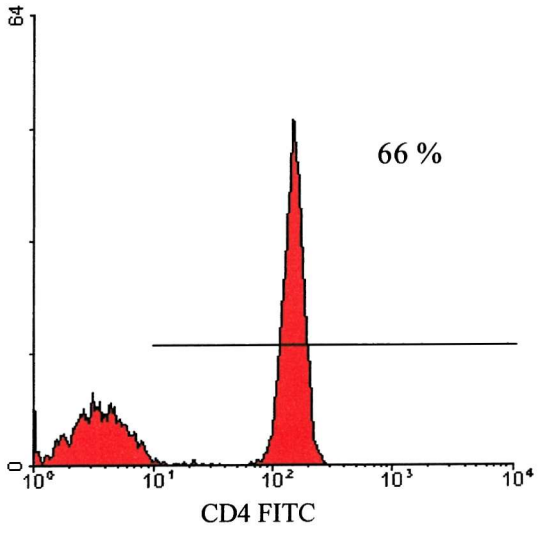


Figure 4.4.1 Phenotype of purified T cell populations

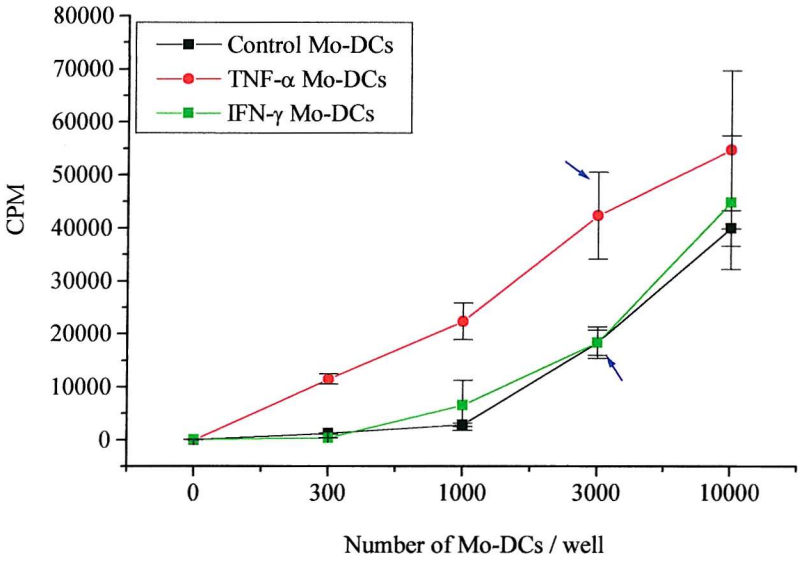
A representative example of a cell population enriched for T cells expressing CD45RA is presented in a). T cells were identified using FITC-conjugated anti-CD5 mAb. The histogram in b) shows CD4 expression in a CD3$^+$ T cell population.

4.4.2.2 Allogeneic CD45RA$^+$ T cell proliferation driven by mediator-treated monocyte-derived dendritic cells

Figure 4.4.2 shows an example of T cell proliferation in response to untreated control Mo-DCs or Mo-DCs treated with TNF-α, IFN-γ, IL-3 or IL-5. TNF-α treated Mo-DCs increased T cell proliferation above the level seen with untreated Mo-DCs even using as few Mo-DCs as 300 per well. IL-5 also resulted in an increase in T cell proliferation although not to the same extent as TNF-α exposed Mo-DCs, whereas IFN-γ and IL-3 did not effect T cell proliferation any more than control Mo-DCs.

Figure 4.4.3 summarises the effects of mediator-treated Mo-DCs from normal and asthmatic subjects on allogeneic T cell proliferation, presented as relative proliferation index. TNF-α exposed Mo-DCs significantly increased T cell proliferation when derived from normal subjects (RPI = 1.1 ± 0.2, n = 15, $p < 0.001$) and markedly increased T cell proliferation when derived from asthmatic subjects (RPI = 1.1 ± 0.3, n = 5). Surprisingly, a similarly enhanced T cell proliferation was not observed by IFN-γ treated Mo-DCs, that like TNF-α treated Mo-DCs express a mature Mo-DC phenotype (4.2.2 and 4.2.3), predicted to significantly enhance T cell proliferation. Although IFN-γ treated Mo-DCs did not significantly enhance T cell proliferation above that seen for control Mo-DCs, statistical analysis revealed that IFN-γ treated normal Mo-DCs were significantly better at driving T cell proliferation than IFN-γ treated asthmatic Mo-DCs. However, this may be a reflection of the low number of asthmatic (n = 3) compared to normal (n = 14) subjects studied. Unlike TNF-α, none of the mediators studied was able to significantly increase the capacity of the Mo-DC to drive T cell proliferation. On the contrary, a slight reduction in T cell proliferation was observed in response to most mediators in asthmatic Mo-DCs and some mediators in normal Mo-DCs. For example, Mo-DCs from normal subjects show significantly reduced relative proliferation index in response to IL-13 treatment (RPI = -0.3 ± 0.1, n = 6, $p < 0.05$).

a) T cell proliferation driven by control, TNF-α and IFN-γ treated Mo-DCs



b) T cell proliferation driven by control, IL-3 and IL-5 treated Mo-DCs

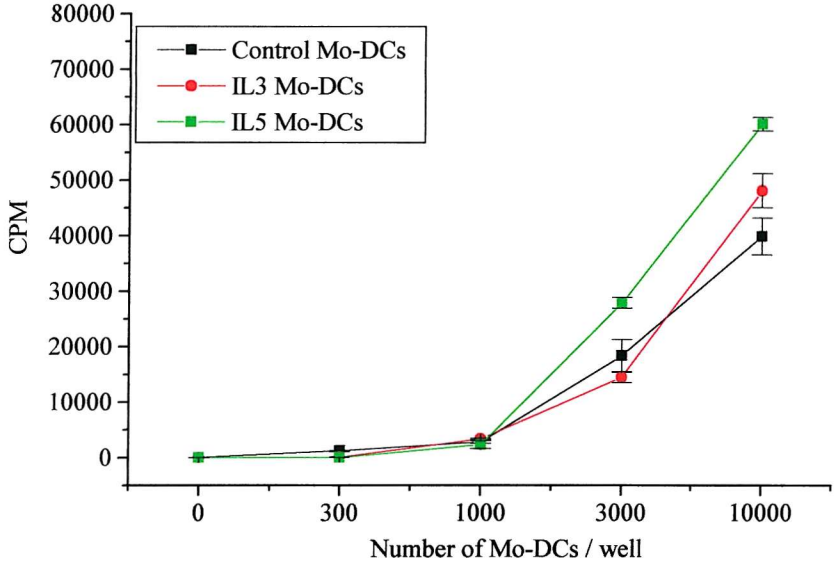
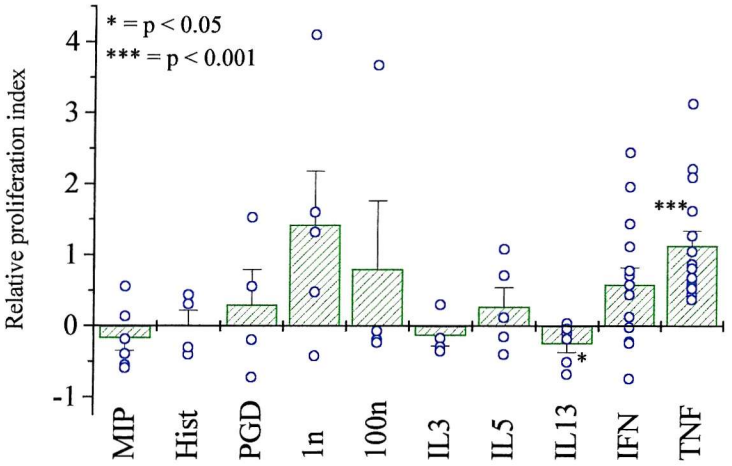


Figure 4.4.2 ^{3}H-thymidine incorporation in Mo-DC : T cell co-cultures

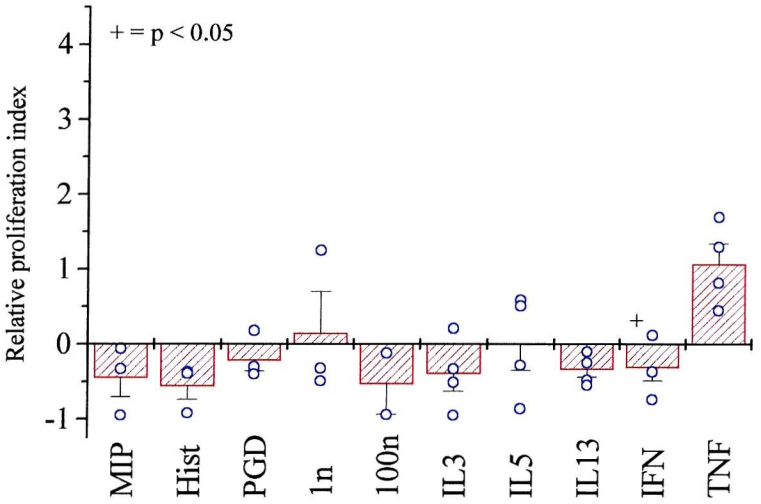
Six day old Mo-DCs from a representative asthmatic subject were exposed to TNF-α, IFN-γ, IL-3 or IL-5 for a further 48 hours then harvested, washed and irradiated. Cultures of 1.0×10^5 allogeneic T cells, enriched for CD45RA expression, and varying numbers of Mo-DCs per well were set up in triplicates. After 5 days, T cell proliferation was assessed by H^3-thymidine incorporation. The graphs present T cell proliferation as CPM against an increasing number of a) control, TNF-α and IFN-γ treated Mo-DCs, and b) control, IL-3 and IL-5 treated Mo-DCs. The blue arrows in a) indicate the CPM values for TNF-α exposed Mo-DCs and control Mo-DCs that are chosen for calculations of the TNF-α relative proliferation index.

a) Normals



n = MIP-1α (6), Histamine (4), PGD_2 (4), IgE 1nM (5), IgE 100nM (4), IL-3 (4), IL-5 (5), IL-13 (6), IFN-γ (14), TNF-α (15).

b) Asthmatics



n = MIP-1α (3), Histamine (3), PGD_2 (4), IgE 1nM (3), IgE 100nM (2), IL-3 (4), IL-5 (4), IL-13 (4), IFN-γ (4), TNF-α (4).

Figure 4.4.3 Allogeneic T cell proliferation in response to treated Mo-DCs

Six day old Mo-DCs from a) normal and b) asthmatic subjects were exposed to allergic mediators for a further 48 hours, then harvested, irradiated and incubated with allogeneic T cells enriched for CD45RA expression. Cultures of 1.0×10^5 T cells and varying numbers of Mo-DCs per well were set up in triplicates. After 5 days, T cell proliferation was assessed by H^3-thymidine incorporation over 6 hours. Each individual RPI value is presented as an open blue circle and the mean of all RPI values for each allergic mediator is shown as a column with error bars (SEM).

* = $p < 0.05$ compared to the RPI of control untreated Mo-DCs.

*** = $p < 0.001$ compared to the RPI of control untreated Mo-DCs.

+ = $p < 0.05$ compared to the RPI of the same treatment in normal Mo-DCs.

4.4.3 Discussion

4.4.3.1 Proliferation of allogeneic CD45RA$^+$ T cells

The aim of this part of the primary screen was to determine whether the microenvironment observed in atopic asthmatic inflammation could influence the ability of DCs to stimulate T cells. The T cells used for the proliferation assay were in parallel used for development of a T cell differentiation assay (4.4.3.3.). As DCs are the only APCs capable of inducing a primary immune response, and therefore are good candidates for initiating the development of atopic disease, the study aimed to use naïve T cells. Human naïve T cells are most commonly distinguishing by their surface expression CD45RA. The best source of naïve CD45RA$^+$ T cells is cord blood. However, since the availability of cord blood was very unreliable, the source of T cells in this study was human adult peripheral blood. CD45RA expression has recently been demonstrated on CD8$^+$ T cells of the memory compartment, possibly as a consequence of conversion of CD45RO$^+$ to CD45RA$^+$ following activation [323]. Hence the CD45RA$^+$ T cell population in adult blood may consist of memory as well as unprimed naïve CD4$^+$ and CD8$^+$ T cells. However, by selecting for CD45RA$^+$ T cells, a population mainly containing quiescent T cells may be obtained that possibly is more easily influenced by Mo-DCs than already activated T cells. A protocol depleting CD45RO$^+$ T cells was therefore developed. However, the current protocol only generated on average 55.4 % ± 6.4 (n = 8) CD45RA$^+$, CD5$^+$ T cells.

To mimic the *in vivo* conditions of T cell priming, this study would ideally have used an autologous system and looked at antigen-driven T cell proliferation. However, the number of antigen-specific naïve T cells in the peripheral blood is expected to be low, resulting in difficulties in measuring DC-influenced autologous naïve T cell proliferation *in vitro*. An alternative approach would be to measure proliferation of allogeneic naïve T cells. Alloreactivity occurs when T cells respond to allogeneic forms of MHC expressed by the APC. Allogeneic T cell proliferation is in general greater than autologous T cell proliferation as allogeneic forms of MHC together with self or foreign peptides present several ligand combinations to be recognised by a higher frequency of the TCR repertoire [324]. It is believed that factors influencing autologous T cell proliferation, such as costimulatory molecules and the presence of cytokines, still may have effects on allogeneic T cell proliferation. For example, TNF-α or IL-1β exposed DCs stimulate a better allogeneic T cell proliferation than unstimulated DCs, most likely due to increased expression of costimulatory molecules [88] [82]. Allogeneic T cells were therefore chosen for the purpose of this study.

Using the current protocol, the T cell purity was on average 76.0 % ± 3.3 (n = 8). The main contaminating cell population was NK-cells that may interact with, and respond to the Mo-DCs and could potentially influence the result. Attempts to increase the T cell purity by including depletion of $CD16^+$ or $CD56^+$ cells in the purification protocol have been unsuccessful.

4.4.3.2 The ability of monocyte-derived dendritic cells treated with allergic mediators to drive allogeneic $CD45RA^+$ T cell proliferation

TNF-α exposed Mo-DCs were found to increase allogeneic T cell proliferation compared to control Mo-DCs, showing a relative proliferation index ranging from 0.4 – 3.1 in normal Mo-DCs and 0.5 – 1.7 in asthmatic Mo-DCs. The magnitude of these relative proliferation indexes are within the range reported for mature Mo-DCs (RPI ranging from 0.5 – 2.8) [82,88].

It was evident that no other mediator treatment was able to modify Mo-DCs, from either normal or asthmatic subjects, to enhance T cell activation. Instead a slight reduction in T cell activation in response to most of the allergic mediators was commonly observed, emphasised by the statistically significant reduction in T cell activation induced by IL-13 treated Mo-DCs from normal subjects. Not even IFN-γ, that induced an even more mature Mo-DC phenotype than TNF-α (4.2), significantly enhanced T cell proliferation. An extended study of the mechanisms underlying this difference between TNF-α and IFN-γ exposed Mo-DCs is presented in Chapter 5. Finally, Mo-DCs from normal and asthmatic subjects differed in their ability of driving T cell proliferation as IFN-γ treated Mo-DCs from normal subjects were found to be significantly more potent at driving T cell proliferation than Mo-DCs from asthmatic subjects treated with IFN-γ.

4.4.3.3 Development of a T cell differentiation assay

As already stressed, the hypothesis underlying the work described in this chapter was that mediators of the allergic asthmatic environment may change the phenotype and function of DCs resulting in initiation or perpetuation the chronic allergic inflammation seen in asthmatic airways. Since the allergic inflammation is characterised by a polarised Th2 response, attempts were made to develop an assay to include in the primary screen that

would reveal whether treatment of Mo-DCs with particular allergic mediators could influence the differentiation of activated T cells.

4.4.3.3.1 The trispecific antibody method

The initial assay that was developed was based on priming allogeneic T cells, enriched for CD45RA expression (4.4.1.3), with irradiated Mo-DC exposed to allergic mediators (4.4.1.2) followed by re-stimulation with trispecific antibody (kind gift from Prof. M. Glennie, Tenovus, Southampton). As the trispecific antibody simultaneously targets CD3, CD2 and CD28 on the T cell, hence simulating the TCR/CD3 and costimulatory signals provided by an APC, this method excluded any need for re-stimulation with treated Mo-DCs. Following 24 hours incubation with trispecific antibody the T cells were harvested, counted and supernatants collected for analysis of cytokine content by ELISA (See Figure 4.1.2). Although measurable levels of cytokines were produced (data not shown), the protocol used may have introduced several factors that potentially could interfere with the Mo-DC-induced T cell response. These included the use of an allogeneic T cell population containing a mix of memory and naïve cells, contaminating NK-cells and re-stimulation with trispecific antibody.

The memory T cells in the mixed naïve and memory allogeneic T cell population will secrete cytokines following interaction with the peptide-MHC class II complex expressed on the allogeneic Mo-DCs. These cytokines may potentially alter the Mo-DC induced differentiation of the naïve T cells. As mentioned in section 4.4.3.1, one way of avoiding this problem would be to purify T cells from cord blood. However, cord blood is not widely available or available in large enough quantities to perform these large T cell proliferation and differentiation assays.

In a recent *in vitro* study of mouse DCs, NK cell cytolytic activity and IFN-γ production was increased following interaction between DCs and resting NK cells [325]. If a similar mechanism can occur in human, NK cell produced IFN-γ may override the Mo-DC-induced differentiation of the naïve T cell and skew the response towards Th1.

The trispecific antibody is very potent at driving T cell proliferation. However, it is currently not known in what direction the trispecific antibody drives a naïve T cell to differentiate, i.e. Th1, Th0 or Th2. It is therefore possible that by re-stimulating the effector T cell population with the trispecific antibody the Mo-DC induced T cell phenotype may be altered.

4.4.3.3.2 The cytokine secretion assay method

To eliminate the need for re-stimulation with trispecific antibody and the associated artefacts this approach may have introduced, evidence for Th1 versus Th2 skewing was sought immediately following the primary response. Cytokine secretion assay kits (Miltenyi Biotech), measuring IFN-γ and IL-4 producing cells by flow cytometry, were used as a more sensitive method of cytokine detection than conventional ELISAs. The naïve T cells were primed with Mo-DCs exposed to allergic mediators for 5 days where after IFN-γ and IL-4 producing cells were analysed by flow cytometry.

This method successfully identified a low frequency of cytokine secreting cells following one round of stimulation (data not shown). It further allowed different cytokine producing cell populations to be distinguished accurately. However, this method was found to be unsuitable for the identification of Th1-like and Th2-like cells as no IL-4 producing T cells were found following primary stimulation. This is in accordance with literature showing that human naïve T cells require at least two rounds of stimulation in the presence of IL-4 to develop a Th2 phenotype [31]. An established Th1 phenotype has on the other hand been demonstrated after only 4 days stimulation of naïve T cells in the presence of IL-12 [31]. Accordingly, IFN-γ producing T cells were consistently found following 5 days in culture with treated Mo-DCs. The level of IFN-γ produced was in addition depending on the nature of the Mo-DC treatment, although no trend for increased or decreased IFN-γ production could be linked to any specific Mo-DC treatment at the low number of experiments performed. The differences between Mo-DC treatments were however more pronounced for both IFN-γ and IL-4 following re-stimulation with trispecific antibody, further indicating the requirement for repeated stimulation for the complete differentiation into an effector T cell.

4.4.3.3.3 Conclusions regarding the T cell differentiation assay

The ability to measure both the typical Th1 cytokine IFN-γ and the typical Th2 cytokine IL-4 is essential for the determination of the nature of an effector T cell population. Both methods discussed above failed to completely satisfy this need, as the effects of the trispecific antibody are unknown at present, and as Th2 differentiation probably requires more than one round of stimulation. It is possible that satisfactory results may be obtained using a method by which allogeneic $CD4^+$, $CD45RA^+$ T cells are primed and re-stimulated

with Mo-DCs generated from the same donor. However, due to time limitations development of a satisfactory T cell differentiation assay was not accomplished during the course of the project. Results from this assay have therefore not been considered when selecting allergic mediators for further investigation.

4.4.3.4 Conclusion of the effects of allergic mediators on monocyte-derived dendritic cells: consequences for T cell function

The ability of Mo-DCs to drive T cell proliferation was investigated after treatment with allergic mediators. Using both statistical analysis and an arbitrary significance limit the results presented in this section show that;

- Mo-DCs from normal subjects responded to TNF-α by significantly enhancing and to IL-13 by significantly suppressing T cell proliferation. None of the other mediators studied significantly or markedly altered the ability of normal Mo-DCs to drive T cell proliferation.
- Mo-DCs from asthmatic subjects responded to TNF-α by markedly enhancing T cell proliferation. None of the other mediators studied significantly or markedly altered the ability of asthmatic Mo-DCs to drive T cell proliferation.
- Mo-DCs from normal subjects were found to be significantly better than Mo-DCs from asthmatic subjects at driving T cell proliferation when treated with IFN-γ.

Section 4.5

Summary and discussion of the results from the primary screen

The hypothesis underlying the work described in this chapter was that mediators of the allergic asthmatic environment bring about phenotypic and functional changes in DCs resulting in initiation or perpetuation of the chronic allergic inflammation seen in asthmatic airways. The primary screen consisted of four assays, evaluating the effects of allergic mediators on Mo-DC 1) phenotype, 2) receptor-mediated endocytosis, 3) cytokine production and 4) ability to drive proliferation of allogeneic $CD45RA^+$ T cells. All four assays were designed to identify allergic mediators resulting in a statistically significant or marked change in Mo-DC phenotype and / or function. Mo-DCs were derived *in vitro* from normal and asthmatic subjects and the effects of 10 mediators were investigated. Using this experimental approach, significant and marked changes in Mo-DC phenotype or function were identified following treatment with TNF-α, IFN-γ, IL-3, IL-5 and IgE. In contrast, only slight effects were observed following treatment with MIP-1α, histamine, PGD_2 and IL-13. This experimental approach also identified four areas of particular interest.

1) Although expressing a seemingly mature phenotype, IFN-γ exposed Mo-DCs fail to induce an enhanced allogeneic T cell proliferation like phenotypically mature TNF-α treated Mo-DCs.
2) CD23 disappears from the surface of Mo-DCs in response to TNF-α, IFN-γ and IgE. The mechanisms underlying this downregulation are unknown.
3) IL-3 and IL-5 frequently induced a mature Mo-DCs phenotype, particularly in Mo-DCs from normal subjects.
4) Mo-DCs from normal and asthmatic subjects respond differently to allergic mediators.

These areas are discussed further in the following sections.

4.5.1 Interferon-γ exposed monocyte-derived dendritic cells

In the primary screen of allergic mediators IFN-γ was shown to significantly increase the surface expression of CD40, CD80, CD83, CD86 and HLA-DR and to reduce the expression of CD23 on Mo-DCs from normal and asthmatic subjects (4.2). Further, IFN-γ reduced receptor-mediated endocytosis in Mo-DCs from normal and asthmatic subjects (4.2) and markedly increased the production of IL-6 in Mo-DCs from asthmatic subjects (4.3). With the exception of the effects seen on IL-6 production, these observations were also noted for Mo-DCs treated with TNF-α. However, in contrast to TNF-α treated Mo-DCs, IFN-γ treated Mo-DCs did not significantly enhanced proliferation of allogeneic T cells (4.4).

Reports of similar IFN-γ induced phenomenon are relatively limited. Mouse spleen DCs cultured for 24 hours in the presence of IFN-γ demonstrated increased expression of CD86 with no change in expression of CD80, ICAM-1, heat stable antigen (HsAg), MHC class II or MHC class I[326]. However, these IFN-γ stimulated DCs showed impaired ability to promote allogeneic T cell proliferation in comparison to control GM-CSF stimulated DCs. A lower level of CD80 expression on the IFN-γ stimulated DCs was suggested to be responsible for the observed difference in T cell proliferation. In human, it has been shown that DCs derived in GM-CSF, IL-4 and IFN-γ over a period of 12 days express elevated levels of HLA-DR and CD11a and reduced levels of CD1a and CD80 [317]. These IFN-γ stimulated DCs demonstrated a reduced ability to drive proliferation of naive as well as memory allogeneic T cells enriched either for CD4 or CD8 expression, as compared to control DCs. This is in contrast to a recent report showing enhanced proliferation of allogeneic T cells stimulated with purified $CD11c^+$ peripheral blood DCs treated with IFN-γ[42]. These IFN-γ treated blood DCs also expressed elevated levels of CD40, CD83, CD80, CD86, HLA-DR and HLA-DQ and were shown to promote Th1 differentiation of allogeneic naïve T cells by producing high levels of IL-12 p70.

The lack of enhanced T cell proliferation despite the mature phenotype of IFN-γ stimulated Mo-DC could be due to several factors. It is possible that Mo-DCs undergo apoptosis following stimulation with IFN-γ or induce apoptosis of a proportion of the responding T cell population. It is also possible that IFN-γ stimulates the secretion of cytokines with known immunosuppressive function, such as TGF-β. Further, since IFN-γ exposed Mo-DCs express even higher levels of costimulatory molecules than TNF-α exposed Mo-DCs, it is possible that T cell proliferation follows faster kinetics in response to IFN-γ exposed Mo-DCs than TNF-α exposed Mo-DCs.

The combination of a very mature DC phenotype yet a moderate capacity to activate T cells may have broad clinical implications for controlling immune responses. The mechanisms underlying the failure of IFN-γ stimulated Mo-DCs to enhance allogeneic T cell proliferation are therefore extensively investigated in Chapter 5.

4.5.2 The fate of CD23 following stimulation with tumour necrosis factor-α, interferon-γ and immunoglobulin E

One of the most dramatic changes in Mo-DC phenotype observed in the primary screen of allergic mediators was the reduction of CD23 surface expression in response to TNF-α, IFN-γ and IgE at 100 nM (4.2). The effect of these mediators was equally striking

on normal as on asthmatic Mo-DCs. The route by which CD23 disappears from the cell surface in response to these mediators is not clear. However, the mechanisms by which TNF-α, IFN-γ and IgE mediate CD23 downregulation are most likely dissimilar as IgE, in contrast to TNF-α and IFN-γ, directly ligates CD23 and further reduced the CD23 surface expression in the absence of concurrent Mo-DC maturation. In cultured human LCs, IFN-γ has been demonstrated to induce the release of soluble CD23 (sCD23)[327] making this an attractive theory for human Mo-DCs as well. Shedding of sCD23 is however not a probable explanation for the disappearance of surface CD23 following stimulation with IgE as IgE bound to CD23 has been shown to stabilise and protect CD23 against proteolysis [112]. IgE is more likely to induce internalisation of CD23 as already mentioned in section 4.2.3.1. Further, CD23 may disappear as a consequence of regulation at the level of CD23 gene transcription, as was shown for IL-10 stimulated monocytes[277]. The fate of CD23 following stimulation with TNF-α, IFN-γ and IgE is further investigated in Chapter 6.

4.5.3 Effects of interleukin-3 and interleukin-5

In the primary screen, IL-3 and IL-5 were frequently shown to induce a mature phenotype in Mo-DCs from both normal and asthmatic subjects (4.2). This effect on Mo-DC maturation was however not reflected in the way these Mo-DCs stimulated T cell proliferation (4.4). Due to time limitation, further investigation of the effects of IL-3 and IL-5 on Mo-DC phenotype and function and consequences for activation and differentiation of T cells was not within the scope of this project. It is therefore not possible to speculate whether or not DCs exposed to IL-3 and IL-5 are more prone to initiate or perpetuate the chronic allergic inflammation seen in asthmatic airways.

4.5.4 Functional differences between monocyte-derived dendritic cells from normal and asthmatic subjects

Differences in the way Mo-DCs from normal and asthmatic subjects respond to TNF-α, IFN-γ, IgE and the Th2 cytokines have been identified analysing the phenotype and function of Mo-DCs.

It appears that Mo-DCs from asthmatic subjects are significantly more responsive to IgE. This was shown as asthmatic Mo-DCs treated with 1 nM IgE expressed significantly higher levels of CD80 and CD86 than normal Mo-DCs (Figure 4.2.2). Although not supported by statistical analysis, the expression level of CD80 and CD86 also appeared

increased in asthmatic, but not normal Mo-DCs following treatment with 100 nM IgE. Further, it was found that Mo-DCs from asthmatic subjects express a generally less mature phenotype in response to TNF-α, IFN-γ, IL-3 and IL-5 than Mo-DCs from normal subjects (Figure 4.2.2). This is also supported by statistical analysis as Mo-DCs from asthmatic subjects express significantly lower levels of CD83 following treatment with IFN-γ (data not shown) and significantly higher levels of CD23 following treatment with IL-3 (Figure 4.2.2a). The generally more immature phenotype of Mo-DCs from asthmatic subjects also affected their function, as IFN-γ treated Mo-DCs from asthmatic subjects were found to drive proliferation of allogeneic T cells significantly less well than IFN-γ treated Mo-DCs from normal subjects (Figure 4.4.3).

The increased surface expression of the costimulatory molecules CD80 and CD86 in response to IgE may result in enhanced antigen presentation of allergens taken up via FcεRI and allergen-specific IgE. This process of allergen focusing, is known to promote very efficient antigen presentation, especially of allergens an individual already has been sensitised to, present at very low concentrations [84,124,234,242,251]. A trend for higher surface expression of CD40, CD80 and HLA-DR on $FcεRI^+$, as compared to $FcεRI^-$ Mo-DCs has also been observed, that moreover was shown more prominent in Mo-DCs from asthmatic subjects (data not shown). This may suggest that Mo-DCs from asthmatic subjects are more prone to perpetuate the allergic inflammation when exposed to an IgE-rich environment than Mo-DCs from normal subjects.

The maturation state induced by TNF-α, IFN-γ, IL-3 and IL-5 in Mo-DCs from asthmatic subjects was more mature than that for untreated, control Mo-DCs from asthmatic subjects, yet less mature than that induced by the same mediator in Mo-DCs from normal subjects. It is possible that these "semi-mature" Mo-DCs from asthmatic subjects may affect differentiation of T cells differently to the more mature Mo-DCs from normal subjects, as the maturation state of the DCs has been shown to influence T cell differentiation. For example, immature DCs have been suggested to preferentially drive differentiation of Tr1-like cells, whereas mature DCs have been suggested to drive differentiation of Th1 cells[328]. Further, functionally and phenotypically immature rat respiratory tract DCs were found to preferentially drive Th2 differentiation [258]. However, following *in vitro* culture in the presence of GM-CSF these rat DCs matured and became capable of driving both Th1 and Th2 differentiation. Without further investigating T cell differentiation driven by Mo-DCs from normal and asthmatic subjects treated with TNF-α, IFN-γ, IL-3 or IL-5 it is difficult to predict if Mo-DCs from asthmatic subjects are more prone to drive allergic inflammation when exposed to an allergic environment.

Finally, it should be noted that the differences observed between normal and asthmatic subjects may be a consequence of the generally low number of subjects investigated, especially in the asthmatic group.

4.5.5 Conclusion concerning the effects of allergic mediators on monocyte-derived dendritic cell phenotype and function

The results presented in this chapter support the proposal that mediators within the allergic asthmatic environment can change the phenotype and function of DCs, as Mo-DCs from both normal and asthmatic subjects were shown to respond to TNF-α, IFN-γ, IL-3, IL-5 and IgE. It was not within the scope of the primary screen to show whether or not these changes consequently resulted in the preferential differentiation of Th2 cells. However, based on the evidence provided, it can be speculated that in particular, DCs from asthmatic subjects exposed to an allergic environment may initiate or perpetuate the chronic allergic inflammation seen in asthmatic airways.

Chapter Five

The effects of interferon-γ on monocyte-derived dendritic cells from normal subjects

Summary

In Chapter 4, IFN-γ was shown to induce strong Mo-DC maturation, and yet IFN-γ-treated Mo-DCs failed to drive T cell proliferation as potently as phenotypically less mature TNF-α-treated Mo-DCs. The work presented in this chapter aimed to investigate the mechanisms underlying this disparity between the phenotype and function of IFN-γ treated Mo-DCs.

Experiments were designed to explore the possible involvement of 1) the direct effects of IFN-γ, including the dose and time of exposure, 2) T cell phenotype and viability, 3) Mo-DC viability, and 4) the production of soluble factors capable of immune suppression including nitric oxide and TGF-β.

IFN-γ treated Mo-DCs were not found to be better at driving T cell proliferation when treated with a reduced concentration of IFN-γ or treated with IFN-γ for a shorter period of time. Further, no link between Mo-DC treatment and T cell phenotype and viability was found that could explain the observed impaired T cell proliferation. IFN-γ was found to enhance surface expression of CD95 on Mo-DCs. Much attention was therefore focused on the possibility that IFN-γ treated Mo-DCs were susceptible to Fas-mediated apoptosis. However, $CD95^+$, IFN-γ treated Mo-DCs were not found to undergo Fas-mediated apoptosis in response to sFasL and further, T cell proliferation was unaffected by blocking Fas-mediated signalling using an anti-CD95 mAb. Nitric oxide-induced apoptosis was also excluded as a major mechanism underlying the impaired T cell proliferation as the nitric oxide content in cultures of IFN-γ treated Mo-DCs and in IFN-γ treated Mo-DC : T cell co-cultures was very low and T cell proliferation was unaffected by inhibiting nitric oxide production using a nitric oxide synthase inhibitor. However, Mo-DC viability could not be excluded as having an effect on T cell proliferation as a lower number of viable Mo-DCs was constantly found in IFN-γ treated Mo-DC : T cell co-cultures and a significantly higher proportion of late apoptotic cells was found when IFN-γ treated Mo-DCs were harvested and then put back into culture. Impaired T cell proliferation may also result from suppression by TGF-β1 that was found at significantly higher levels in IFN-γ treated Mo-DC : T cell co-cultures.

5.1 Introduction

5.1.1 Aim

In Chapter 4, it was shown that IFN-γ potently induced a mature Mo-DC phenotype without significantly enhancing T cell proliferation. As this may have clinical implications in controlling immune responses, the work presented in this chapter aimed to investigate the possible mechanisms underlying the failure of IFN-γ-treated Mo-DCs to enhance T cell proliferation.

5.1.2 Experimental approach

In light of existing reports of the effects of IFN-γ on DC phenotype and function, the lack of significantly enhanced T cell proliferation could be due to several factors. These include; 1) direct effects of IFN-γ, 2) the effects of IFN-γ treated Mo-DCs on T cell phenotype and viability, 3) the effects of IFN-γ on Mo-DC viability, and 4) the effects of IFN-γ on the production of soluble factors capable of immune suppression. These areas are reviewed and discussed further below.

5.1.2.1 Direct effects of IFN-γ

A more mature Mo-DC would be expected to drive stronger T cell proliferation than a less mature Mo-DCs. Further, T cell proliferation in response to a mature Mo-DC is commonly observed at a lower DC : T cell ratio and at an earlier time point during the T cell proliferation assay. In Chapter 4, the effects of IFN-γ treated Mo-DCs on T cell proliferation was studied using Mo-DCs treated with 1.9 nM IFN-γ for 48 hours. As IFN-γ treated Mo-DCs appeared to express an even more mature phenotype than TNF-α treated Mo-DCs, it is possible that the IFN-γ treated Mo-DC may be functionally debilitated at 1.9 nM IFN-γ due to high potency of IFN-γ or the length of treatment with IFN-γ. In accordance, functional exhaustion has been reported for Mo-DCs stimulated with LPS and CD40L that no longer produce IL-12, IL-10 or TNF-α[153].

The influence of IFN-γ and TNF-α at varying concentrations and for varying length of treatment, has therefore been investigated in the work presented in this chapter. Further,

in Chapter 4, T cell proliferation was measured following 5 days incubation with Mo-DCs. Due to the exceptionally high levels of costimulatory molecule expression on IFN-γ treated Mo-DCs, it is possible that T cell proliferation had reached its maximum before day 5 and was declining when proliferation was measured. T cell proliferation has therefore been measured at intervals up to and including 5 days of culture.

The series of experiments investigating the direct effects of IFN-γ are presented in section 5.3.2.

5.1.2.2 Effects of IFN-γ treated monocyte-derived dendritic cell on T cell phenotype and viability

All proliferation assays in Chapter 4 were performed using a mixture of $CD4^+$ and $CD8^+$ T cells enriched for quiescent CD45RA expressing cells. Selective suppression, or deletion, of $CD4^+$ or $CD8^+$ T cells could be responsible for the lack of enhanced T cell proliferation seen in co-cultures of IFN-γ treated Mo-DCs and T cells. Suppression or deletion of $CD4^+$ T cells has been suggested in the non-obese diabetes (NOD) mouse. When spleen DCs stimulated with IFN-γ *ex vivo*, were transferred into 1- or 4-week old NOD mice the onset of insulin-dependent diabetes mellitus (IDDM), that is dependent on $CD4^+$ T cells, was delayed [329]. To determine if proliferation of the CD4 or CD8 subpopulation was being preferentially targeted, the proportion of $CD4^+$ and $CD8^+$ T cells in the co-cultures has been investigated. Further, in trying to resolve whether T cell stimulated with IFN-γ treated Mo-DCs receive different, or not as many or strong activation signals as T cells stimulated with TNF-α treated Mo-DCs, the progression of T cell activation was estimated by analysing the proportion of quiescent $CD45RA^+$ to activated $CD45RO^+$ T cells.

Murine DC have been shown to express CD95L and to induce Fas-mediated apoptosis of Fas (CD95) expressing T cells[330]. Therefore, surface expression of CD95 on T cells stimulated with IFN-γ treated Mo-DCs was investigated. The apoptotic state of the cells in these co-cultures was also monitored by studying annexin-V binding using flow cytometry.

The experiments investigating T cell phenotype and viability are presented in section 5.3.3.

5.1.2.3 Effects of IFN-γ on monocyte-derived dendritic cell viability

To receive adequate TCR-stimulation for T cell activation, it is in the T cells' interest to keep the DC alive during antigen presentation. The T cell therefore provides the DC with survival signals mediated via surface molecules expressed on DCs such as tumour necrosis factor-related activation-induced cytokine receptor (TRANCE-R) and CD40. Signals via TRANCE-R and CD40 have been shown to lead to an increase in expression of genes belonging to the bcl-2 family that have anti-apoptotic functions in DCs [104,331]. After a few days of antigen presentation to T cells, DCs undergo apoptosis, possibly as a mechanism for keeping homeostasis of the immune response. Apoptotic cell death is tightly controlled by a series of proteolytic enzymes of the caspase family, culminating in condensation and fragmentation of the cells into apoptotic bodies that are rapidly removed by phagocytes[332]. Activation of the caspase cascade may be initiated by intracellular factors such as radiation or chemicals. However, at the end of antigen presentation it is more likely induced by activation of death domain containing surface receptors of the TNF receptor family expressed on the DCs. Fas (CD95) is a 36 kD member of the TNF receptor family that following ligation by Fas ligand (FasL), typically expressed on activated T cells, induces apoptosis of Fas expressing cells. However, although both murine and human DCs can express CD95 they appear largely resistant to Fas-mediated apoptosis, especially when mature[105-107]. Resistance to Fas-mediated apoptosis is thought to prolong the DC : T cell interaction and contribute to the superiority of the DC as an APC since macrophages and B cells are not resistant to Fas-mediated apoptosis[333]. However, Fas-resistance in DCs have recently been shown to be reversed following infection with measle virus (MV) [109]. MV-inflected Mo-DCs have been shown to undergo Fas-mediated apoptosis in co-culture with T cells [109] possibly as a consequence of the concurrent change in CD40 signaling evident as impaired CD40-mediated maturation in these cells[334]. It is therefore possible that $CD95^+$ DCs may indeed undergo Fas-mediated apoptosis under circumstances where one or more signaling pathways are modified. With the exception of the study of MV-infected Mo-DCs, Fas-mediated apoptosis in human DCs have been investigated using untreated DCs. There are no reports on the effects of IFN-γ on the expression of CD95 by human DCs or on their susceptibility to Fas-mediated apoptosis. Although IFN-γ has got no reported effects on surface expression of CD95 or Fas-mediated apoptosis in murine DCs [107], IFN-γ has been shown to up-regulate surface expression of CD95, leading to enhanced Fas-mediated apoptosis, in several other cell types including murine epidermal LC [335], murine microglia [336], human kerotinocytes [337] and human eosinophils [338].

The experiments presented in section 5.3.4 were directed at determining if 1) IFN-γ treatment of Mo-DCs alters the survival signals provided by the T cell, consequently affecting Mo-DC viability, 2) if IFN-γ induces CD95 expression on Mo-DCs and 3) if IFN-γ renders $CD95^+$ Mo-DCs susceptible to Fas-mediated apoptosis.

5.1.2.4 Effects of IFN-γ treatment on the production of soluble factors by monocyte-derived dendritic cells and monocyte-derived dendritic cell: T cell co-cultures

Nitric oxide is a multifunctional signalling molecule produced from L-Arginine and O_2 by one of three nitric oxide synthase (NOS) isoforms, neural NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS)[339]. In murine DCs, NO is produced by iNOS in response to IFN-γ, sCD40L, LPS or upon encounter with allogeneic, but not syngeneic T cells[340]. This NO has been shown to induce apoptosis of the murine DCs, resulting in reduced proliferation of the T cells in the allogeneic DC : T cell co-culture. NO has also been known to induce T cell apoptosis[340]. However, NO-induced T cell apoptosis have been shown to be preventable in the presence of IL-2 [340]. Nonetheless, these T cells have lost their ability to proliferate, possibly also explaining the reduced T cell proliferation seen. Since IFN-γ-induced NO production with concurrent NO-induced DC and T cell apoptosis may also characterise human DCs, the NO content in the supernatants of Mo-DCs and in Mo-DC : T cell co-cultures was investigated. T cell proliferation assays in the presence of the NOS inhibitor Nomega-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME) have also been performed to further unravel the possible involvement of NO in T cell proliferation driven by IFN-γ treated Mo-DCs.

The members of the TGF-β family (TGF-β1, 2 and 3) are multifunctional secreted peptides that act on a wide range of cell types, including T cells, B cells, DCs, neutrophils and epithelial cells, by controlling such diverse functions as cell growth, differentiation and state of activation. However, it is the immunosuppressive function of TGF-β that has caught much attention in recent literature. For example, it has been shown that DC-mediated autologous and allogeneic T cell proliferation is inhibited in the presence of rheumatoid synovial fluid (SF) [341]. Immunosuppression was ascribed to the presence of TGF-β in the SF as T cell proliferation was restored in the additional presence of a neutralising anti-TGF-β mAb. Negative regulation of inflammatory responses has further been connected to TGF-β *in vivo*, as transgenic mice expressing an intracellular antagonist of TGF-β signalling, show enhanced airway inflammation and reactivity [342]. TGF-β is produced by most

leukocyte lineages but is perhaps most commonly associated with the phenotype of T regulatory cells[343]. Human CD34-derived DCs have been shown to express mRNA for TGF-β [32]. To explore the possibility that TGF-β may be involved in impairing T cell proliferation driven by IFN-γ treated Mo-DCs, the presence of TGF-β1 in Mo-DC cultures and Mo-DC : T cell co-cultures was investigated.

Experiments investigating the presence of TGF-β and NO are presented in section 5.3.5.

5.2 Materials and methods

5.2.1 Subjects

24 non-atopic, non-asthmatic normal subjects (12 female, 12 male) took part in this study.

5.2.2 Effects of TNF-α and IFN-γ on monocyte-derived dendritic cell and T cell phenotype

To assess the effects of TNF-α and IFN-γ on the phenotype of Mo-DCs, 8 day old Mo-DCs, exposed to TNF-α and IFN-γ for varying lengths of time, were harvested and stained for surface expression of CD1a, CD14, CD95 and HLA-DR as described (2.2.5.2).

To assess the effects of TNF-α and IFN-γ treated Mo-DCs on the phenotype of T cells in Mo-DC : T cell co-cultures, 8 day old Mo-DCs, exposed to TNF-α and IFN-γ for 48 hours, were harvested, irradiated and put back into culture with allogeneic T cells enriched for CD45RA expression as described (2.2.4.3). Following 5 days incubation the cells were harvested and stained for surface expression of CD3, CD5, CD4, CD8, CD45RA, CD45RO, CD95 and HLA-DR as described (2.2.5.2).

During flow cytometric analysis dead cells were identified using 7-AAD and were excluded from the events collected (2.2.5.4). A total of 5 000 events were acquired.

The level of $CD95^+$ Mo-DCs was assessed by analysing both percentage labelled cells on a gated cell population and the geometric MFI on the whole cell population. The resulting percentage labelled cells or MFI value for a specific Mo-DC treatment was used to calculating the percentage change relative to untreated control Mo-DCs according to the following equation;

$$((\text{Treated Mo-DC \% or MFI value} / \text{Control Mo-DC \% or MFI value}) - 1) \times 100$$

The level of $CD4^+$, $CD8^+$, $CD45RA^+$, $CD45RO^+$, $CD95^+$ and $HLA\text{-}DR^+$ T cells in Mo-DC : T cell co-cultures was assessed by analysing percentage labelled cells on a gated T cell population expressing CD5 or CD3. The resulting percentage labelled T cells was used for calculating the percentage change relative to the starting T cell population on day 0

according to the following equation;

((T cell sample % value on day 5 / T cell sample % value on day 0) -1) × 100

5.2.3 Identification of apoptotic cells using annexin-V staining

In all culture systems described below, early and late apoptotic cells, live and dead cells were identified according to their surface binding of annexin-V and permeability for PI as described (5.1.2.2). Dead cells were not included in the analysis of apoptotic cells.

5.2.3.1 Identification of apoptotic cells in monocyte-derived dendritic cell cultures

For analysis of apoptotic cells in Mo-DC cultures on day 8, cells were stained using the annexin-V staining method as described (2.2.7.1). In brief, cultured cells were harvested and washed in PBS before they were incubated in biotinylated annexin-V in annexin-V binding buffer for 15 minutes at room temperature in the dark. Following two washes in annexin-V binding buffer the cells were incubated in FITC-conjugated streptavidin in annexin-V buffer for 15 minutes at RT in the dark. Following two washes in annexin-V binding buffer the cells were resuspended in FACS buffer, transferred to FACS tubes, and cells were analysed by flow cytometry within two hours (2.2.7.2).

5.2.3.2 Identification of apoptotic cells in monocyte-derived dendritic cell cultures on day 8 plus 1 day with or without FasL

For analysis of apoptotic cells in Mo-DC cultures on day 8 plus 1 day, Mo-DCs were harvested on day 8 and washed twice in RPMI. 1.5×10^5 Mo-DCs were resuspended in 150 µL complete RPMI supplemented with 1 000 U/mL rhIL-4 and 35 ng/mL rhGM-CSF with or without 500 ng/mL rh soluble *SUPER*Fas Ligand (sFasL) (Alexis Corporation, Nottingham, UK). The cells were put back into the wells of a 48-well plate and following 24 hours incubation at 37 °C, 5% CO_2 the cells were harvested and stained using the annexin-V staining method described (2.2.7 and 5.2.3.1).

5.2.3.3 Identification of apoptotic cells in monocyte-derived dendritic cell: T cell co-cultures

For analysis of apoptotic cells in Mo-DC : T cell co-cultures, cultures of irradiated Mo-DCs and T cells were set up as described (2.2.4.3). Following 5 days incubation the cells were harvested and stained using the annexin V staining method described (2.2.7).

5.2.4 T cell proliferation assay in the presence of blocking anti-CD95 mAb or L-NAME

T cell proliferation assays were set-up in 96-well plates as described (2.2.4.1) with or without the additional presence of 1) 0.01 mM or 1.0 mM L-NAME (Sigma), or 2) 1 μg/mL blocking anti-CD95 mAb (IgG1, clone ZB4, Beckman, High Wycombe, UK) or 3) 1 μg/mL purified mouse IgG1 κ isotype control (PharMingen International, Becton Dickinson, Oxford, UK). In brief, 1.0×10^5 T cells were co-cultured with serially diluted irradiated Mo-DCs in triplicates for 5 days, then pulsed with H^3-thymidine for a further 6 hours before thymidine incorporation was stopped by harvesting the plates onto glass fibre filters. T cell proliferation was quantified by measuring H^3-thymidine incorporation as counts per minute (CPM) following counting of the glass fibre filters in a plate reader.

Calculation of relative proliferation index was performed as described (4.4.1.4).

5.2.5 Co-culture of CFSE stained monocyte-derived dendritic cells and allogeneic T cells

On day 8, treated or untreated Mo-DCs were stained with CFSE and put back into culture with allogeneic T cells (2.2.4.2). Daily, over a period of 5 days one co-culture for each condition was harvested, live cells were counted based on exclusion of trypan blue and the percentage of viable CFSE-labelled Mo-DCs estimated by flow cytometry (2.2.8).

During flow cytometric analysis dead cells were identified using PI and were excluded from the events collected (2.2.8). A total of 5 000 events were acquired.

A viability index has been calculated to describe the viability of TNF-α and IFN-γ treated Mo-DCs. The viability index shows the magnitude of the difference between viable TNF-α or IFN-γ treated Mo-DCs and control Mo-DCs at a given time point. The viability index is based on the number of viable Mo-DCs, calculated using the total number of viable

cells and the percentage of viable $CFSE^+$ Mo-DCs, as determined by exclusion of trypan blue and flow cytometry, respectively. The viability index was calculated according to the following equation;

Viability index = (number of viable TNF-α or IFN-γ Mo-DCs - number of viable control Mo-DCs) / number of viable control Mo-DCs

The numbers of viable cells used in this equation were taken from the time point at which the biggest difference between viable control and TNF-α or IFN-γ Mo-DCs was observed.

5.2.6 Measurement of nitric oxide production

For analysis of NO content in Mo-DC supernatants, 6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 48 hours then harvested, counted and the total volume of supernatant measured. The supernatants were stored at -40°C pending analysis of NO content.

For analysis of NO content in Mo-DC : T cell co-cultures, cell cultures were set up as described (2.2.4.3). In brief, 6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 48 hours then harvested, irradiated and put back into culture with allogeneic T cells enriched for CD45RA expression. Following 5 days incubation the cells were harvested, counted and the total volume of supernatant measured and saved for analysis of NO content.

The NO content in the stored supernatants was measured in duplicates using a nitric oxide analyser as described (2.2.10). The NO concentration in the undiluted supernatants was calculated by relating the emission (mV) generated by the chemiluminescent reaction of NO with O_3, to a standard curve. The sensitivity for measurement of NO in liquid samples was approximately 1 pmol.

5.2.7 Measurement of TGF-β1 production by ELISA

For analysis of TGF-β1 content in Mo-DC supernatants, 6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 24 hours when 500 μL supernatant was removed from the Mo-DC cultures and stored at -40°C pending analysis of TGF-β1 content by

ELISA. On day 8 the Mo-DCs were harvested, counted and the total volume of supernatant measured.

For analysis of TGF-β1 content in Mo-DC : T cell co-cultures, cell cultures were set up as described (2.2.4.3). In brief, 6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 48 hours then harvested, irradiated and put back into culture with allogeneic T cells enriched for CD45RA expression. Following 5 days incubation the cells were harvested, counted and the total volume of supernatant measured and saved for analysis of TGF-β1 by ELISA.

TGF-β content in Mo-DC supernatants was measured by ELISA according to the supplier's (R&D Systems) instructions in duplicates (2.2.9). Supernatants for measuring TGF-β1 content were diluted 1:5 in the appropriate reagent buffer to fall within the range of the standard curve (31.25 pg/mL – 2 000 pg/mL). The TGF-β1 ELISA was design for use with streptavidin-conjugated HRP with TMB substrate and the plates were subsequently read at 450 nm on an ELISA plate reader.

The TGF-β1 concentration in the supernatants was calculated by relating the mean absorbance value of the duplicates to a standard curve. The TGF-β1 content has been presented as pg / 5.0×10^5 Mo-DCs by accounting for the 1:5 dilution factor and by adjusting the supernatant concentration to the estimated volume of medium on day 7 and the final number of cells present on day 8.

5.2.8 Statistical analysis

Using SPSS 10.0 for Windows, the non-parametric Wilcoxon test was used where possible ($n \geq 5$) to compare two related samples. A p value of less than 0.05 was considered statistically significant.

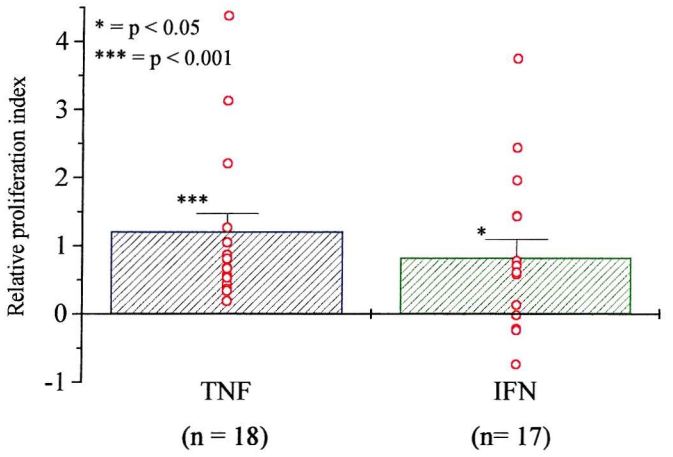
5.3 Results

5.3.1 Effects of TNF-α and IFN-γ on monocyte-derived dendritic cell driven T cell proliferation

6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 48 hours, then harvested, irradiated and incubated with allogeneic T cells for 5 days before T cell proliferation was assessed by H^3-thymidine incorporation.

Figure 5.1a shows allogeneic T cell proliferation driven by TNF-α and IFN-γ treated Mo-DCs presented as a relative proliferation index (RPI). It is evident that both TNF-α and IFN-γ exposed Mo-DCs significantly enhanced T cell proliferation although not to the same extent (TNF-α; RPI = 1.2 ± 0.3, n = 18, $p < 0.001$ and IFN-γ; RPI = 0.8 ± 0.3, n = 17, $p < 0.05$). In Figure 5.1b, for each subject the RPI obtained from TNF-α treated Mo-DCs has been paired with the RPI obtained from IFN-γ treated Mo-DCs to show the trend for individual Mo-DC preparations. It is evident that in 11 out of 17 cases TNF-α treated Mo-DCs were better at driving T cell proliferation than IFN-γ treated Mo-DCs.

a) T cell proliferation driven by TNF-α and IFN-γ treated Mo-DCs



b) The connection between the TNF-α and IFN-γ derived RPI for each subject

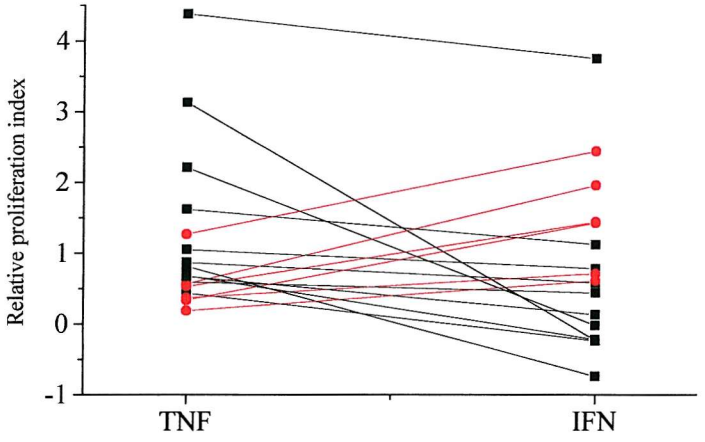


Figure 5.1 Effects of TNF-α and IFN-γ on Mo-DC driven allogeneic T cell proliferation

6 day old Mo-DCs from normal subjects were exposed to TNF-α or IFN-γ for a further 48 hours, then harvested, irradiated and incubated with allogeneic T cells enriched for CD45RA expression. Cultures of 1.0×10^5 T cells and varying numbers of Mo-DCs per well were set up in triplicates. After 5 days, T cell proliferation was assessed by H^3-thymidine incorporation over 6 hours. In a) each individual RPI value is presented as an open red circle and the mean of all RPI values for TNF-α and IFN-γ is shown as a column with error bars (SEM). In b), for 17 normal subjects, a line to indicate their relation has joined theTNF-α and IFN-γ RPI. A black line indicates a relation where TNF-α is inducing more T cell proliferation than IFN-γ, whereas a red line indicates the reverse.

* = $p < 0.05$ and *** = $p < 0.001$ compared to control Mo-DCs.

5.3.2 Direct effects of IFN-γ - dose, time of exposure and kinetics of T cell proliferation

6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 48 hours, then harvested, irradiated and incubated with allogeneic T cells for 5 days before T cell proliferation was assessed by H^3-thymidine incorporation.

To investigate if super-optimal or sub-optimal levels of IFN-γ were responsible for the impaired function of IFN-γ treated Mo-DCs, the effects on T cell proliferation of Mo-DCs treated with different concentrations of both TNF-α and IFN-γ, were studied. A trend for reduced T cell proliferation in response to Mo-DCs treated with 10- or 100-fold less TNF-α or IFN-γ was observed (data not shown). The effect of exposing Mo-DCs to TNF-α or IFN-γ for a shorter period of time was also investigated. A trend for reduced T cell proliferation in response to Mo-DCs treated with TNF-α or IFN-γ for 24 hour was also observed (data not shown). Both these observations are consistent with a less mature phenotype of these Mo-DCs (data not shown).

The effects of different concentrations of TNF-α and IFN-γ were also explored in an attempt to standardise the protocol. The purpose of this was to find one concentration of TNF-α that resulted in a similar Mo-DC phenotype as one concentration of IFN-γ. This would eliminate possible effects of differences in Mo-DCs phenotype when investigating the effects of TNF-α and IFN-γ on Mo-DC driven T cell proliferation. However, no one concentration of TNF-α induced the same Mo-DC phenotype as one concentration of IFN-γ (data not shown). For example, at concentrations of TNF-α and IFN-γ where surface expression of HLA-DR was similar, IFN-γ treated Mo-DCs expressed higher levels of CD86 and lower levels of CD23 than TNF-α treated Mo-DCs (data not shown). The original concentrations of TNF-α and IFN-γ, determined as described in Chapter 4 (4.1.2.8 and 4.1.3), were therefore kept for all subsequent experiments.

Figure 5.2 shows a representative example of allogeneic T cell proliferation in response to control, TNF-α and IFN-γ treated Mo-DCs for the last 4 days of the 5 day proliferation assay. TNF-α treated Mo-DCs were most potent at driving T cell proliferation in this subject. By 3 days in culture they already showed a marked increase above the levels of proliferation obtained with control and IFN-γ treated Mo-DCs. TNF-α treated Mo-DCs continued to be superior at driving T cell proliferation throughout the time course. Control Mo-DCs supported more T cell proliferation than IFN-γ treated Mo-DCs throughout the

time course in this subject. Although IFN-γ treated Mo-DCs were shown to be better at driving T cell proliferation than TNF-α treated Mo-DCs in one of the other two subjects studied (data not shown), the order of superiority was consistent throughout all time courses.

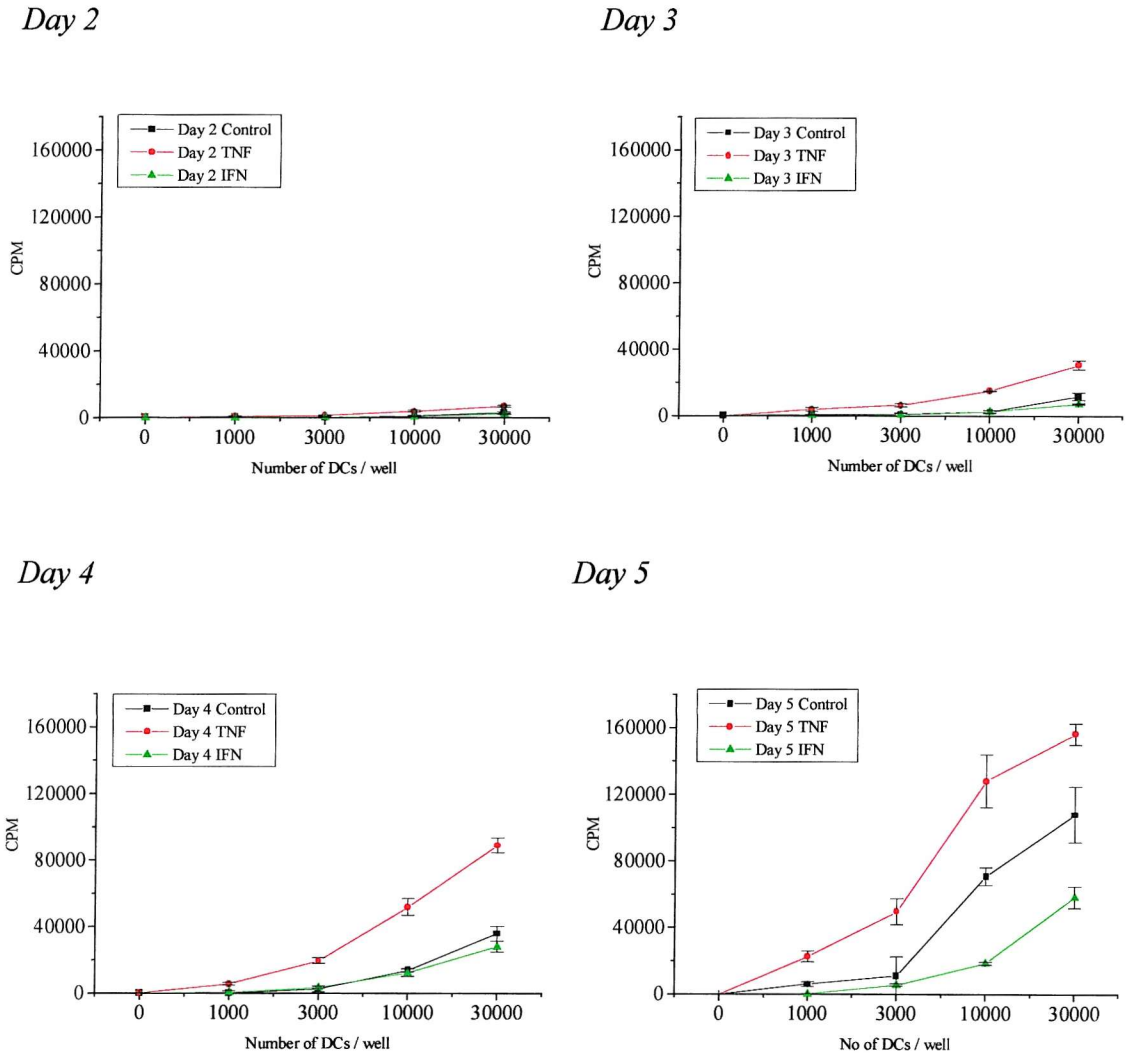


Figure 5.2 Kinetics of T cell proliferation in response to control, TNF-α and IFN-γ treated Mo-DCs

6 day old Mo-DCs from a representative subject were left untreated (control) or exposed to TNF-α or IFN-γ for a further 48 hours then harvested, washed and irradiated. Cultures of 1.0×10^5 allogeneic T cells, enriched for CD45RA expression, and varying numbers of Mo-DCs per well were set up in triplicates. Following 2, 3, 4 and 5 days of incubations at 37 °C, 5 % CO_2, T cell proliferation was assessed by H^3 thymidine incorporation. T cell proliferation is presented as CPM against an increasing number of control, TNF-α or IFN-γ treated Mo-DCs.

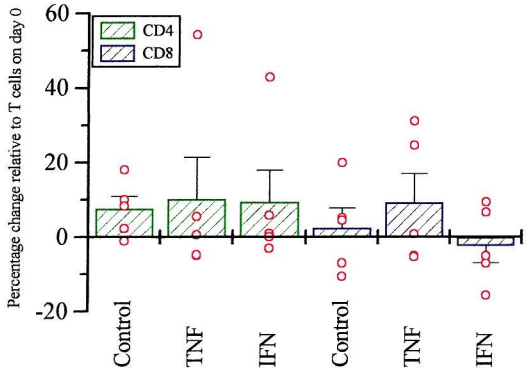
5.3.3 Effects of TNF-α and IFN-γ treated monocyte-derived dendritic cells on T cell activation and viability

Surface expression of CD4, CD8, CD45RA, CD45RO and CD95 on allogeneic T cells following 5 days incubation with control, TNF-α or IFN-γ stimulated Mo-DCs were analysed by flow cytometry gating on $CD5^+$or $CD3^+$ cells.

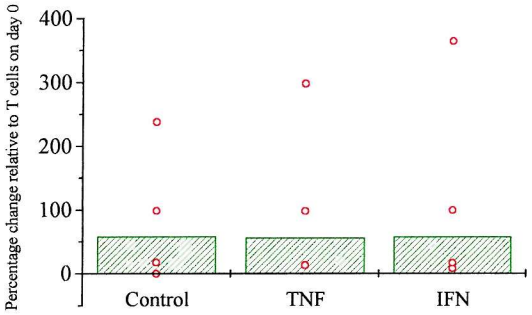
Figure 5.3a -c show the effects of control, TNF-α and IFN-γ exposed Mo-DCs on T cell CD4, CD8, CD95, CD45RA and CD45RO expression presented as percentage change relative to the starting T cell population on day 0. Although exposure to Mo-DCs did change the phenotype of the responding T cells, no difference were found in the phenotype of the T cells co-cultured with control Mo-DCs or Mo-DCs treated with TNF-α or IFN-γ. In 5 donors studied, neither control, TNF-α nor IFN-γ exposed Mo-DCs significantly changed the percentage of $CD4^+$ or $CD8^+$ T cells in the Mo-DC : T cell co-cultures (Figure 5.3a). A general trend for increased percentage $CD95^+$ T cells was however observed for all three Mo-DC treatments (Figure 5.3 b). A significant decrease ($p < 0.05$) in $CD45RA^+$ T cells and significant increase ($p < 0.05$) in $CD45RO^+$ T cells were also observed in response to all three Mo-DC treatments (Figure 5.3c), reflecting the state of activation of the T cell population following 5 days in culture with Mo-DCs.

After 5 days, cells from Mo-DC : T cell co-cultures were stained with biotinylated annexin V (detected with SA-FITC) and PI. Using this approach cells that were live, early apoptotic, late apoptotic and dead could be identified (Figure 5.4a) based on the properties of their cell membrane. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the membrane[344]. PS is thereby exposed for binding by phospholipid-binding proteins such as annexin-V. As apoptosis proceeds the cell membrane becomes permeable to PI that thereby may be used for distinguishing early from late apoptotic cells. Hence, early apoptotic cells show surface staining for annexin-V but are impermeable to PI (the red square in Figure 5.4a), whereas late apoptotic cells show surface staining for annexin-V and are PI permeable (the green square in Figure 5.4a). Live cells with intact cell membrane show no surface staining for annexin-V and exclude PI (the blue square in Figure 5.4a). Dead cells have a highly permeable cell membrane resulting in high uptake of PI (the yellow square in Figure 5.4a). Compared to control Mo-DCs, both TNF-α and IFN-γ treated Mo-DCs concurrently reduced the total cell viability and early and late apoptosis in the co-cultures (Figure 5.4b), however these changes were not supported by statistical analysis.

a) T cell CD4 and CD8 expression



b) T cell CD95 expression



c) T cell CD45RA and CD45RO expression

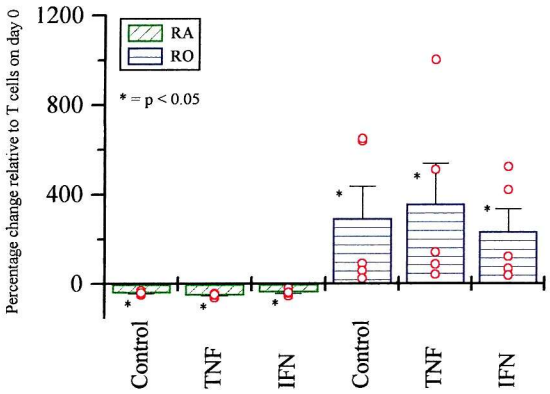
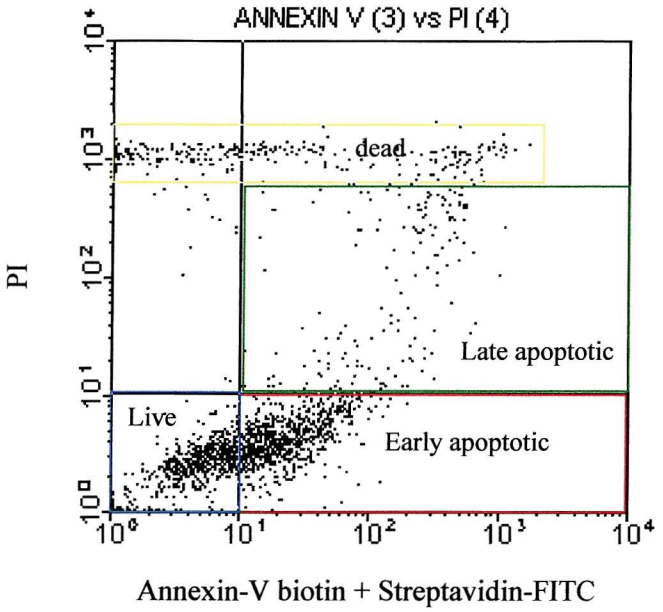


Figure 5.3 T cell phenotype following 5 days in culture with Mo-DCs

6 day old Mo-DCs were left untreated (control) or exposed to TNF-α or IFN-γ for a further 48 hours then harvested, washed and irradiated. Cultures of allogeneic T cells, enriched for CD45RA expression and Mo-DCs were set up. Following 5 days incubation the phenotype of the T cells was analysed by flow cytometry. 5 000 events per sample were collected from a live, $CD5^+$ or $CD3^+$ population. The effects of control, TNF-α and IFN-γ treated Mo-DCs on T cell expression of a) CD4 and CD8 (n = 5), b) CD95 (n = 4) and c) CD45RA and CD45RO (n = 5) are presented as percentage change relative to the phenotype of the starting T cell population on day 0. The percentage change values are based on the percentage of cells positively stained for each marker. Each individual value (percentage change) is presented as an open red circle. In a) and c) the mean of all values is shown as a column with error bars (SEM). In b) the column represents the median. * = $p < 0.05$ compared to starting T cell population on day 0.

a) Example of annexin V and PI staining



b) Apoptosis in Mo-DC : T cell co-cultures

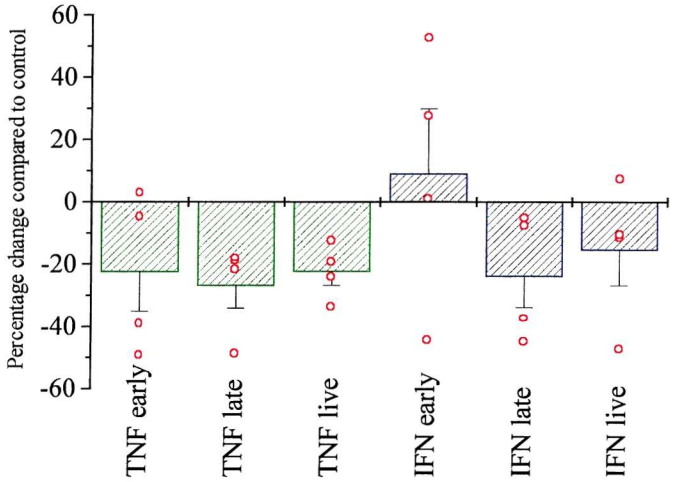


Figure 5.4 Apoptosis in Mo-DC : T cell co-cultures

6 day old Mo-DCs were left untreated (control) or exposed to TNF-α or IFN-γ for a further 48 hours then harvested, washed and irradiated. Cultures of allogeneic T cells, enriched for CD45RA expression and Mo-DCs were set up. Following 5 days incubation apoptotic cells in the Mo-DC : T cell co-cultures were analysed by flow cytometry. In a) a representative example of cells labelled with annexin-V biotin + streptavidin-FITC and PI is presented in a flow cytometry dotplot. Coloured boxes are distinguishing early apoptotic (red), late apoptotic (green), dead (yellow) and live cells (blue). In b) the effects of TNF-α and IFN-γ exposed Mo-DCs on apoptosis in the co-cultures (n = 4) is presented as percentage change relative to control untreated Mo-DC. Each individual value (percentage change) is presented as an open red circle and the mean of all values as a column with error bars (SEM). The percentage change is based on the percentage early, late or live cells.

5.3.4 Effects of TNF-α and IFN-γ on monocyte-derived dendritic cell viability

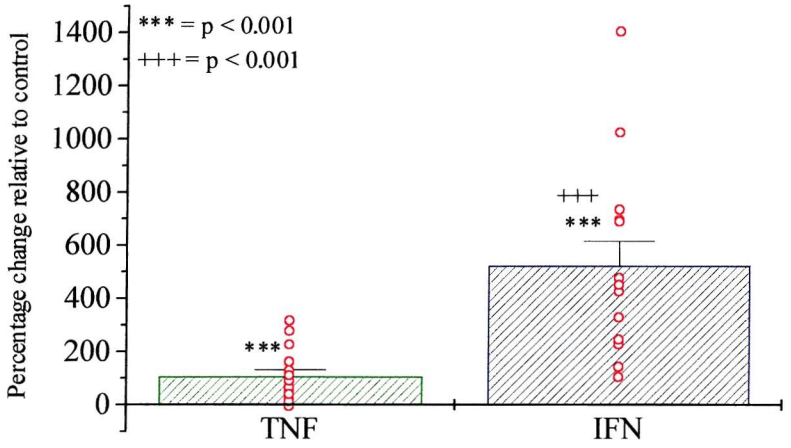
5.3.4.1 Monocyte-derived dendritic cell surface expression of CD95

8 day old Mo-DCs, treated with TNF-α or IFN-γ for a varying length of time, were harvested and stained for surface expression of CD95.

Based on the percentage $CD95^+$ Mo-DCs, Figure 5.5a shows the effects of TNF-α and IFN-γ treatment on Mo-DC surface expression of CD95, presented as percentage change compared to control Mo-DCs. Compared to control Mo-DCs the percentage $CD95^+$ Mo-DCs increased significantly following 48 hours incubation with either TNF-α (105 % ± 26, $p < 0.001$, $n = 15$) or IFN-γ (522 % ± 92, $p < 0.001$, $n = 15$). Furthermore, in IFN-γ treated Mo-DC cultures a significantly higher proportion of the cells were $CD95^+$ compared to that found in TNF-α treated Mo-DC cultures ($p < 0.001$). Also the intensity (MFI) of CD95 on TNF-α and IFN-γ Mo-DCs was significantly increased (both $p < 0.001$, $n = 15$) compared to control Mo- DCs at this time point (data not shown).

Figure 5.5b shows the kinetics of CD95 surface expression on Mo-DCs in response to TNF-α or IFN-γ. The rate of induction of CD95 surface expression differed in response to TNF-α or IFN-γ. The percentage of $CD95^+$ Mo-DCs was already significantly increased ($p < 0.05$) following 12 hours incubation with IFN-γ but not following 12 hours incubation with TNF-α. Surface expression of CD95 on IFN-γ treated Mo-DCs increased further over the following 36 hours, reaching an average level of 83 % ± 3 ($n = 15$) $CD95^+$ cells following 48 hours incubation with IFN-γ. On the contrary, TNF-α treated Mo-DCs appeared to respond by only slightly increasing CD95 expression beyond that observed following 12 hours. Consequently, the difference in CD95 surface expression between TNF-α and IFN-γ treated Mo-DCs is significant at both the 24 ($p < 0.05$) and 48 hour ($p < 0.001$) time points.

a) CD95 expression at 48 hours



b) Kinetics of CD95 expression

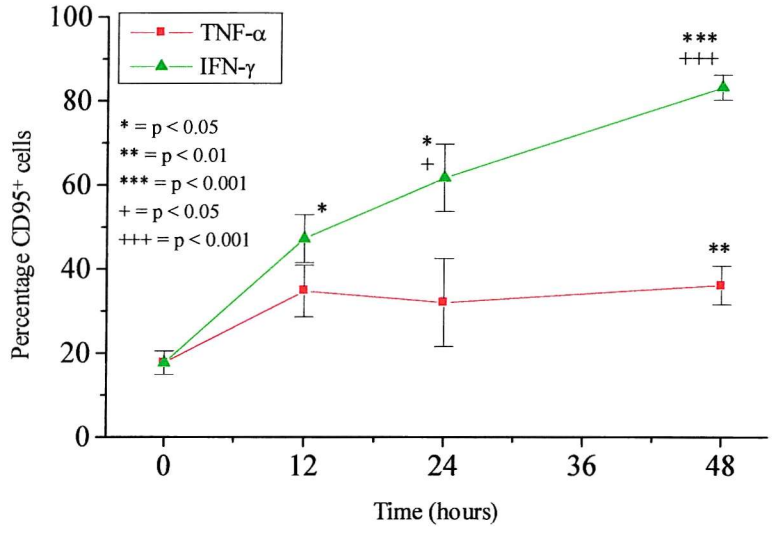


Figure 5.5 CD95 expression on Mo-DCs

8 day old Mo-DCs exposed to TNF-α or IFN-γ for 12, 24 or 48 hours were harvested and stained for surface expression of CD95. In a) the effects of TNF-α and IFN-γ following 48 hours incubation are presented as percentage change relative to control based on the percentage of cells positively stained for CD95. Each individual value (percentage change) is presented as an open red circle and the mean of all values is shown as a column with error bars (SEM). In b) the kinetics of CD95 expression in response to TNF-α and IFN-γ is presented as percentage $CD95^+$ cells at 12, 24, or 48 hours. For both TNF-α and IFN-γ, n = 6 at 0, 24 and 48 hours and n = 4 at 12 hours. The mean of all subjects studied is presented as a triangle (IFN-γ) or square (TNF-α) with error bars (SEM) and a line to indicate the trend joins each time point.

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to control Mo-DCs.

+ = $p < 0.05$, +++ = $p < 0.001$ compared to TNF-α Mo-DCs.

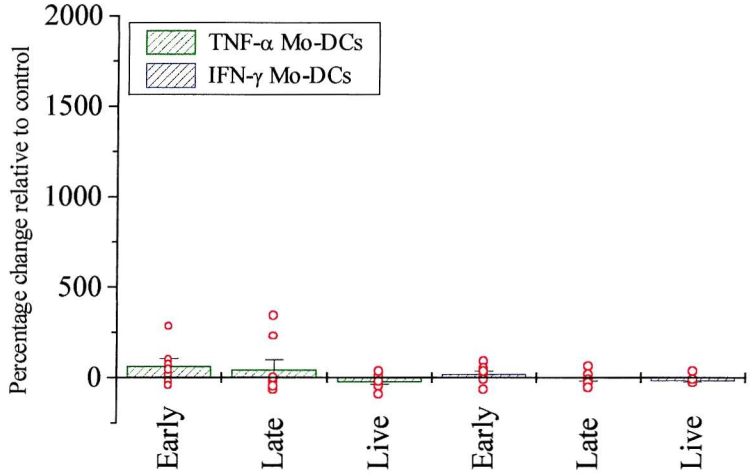
5.3.4.2 Monocyte-derived dendritic cell apoptosis

8 day old Mo-DCs, treated with TNF-α or IFN-γ for varying lengths of time, were harvested and either analysed immediately for apoptotic cells by staining with annexin-V, or put back into culture for another 24 hours then harvested and analysed for apoptotic cells by staining with annexin-V.

Figures 5.6a and b show the effects of TNF-α and IFN-γ on apoptosis in the Mo-DC population on day 8 and day 8+ 1, respectively. On day 8, no significant change in cell viability or apoptosis was observed in Mo-DC populations treated with either TNF-α or IFN-γ as compared to control Mo-DC populations. Further, the level of cell viability and apoptosis was not significantly different between TNF-α or IFN-γ treated Mo-DCs. However, when these 8 day old Mo-DCs were put back into culture for a further 24 hours in the absence of TNF-α or IFN-γ, the percentage late apoptotic cells increased significantly in TNF-α and IFN-γ treated Mo-DC populations compared to control Mo-DC populations ($p < 0.05$) (Figure 5.6b). Although not statistically significant, a trend was observed for IFN-γ treatment to induce more late apoptosis and cell death than TNF-α. Control Mo-DCs showed similar levels of early and late apoptotic Mo-DCs as well as live cells on day 8 + 1 compared to that found on day 8 (data not shown).

The extent to which TNF-α and IFN-γ induced apoptosis in Mo-DC cultures on day 8 + 1 was further found to be dependent on the length of exposure to TNF-α and IFN-γ leading up to day 8 (data not shown). Mo-DC cultures exposed to TNF-α or IFN-γ for 12 or 24 hours showed a lower percentage of late apoptotic cells and a higher percentage of early apoptotic cells and live cells compared to Mo-DC cultures exposed to TNF-α and IFN-γ for 48 hours.

a) Mo-DC apoptosis on day 8



b) Mo-DC apoptosis on day 8 + 1 day

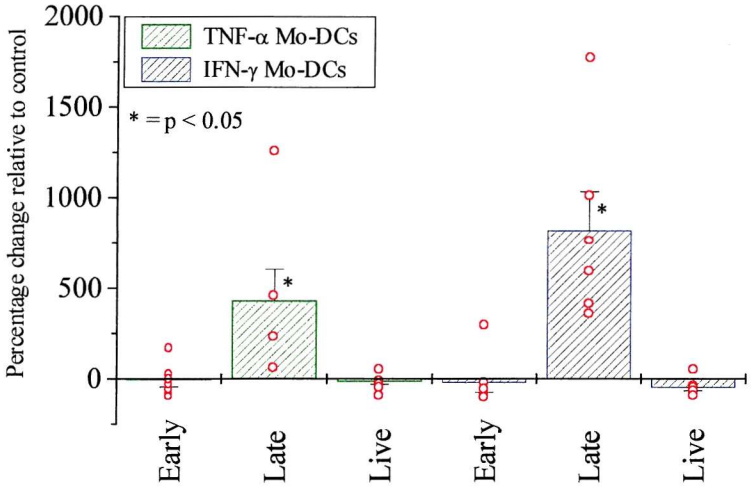


Figure 5.6 Effects of TNF-α and IFN-γ on Mo-DC apoptosis

6 day old Mo-DCs were left untreated (control) or exposed to TNF-α or IFN-γ for a further 48 hours. On day 8 the cells were harvested and either a) analysed for apoptosis by flow cytometry (n = 8) b) or put back into culture for another 24 hours and then harvested and analysed for apoptosis (n = 7). Based on the percentage early, late or live cells, the effects of TNF-α and IFN-γ on Mo-DC apoptosis are presented as percentage change relative to control untreated Mo-DCs. Each individual value (percentage change) is presented as an open red circle and the mean of all values is shown as a column with error bars (SEM).

* = $p < 0.05$ compared to control Mo-DCs.

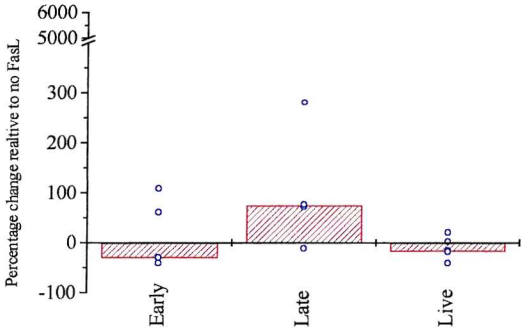
5.3.4.3 Effects of soluble FasL on monocyte-derived dendritic cell apoptosis

8 day old Mo-DCs, treated with TNF-α or IFN-γ for 48 hours, were harvested and washed, then put back into culture for another 24 hours in the presence or absence of sFasL. On day 8 + 1 the Mo-DCs were harvested and analysed for apoptotic cells by staining with annexin-V.

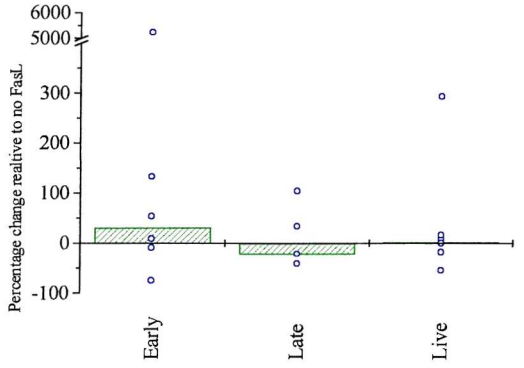
Figures 5.7a - c show the effects of sFasL on apoptosis in cultures of control, TNF-α and IFN-γ treated Mo-DCs, presented as percentage change relative to no FasL. The Fas-sensitive cell line HUT78 was exposed to sFasL to verify the potency of sFasL as an apoptosis-inducing agent. Within 24 hours sFasL was shown to kill virtually all HUT78 cells (data not shown). Only a proportion of control Mo-DCs were shown to undergo Fas-mediated apoptosis as a trend for increased late apoptotic cells was observed following treatment with sFasL compared to no sFasL (Figure 5.7a). Further, sFasL had no effect on TNF-α treated Mo-DCs (Figure 5.7b), whereas sFasL significantly reduced the percentage late apoptotic cells in IFN-γ treated Mo-DC populations ($p < 0.05$, $n = 5$) (Figure 5.7c). Although this effect of sFasL on IFN-γ treated Mo-DCs is very small, this may suggest that sFasL reverses the apoptotic process of IFN-γ treated Mo-DCs as a concurrent trend for an increased percentage of early apoptotic cells was seen in these cultures.

Due to reports indicating anti-apoptotic properties of soluble CD95 (sCD95)[345,346], the sCD95 content in Mo-DCs supernatants was investigated using a commercial ELISA kit. However, no sCD95 could be detected in Mo-DC supernatants regardless of the Mo-DC treatment (data not shown).

a) Control Mo-DCs



b) TNF-α treated Mo-DCs



c) IFN-γ treated Mo-DCs

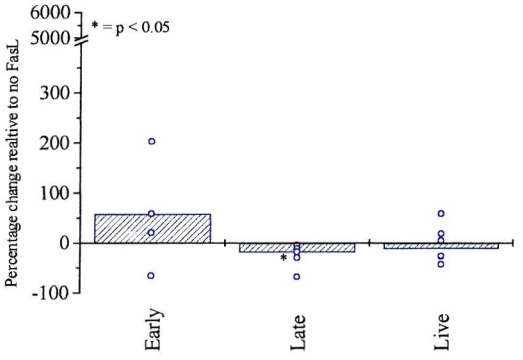


Figure 5.7 Effects of sFasL on Mo-DC apoptosis

8 day old Mo-DCs stimulated with TNF-α or IFN-γ for 48 hours were harvested and washed then put back into culture for another 24 hours with or without sFasL. On day 8 + 1 day apoptotic cells were analysed by flow cytometry. The effects of sFasL on a) control, b) TNF-α and c) IFN-γ treated Mo-DCs is presented as percentage change related to no sFasL treatment. Each individual value (percentage change) is presented as an open blue circle with the median of all values shown as a column. The percentage change is based on the percentage early, late or live cells.

* = $p < 0.05$ relative to no sFasL treatment.

5.3.4.4 Survival of monocyte-derived dendritic cells in culture with T cells

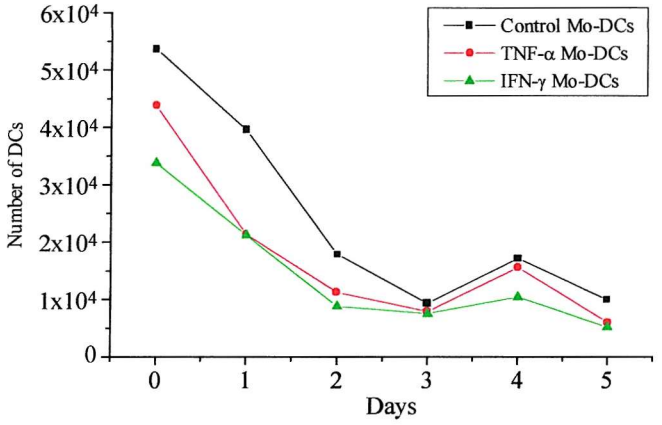
8 day old Mo-DCs treated with TNF-α or IFN-γ for 48 hours were harvested, stained with CFSE and put back into culture with allogeneic T cells in the presence or absence of blocking anti-CD95 mAb. Over a period of 5 days the Mo-DC : T cell co-cultures were harvested, total cell number counted and viable CFSE labelled cells analysed by flow cytometry.

Figure 5.8 a) and b) show a representative example of the number of viable Mo-DCs and T cells in a Mo-DC : T cell co-culture. Regardless of the treatment of the Mo-DC, the number of Mo-DCs is generally reduced to only a few thousand following 2-3 days in culture with allogeneic T cells. The rate of disappearance of Mo-DCs from the co-culture was independent of Fas-mediated apoptosis as this reduction following 2-3 days was also found in the presence of a blocking anti-CD95 mAb (data not shown). Further, regardless of Mo-DC treatment, the number of T cells in these Mo-DC : T cell co-cultures generally decline over the first 3 days followed by a sharp increase in cell number during the fourth or fifth day of culture. Although not supported by statistical analysis, the number of viable IFN-γ treated Mo-DCs was constantly found to be lower than the number of viable control or TNF-α treated Mo-DCs when analysed a few hours later. This was despite careful counting of cells to assure that an equal number of viable Mo-DCs for each treatment were seeded.

The influence of Fas-mediated apoptosis of Mo-DCs in Mo-DC : T cell co-cultures were investigated by using a blocking anti-CD95 mAb. To verify the effectiveness of the blocking anti-CD95 mAb, a population of HUT78 cells was incubated with sFasL in the presence or absence of the anti-CD95 mAb. In the presence, but not in the absence of the anti-CD95 mAb, most HUT78 cells were viable (data not shown) indicating inhibition of sFasL-induced apoptosis by the blocking anti-CD95 mAb. Figure 5.9 a) and b) show the viability index for TNF-α and IFN-γ treated Mo-DCs in the presence or absence of blocking anti-CD95 mAb. The viability index is comparing the number of viable TNF-α or IFN-γ treated Mo-DCs cultured in the presence or absence of blocking anti-CD95 mAb to the number of viable control Mo-DCs cultured under the same conditions. The number of viable control Mo-DCs was not significantly changed when cultured in the presence of blocking anti-CD95 mAb (data not shown). As the viability index for TNF-α treated Mo-DCs was close to zero either in the presence or absence of anti-CD95 mAb, the survival of TNF-α treated Mo-DCs in culture with allogeneic T cells was equivalent to that of control

Mo-DCs cultured under the same conditions. However, in the absence of anti-CD95 mAb IFN-γ treated Mo-DCs survived significantly less well in culture with allogeneic T cells than control Mo-DCs (viability index -0.6 ± 0.1, $n = 6$, $p < 0.05$). In the presence of anti-CD95 the survival of IFN-γ treated Mo-DCs was enhanced, but not to levels equivalent to that of control Mo-DCs (viability index -0.3 ± 0.2, $n = 5$). Although having slight effects on the number of viable IFN-γ treated Mo-DCs, the presence of blocking anti-CD95 mAb during the 5 day T cell proliferation assay was shown to have no effect on T cell proliferation driven by either control, TNF-α or IFN-γ treated Mo-DCs (data not shown).

a) Number of Mo-DCs



b) Number of T cells

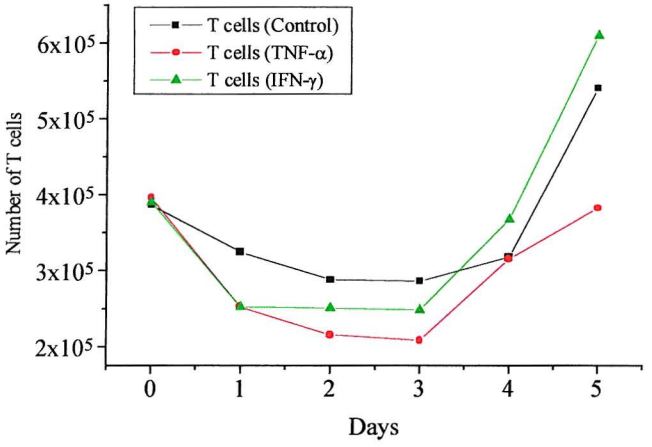
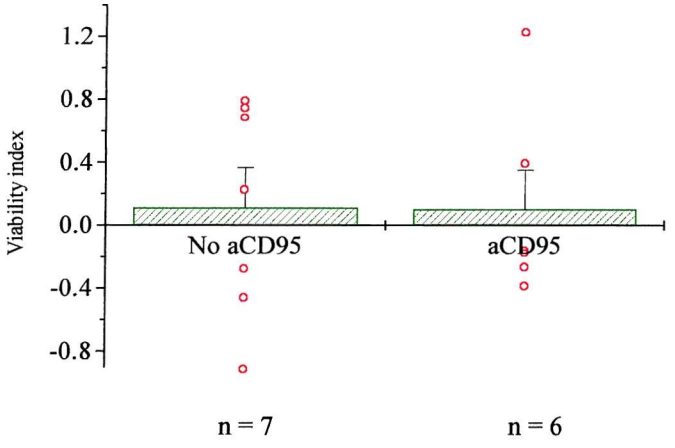


Figure 5.8 Survival of Mo-DCs and T cells in co-culture over a 5 day period

Mo-DCs were left untreated (control) or exposed to TNF-α or IFN-γ for 48 hours then harvested, stained with CFSE and put back into culture with allogeneic T cells. Over a period of 5 days the Mo-DC : T cell co-cultures were harvested, total cell number counted and viable CFSE labelled cells analysed by flow cytometry. One out of seven CFSE-labelled Mo-DC : T cell co-culture is presented. In a), the number of live control, TNF-α and IFN-γ Mo-DCs in the Mo-DC : T cell co-culture is presented. In b), the number of live T cells cultured with control, TNF-α and IFN-γ Mo-DCs is presented.

a) Survival of TNF-α treated Mo-DCs in culture with T cells



b) Survival of IFN-γ treated Mo-DCs in culture with T cells

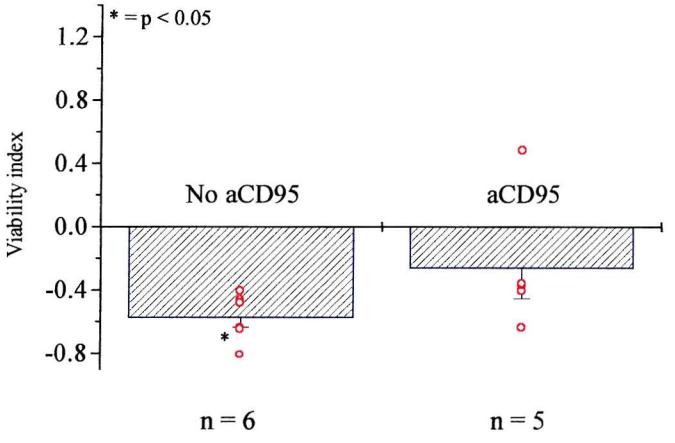


Figure 5.9 Effects of blocking CD95 on Mo-DC survival in culture with T cells

Mo-DCs were left untreated (control) or exposed to TNF-α or IFN-γ for 48 hours then harvested, stained with CFSE and put back into culture with allogeneic T cells. Over a period of 5 days the Mo-DC : T cell co-cultures were harvested, total cell number counted and viable CFSE labelled cells analysed by flow cytometry. The effects of a blocking anti-CD95 mAb on the number of viable a) TNF-α and b) IFN-γ treated Mo-DCs in Mo-DC : T cell co-cultures is presented as viability index. The viability index is relating the number of viable TNF-α or IFN-γ treated Mo-DCs to viable control Mo-DCs. The numbers of viable cells used to calculate the viability index were taken from the time point at which the biggest difference between viable control and TNF-α or IFN-γ Mo-DCs was observed.

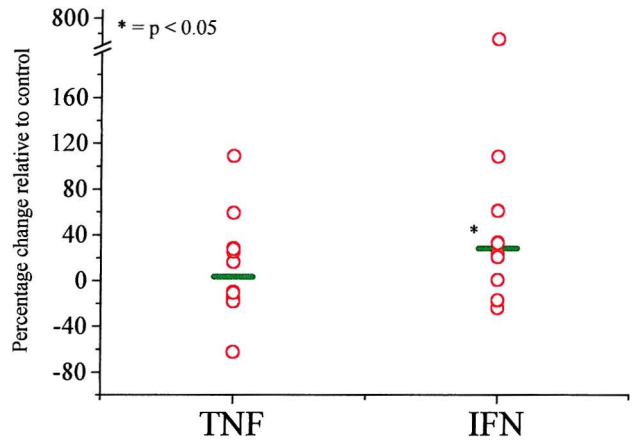
5.3.5 Effects of TNF-α and IFN-γ on the production of soluble factors by monocyte-derived dendritic cells and monocyte-derived dendritic cell : T cell co-cultures

To assess the effects of IFN-γ and TNF-α on NO and TGF-β production in Mo-DC cultures and Mo-DC : T cell co-cultures, supernatants were collected and NO and TGF-β content analysed.

Figures 5.10 a) and b) show NO content in the supernatants of Mo-DC cultures and Mo-DC : T cell co-cultures, respectively. A small but significant increase in NO production was evident in the supernatants of IFN-γ treated Mo-DCs (67.5 % ± 43.2, $p < 0.05$) compared to control Mo-DC (Figure 5.10a). However, no change in NO content was observed in the supernatants from TNF-α treated Mo-DCs (Figure 5.10a) or from TNF-α or IFN-γ treated Mo-DC : T cell co-cultures (Figure 5.10b). The lack of influence of NO in the human Mo-DC driven T cell proliferation assay was further supported by the observation that the NOS inhibitor L-NAME was unable to affect T cell proliferation driven by control, TNF-α or IFN-γ treated Mo-DCs (data not shown).

Figure 5.11 a) and b) show TGF-β1 content in the supernatants of Mo-DC cultures and Mo-DC : T cell co-cultures, respectively. No difference in the amount of TGF-β1 produced by TNF-α and IFN-γ treated Mo-DCs was found (Figure 5.11a). Further, Mo-DCs did not produce significantly more TGF-β1 following TNF-α or IFN-γ stimulation compared to control Mo-DCs. The amount of TGF-β1 produced corresponded to 2.8 ± 0.6 ng/ 5.0×10^5 control Mo-DCs, 3.3 ± 1.3 ng/ 5.0×10^5 TNF-α treated Mo-DCs and 3.7 ± 1.0 pg/ 5.0×10^5 IFN-γ treated Mo-DCs (data not shown). However, in Mo-DC : T cell co-cultures IFN-γ treated Mo-DCs induced significantly increased TGF-β1 production (16.4 % ± 4.2, $p < 0.05$) whereas TNF-α treated Mo-DCs significantly decreased TGF-β1 production (–20.0 % ± 4.7, $p < 0.05$) compared to control Mo-DCs (Figure 5.11b). The amount of TGF-β1 produced corresponded to 275 ± 81 pg/ 5.0×10^5 cells in control Mo-DC : T cell co-cultures, 221 ± 67 pg/ 5.0×10^5 cells in TNF-α Mo-DC : T cell co-cultures and 310 ± 79 pg/ 5.0×10^5 cells in IFN-γ Mo-DC : T cell co-cultures (data not shown). The amount of TGF-β1 produced in TNF-α Mo-DC : T cell co-cultures was significantly different ($p < 0.05$) to that observed in IFN-γ Mo-DC : T cell co-cultures (data not shown).

a) Nitric oxide content in Mo-DC cultures



b) Nitric oxide content in Mo-DC : T cell co-cultures

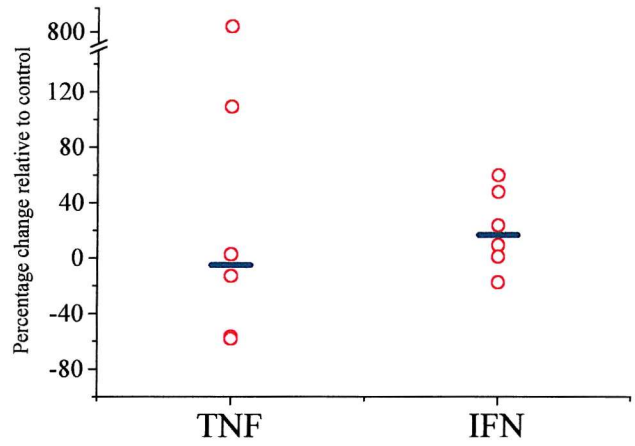
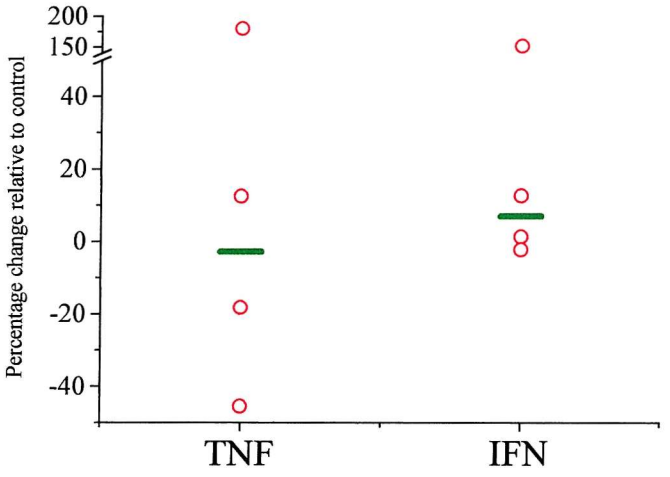


Figure 5.10 Nitric oxide content in culture supernatants

In a) 6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 48 hours followed by collection of the supernatant for measurement of NO by a nitric oxide analyse (n = 12). In b) 6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 48 hours, then harvested, irradiated and put back into culture with allogeneic T cells. Following 5 days incubation the supernatant was collected for measurement of NO by a nitric oxide analyser (n = 6). The effects of TNF-α and IFN-γ are presented as percentage change relative to control Mo-DCs. Each individual value (percentage change) is presented as an open red circle and the median is represented as the horizontal line.

* = $p < 0.05$ compared to control Mo-DCs.

a) TGF-β1 content in Mo-DC cultures



b) TGF-β1 content in Mo-DC : T cell co-cultures

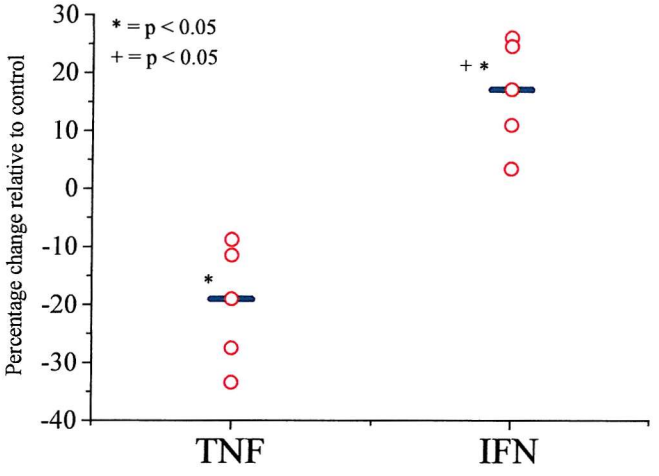


Figure 5.11 TGF-β1 content in culture supernatants

In a) 6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 24 hours followed by collection of a small volume of supernatant for measurement of TGF-β1 content by ELISA (n = 4). In b) 6 day old Mo-DCs were exposed to TNF-α or IFN-γ for a further 48 hours, then harvested, irradiated and put back into culture with allogeneic T cells. Following 5 days incubation the supernatant was collected for measurement of TGF-β1 content by ELISA (n = 5). The effects of TNF-α and IFN-γ are presented as percentage change relative to control Mo-DCs. The median is represented as the horizontal line.

* = $p < 0.05$ compared to control untreated Mo-DCs.

+ = $p < 0.05$ compared to TNF-α Mo-DCs.

5.4 Discussion

Both TNF-α and IFN-γ treated Mo-DCs drive significantly enhanced proliferation of allogeneic T cells. However, the phenotypically more mature IFN-γ treated Mo-DCs are less efficient at driving T cell proliferation than the less mature TNF-α treated Mo-DC. Since differences such as gender, atopic state, viability and activation state of the T cell population at the start of culture as well as the influence of contaminating cell populations have been excluded (data not shown), the experiments described in this chapter aimed to explore possible mechanisms underlying the frequently impaired T cell proliferation in response to IFN-γ treated Mo-DCs.

5.4.1 Direct effects of IFN-γ

Two aspects of the direct effects of IFN-γ in influencing Mo-DC driven T cell proliferation have been investigated (5.3.2) and consequently excluded as possible mechanisms underlying the impairing T cell proliferation. Firstly, Mo-DCs may be functionally debilitated at certain doses or following a certain period of exposure. Altering the dose or the length of treatment with IFN-γ did not enhance T cell proliferation. On the contrary, a reduction in T cell proliferation was observed in accordance with a less mature phenotype observed for these Mo-DCs. Secondly, the kinetics of allogeneic T cell proliferation induced by IFN-γ compared to TNF-α treated Mo-DCs may vary. However, untreated Mo-DCs and Mo-DCs treated with TNF-α or IFN-γ were shown to drive allogeneic T cell proliferation at similar rates, with detectable proliferation appearing at 3 days in all cultures.

It may be considered incorrect to compare the effects of a single dose of TNF-α to a single dose of IFN-γ, but rather, that the effects of TNF-α and IFN-γ on Mo-DC driven T cell proliferation should be studied using concentrations of TNF-α and IFN-γ that result in a similar phenotype. In an attempt to standardise the protocol, it was found that TNF-α and IFN-γ were inducing different patterns of surface molecules irrespective of the dose used. For example, at concentrations of TNF-α and IFN-γ where surface expression of HLA-DR was similar, IFN-γ treated Mo-DCs expressed higher levels of CD86 and lower levels of CD23 than TNF-α treated Mo-DCs (data not shown). Thus TNF-α and IFN-γ treated Mo-DCs may be qualitatively different. This may result from independent signalling pathways leading from the TNF-α and the IFN-γ receptors. Ligation of IFN-γ to the IFN-R results in

activation of the transcription factor Stat1 via the JAK-STAT pathway, leading to transcription of IFN-γ inducible genes [314]. Signalling pathways leading from the TNF-α receptors (TNFR1 are TNFR2) are more complex. In general, activation of the caspase and JUN kinase pathways result in apoptosis whereas activation of signalling pathways via NF-κB result in prolonged survival and transcription of TNF-α inducible genes [347,348]. By engaging different groups of transcription factors different groups of genes are transcribed that may explain the different patterns of surface molecules seen on Mo-DCs following treatment with TNF-α and IFN-γ. The original protocol with the original TNF-α and IFN-γ concentrations was consequently kept for following experiments.

5.4.2 Effects of TNF-α and IFN-γ treated monocyte-derived dendritic cells on T cell phenotype and viability

To determine if T cells were responding differently to TNF-α and IFN-γ treated Mo-DCs, the phenotype and viability of the T cell populations in Mo-DC : T cell co-cultures was investigated (5.3.3). It was shown that T cell populations reached a similar level of expression of CD4, CD8, CD45RA, CD45RO and CD95 and similar level of viability regardless of the treatment of the Mo-DC. Mo-DCs were shown to drive the expansion of both $CD4^+$ and $CD8^+$ T cells as the proportions of both T cell populations were increased in co-culture with control, TNF-α or IFN-γ treated Mo-DCs. This excludes the possibility that IFN-γ treated Mo-DCs may preferentially reduce $CD4^+$ T cell activation, or even deplete $CD4^+$ T cell populations, as has been suggested *in vivo* for IFN-γ treated spleen DCs from NOD mice[329]. As expected, CD45RA surface expression was reduced and CD45RO expression increased in all Mo-DC stimulated T cell populations, reflecting the activation and expansion of T cells from the starting population that was enriched for resting T cell ($CD45RA^+$). Marked upregulation of surface expression of CD95 was seen on T cell populations irrespective of Mo-DC treatment, suggesting that no one Mo-DC treatment leads to preferential susceptibility of the responding T cells to Fas-mediated apoptosis. Accordingly, no difference in early or late apoptosis or in the proportion of viable cells in these co-cultures was detected. Further, no change in T cell proliferation was observed when blocking CD95 using a mAb (data not shown). Surface expression of CD95L on Mo-DCs and T cells could not be investigated to predict their ability to induce Fas-mediated apoptosis, as all commercially available mAbs for human CD95L failed to work in this system.

In summary, of the T cell markers studied no differences in phenotype between the T cells responding to TNF-α or IFN-γ treated Mo-DCs were noted. This may suggest that suppression or impairment of T cell proliferation is an event separate from T cell activation, as assessed by phenotype. Alternatively, these cultures may involve a different less proliferative T cell population that cannot be discriminated by the markers studied here.

5.4.3 Effects of TNF-α and IFN-γ on monocyte-derived dendritic cell viability

Various aspects of Mo-DC viability in response to TNF-α and IFN-γ were investigated (5.3.4) to assess the possibility of any involvement in suppression of Mo-DC driven T cell proliferation. These aspects included; 1) effects on CD95 surface expression, 2) effects on Fas-independent and dependent apoptosis, and 3) effects on Mo-DC survival in culture with allogeneic T cells.

5.4.3.1 Effects on CD95 surface expression

It was shown that surface expression of CD95 was significantly increased on Mo-DCs in response to both TNF-α and IFN-γ (5.3.4.1). This is in contrast to a previous report by Ashany *et.al.* showing no effect on CD95 surface expression by TNF-α or IFN-γ on murine DCs[107]. This lack of correlation may be due to intrinsic differences between human and murine DCs, however since Mo-DCs appear sensitive to changes in IFN-γ concentration (data not shown, see 5.4.1), the difference is perhaps more likely due to variation in the concentration and length of treatment with TNF-α and IFN-γ. Ashany *et. al.* treated bone marrow-derived and spleen-derived DCs for 24 hours with 10 ng/mL TNF-α or 100 U/mL IFN-γ. This corresponds to approximately 7 fold less TNF-α and 100 fold less IFN-γ compared to that used in the current study. Accordingly, when similar concentrations of TNF-α (7.5 ng/mL) and IFN-γ (95 U/mL) were used in this study, only a slight increase in CD95 expression was observed 48 hours later (data not shown).

Importantly, IFN-γ was shown to induce significantly higher levels of CD95 on Mo-DCs than TNF-α (Figure 5.5a). As already mentioned (5.4.1), TNF-α and IFN-γ induce different patterns of surface molecule expression on Mo-DCs independent of the concentration used, most likely resulting from involvement of separate signalling pathways.

5.4.3.2 Effects on spontaneous and Fas-dependent apoptosis

No change in spontaneous Mo-DC apoptosis was observed following 48 hours incubation with TNF-α or IFN-γ compared to control Mo-DCs (5.3.4.2). However, when harvested, washed and put back into culture for another 24 hours, the proportion of late apoptotic cells was significantly increased in both TNF-α and IFN-γ treated Mo-DCs. It is possible that the Mo-DCs die as a consequence of lack of survival signals usually provided by the T cell. Control Mo-DCs may on the other hand have a longer life span in the absence of survival signals than the more mature TNF-α and IFN-γ treated Mo-DCs, as they have not progressed as far in their life cycle. This is further supported by the observation that the phenotypically less mature Mo-DCs treated with TNF-α or IFN-γ for only 12 or 24 hours leading up to day 8 have a smaller proportion of late apoptotic cells on day 8 + 1 than the more mature Mo-DCs treated with TNF-α or IFN-γ for 48 hours leading up to day 8 (data not shown). Moreover, physical handling of the Mo-DCs on day 8 may further abrogate apoptosis by activating the Mo-DCs.

The mechanism responsible for the increased apoptosis on day 8 + 1 day may also be specifically mediated by certain mediator or surface receptor. For example, signalling from TNFR1 and TNFR2 can directly result in apoptosis in various cell types[349] and IFN-γ mediated apoptosis is primarily induced following production of NO or ligation of CD95. NO-induced apoptosis cannot be excluded on day 8 + 1 as the NO content in these cultures was not measured. Whilst investigating the susceptibility of control Mo-DCs and Mo-DCs treated with TNF-α or IFN-γ to Fas-mediated apoptosis it was found that the proportion of late apoptotic cells increased slightly in control Mo-DC populations in response to sFasL (Figure 5.7a). In contrast, TNF-α treated Mo-DCs showed no signs of responding to sFasL. This is in accordance with literature showing that immature Mo-DCs are partially sensitive, whereas mature Mo-DCs are insensitive to Fas-mediated apoptosis [105]. The level of resistance to Fas-mediated apoptosis in DCs has been reported to correlating with the level of expression of Fas-associated death domain-like IL-1β-converting enzyme-inhibitory protein ligand (c-$FLIP_L$), expressed at high and low levels in mature and immature DCs, respectively[105-107]. c-$FLIP_L$ blocks early event in the Fas signaling pathway by inhibiting the recruitment of pro-caspase-8 to the Fas : Fas associated protein with death domain (FADD) complex (Figure 5.12). IFN-γ treated Mo-DCs did not undergo apoptosis in response to sFasL (Figure 5.7c). On the contrary, IFN-γ treated Mo-DC populations were shown to contain a significantly smaller proportion of late apoptotic cells following 24 hours incubation with sFasL. This may suggest that IFN-γ treated Mo-DCs not only are resistant

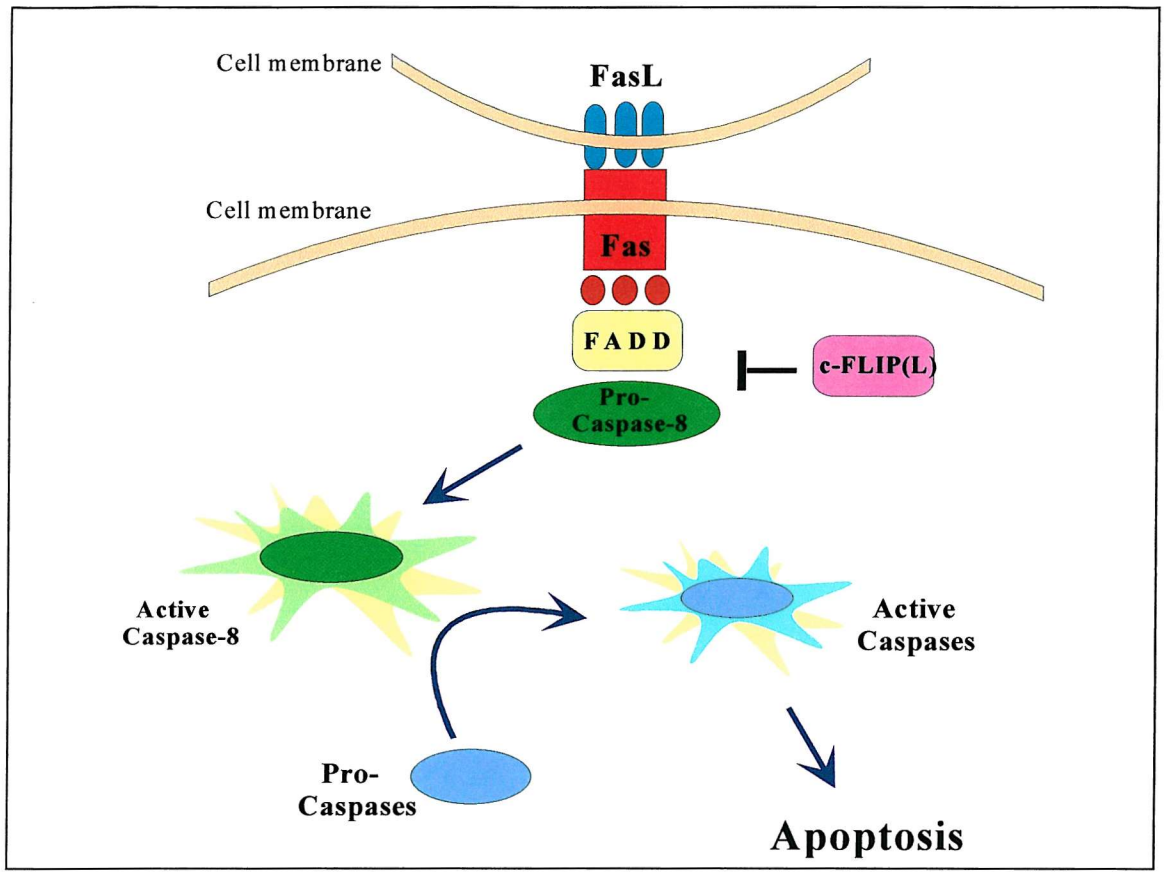


Figure 5.12 Fas signaling pathway

Fas is a member of the TNF receptor superfamily that has been shown to be an important mediator of apoptotic cell death. Binding of FasL induces trimerisation of Fas in the target cell membrane and activation of Fas. Activated Fas recruits FADD via interaction between death domains on both Fas and FADD. Inactive pro-caspase-8 is then recruited to the Fas-FADD complex, leading to its activation by auto-proteolysis. Active caspase-8 catalyses the activation of several effector caspases that cleave cellular components ultimately leading to apoptosis of the cell. Under non-infected conditions the Fas signaling pathway is stopped in DCs by c-FLIP$_L$ that inhibits the recruitment of pro-caspase-8 to the Fas-FADD complex. (Adaptation of a picture taken from the Sigma catalogue for Cell signaling and neuroscience 2000/2001).

to Fas-mediated apoptosis but that stimulation via Fas also enhances the viability of these cells as a concurrent trend for increased early apoptosis was seen in these cultures. Any involvement of anti-apoptotic sCD95 in preventing and reversing the apoptotic process in IFN-γ treated Mo-DCs was excluded as Mo-DCs were shown unable to produce sCD95 under any circumstances investigated (data not shown). However, under certain circumstances signaling via Fas has been shown to protect against Fas-mediated death by activating NF-κB [350,351]. It is therefore possible that similar NF-κB protection apply in IFN-γ treated Mo-DCs as a consequence of stimulation with sFasL. In trying to understand the puzzling effects of sFasL on IFN-γ treated Mo-DCs, it would further be interesting to investigate the level of $cFLIP_L$ in these Mo-DCs.

5.4.3.3 Effects on monocyte-derived dendritic cell survival in culture with allogeneic T cells

During antigen presentation T cells provide DCs with survival signals. *In vivo*, enhanced antigen presentation has been suggested to result from increased survival of DCs [352]. However, despite the occurrence of survival signals, the DC eventually disappears from the regional lymph nodes[353], terminating antigen presentation.

To study the effects of TNF-α and IFN-γ on the survival of Mo-DCs in culture with allogeneic T cells, CFSE labelled Mo-DCs were co-cultured with unlabelled T cells and the number of viable cells estimated over 5 days (5.3.4.4). In agreement with findings for murine DCs *in vivo*[353], viable Mo-DCs had essentially disappeared following 2 – 3 days in culture with allogeneic T cells (Figure 5.8). The rate of disappearance of viable Mo-DCs was independent of the treatment of the Mo-DCs, indicating that the survival signals provided by the T cell are no different. The increase in number of T cells following 4 – 5 days in culture with Mo-DCs is reflecting the clonal expansion detected as thymidine up-take in T cell proliferation assays at this time point.

As an equal number of viable Mo-DCs was seeded at the start of each co-culture, the lower number of viable IFN-γ treated Mo-DCs generally observed a few hours later may suggest that IFN-γ treated Mo-DCs are sensitive to physical handling and die more easily than control or TNF-α treated Mo-DCs. The lower number of viable IFN-γ treated Mo-DCs in these Mo-DC : T cell co-cultures may consequently result in a lower level of T cell proliferation. The fragile nature of IFN-γ treated Mo-DCs may be due to increased sensitivity to ATP-induced cell death as IFN-γ has been reported to increase mRNA

expression of the purinergic receptor P2X7 and to increase ATP-induced lysis of the monocytic cell line THP-1[354] and macrophages [355]. Functional P2X7 was also recently reported on human DCs[356]. P2X7 is a low affinity ATP receptor requiring mM concentrations of ATP to become active. Such high concentrations of extracellular ATP may be obtained *in vitro* as a result of cell lysis due to physical handling. Evidence of plasma membrane blebbing, typical of P2X7 activation, in IFN-γ treated Mo-DC cultures (data not shown) support the possibility that ATP and P2X7 are involved in reducing the number of viable IFN-γ treated Mo-DCs.

To evaluate the possible involvement of Fas-mediated apoptosis in the disappearance of Mo-DCs from Mo-DC : T cell co-cultures, co-cultures were set up in the additional presence of a blocking anti-CD95 mAb. It was shown that the rate of disappearance of Mo-DCs from the co-cultures was unaffected by blocking CD95 signalling in Mo-DCs (data not shown). However, as shown by the viability index, the number of viable IFN-γ treated Mo-DCs in co-cultures was slightly increased by blocking CD95 (Figure 5.9b). Because these effects were only slight, it is not surprising that no effect on T cell proliferation was observed in the presence of the blocking anti-CD95 mAb (data not shown). The viability of control or TNF-α treated Mo-DCs were completely unaffected by blocking CD95 in accordance with the relative Fas-resistance of Mo-DCs.

The increased viability of IFN-γ treated Mo-DCs seen when blocking Fas in culture with T cells contradicts the suggested enhanced survival of IFN-γ treated Mo-DCs stimulated with sFasL (5.4.3.2). This may be due to the design of these *in vitro* systems. When blocking CD95 in the Mo-DC : T cell co-culture the T cell can still influence the Mo-DC via several other Mo-DC expressed surface receptors, whereas only Fas-mediated signals are implicated when stimulating Mo-DCs with sFasL. However, it should be stressed that in both *in vitro* systems the effects of blocking or stimulating Fas are small.

5.4.4 Effects of TNF-α and IFN-γ on the production of soluble factors

Production of nitric oxide and TGF-β1 in Mo-DC cultures as well as Mo-DC : T cell co-cultures were investigated (5.3.5) as they have known immunosuppressive functions and hence may be involved in suppressing Mo-DC driven T cell proliferation.

5.4.4.1 Nitric oxide

Whereas no significant increase in NO content was observed in supernatants of TNF-α treated Mo-DCs, a small but significant increase in NO content was observed in Mo-DC supernatants following treatment with IFN-γ. However, it is unlikely that such low levels of NO (0.6 ± 0.1 μM, n = 12) would have any effect on the viability of Mo-DCs since 80 μM NO_2^-, the soluble form of NO, only induced 2 % apoptosis in murine DCs[340]. This is also supported by the lack of increased apoptosis in IFN-γ treated Mo-DC populations on day 8 as compared to control Mo-DC populations (Figure 5.6a). No linkage between the significant increase in late apoptosis observed on day 8 + 1 day following TNF-α and IFN-γ treatment (Figure 5.6b) and NO can be concluded as the NO content in these supernatants was not measured.

In contrast to murine DCs, no significant increase in NO content in co-cultures of IFN-γ treated Mo-DC and allogeneic T cells was observed. This may be due to the concurrent increase in TGF-β1 content seen in these co-cultures (Figure 5.11b) as TGF-β has been shown to reduce IFN-γ induced NO production by decreasing the stability and translation of iNOS mRNA, and increasing degradation of iNOS protein [357]. It is therefore not surprising that no enhancement in T cell proliferation was seen in the presence of the NOS inhibitor L-NAME in these co-cultures (data not shown).

No significant increase in NO content was observed in co-culture supernatants of allogeneic T cells and TNF-α treated Mo-DCs.

5.4.4.2 TGF-β1

Whereas no significant increase in production of TGF-β1 could be detected in Mo-DC supernatants following treatment with TNF-α or IFN-γ, a significant increase in TGF-β1 production was detected in co-cultures of IFN-γ treated Mo-DCs and T cells. As only TGF-β mRNA and not protein has been reported in DCs so far, the TGF-β1 in these co-cultures is more likely to be produced by the T cell population. However, as interaction with T cells has been shown to alter many functions in DCs, it cannot be excluded that Mo-DCs translate the message and release TGF-β following T cell interaction. Intracellular staining for flow cytometry could be used to ascertain the source of TGF-β in these co-cultures.

The presence of TGF-β1 in the co-culture supernatant is of interest due to its reported role as a regulatory cytokine produced by Tr1 cells. TGF-β produced by murine $CD4^+$, $CD25^+$, $CD45RB^{low}$ Tr1 cells following ligation of CTLA-4 *in vivo* has recently been shown to inhibit activation of responsive $CD45RB^{high}$, $CD4^+$ T cells resulting in abrogation of colitis in these animals [358]. A similar connection between CTLA-4, TGF-β and immune suppression has also been reported in human self-MHC-reactive T cell clones [359]. These self-MHC-reactive T cells demonstrated suppressor function in culture with CD40L-stimulated non-T cells that further was shown to be dependent on the production of TGF-β.

Production of TGF-β has been reported to be regulated by IL-12. Only in the presence of a neutralising IL-12 mAb were OVA-specific TCR-transgenic T cells shown to produce TGF-β when stimulated with OVA-pulsed DCs *in vitro* [360]. Following 48 hours treatment with IFN-γ, Mo-DCs produce extremely low levels of IL-12 shown both by ELISA and by intracellular staining for flow cytometry (Dr A. Semper, unpublished data). It is therefore possible that human Mo-DCs treated with IFN-γ for 48 hours, producing very low levels of IL-12, skew the development of naïve T cells into a Tr1-like population that produce TGF-β, possibly as a consequence of CTLA-4 ligation. The TGF-β produced by this T cell population may suppress activation of bystander responsive T cells, shown as reduced proliferation in the Mo-DC : T cell co-cultures. TGF-β may further affect the Mo-DC population by reducing their maturation, as has been shown *in vitro* at TGF-β concentrations above 1 ng/mL (unpublished data, Dr A. Semper). These Mo-DCs exposed to TGF-β may hence be less potent at driving T cell proliferation.

In contrast to IFN-γ treated Mo-DCs, TNF-α treated Mo-DCs induced significantly reduced TGF-β1 production in Mo-DC : T cell co-cultures compared to control Mo-DCs. As TNF-α treated Mo-DCs have been shown to produce higher levels of IL-12 than IFN-γ treated Mo-DCs (unpublished data, Dr A. Semper), this difference between IFN-γ and TNF-α treated Mo-DCs may be due to their respective inability and ability to produce IL-12. The IL-12 produced by TNF-α treated Mo-DCs may hence suppress TGF-β production by T cells.

Although TNF-α and IFN-γ treated Mo-DCs induced significantly different TGF-β1 production in Mo-DC : T cell co-cultures, the difference in amount of TGF-β1 found was small (5.3.5). However, these small changes in amount of TGF-β1 observed *in vitro* may have huge functional consequences when secreted *in vivo* where the TGF-β1 will be concentrated and focused on the proximal cells in the local microenvironment.

5.4.5 Conclusion of the effects of IFN-γ on monocyte-derived dendritic cells

The aim of the work presented in this chapter was to investigate possible mechanisms underlying the impaired T cell proliferation that occurs in response to phenotypically mature IFN-γ treated Mo-DCs. From the results presented in this chapter two possible explanations can be suggested.

1. IFN-γ treated Mo-DCs drive impaired T cell proliferation due to a fewer number of viable Mo-DCs present during the proliferation assay. The reason for the reduced viability of the IFN-γ treated Mo-DCs was not determined. However, possible mechanisms leading to reduced Mo-DC viability can be excluded including;
    - Fas-mediated apoptosis, as IFN-γ treated Mo-DCs did not apoptose in response to sFasL. Further, blocking CD95 using an anti-CD95 mAb only slightly increased the number of viable IFN-γ treated Mo-DCs in Mo-DC : T cell co-cultures without affecting T cell proliferation.
    - NO-induced apoptosis, as the NO content in cultures of IFN-γ treated Mo-DCs and in IFN-γ treated Mo-DC : T cell co-cultures was low and blocking the production of NO in Mo-DC : T cell co-cultures did not affect T cell proliferation.
2. IFN-γ treated Mo-DCs drive impaired T cell proliferation by inducing TGF-β1 production in IFN-γ treated Mo-DC : T cell co-cultures. However, the TGF-β1 has not yet been shown to be responsible for the impaired T cell proliferation and the cell responsible for the production of TGF-β1 has yet to be identified.

Chapter Six

The mechanisms underlying the disappearance of CD23 from the surface of treated monocyte-derived dendritic cells from normal subjects

Summary

One of the most marked changes in Mo-DC phenotype observed in Chapter 4 was the near to complete elimination of surface expressed CD23 in response to TNF-α, IFN-γ and 100 nM IgE. Because $CD23^+$ DCs could play a role in allergic inflammation, the work presented in this chapter aimed to identify the routes by which these three very different agents downregulate CD23 on the surface of Mo-DCs.

Experiments were designed to investigate the effects of TNF-α, IFN-γ and 100 nM IgE on the kinetics of CD23 downregulation and CD23 internalisation using flow cytometry, CD23 gene transcription using TaqMan RT-PCR, the cellular distribution of CD23 using confocal microscopy and sCD23 shedding using ELISA.

These experiments showed that untreated Mo-DCs maintain a stable level of surface expressed CD23 by constant CD23 turnover, involving exposure of new CD23 at the surface and the clearance of CD23 from the surface by CD23 internalisation and release of sCD23 into the culture supernatant. Further, downregulation of surface expressed CD23 in response to TNF-α, IFN-γ and 100 nM IgE was shown to follow different kinetics. Exposure to 100 nM IgE resulted in a rapid reduction in CD23 surface expression already visible after 3 hours incubation, whereas TNF-α and IFN-γ required around 24 hours to reduce CD23 surface expression significantly. TNF-α and IFN-γ treatment reduced surface expression of CD23 by increasing shedding of sCD23 and by reducing the rate of appearance of new CD23 at the surface. In contrast, IgE protected surface expressed CD23 against proteolytic cleavage and instead rapidly reduced CD23 surface expression by CD23 internalisation and by blocking the appearance of new CD23 at the surface.

6.1 Introduction

6.1.1 Aim

One of the most marked changes in Mo-DC phenotype observed in Chapter 4 was the near to complete elimination of surface expressed CD23 in response to TNF-α, IFN-γ and 100 nM IgE. As CD23 expressed on B cells has been shown to promote IgE-dependent antigen focusing [361,362], it is possible that CD23 may also contribute to allergen uptake and presentation by DCs. Downregulation of CD23 in TNF-α and IFN-γ treated Mo-DCs may on the other hand function to terminate allergen capturing as the Mo-DC matures. TNF-α, IFN-γ and IgE may hence downregulate surface expressed CD23 by different mechanisms. The work presented in this chapter aimed to identify the processes underlying the disappearance of CD23 from the surface of Mo-DCs in response to TNF-α, IFN-γ and 100 nM IgE.

6.1.2 CD23 - the low-affinity IgE receptor

6.1.2.1 Biochemical characterisation and expression of CD23

The low affinity IgE receptor CD23 is a 45 kDa transmembrane glycoprotein of the C-type lectin family[363]. CD23 binds monomeric IgE with two of its three lectin-like head regions associated with the two Cε3 domains on IgE [112]. CD23 exists in two isoforms. CD23a is only expressed on IgD^+, IgM^+ B cells prior to Ig switching during differentiation into plasma cells, whereas CD23b is widely expressed on hematopoetic cells such as alveolar macrophages [279], monocytes [279] and eosinophils [278]. In human serum, CD23 also exists in a sCD23 form resulting from proteolytic cleavage of the membrane expressed CD23 by metalloproteases into fragments ranging from 16 kDa to 37 kDa [112,364]. In the presence of Ca^{2+} these fragments retain their IgE binding capacity and have been shown to be involved in the regulation of IgE synthesis in B cells [112]as well as regulation of TNF-α and NO production by monocytes[365,366].

IL-4 and IL-13 have been demonstrated to induce CD23b expression on B cells, DCs[367,368] and monocytes [279,369]. Since the allergic environment contains high levels of IL-4 and IL-13, it is not surprising that alveolar macrophages[279], monocytes[279] and circulating B-cells[370,371] in patients with atopic asthma show increased surface expression of CD23.

6.1.2.2 IgE binding to CD23 - functional consequences

The effect of IgE binding to CD23 varies hugely depending on the cell type. For example, in kerotinocytes, IgE ligation of CD23 results in the production of TNF-α and IL-6 [372] and in alveolar macrophages and peripheral blood monocytes in the production of TNF-α and IL-1β [373]. In B cells, binding of CD23 result in downregulation of IgE production [374] and inhibition of CD23 proteolytic cleavage[112]. Moreover, both murine and human B cells efficiently present antigen to antigen specific T cells following antigen focusing via CD23[361,362]. Phagocytosis of IgE-coated particles mediated by CD23 has also been reported in macrophages and monocytes [278,362].

6.1.2.3 Effects of IFN-γ on CD23 expression

Although there have been some reports of IFN-γ enhancing IL-4 induced CD23 surface expression [279,375,376], more recent reports indicate an IFN-γ dependent reduction in IL-4 induced CD23 surface expression[369,377-379]. There are further conflicting reports in the literature regarding the level at which IFN-γ regulates CD23 surface expression. It has been shown that IFN-γ promotes the release of sCD23 in both human LCs[327] and the human B cell line JIJOYE[376] leading to reduced surface expression of CD23 despite a concurrent increase in CD23 mRNA. In contrast, IFN-γ has also been suggested to block CD23 transcription by activation of STAT1 that forms homodimers able to compete with STAT6 homodimers for binding to the IL-4 response element in the CD23b promotor in B cells and monocytes [380]. Further, IFN-γ has been suggested to inhibit transcription of IL-4 induced genes including CD23 by inducing *de novo* synthesis of phosphatases, possibly of the SOCS/ SSI/CIS gene family, able to dephosphorylate activated STAT6 [377]. In contrast, IFN-γ has been reported not to affect STAT6 activation but to reduce responsiveness to IL-4 in human mononuclear cells and B cells by reducing the stability of the IL-4R mRNA[381].

6.1.2.4 Effects of TNF-α on CD23 expression

Reports on the effects of TNF-α on CD23 surface expression are few. However, it has been shown that TNF-α reduces CD23 surface expression in monocytes partly by

decreasing CD23 transcription and by enhancing the release of sCD23[382]. TNF-α mediated reduction in CD23 surface expression has also been demonstrated in human LCs, however, in this system TNF-α was shown not to affect the release of sCD23 [327].

6.1.3 Experimental approach

In light of the current literature concerning the effects of TNF-α, IFN-γ and IgE on CD23 expression, experiments were designed to investigate the kinetics of CD23 downregulation and CD23 internalisation using flow cytometry, transcriptional regulation of CD23 using TaqMan RT-PCR, the cellular distribution of CD23 using confocal microscopy and the release of sCD23 by ELISA.

As Mo-DCs not only express CD23 but also the high affinity IgE receptor, FcεRI, it is possible that the observed effects of 100 nM IgE are due to concurrent ligation of IgE to FcεRI and CD23. To distinguish between effects mediated by FcεRI alone and by FcεRI and / or CD23, the route of disappearance of surface expressed CD23 on Mo-DCs was also investigated in the presence of 1 nM IgE, primarily targeting FcεRI.

6.2 Materials and methods

6.2.1 Subjects

21 non-atopic, non-asthmatic normal subjects (12 female, 9 male) took part in this study.

6.2.2 Effects of IgE, TNF-α and IFN-γ on monocyte-derived dendritic cell phenotype

To assess the effects of TNF-α, IFN-γ and IgE on the phenotype of Mo-DCs, 8 day old Mo-DCs, exposed to TNF-α, IFN-γ, 100 nM IgE or 1 nM IgE for 3, 6, 12, 24 or 48 hours, were harvested and stained for surface expression of CD1a, CD14, CD23 and HLA-DR as described (2.2.5.2.1).

During flow cytometric analysis dead cells were identified using 7-AAD and were excluded from the events collected (2.2.5.4). A total of 5 000 live events were acquired.

The level of $CD23^+$ Mo-DCs was assessed by analysing the percentage labelled cells on a gated cell population. The resulting percentage labelled cells for a specific Mo-DC treatment was used for calculating the percentage change relative to untreated control Mo-DCs according to the following equation;

$$((\text{Treated Mo-DC \%} / \text{Control Mo-DC \%}) - 1) \times 100$$

6.2.3 Flow cytometric analysis of CD23 internalisation

To assess the effects of TNF-α, IFN-γ and IgE on internalisation of CD23 in Mo-DCs, surface expressed CD23 on 8 day old Mo-DCs was pre-labelled with FITC-conjugated anti-CD23 (clone BU38) or isotype control mAb as described (2.2.3.2.1). Mo-DCs pre-labelled with BU38 were cultured with TNF-α, IFN-γ or 100 nM IgE for 6, 12 or 24 hours (2.2.3.2.2.) and then harvested and immunostained with PE-conjugated mAbs specific for HLA-DR, CD23 (clone EBVCS-5) or GAM as described (2.2.5.2.1). A proportion of each culture condition was in addition treated with trypsin (2.2.3.2.3) to remove surface expressed CD23 prior to immunostaining with the anti-GAM or anti-CD23

mAb. Mo-DCs that were not pre-labelled with BU38-FITC were in parallel stained for surface expression of CD1a and CD14 (2.2.5.2.1) to confirm that the monocytes had converted to Mo-DCs.

During flow cytometric analysis dead cells were identified using 7-AAD and were excluded from the events collected (2.2.5.4). A total of 5 000 live events were acquired.

The level of internalised CD23 was estimated by analysing the intensity of BU38-FITC fluorescence (MFI) remaining following removal of surface expressed BU38-labelled CD23 by trypsin.

6.2.4 Confocal microscopy of CD23 distribution

To determine the distribution of CD23 in Mo-DCs, 8 day old Mo-DCs, exposed to TNF-α, IFN-γ, 100 nM IgE or 1 nM IgE for 24 hours, were harvested, cytospun onto polylysine coated slides then stained with unconjugated mouse anti-human CD23 mAb (clone BU38) and RRX-conjugated GAM mAb as described (2.2.12.1). Surface and intracellular immunostaining for BU38 was detected using a confocal microscope (2.2.12.2). A series of confocal sections was taken at 0.5 μm intervals.

6.2.5 TaqMan RT-PCR to quantify CD23 transcription

To assess the effects of TNF-α, IFN-γ and IgE on Mo-DC transcription of CD23, 8 day old Mo-DCs, exposed to TNF-α, IFN-γ, 100 nM IgE or 1 nM IgE for 6, 12, 24 or 48 hours, were harvested and total RNA purified using QIAShredder columns and RNeasy mini spin columns as described (2.2.11.1). Total RNA was reverse transcribed using random hexamer primers (2.2.11.3) before the CD23 cDNA content in duplicate reactions was estimated by multiplex TaqMan PCR using pre-developed TaqMan primers and probes (2.2.11.4). Relative quantification, comparing the CD23 content in treated to untreated Mo-DC cDNA preparations, was calculated using the equation described (2.2.10.4) after the C_T value for CD23, for each duplicate, had been normalised to the C_T value for the endogenous internal standard 18S rRNA.

6.2.6 Measurement of sCD23 production by ELISA

For analysis of sCD23 content in Mo-DC supernatants, 8 day old Mo-DCs, exposed to TNF-α, IFN-γ, 100 nM IgE or 1 nM IgE for 6, 12, 24 or 48 hours were harvested, counted and the total volume of supernatant measured and stored at -40°C for analysis of sCD23 by ELISA at a later date.

sCD23 content in Mo-DC supernatants was measured by ELISA according to the supplier's (Biosource Europe S.A) instructions in duplicates (2.2.9). Supernatants for measuring sCD23 content were diluted 1:5 in the supplied diluent, to fall within the range of the standard curve (1U/mL – 20 U/mL). The sCD23 was detected by an anti-sCD23-HRP conjugate and TMB substrate and the plates were subsequently read at 450 nm on an ELISA plate reader.

The sCD23 concentration in the supernatants was calculated by relating the mean absorbance value of the duplicates to a standard curve. The concentration was further converted to U / 5.0×10^5 cells by adjusting for the volume of medium and number of cells counted at the end of culture.

6.2.7 Statistical analysis

Using SPSS 10.0 for Windows, the non-parametric Wilcoxon test was used where appropriate to compare responses within a subject group. A p value of less than 0.05 was considered statistically significant.

6.3 Results

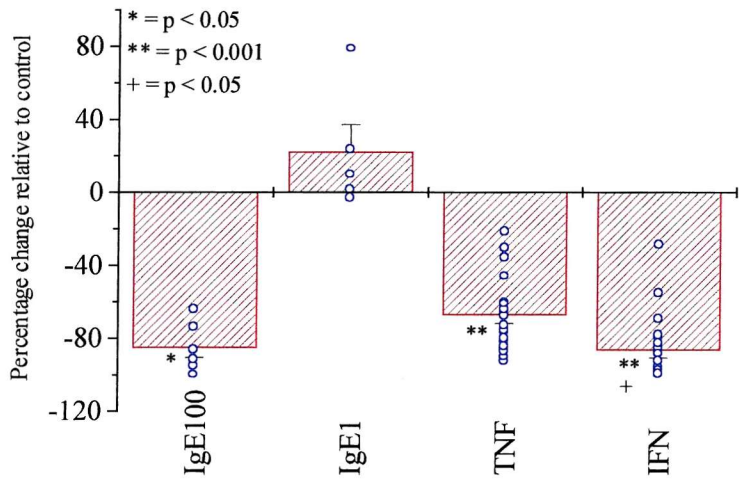
6.3.1 Surface expression of CD23 on treated monocyte-derived dendritic cells

8 day old Mo-DCs, treated with TNF-α, IFN-γ, 100 nM IgE or 1nM IgE for varying lengths of time, were harvested and stained for surface expression of CD23.

Figure 6.1 a) and b) show the effects of 48 hours treatment with TNF-α, IFN-γ and IgE on Mo-DC CD23 surface expression. Compared to control Mo-DCs, the percentage $CD23^+$ cells was significantly reduced following incubation with either 100 nM IgE (-85 % ± 6, $p < 0.05$), TNF-α (-67 % ± 5, $p < 0.001$) or IFN-γ (-86 % ± 4, $p < 0.001$) (Figure 6.1a). At this time point surface expression of CD23 was also significantly lower in Mo-DCs treated with IFN-γ as compared to TNF-α ($p < 0.05$). The percentage $CD23^+$ Mo-DCs did not change significantly in response to 48 hours treatment with 1 nM IgE. On the contrary, a trend for increased CD23 expression was observed.

The kinetics of CD23 disappearance in response to TNF-α, IFN-γ and 100 nM IgE was found to differ (Figure 6.1b). Although not statistically significant due to low number of IgE-treated Mo-DCs studied (n between 3 and 5 depending on time point), a reduction in CD23 surface expression was noticeable already following 3 hours in culture with 100 nM IgE ($n = 3$). Despite the higher numbers of TNF-α and IFN-γ treated Mo-DCs studied (n between 7 and 20 depending on time point), TNF-α or IFN-γ only led to a significant reduction in CD23 expression ($p < 0.05$) following 24 hours incubation (TNF-α $n = 10$, IFN-γ $n = 9$). It appears that IFN-γ reduced CD23 surface expression to a significantly higher extent ($p < 0.05$) than TNF-α following 48 hours incubation. Hence, the average percentage $CD23^+$ Mo-DCs following 48 hours treatment with TNF-α was 15 % ± 2 ($n = 20$), whereas the same length of treatment with IFN-γ resulted in 7 % ± 3 ($n = 19$) and 100 nM IgE resulted in 8 % ± 3 ($n = 5$) $CD23^+$ Mo-DCs. A slight increase in CD23 surface expression was observed following 12 hours incubation with TNF-α ($n = 7$).

a) CD23 expression at 48 hours



b) Kinetics of CD23 downregulation

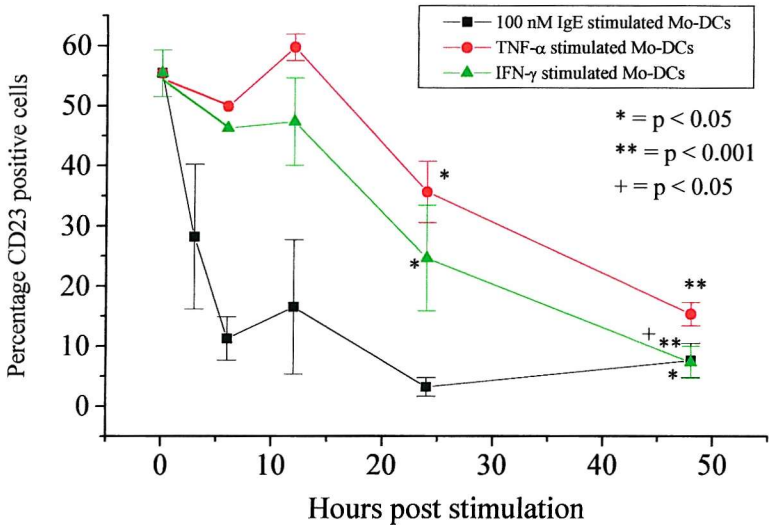


Figure 6.1 Mo-DC surface expression of CD23

8 day old Mo-DCs, exposed to TNF-α, IFN-γ and IgE for varying lengths of time, were harvested and stained for surface expression of CD23. In a) the effects of 100 nM IgE (n = 6), 1n nM IgE (n = 5), TNF-α (n = 19) and IFN-γ (n = 19) following 48 hours incubation are presented as percentage change relative to control. Each individual value (percentage change) is presented as an open blue circle and the mean of all values is shown as a column with error bars (SEM). In b) the kinetics of CD23 downregulation in response to 100 nM IgE, TNF-α and IFN-γ is presented as percentage $CD23^+$ cells at 3, 6, 12, 24 or 48 hours. The mean of all subjects studied (n varies between 1 and 20) is presented as a square (IgE), triangle (IFN-γ) or circle (TNF-α) with error bars (SEM) and a line to indicate the trend joins each time point.

* = $p < 0.05$, ** = $p < 0.001$ compared to control Mo-DCs.

+ = $p < 0.05$ compared to TNF-α treated Mo-DCs.

6.3.2 Internalisation of CD23 in control and treated monocyte-derived dendritic cells

To investigate the effects of TNF-α, IFN-γ and 100 nM IgE on internalisation of CD23, Mo-DCs were pre-labelled with a FITC conjugate of the anti-CD23 mAb BU38, then treated with TNF-α, IFN-γ or 100 nM IgE for 6, 12, or 24 hours. Flow cytometry was then used to determine the internalisation of previously surface-expressed CD23 (labelled with FITC-conjugated BU38) by measuring the intensity of BU38-FITC fluorescence remaining after removal of surface expressed BU38-labelled CD23 by trypsin. The disappearance of BU38-labelled CD23 from the cell surface was in parallel monitored by staining the non-trypsinised cells with a PE-conjugated secondary GAM antibody. The appearance of previously unexposed surface CD23 was also detected by staining BU38 pre-labelled, non-trypsinised cells with a second PE-conjugated anti-CD23 mAb (clone EBVSC-5) that competes with BU38 for binding to CD23.

The interpretation of the fluorescence intensity following different treatments is presented in Table 6.1.

Table 6.1 The interpretation of the fluorescence intensity for the CD23 internalisation assay

Treatment	Immunostaining	Interpretation of fluorescence
- Trypsin	BU38-FITC	Total presence of CD23 pre-labelled with BU38, surface and intracellular
+ Trypsin	BU38-FITC	Intracellular CD23 pre-labelled with BU38
- Trypsin	GAM-PE	Surface expressed BU38-labelled CD23
+ Trypsin	GAM-PE	Surface expressed BU38-labelled CD23 remaining after removal of surface expressed CD23
- Trypsin	EBVCS-5 PE	New surface expressed CD23 not pre-labelled with BU38.

6.3.2.1 The fate of BU38-FITC labelled CD23

The persistence of BU38-FITC labelled CD23 at the cell surface was assessed by staining with a PE-conjugated anti-mouse secondary antibody ("Surface BU38 (GAM)" in Figure 6.2). In all Mo-DC treatments, surface expressed CD23 labelled with BU38 declined

over time. The kinetics of this disappearance was more rapid in TNF-α treated Mo-DCs (Figure 6.2b) than in control cells (Figure 6.2a). However, the most pronounced decrease in BU38-labelled CD23 occurred in response to 100 nM IgE, with virtually all surface expressed BU38-labelled CD23 having disappeared after only 6 hours in culture (Figure 6.2c). The kinetics of the disappearance of BU38-labelled CD23 in response to IFN-γ was similar to that found for TNF-α treatment (data not shown).

To discriminate between CD23 labelled with BU38-FITC remaining at the cell surface and that that had been internalised, it was necessary to remove surface expressed BU38-labelled CD23 by trypsin. To test the efficacy of the trypsin treatment, cells pre-labelled with BU38-FITC were stained with the GAM secondary antibody before and after trypsin treatment. In all Mo-DC cultures, after trypsin treatment no BU38 could be detected with the secondary antibody (Figure 6.2). This is believed to result from trypsin removing the CD23-BU38-FITC complex, since, in separate experiments (data not shown) trypsin removed unlabelled CD23 from the surface of Mo-DCs.

Having shown that trypsin removes BU38-labelled CD23 from the surface, the fluorescence intensity of FITC was compared before and after trypsin treatment. In the absence of trypsin the overall intensity of BU38-FITC fluorescence declined slightly over time ("Total BU38 FITC" in Figure 6.2), being apparent within 6 hours of treatment with 100 nM IgE (Figure 6.2c) and within 12 hours of treatment with TNF-α (Figure 6.2b) in the subject presented. In comparison, the overall fluorescence intensity of cells pre-labelled with the FITC-conjugated isotype control mAb was very weak (MFI around 2.0) throughout the chase period (data not shown). After trypsin treatment, some FITC fluorescence from BU38 could still be detected in all conditions (Figure 6.2), suggesting that a small proportion of the surface-expressed BU38-labelled CD23 was present intracellularly. In control and TNF-α treated Mo-DCs, the mean intensity of FITC fluorescence after a 6-hour chase period in non-trypsinised cells was 21.0 and 15.7, respectively, and 4.6 in trypsinised cells for both culture conditions. In contrast, at the same time point, the mean intensity of FITC fluorescence in cells treated with 100 nM IgE was 10.2 for non-trypsinised cells and 9.2 after trypsinisation. This suggests that internalised BU38-FITC was contributing more to the total BU38-FITC fluorescence in cells exposed to 100 nM IgE. The mean intensity of FITC fluorescence after trypsinisation in IFN-γ treated Mo-DCs was similar to that observed in control and TNF-α treated Mo-DCs (data not shown).

Considering that the intensity of BU38-FITC fluorescence on non-trypsinised cells reflects both surface and internalised CD23, consistently higher MFI values (0 hours in

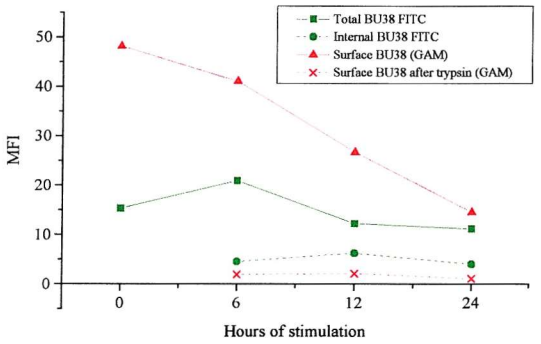
Figure 6.2) and more $BU38^+$ Mo-DCs (data not shown) were identified using the GAM secondary antibody to detect cell surface BU38.

6.3.2.2 Export of new CD23 to the cell surface

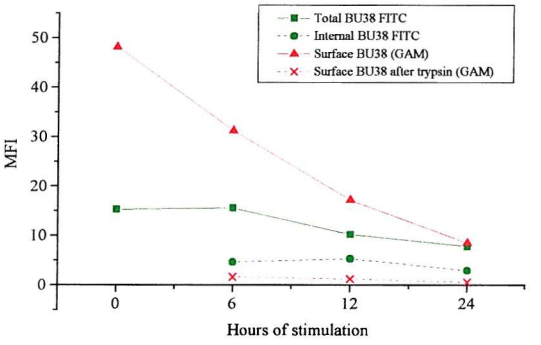
The anti-CD23 mAb BU38 was found to block the binding of a second anti-CD23 mAb EBVCS-5 to the receptor (Figure 6.3a). This strategy was used to detect previously unexposed CD23 on the surface of BU38 pre-labelled Mo-DCs.

Already following 3 hours in culture, detectable levels of new CD23 were found on the surface of BU38 pre-labelled control Mo-DCs using EBVCS-5 (Figure 6.3b). However, this did not affect the overall level of CD23 surface expression on control Mo-DCs that was found to be relatively constant over a period of 48 hours (data not shown). New CD23 was also detected on BU38 pre-labelled Mo-DCs treated with TNF-α for 6 hours (Figure 6.3c and d), although the amount of new CD23 was lower than that found on control Mo-DCs at the same time point. In contrast to control Mo-DCs, that exposed a constant amount of new CD23 at the cell surface at all time points investigated, the amount of new CD23 appearing at the surface of TNF-α treated Mo-DCs was reduced with time (Figure 6.3d). IFN-γ treated Mo-DCs showed a similar pattern of exposing new CD23 to TNF-α treated Mo-DCs (Figure 6.3d), although these cells exposed slightly more new CD23 on the surface than TNF-α treated Mo-DCs in the subject presented. No new CD23 was found on BU38 pre-labelled Mo-DCs cultured with 100 nM IgE at any time point investigated (Figure 6.3c and d). IgE was found not to block the binding of EBVCS-5 to CD23 as determined by removal of surface bound IgE with lactic acid (data not shown). Similar kinetics of the appearance of new CD23 at the cell surface of control Mo-DCs and Mo-DCs treated with TNF-α, IFN-γ or 100 nM IgE was also found in the other two experiments conducted (data not shown).

a) Control Mo-DCs



b) TNF-α treated Mo-DCs



c) 100 nM IgE treated Mo-DCs

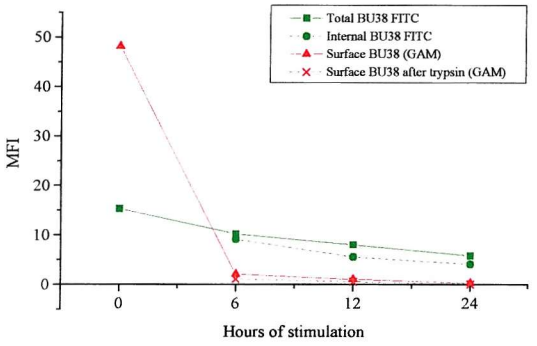
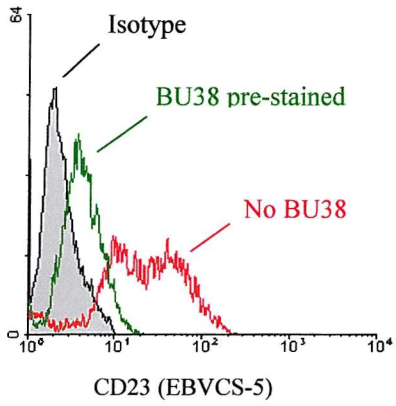


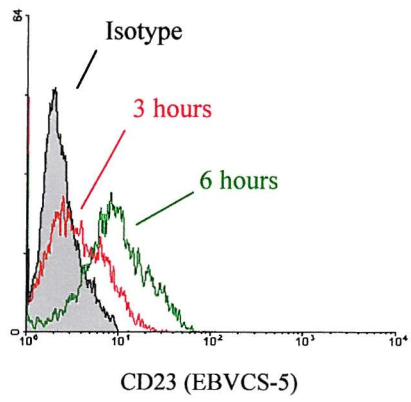
Figure 6.2 Disappearance of BU38-FITC labelled CD23 from control Mo-DCs and Mo-DCs treated with TNF-α or 100 nM IgE

Representative results from one of three experiments conducted to investigate the fate of CD23 labelled with a pulse of BU38-FITC mAb. Eight day old Mo-DCs, pre-labelled with BU38, were a) left untreated (control) or exposed to b) TNF-α or c) 100 nM IgE for 6, 12, or 24 hours before harvest. A proportion of each culture condition was treated with trypsin and both trysinised and untrypsinised Mo-DCs were stained with a PE-conjugated GAM mAb. Surface and intracellular CD23 pre-labelled with BU38 (■), internalised CD23 pre-labelled with BU38 (•), surface expressed CD23 pre-labelled with BU38 (▲) and surface expressed CD23 pre-labelled with BU38 after trypsinisation (×) are presented as mean fluorescence intensity (MFI). The 0 hour time point shows CD23 surface expression on control Mo-DCs (no BU38 pre-label) identified directly by BU38-FITC or indirectly by BU38-FITC followed by GAM-PE.

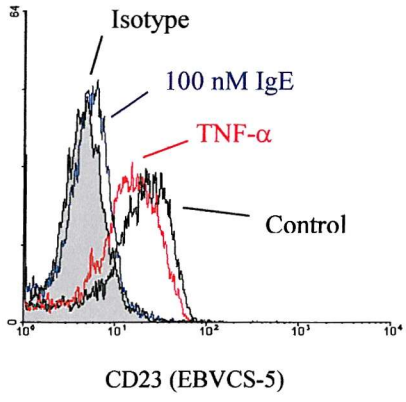
a) BU38 blocks EBVCS-5



b) New CD23 on control Mo-DCs



c) New CD23 at 6 hours



d) New CD23 at 6, 12 and 24 hours

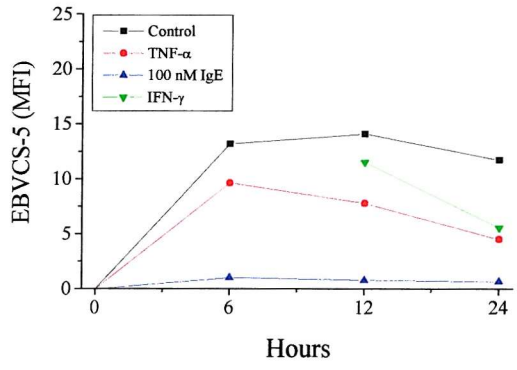


Figure 6.3 Appearance of new CD23 at the cell surface of Mo-DCs

Representative results from 3 separate experiments conducted to determine if new CD23 is exposed to the cell surface of Mo-DCs. In a) binding of the anti-CD23 mAb EBVCS-5 was prevented on 8 day-old control Mo-DCs, pre-stained with the anti-CD23 mAb BU38. In b) control Mo-DCs were pre-labelled with BU38, then cultured for 3 or 6 hours before previously unexposed surface CD23 was identified using EBVCS-5. In c) and d), Mo-DC were pre-labelled with BU38, then cultured for c) 6 hours or d) 6, 12 or 24 in the absence (control) or presence of TNF-α, IFN-γ or 100 nM IgE before previously unexposed surface CD23 was identified using EBVCS-5.

6.3.3 Distribution of CD23 in monocyte-derived dendritic cells

To further reveal the location of CD23 in Mo-DCs treated with TNF-α, IFN-γ and IgE, the distribution of CD23 in Mo-DCs was investigated by confocal microscopy. For this purpose, 8 day old Mo-DCs treated with TNF-α, IFN-γ, 100 nM IgE or 1nM IgE for 24h hours, were harvested, cytospun onto polylysine coated slides then fixed, permeabilised and stained with mouse anti-human CD23 mAb followed by fluorochrome conjugated GAM mAb before analysis by confocal microscopy.

Figure 6.4 shows CD23 staining in a series of sequential confocal sections taken at 0.5 μm intervals through untreated control Mo-DCs. Although the Mo-DCs are severely deformed during the cytospin process (around 5 μm thick and 60 μm wide), staining for CD23 was evident both on the surface, demonstrated by clear contours of dendrites in sections 4 and 5, and intracellularly, demonstrated by staining throughout the cell cytoplasm in sections 3 – 6.

Figure 6.5 shows the maximal projection images, in which, from each series, all sequential sections have been digitally recombined. Over half the untreated control Mo-DCs showed strong CD23 immunoreactivity with a localised accumulation of intracellular CD23 in close proximity to the cell nucleus. Mo-DCs treated with TNF-α or 100 nM IgE showed only weak CD23 immunoreactivity, equivalent to background levels as determined by staining with an isotype control mAb (data not shown), although in some cells a weak accumulation of CD23 staining could still be discerned adjacent to the nucleus. Mo-DCs treated with IFN-γ or 1nM IgE showed moderate levels of CD23 immunoreactivity that was localised both to the surface and cytoplasm of the Mo-DC as determined by studying the individual confocal images in each series (data not shown).

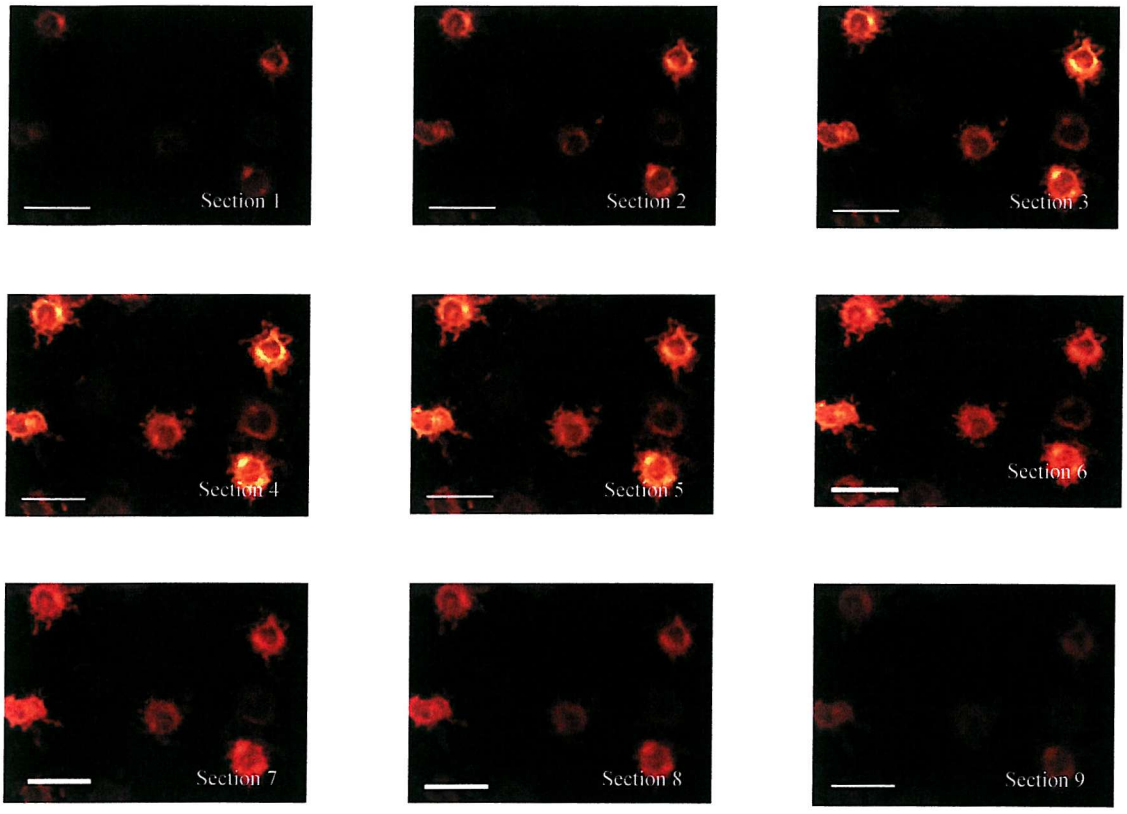


Figure 6.4 The distribution of CD23 in control Mo-DCs

8 day old untreated Mo-DCs were harvested, cytospun onto polylysine coated slides then fixed, permeabilised and stained with unconjugated CD23 mAb followed by RRX-conjugated GAM mAb. This series of images shows CD23 staining in sequential confocal sections taken at 0.5 μm intervals from the top to the bottom of the cells. All images are presented using a glowing colour "look-up table" for clear definition of CD23 intensity. Scale bar = 80 μm.

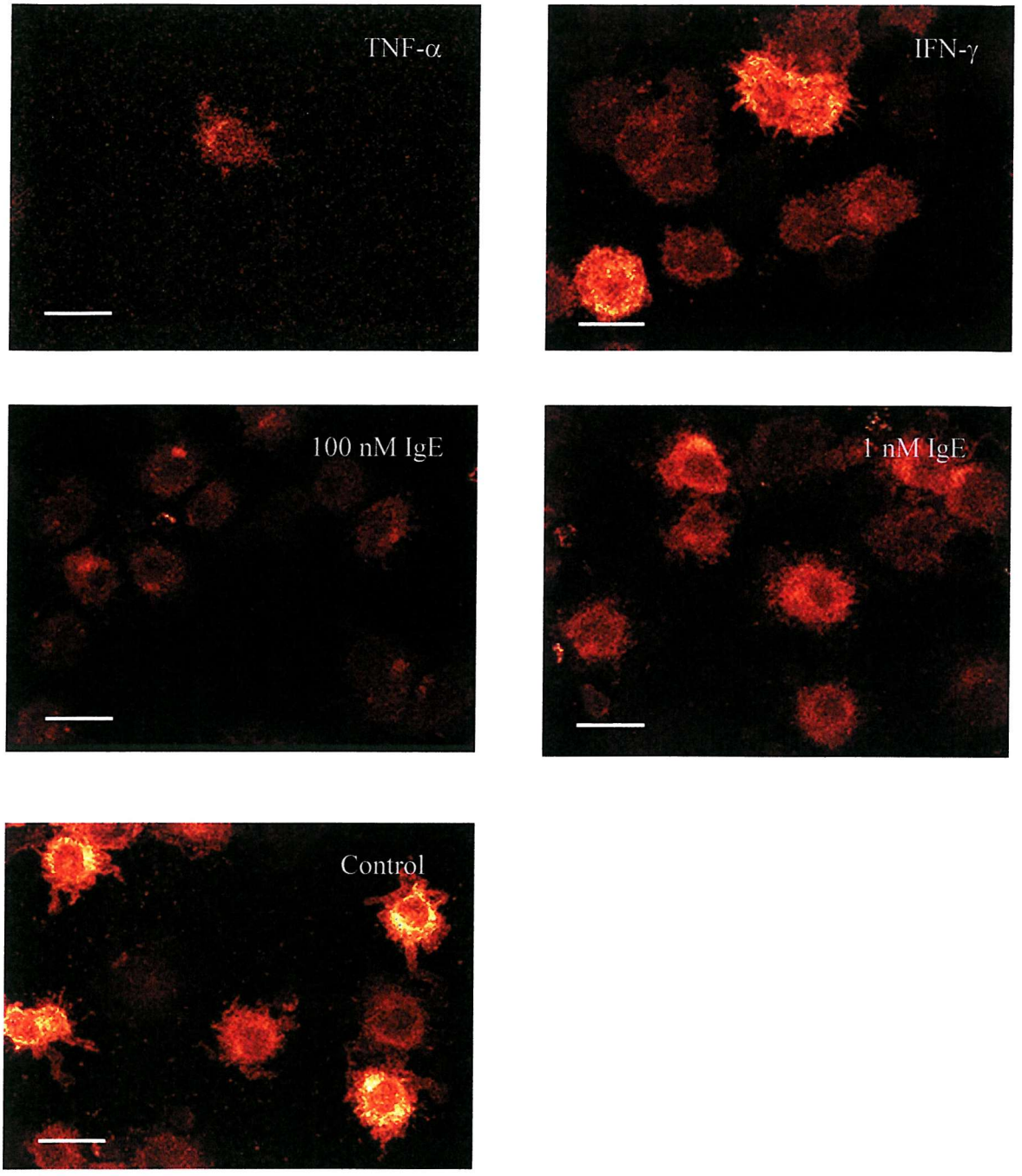


Figure 6.5 The effects of TNF-α, IFN-γ and IgE on CD23 distribution in Mo-DCs

8 day old Mo-DCs, treated with TNF-α, IFN-γ, 100 nM IgE or 1 nM IgE for 24 hours, were harvested and stained for detection of CD23 as described in Figure 6.4. For each image, the series of sequential confocal sections has been recombined as a maximal projection image. All preparations were scanned using the same instrument settings. All images are presented using a glowing colour "look-up table" for clear definition of CD23 intensity. Scale bar = 80 μm.

6.3.4 Transcriptional regulation of CD23 in response to TNF-α, IFN-γ and IgE

The level of CD23 gene transcription in 8 day old Mo-DCs treated with TNF-α, IFN-γ, 100 nM IgE or 1 nM IgE for 6- 48 hours was investigated by TaqMan RT-PCR.

An example of real-time amplification of CD23 and 18S rRNA cDNA, reverse transcribed from total RNA purified from Mo-DCs, is presented in Figure 6.6. The Mo-DC preparation contained more 18S cDNA than CD23 cDNA, evident by the lower C_T values for 18S. By normalising the CD23 C_T values to the corresponding 18S C_T value for each treatment, relative quantification of CD23 transcripts could be performed (2.2.11.4).

In Figure 6.7 the relative quantification values of Mo-DCs treated with TNF-α, IFN-γ or IgE is presented. Relative quantification is a unit used to relate the level of CD23 transcripts in treated Mo-DC preparations to that of untreated control Mo-DC preparations. A relative quantification value of over 1.0 indicates more CD23 transcript in the treated samples than control sample whereas a relative quantification value below 1.0 indicates the reverse. In response to TNF-α, a general trend for reduced CD23 transcription, already starting following 6 hours incubation with TNF-α was observed (Figure 6.7a). In contrast to TNF-α, no consistent change in CD23 transcription could be determined in response to IFN-γ: the CD23 mRNA content was decreased following 6 and 24 hours but increased following 12 and 48 hours treatment (Figure 6.7b). An initial increase in CD23 transcription occurred after 6 – 12 hours incubation with 100 nM IgE (Figure 6.7c). This increase was followed by a marked reduction in CD23 transcription after 24 –48 hours incubation. Finally, although only repeated twice, a sharp increase in CD23 transcription was observed following 6 hours treatment with 1nM IgE (Figure 6.7d). At later time points no change in level of CD23 mRNA was observed between untreated Mo-DCs and Mo-DCs treated with 1 nM IgE.

Relative quantification values below 1.0 were particularly evident for Mo-DCs treated for 48 hours with TNF-α, IFN-γ or 100 nM IgE. By recalculating the relative quantification values at the 48 hour time points it was possible to quantify how many times more mRNA the control Mo-DC preparation contained. This quantification value is referred to as reverse relative quantification value and is presented together with its corresponding relative quantification value in Table 6.2. Hence, control Mo-DC preparations contained 5.2 times more CD23 mRNA than Mo-DC preparations treated with TNF-α for 48 hours (Table 6.2). Similarly, control Mo-DC preparations contain 3.8 and 2.3 times more CD23 mRNA than Mo-DCs treated with IFN-γ or 100 nM IgE for 48 hours, respectively.

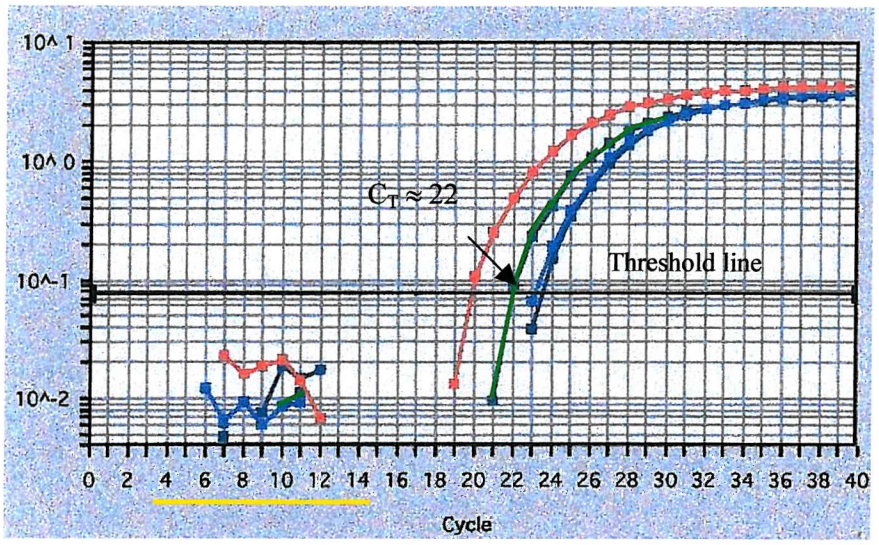
Table 6.2 Relative quantification and reverse relative quantification values for Mo-DC preparations 48 hours after treatment

Mo-DC treatment	Relative Quantification value[+]	Reverse Relative Quantification value[++]
TNF-α	0.2	5.2
IFN-γ	0.3	3.8
100 nM IgE	0.4	2.3

[+] Relative quantification values for CD23 transcript in Mo-DC preparations were calculated as described (2.2.11.4).

[++] By subtracting the average ΔC_T for treated preparations from the average ΔC_T from the control preparation a "reverse $\Delta\Delta C_T$" was obtained that was used instead of $\Delta\Delta C_T$ in equation 2 (2.2.11.4) to calculate the reverse relative quantification. The reverse relative quantification value shows how many times more CD23 mRNA was found in the control Mo-DC preparation compared to the treated Mo-DC preparations.

a) CD23



b) 18S

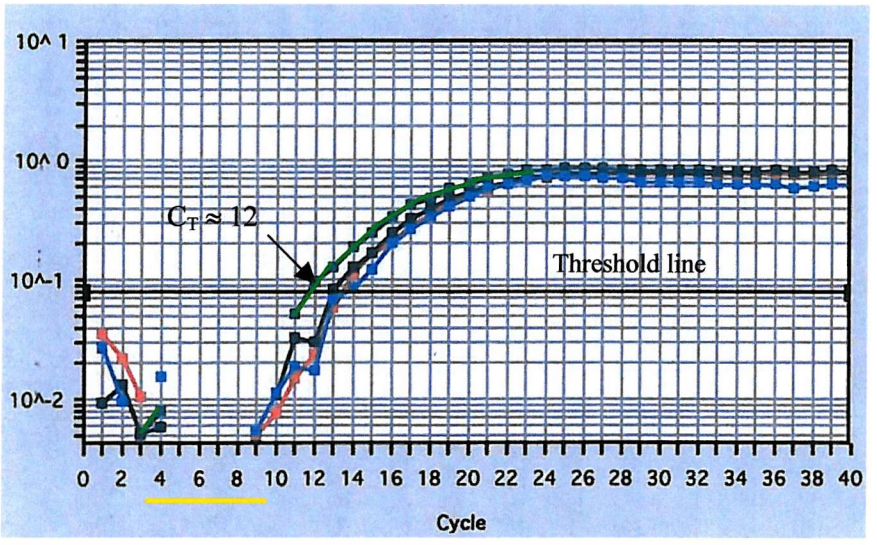
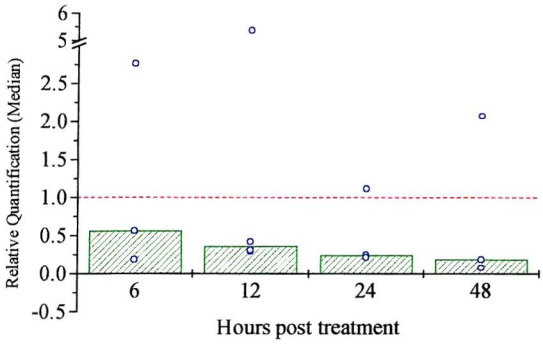


Figure 6.6 Example of a logarithmic scale amplification plot for CD23 and 18S rRNA in Mo-DCs

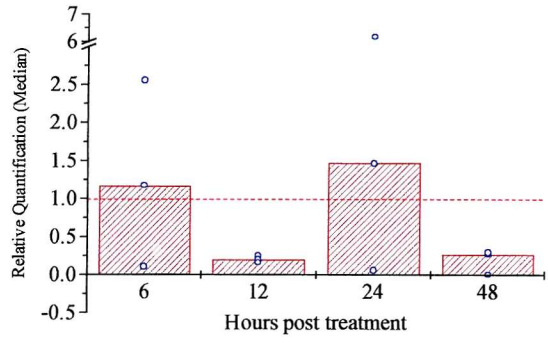
mRNA from 8 day old Mo-DCs left untreated (•) or cultured for 24 hours with TNF-α (•), IFN-γ (•) or 100 nM IgE (•), were purified and reverse transcribed before the a) CD23 and b) 18S cDNA content was measured by TaqMan PCR. The yellow lines define the range of cycles chosen as the baseline for CD23 and 18S amplification. The threshold lines were set within the exponential amplification phase. The black arrows indicate the approximate C_T value of CD23 and 18S for Mo-DCs exposed to TNF-α for 6 hours.

a) TNF-α



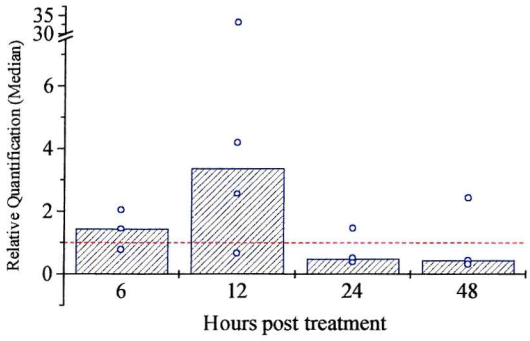
6 hours n = 3, 12 hours n = 4, 24 hours n = 3, 48 hours n = 3.

b) IFN-γ



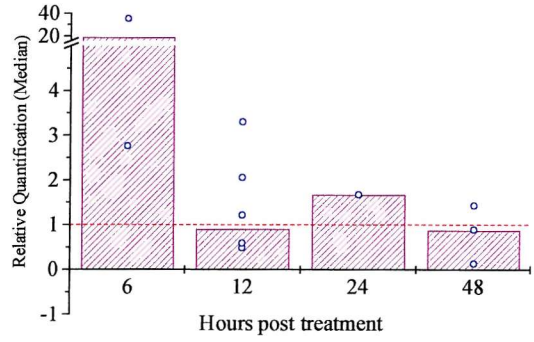
6 hours n = 3, 12 hours n = 3, 24 hours n = 3, 48 hours n = 3.

c) 100 nM IgE



6 hours n = 3, 12 hours n = 4, 24 hours n = 3, 48 hours n = 3.

d) 1 nM IgE



6 hours n = 2, 12 hours n = 6, 24 hours n = 1, 48 hours n = 3.

Figure 6.7 Effects of TNF-α, IFN-γ and IgE on CD23 transcription

mRNA from 8 day old Mo-DCs exposed to TNF-α, IFN-γ or IgE for 6, 12, 24 or 48 hours, were purified and reverse transcribed before the CD23 cDNA content was measured by TaqMan PCR. The effects of a) TNF-α, b) IFN-γ, c) 100 nM IgE and d) 1 nM IgE are presented as relative quantification values. Each individual value (relative quantification) is presented as an open blue circle and the median of all values is shown as a column. The dashed red line indicates the level of CD23 cDNA found in control Mo-DC samples, corresponding to a relative quantification value of 1.

6.3.5 Effects of TNF-α, IFN-γ and IgE on shedding of sCD23

8 day old Mo-DCs, treated with TNF-α, IFN-γ, 100 nM IgE or 1 nM IgE for 6 –48 hours, were harvested, the supernatants saved and the sCD23 content in the supernatants analysed by ELISA.

Figure 6.8 shows the effects of TNF-α, IFN-γ, 100 nM IgE and 1 nM IgE on the shedding of sCD23 from Mo-DCs into the culture supernatants. Compared to control Mo-DC supernatants there was a trend for decreased sCD23 content in Mo-DC supernatants treated with 100 nM IgE at all time points investigated (Figure 6.8c). This reached statistical significance in supernatants from Mo-DCs treated with 100 nM IgE for 24 hours (-33 ± 4 %, $n = 6$, $p < 0.05$). This decrease, expressed as percentage change relative to untreated control samples, was also reflected as a significant decrease ($p < 0.05$) in the mean absolute amount of sCD23 found in Mo-DC supernatants treated with 100 nM IgE for 24 hours (15 ± 2 U per 5.0×10^5 cells, $n = 6$, $p < 0.05$) compared to that found in control Mo-DC supernatants (24 ± 2 U sCD23 per 5.0×10^5 Mo-DCs, $n = 16$) (data not shown). A trend for increased levels of sCD23 in supernatants from Mo-DC treated with TNF-α at all time points was noted (Figure 6.8a). This reached statistical significance in supernatants from Mo-DCs treated with TNF-α for 48 hours (25 ± 11 %, $n = 7$, $p < 0.05$). However, although an increase expressed as percentage change relative to untreated control samples was observed, no increase in the mean absolute amount of sCD23 in Mo-DC supernatants treated with TNF-α for 48 hours was found compared to control Mo-DC supernatants. Thus, following 48 hours treatment with TNF-α, Mo-DC supernatants contained on average 25 ± 2 U sCD23 per 5.0×10^5 Mo-DCs ($n = 7$), that is virtually equivalent to the levels found in control Mo-DC supernatants (see above). Similarly, although there also appeared to be a trend for increased levels of sCD23 in the supernatants from Mo-DCs treated with IFN-γ (Figure 6.8b), no difference was evident in the absolute levels of sCD23 between supernatants from IFN-γ treated Mo-DCs (23 ± 2 U sCD23 per 5.0×10^5 Mo-DCs, $n = 7$, data not shown) and control Mo-DCs (see above). No change in sCD23 content in Mo-DC supernatants treated with 1 nM IgE was observed (Figure 6.8d).

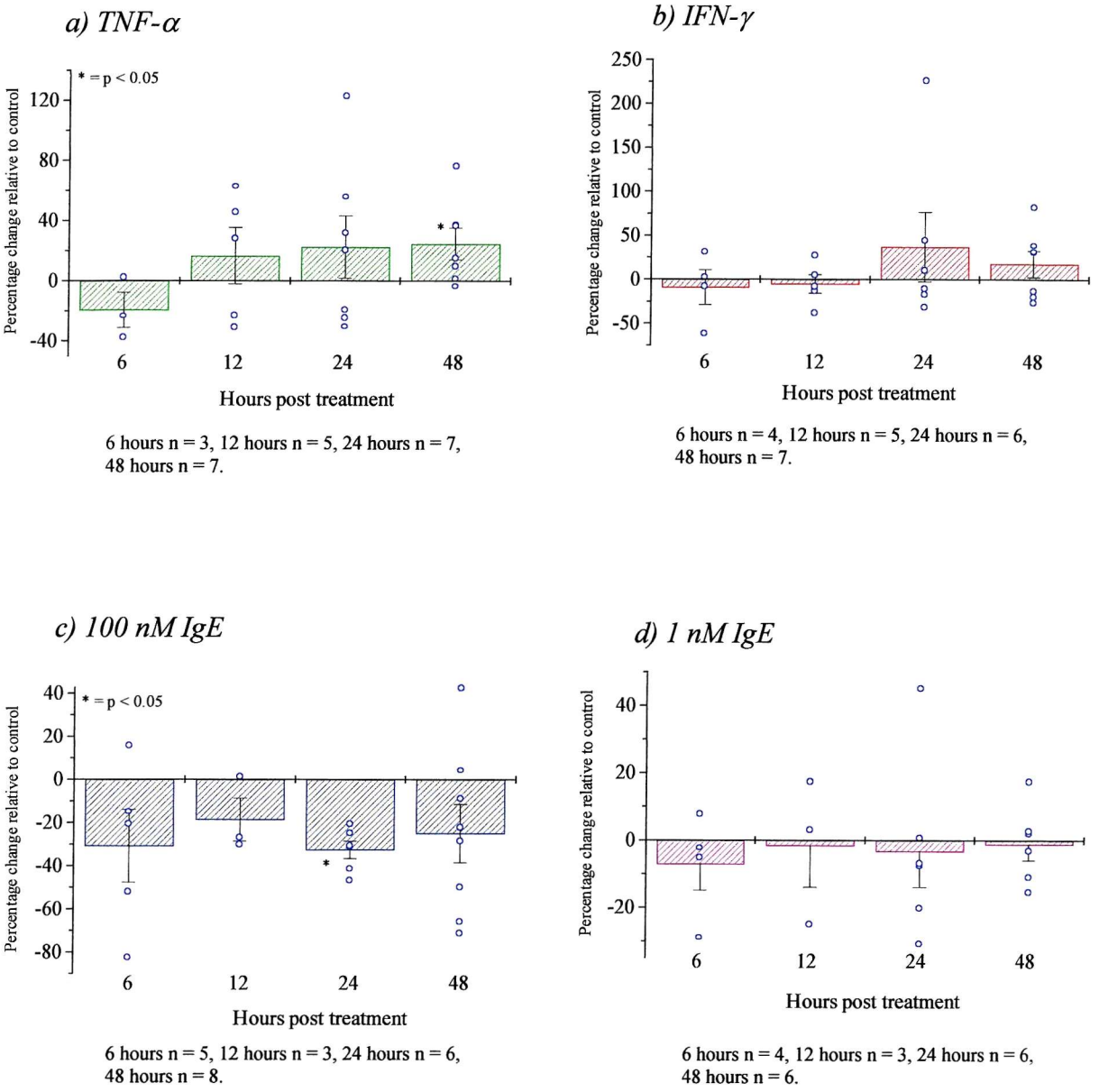


Figure 6.8 Soluble CD23 in Mo-DC supernatants

8 day old Mo-DCs exposed to TNF-α, IFN-γ or IgE for 6, 12, 24 or 48 hours, were harvested and the supernatants collected for measurement of sCD23 content by ELISA. The effects of a) TNF-α, b) IFN-γ, c) 100 nM IgE and d) 1 nM IgE are presented as percentage change relative to control. Each individual value (percentage change) is presented as an open blue circle and the mean of all values is shown as a column with error bars (SEM).

* = $p < 0.05$ compared to control Mo-DCs.

6.4 Discussion

In the work reported in Chapter 4, CD23 was shown to disappear from the surface of Mo-DCs treated for 48 hours with TNF-α, IFN-γ and 100 nM IgE. In this chapter the effects of TNF-α, IFN-γ and IgE treatment have been further investigated by studying the kinetics of CD23 downregulation, CD23 internalisation, CD23 cell distribution, CD23 transcription and the release of sCD23 into the culture supernatants.

6.4.1 The kinetics of CD23 disappearance

The disappearance of CD23 from the cell surface of Mo-DCs was found to follow different kinetics dependent on the treatment of the cells. Although, not supported by statistical analysis due to a low number of samples studied, treatment with 100 nM IgE was shown to result in the fastest disappearance of surface expressed CD23 on Mo-DCs, with noticeable reduction already after 3 hours in culture. In contrast, significant reduction in CD23 surface expression was observed after 12 hours in culture with TNF-α or IFN-γ. IFN-γ treatment resulted in slightly faster clearance of surface expressed CD23 than TNF-α treatment. TNF-α treatment also differed by increasing rather than decreasing the proportion of $CD23^+$ Mo-DCs at the 12-hour time point. This increase was observed in parallel with an increase in surface expression of HLA-DR and CD86 (data not shown).

The very rapid reduction in CD23 surface expression on Mo-DCs following ligation of IgE was consistent with that found following treatment of CD23 expressing RPMI 8866 cells (murine B cell line) with a stimulatory anti-CD23 mAb[322]. Already following 1 hour in culture with the stimulatory anti-CD23 mAb the surface expression of CD23 on the RPMI 8866 cells was considerably reduced [322]. The effect of TNF-α on CD23 surface expression observed here was also consistent with reports in the literature, as the IL-4-induced CD23 surface expression on monocytes was found reduced after one day in culture with TNF-α[382]. Surface expression of CD23 on these monocytes was reported to further decrease by extending the culture period beyond 24 hours. In contrast, although IFN-γ has been reported to reduce the surface expression of CD23 in various cell types[369,378,379], no detailed study of the kinetics of clearance of CD23 in response to IFN-γ exists to date.

As TNF-α, IFN-γ and 100 nM IgE clearly differ in their ability to physically interact with CD23 it is not surprising that they also differ in their kinetics of clearing surface expressed CD23. It is therefore also likely that these mediators clear CD23 surface

expression by different mechanisms. Reports on the effect of TNF-α, IFN-γ and IgE on various cells types indicate the possible involvement of CD23 internalisation[322], altered CD23 transcription and / or translation[377,380,382] or shedding of sCD23 into the culture supernatants[327,369,376,382] in reducing surface expression of CD23. To determine the relative contribution of these means of clearing CD23 in Mo-DCs treated with TNF-α, IFN-γ or 100 nM IgE, it was necessary to develop an assay for measuring CD23 internalisation.

6.4.2 The CD23 internalisation assay

In a study of CD23 internalisation by B cells, surface expressed CD23 was labelled with ^{125}I and internalisation monitored by immunoprecipitaion after the removal of surface expressed CD23 by trypsin[322]. Since ^{125}I binds to tyrosine residues present in the IgE binding lectin-like head region of CD23, potentially blocking the binding of IgE, this approach was considered unsuitable for the present application. Therefore a flow cytometric assay was devised in which the surface of Mo-DCs was labelled with a FITC-conjugated, non-competitive anti-CD23 mAb (clone BU38). The fate of the BU38-CD23 complex was followed after various time intervals by monitoring the intensity of FITC fluorescence. To discriminate between internalised BU38-labelled CD23 and that remaining at the cell surface, comparison was made to cells from which surface expressed BU38-labelled CD23 was removed by trypsin treatment. A PE-conjugated secondary GAM antibody was also used to identify BU38-CD23 complexes remaining on the surface of non-trypsinised cells. Moreover, this technique allowed the detection of new CD23 at the cell surface of BU38 pre-labelled, non-trypsinised cells as BU38 was found to block the binding of a second anti-CD23 mAb (clone EBVCS-5).

By using the GAM secondary antibody it was found that trypsin successfully removed surface expressed BU38-FITC, presumably by cleaving off the CD23-BU38 complex. The secondary GAM antibody also proved to be a useful tool for monitoring the disappearance of BU38 pre-labelled CD23 from the cell surface. The GAM antibody showed that the rate of disappearance of BU38-labelled CD23 was dependent on the culture condition, with faster clearance of BU38-CD23 complexes from the surface of Mo-DCs treated with 100 nM IgE than TNF-α, IFN-γ or control Mo-DCs. The fluorescence intensity of the GAM antibody (PE) consistently exceeded that of BU38 (FITC). This is most probably due to the emission from PE being more intense than that from FITC and to the

fact that the secondary antibody amplifies the CD23 signal by potentially binding to several epitopes on BU38.

Comparing the level of FITC fluorescence from cells with or without trypsin treatment, suggested that a proportion of BU38-FITC was internalised, especially following exposure to 100 nM IgE. This is in agreement with the increased rate of internalisation of CD23 in B cells treated with a stimulatory anti-CD23 mAb[322]. However, the overall fluorescence intensity of FITC was found to decline over time making it difficult to accurately quantify the level of internalised BU38-labelled CD23. As CD23 has been shown to be internalised into acidic organelles in B cells where it is degraded into fragments[322], this decline in FITC intensity may be due to degradation or quenching of FITC in similar organelles. It is also possible that FITC disengages from BU38 in culture. This is supported by the finding that the GAM secondary antibody consistently identified more $CD23^+$ Mo-DCs than was evident by the presence of BU38-FITC (data note shown). Consequently, detached FITC may have been taken up non-specifically by macropinocytosis rather than via endocytosis of BU38-CD23 complexes.

This method also successfully identified previously unexposed CD23 at the cell surface. Like the rate of disappearance of BU38-labelled CD23, the rate of appearance of new CD23 was shown to depend on the culture condition, with more new CD23 appearing on the surface of control Mo-DCs than on TNF-α or IFN-γ treated Mo-DCs, and no new CD23 appearing on Mo-DCs treated with 100 nM IgE.

6.4.3 The expression of CD23 in control monocyte-derived dendritic cells

Using confocal microscopy, CD23 was identified in the cytoplasm as well as on the cell surface of untreated control Mo-DCs. In the cytoplasm, CD23 immunoreactivity concentrated in the juxtanuclear location but was also present at low levels throughout the cytoplasm (Figure 6.4). The accumulation of CD23 near the nucleus suggests the presence of newly synthesised CD23, possibly localised to the endoplasmic reticulum or the Golgi complex.

The level of surface expressed CD23 was found to be relatively constant between day 6 and 8 in culture (data not shown). However, using the internalisation assay, CD23 was found to disappear from the cell surface whilst previously unexposed CD23 appeared at the surface. This suggest that the constant level of surface CD23 expression is maintained by a balanced clearance of the receptor and the arrival of new receptor at the cell surface.

Whereas the new appearance of CD23 at the surface may result from both recycling of CD23 and exposure of newly synthesised CD23 exported from the Golgi complex, shedding of receptor is likely to contribute to the removal of CD23 from the surface as the supernatants of control Mo-DCs contain moderate levels of sCD23 (24 ± 9 U sCD23 / 5.0 × 10^5 cells). Moreover, a proportion of BU38 pre-labelled CD23 was protected from treatment with trypsin (Figure 6.3a) and confocal microscopy revealed the presence of evenly dispersed CD23 staining throughout the cell cytoplasm, both suggesting that internalisation of CD23 also contributes to keeping the level of surface expressed CD23 constant in control Mo-DCs. Hence, control Mo-DCs appear to have a constitutive turnover of CD23 that further may be altered by stimulation with TNF-α, IFN-γ or IgE, modulating the balance between appearance and disappearance of CD23 on the surface, leading to an overall reduction of CD23 surface expression.

6.4.4 Possible routes by which TNF-α affects CD23 surface expression

A significant decrease in surface expression of CD23 was observed in Mo-DCs treated with TNF-α for 24 and 48 hours as compared to control Mo-DCs. This decrease was shown to result from increased disappearance of surface expressed CD23 as well as decreased appearance of new CD23 at the cell surface (Figure 6.2b, 6.3c and d).

The level of sCD23 in the supernatants of TNF-α treated Mo-DCs was increased above that observed in control Mo-DC supernatants 12 to 48 hours following exposure to TNF-α. This suggests that the disappearance of CD23 from the cell surface may result from increased shedding of sCD23. This is consistent with a report of the effects of TNF-α on CD23 expression by monocytes [382]. CD23 internalisation is not expected to contribute to the increased disappearance of surface expressed CD23 seen in TNF-α treated Mo-DCs, as the level of CD23 internalisation (reduction in FITC fluorescence following trypsin treatment) in these cells was very similar to that found in control Mo-DCs.

The appearance of new CD23 at the surface of Mo-DCs was also reduced after TNF-α treatment. This may result from reduced CD23 gene transcription since reduced levels of CD23 mRNA were detected 24 to 48 hours post treatment with TNF-α. However, it is difficult to predict protein levels based on levels of CD23 transcription since the stability of CD23 mRNA is unknown. However, only weak cytoplasmic staining for CD23 was detected in TNF-α treated Mo-DCs by confocal microscopy, suggesting only low levels of translation into CD23 protein. It should be noted that this lack of CD23 detection

by confocal microscopy may merely reflect the level of sensitivity of this technique as around 40 % of the TNF-α treated Mo-DC population was shown to express CD23 at low intensity by flow cytometry, a more sensitive technique. Further, CD23 confocal microscopy was performed on only one subject after 24 hours of treatment with TNF-α. This subject may misrepresent the broader population.

A slight increase in CD23 surface expression was observed following 12 hours incubation with TNF-α. This increase was also accompanied by an increase in surface expression of HLA-DR and CD86 (data not shown), indicating activation of the Mo-DCs. As reduced CD23 gene transcription and increased levels of sCD23 were found at this time point, these mechanisms are unlikely to account for the observed increase in CD23 surface expression. However, an increased rate of transport of CD23 protein to the cell surface may account for this increase in CD23 surface expression. This may be confirmed by a detailed study of the kinetics of appearance of new CD23 at the surface.

Taken together, these results suggest that TNF-α reduces the overall surface expression of CD23 by increasing shedding of sCD23 into the supernatant and by reducing the appearance of new CD23 at the cell surface as a consequence of reduced transport of CD23 protein to the surface, reduced CD23 translation, reduced CD23 transcription or by a combination of these processes.

6.4.5 Possible routes by which IFN-γ affects CD23 surface expression

Significant reduction in surface expression of CD23 was observed following 24 hours in culture with IFN-γ. As for TNF-α treatment, this decrease was shown to result from increased disappearance of surface expressed CD23 (data not shown), as well as decreased appearance of new CD23 at the cell surface (Figure 6.4c and d).

The increased disappearance of CD23 from the cell surface may result from shedding of sCD23 as the level of sCD23 was found to increase above that observed in control Mo-DC supernatants following 24 hours incubation with IFN-γ. This is consistent with a report showing increased levels of sCD23 in the supernatants of IFN-γ treated monocytes [369]. As with TNF-α treatment, the level of internalised CD23 in IFN-γ treated Mo-DCs was very similar to that found in control Mo-DCs suggesting that altered CD23 internalisation is unlikely to contribute significantly to the increased disappearance of surface expressed CD23 from IFN-γ treated Mo-DCs.

Over the time period investigated, no consistent trend indicating reduced CD23 transcription in response to IFN-γ was observed. However, the absence of a localised accumulation of CD23 immunoreactivity near the nucleus in IFN-γ treated Mo-DCs, as seen in control Mo-DCs, may suggest reduced CD23 synthesis. This could account for the reduced appearance of new CD23 at the surface of IFN-γ treated Mo-DCs (Figure 6.3d). Reduced translation may result from decreased stability of the CD23 mRNA. Accordingly, IFN-γ has been reported to reduce the stability of CD23 mRNA in B cells[378]. However, reduced protein production may also result from control of other post-transcriptional events.

Taken together, these results suggest that IFN-γ reduces surface expression of CD23 by increasing the release of sCD23 into the supernatant and by reducing the appearance of new CD23 at the cell surface as a consequence of reduced transport of CD23 protein to the surface or reduced new synthesis of CD23 by inhibition of CD23 translation.

6.4.6 Possible routes by which high concentration IgE affects CD23 surface expression

In contrast to the effects of TNF-α and IFN-γ, the kinetics of downregulation of CD23 in response to 100 nM IgE was very rapid, showing a marked reduction in overall CD23 surface expression already following 3 hours. This also correlated with the accelerated disappearance of BU38-labelled CD23 from the surface, as shown by the secondary GAM antibody (Figure 6.2c). Interestingly, no new CD23 had appeared at the cell surface 6 hours after stimulation with 100 nM IgE (Figure 6.3c and d). Hence, 100 nM IgE very efficiently and quickly clears CD23 from the surface and at the same time prevents the appearance of new CD23.

The rapid disappearance of CD23 from the cell surface is not due to increased shedding of sCD23, as the sCD23 content in Mo-DC supernatants treated with 100 nM IgE was reduced compared to that found in control Mo-DCs supernatants at all time points investigated (Figure 6.8). In accordance, binding of IgE to CD23 has been reported to protect against proteolytic cleavage[112]. A large proportion of BU38-labelled CD23 was protected from trypsin treatment, suggesting substantial levels of CD23 internalisation in Mo-DCs treated with 100 nM IgE. This could account for the rapid disappearance of surface expressed CD23 in these cells, which further is supported by a report identifying fragments of CD23 intracellularly in B cells as early as 3 hours following binding of an anti-CD23 mAb [322].

At the early time points investigated, the absence of new CD23 at the surface of Mo-DCs treated with 100 nM IgE is not likely due to inhibition of CD23 transcription or translation (Figure 6.3d), since such regulation would require several hours to have an effect on surface protein expression. Indeed, at 6 – 12 hours in culture with 100 nM IgE, CD23 transcription was slightly increased (Figure 6.7c) although only low levels of CD23 could be detected in the cytoplasm of Mo-DCs treated with IgE by confocal microscopy (Figure 6.5) or on the surface during or after this time interval (Figure 6.1b and 6.3d). Reduced levels of CD23 mRNA were noted 24 to 48 hours post treatment that could contribute to the lack of new CD23 at the cell surface at later time points. However, with no data indicating the stability of CD23 mRNA it is again difficult to predict what affect reduced CD23 transcription may have on CD23 surface expression.

Taken together, these results suggest that in contrast to TNF-α or IFN-γ, 100 nM IgE protects CD23 from proteolytic cleavage. Further it appears that 100 nM IgE reduces the overall surface expression of CD23 by rapidly clearing surface expressed CD23 by internalisation and by blocking the appearance of new CD23 at the cell surface as a consequence of reduced transport of CD23 protein to the surface, reduced CD23 translation, reduced CD23 transcription or by a combination of these processes.

6.4.7 Effects of low concentration of IgE – events mediated by FcεRI and / or CD23

In order to control for any IgE effects on CD23 surface expression being mediated by FcεRI, IgE at 1 nM, primarily targeting FcεRI, was applied in parallel experiments.

Although not causing the same effects on Mo-DCs as 100 nM IgE, 1 nM IgE did indeed affect Mo-DCs. For example, a marked increase in CD23 transcription was observed following 6 hours incubation with 1 nM IgE (Figure 6.7). Moreover, 1 nM IgE was shown to slightly increase rather than reduce CD23 surface expression at the 48 hour time point (Figure 6.1), although this may result from protection against proteolytic cleavage of CD23 by IgE as the level of sCD23 in these Mo-DCs supernatants was simultaneously slightly reduced (Figure 6.8). The observed effects of 1 nM IgE may result from parallel or combined signals from FcεRI and CD23 since binding of IgE to CD23, even at the 1 nM IgE concentration cannot be excluded (Figure 6.9). The presence of parallel or combined signalling pathways leading from CD23 and FcεRI are also supported by the observation that $FcεRI^+$, $CD23^+$ Mo-DCs, but not $FcεRI^-$, $CD23^+$ Mo-DCs reduced their surface

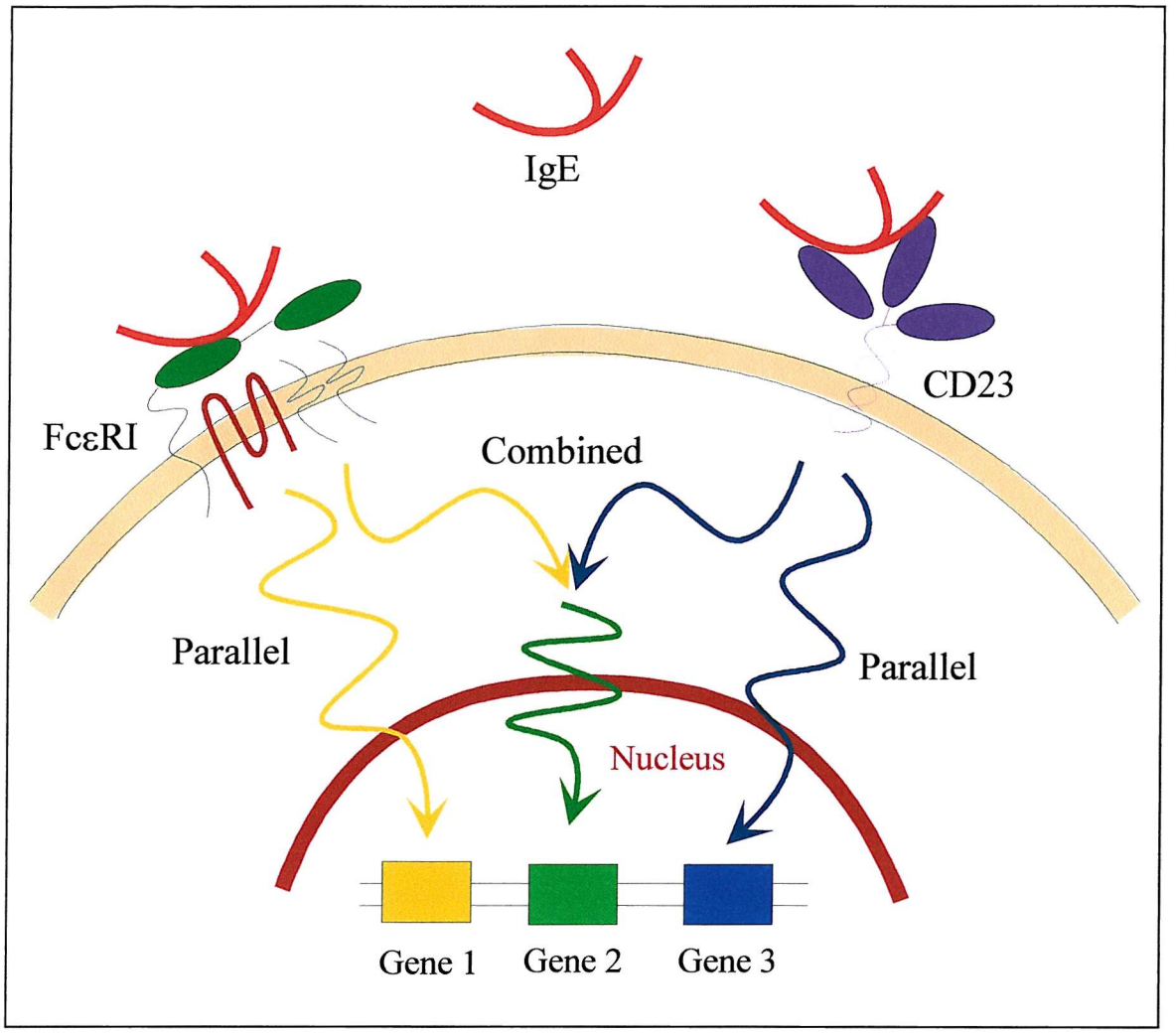


Figure 6.9 Parallel and combined signalling pathways potentially leading from FcεRI and CD23.

expression of CD86 and HLA-DR when increasing the IgE concentration from 6.4 nM to 25.7 nM (data not shown). As 6.4 nM IgE correspond to approximately 64 × K_d of FcεRI, IgE at this concentration is predicted to occupy most surface expressed FcεRI with no further effects on Mo-DC function expected following an additional increase in IgE concentration. The enhanced effects of surface molecule expression may therefore result from concurrent binding of a portion of CD23 as the IgE concentration was increased (CD23 K_d 10^{-6} to 10^{-7} M).

As the IgE preparations used in this study contain very little complexed IgE (data not shown) able to cross-link FcεRI when bound, and no cross-linking antigen, these effects are most likely resulting from ligation rather than cross-linking of receptors. As all reported studies on the effects of IgE binding to FcεRI on DCs focus on the effects of subsequent IgE cross-linking [84,124], these results are novel.

To further investigate common and separate CD23 and FcεRI signalling pathways in Mo-DCs, Mo-DCs may be treated with rat IgE that has been reported to bind human FcεRI but not human CD23 [383]. Alternatively, Mo-DCs may be exposed to chimaeric human IgE in the presence of an anti-CD23 mAb of the clone MHM6 that blocks IgE binding to CD23 [269].

6.4.8 Conclusion

The aim of the work presented in this chapter was to identify the mechanisms by which TNF-α, IFN-γ and 100 nM IgE downregulate CD23 on the surface of Mo-DCs. The results presented in this chapter show that;

- TNF-α and IFN-γ reduced CD23 surface expression by increasing shedding of sCD23 and reducing the rate of appearance of new CD23 at the surface.
- 100 nM IgE reduced CD23 surface expression by rapidly clearing surface expressed CD23 by internalisation and efficiently blocking the exposure of new CD23 at the surface.

The results presented in this chapter also show that;

- Control Mo-DCs maintain a steady-state level of CD23 surface expression by a balanced clearance of surface CD23, involving internalisation and shedding, and appearance of new CD23 at the surface.
- Downregulation of surface expressed CD23 in response to TNF-α, IFN-γ and 100 nM IgE follow different kinetics. Whereas clearance of CD23 in response to 100

nM IgE was visible already after 3 hours, TNF-α and IFN-γ required around 24 hours to reduce surface expression of CD23 significantly.

- 100 nM IgE prevented shedding of sCD23 into the culture supernatant.

Chapter Seven

General Discussion and Future Directions

7.1 The central role of the dendritic cell in immunity and disease

Due to its central role in innate and acquired immunity, much attention has focused on the DC in the progression, and its modification for the prevention, of diseases such as cancer[384], HIV[385] and autoimmune diseases[386,387], but also in transplantation[388].

In this project, the role the DC may play in allergic asthma was studied. The hypothesis explored was that **components within the local microenvironment in the atopic asthmatic lung, including elevated levels of IgE and Th2 cytokines, brings about phenotypic and functional changes in DCs. These changes in turn modify the DC - T cell interaction, resulting in initiation or perpetuation of the chronic allergic inflammation seen in asthmatic airways.** In the following sections, the findings made during the course of this project are summarised and discussed in the context of general DC biology and specifically in terms of their implications for allergic asthma.

7.2 Chapter 3: Characterisation of monocyte-derived dendritic cells from normal and asthmatic subjects

In Chapter 3, Mo-DCs derived from non-atopic (normal) and atopic, asthmatic subjects were characterised and compared to reveal possible inherent tendencies to promote an atopic asthmatic disease phenotype.

7.2.1 Original observations and interpretation

Mo-DCs derived from normal and asthmatic subjects were found to be strikingly similar with the exception of a significantly smaller proportion of the cells being $CD23^+$ in Mo-DC populations from asthmatic subjects. This is in contrast to the only other published report comparing the phenotype of Mo-DCs derived from these two subject groups[262]. In addition to reporting no difference in CD23 surface expression between the two subject groups, this report also contrasted by showing significantly higher levels of HLA-DR and HLA-DQ and significantly lower levels of FcεRI in Mo-DCs derived from atopic, asthmatic subjects. As already discussed in Chapter 3 (3.4.2), these differences between the two studies may result from differences in the developmental stages of the Mo-DCs used, the presence of contaminating cell populations as well as to differences in data analysis.

Although reports of $CD23^+$ human dendritic cell populations *in vivo* are few, $CD23^+$ LCs and dermal dendritic cells have been observed in aeroallergen stimulated skin biopsies

from atopic dermatitis patients[389]. $CD23^+$ inflammatory dendritic epidermal cells (IDEC) have also been found in the lesional skin of subjects with atopic eczema[390]. It is therefore appropriate to consider what mechanisms may lead to the difference in level of CD23 expression in Mo-DCs from normal and asthmatic subjects observed in the current study and what consequences this difference may have on DC function in asthma.

Although it has been proposed that CD23 expressed on IDECs may not result from synthesis of CD23 but rather from binding of sCD23 to the cell surface[391,392], CD23 transcripts and protein have been found in Mo-DCs in this study (Chapter 6). Thus, the lower levels of CD23 found on the surface of Mo-DCs derived from asthmatic subjects may result from reduced CD23 production. This could arise as a result of differences in IL-4-mediated signalling pathways in Mo-DCs from asthmatic subjects, leading to reduced CD23 transcription, or differences in the regulation of factors influencing the stability of CD23 mRNA. Mo-DCs from asthmatic subjects may also differently regulate the export of CD23 protein to the surface compared to Mo-DCs from normal subjects. Reduced CD23 surface expression may affect the antigen capturing capacity of Mo-DCs from asthmatic subjects as CD23 is involved in antigen uptake by $CD23^+$ B cells[361,362] and also since Mo-DCs were found to internalise CD23 in this study (Chapter 6). In addition to having a significantly smaller proportion of $CD23^+$ cells, $CD23^+$ Mo-DC populations from asthmatic subjects were also found to express less CD23 on their surface (data not shown), however, this difference was not supported by statistical analysis. It is therefore possible that in the presence of IgE, the strength of parallel and combined signalling pathways leading from both low and high affinity IgE receptors (Figure 6.9) may induce different groups of gene products in Mo-DCs from normal and asthmatic subjects. This is further supported as IgE treated Mo-DCs from asthmatic subjects were found to express significantly higher levels of CD80 and CD86 than IgE treated Mo-DCs from normal subjects (Chapter 4.2).

The lower levels of CD23 found on the surface of Mo-DCs derived from asthmatic subjects may also result from increased shedding of sCD23 from the cell surface in response to the IL-4 present in the culture supernatant[276]. In support of this, sCD23 was detected in Mo-DC culture supernatants in Chapter 6. Mo-DCs from normal and asthmatic subjects may hence respond differently to IL-4 possibly leading to increased production of metalloproteases in Mo-DCs from asthmatic subjects resulting in increased cleavage of CD23 from the surface. Since sCD23 has been shown to promote the production of IgE in B cells[280-282], DCs in atopic asthmatic subjects may hence be prone to perpetuate the allergic asthmatic response *in vivo* when exposed to an environment containing elevated levels of IL-4, for example the inflamed asthmatic airways[292]. However, since lung DCs are a trace population, the increase in sCD23 is likely to be very local. Further, to date no B cells have

been identified in the epithelium where most lung DCs reside[241,242]. However, increased shedding of sCD23 in Mo-DCs from asthmatic subjects may also activate monocytes, that in contrast to B cells have been detected in the epithelium and hence may be affected by a local increase in sCD23[241,242]. sCD23-dependent activation of monocytes leads to the production of pro-inflammatory cytokines[393,394] that in turn may augment the allergic inflammation by activating cells present in the asthmatic lung including T cells, eosinophils and mast cells.

7.2.2 Future Directions

Since sCD23 released from DCs in vivo might influence neighbouring cells such as monocytes, macrophages and B cells, leading to perpetuation of allergic inflammation, it will be important to compare the level of sCD23 in culture supernatants from Mo-DCs derived from normal and asthmatic subjects. This may be done by ELISA. It will further be interesting to investigate the level of CD23 transcripts in Mo-DCs from these two subjects groups as this may reveal differences in their response to IL-4 which could explain the difference in level of CD23 seen on these Mo-DCs.

7.3 Chapter 4: The effects of allergic mediators on dendritic cell phenotype and function

In Chapter 4, a primary screen was developed and applied to address the hypothesis that mediators of the allergic asthmatic environment bring about phenotypic and functional changes in DCs resulting in the initiation or perpetuation of the chronic allergic inflammation seen in asthmatic airways. Both Mo-DCs derived from normal and atopic, asthmatic subjects were exposed to MIP-1α, histamine, PGD_2, IgE, IL-3, IL-5, IL-13, TNF-α and IFN-γ to identify individual mediators resulting in a statistically significant or marked change in Mo-DC phenotype or function, for further in-depth investigation of their effects on Mo-DCs and possible relevance in allergic asthma.

Using this experimental approach, significant or marked changes in Mo-DC phenotype or function were identified following treatment with TNF-α, IFN-γ, IgE, IL-3, IL-5 and IL-13. In contrast, only slight effects were observed following treatment with histamine, MIP-1α and PGD_2.

7.3.1 Original observations and interpretation

Whereas several reports of the effects of TNF-α[72,86,395] and a smaller number of reports of the effects of IFN-γ[42,317,396] on DCs have been published, only a very few reports concern the effects of IL-3, IL-5, IL-13, IgE, MIP-1α, histamine and PGD_2 on DC phenotype, cytokine production and ability to drive T cell proliferation.

7.3.1.1 Mediators found to affect monocyte-derived dendritic cells; IL-3, IL-5, IL-13 and IgE

In this study, IL-3 was shown to increase surface expression of all surface markers studied in Mo-DCs from normal subjects without affecting their capacity to drive T cell proliferation. Although similar phenotypic changes have been reported on lymphoid peripheral blood DCs treated with IL-3[42], no studies on the effects of IL-3 on myeloid DCs exist. Hence, these effects of IL-3 on Mo-DCs are novel. Similarly, the increase in surface expression of CD80, CD86 and HLA-DR in Mo-DCs from normal subjects treated with IL-5 was also novel, as no studies of the effects of IL-5 on either lymphoid or myeloid DCs have been reported.

Most reports on the effects of IgE on DCs concern FcεRI-mediated allergen focusing [124,270,271], where DCs were found to present antigen to antigen-specific T cells 10 times more efficiently in the presence of IgE [270]. The increased expression of CD80 and CD86 in Mo-DCs from asthmatic subjects and the decreased expression of CD23 in both Mo-DCs from normal and asthmatic subjects in response to IgE shown here are hence novel.

Most studies of the effects of IL-13 on DCs concern the ability of IL-13 to substitute for IL-4 in the generation of immature Mo-DCs[70,312,397]. However, in a study by Sato *et al.*, IL-13 was reported to increase CD80 expression on Mo-DCs derived from two normal subjects [398], consistent with the marked increase in CD80 expression seen here on Mo-DCs from asthmatic subjects. However, in contrast to the current study, Sato *et. al.* also reported increased surface expression of CD1a, CD86 and HLA-DR. These differences between the two studies are most likely due to the low number of subjects presented by Sato *et. al.*

7.3.1.2 Mediators with no effect on monocyte-derived dendritic cells; MIP-1α, histamine and PGD_2

In agreement with the results of the current study, MIP-1α did not effect receptor-mediated endocytosis of FITC-dextran in Mo-DCs or the capacity of these Mo-DCs to drive the proliferation of allogeneic T cells[300]. However, no data has been reported that can confirm the lack of effect of MIP-1α on DC phenotype or cytokine production seen here. Most reports on MIP-1α and DCs primarily focus on the effects of MIP-1α of DC chemotaxis[96,300,399] and downregulation of the MIP-1α receptor CCR1[139].

At nano molar levels of histamine, no significant or marked changes in Mo-DC phenotype or function were found in the current study. This is consistent with a recent study showing no effects of histamine at this concentration[400]. However, at micro molar levels, histamine was found to ligate H_1- and H_2-receptors on Mo-DCs resulting in increased surface expression of CD40, CD80, CD86, CD49d, CD54 and HLA-DR and induced production of IL-6, IL-8, MCP-1 and MIP-1α, but not IL-10, IL-12 or TNF-α. Mo-DCs treated with micro molar levels of histamine were also shown to significantly enhance the proliferation of memory, but not naïve T cells and have therefore been suggested to play an important role in the activation of T cells during the late-phase allergic response.

A similar scenario was evident for the effects of PGD_2. In agreement with the current study, no effects of PGD_2 applied to Mo-DCs at nano molar levels were found in a recent study [401]. However, at micro molar levels, PGD_2 was found to increase surface expression of CD40, CD83 and HLA-DR on Mo-DCs and to enhance their ability to drive the proliferation of allogeneic T cells. Thus, for both histamine and PGD_2, the choice of concentration at the start of this project was unfortunate. However, due to the limited number of Mo-DCs available for these experiments, it was not feasible to investigate at what concentration each mediator induce a maximal response in Mo-DCs. A single concentration had to be selected which was justified in Chapter 4 (Section 4.1).

7.3.2 Future directions

The statistically significant or marked changes in Mo-DC phenotype and function imposed by TNF-α, IFN-γ, IgE, IL-3 and IL-5 was segregated into four areas of particular interest.

1) The effects of IFN-γ on Mo-DCs.

2) The disappearance of CD23 in response to TNF-α, IFN-γ and 100 nM IgE.
3) The mature Mo-DC phenotype induced by IL-3 and IL-5.
4) The phenotypic differences induced by in particular IL-3, IL-5 and IgE in Mo-DCs from normal and asthmatic subjects.

The effects of IFN-γ on Mo-DCs and the disappearance of CD23 in response to TNF-α, IFN-γ and 100 nM IgE were investigated in more detail in Chapter 5 and Chapter 6, respectively, and are discussed later in this chapter (7.4 and 7.5, respectively). Future directions for the remaining two areas of interest are discussed below.

7.3.2.1 Future directions for the effects of IL-3 and IL-5 on monocyte-derived dendritic cells

In this study, IL-3 was found to significantly increase the expression of CD80, CD83, CD86 and HLA-DR and significantly decrease the expression of CD23 on Mo-DCs from normal subjects without affecting their capacity to drive T cell proliferation. As already mentioned, similar phenotypic changes were reported on human $CD123^+$, lymphoid peripheral blood DCs treated with IL-3[42]. However, these lymphoid DCs contrasted to the myeloid Mo-DCs studied here as IL-3 simultaneously enhanced their survival and their capacity to drive proliferation of allogeneic T cells. Further, the T cells stimulated with IL-3 treated lymphoid DCs produced increased levels of IL-10 and IL-4[42]. Although the IL3-R α-chain has been identified on Mo-DCs[78], no studies on the effects of IL-3 on myeloid DCs have been reported. It will be interesting to extend the current study of IL-3 on Mo-DCs to analyse the effects on T cell differentiation. During the course of this project steps were taken to develop an assay to monitor T cell differentiation (Chapter 4, section 4.4). For future work, this assay would be modified to monitor the cytokines produced by allogeneic $CD4^+$, $CD45RA^+$ T cells primed and re-stimulated with Mo-DCs generated from the same donor. An extended study of the effects of IL-3 on Mo-DCs could also include measurement of DC produced IL-12 p70 that can now be detected in the supernatants of Mo-DCs exposed to CD40L-transfected CHO cells.

IL-5 was found to significantly increase the expression of CD80, CD83 and CD86 on Mo-DCs from normal subjects without affecting their capacity to drive T cell proliferation. No reports exist to confirm these effects of IL-5 on Mo-DCs and the IL-5Rα chain could not be detected on the surface of peripheral blood DCs [42]. It will therefore be important to identify the surface receptor used by IL-5 to mediate its effects on Mo-DCs

before further investigations of the effects of IL-5 on Mo-DCs phenotype and function are conducted according to a similar plan as described for IL-3.

Finally, as DCs in the allergic asthmatic lung would encounter a range of mediators, the effects of simultaneously exposing Mo-DCs to IL-3 and IL-5 would also be a valuable approach in further investigation of their effects on Mo-DC phenotype and function and consequences for allergic asthma.

7.3.2.2 Future directions to study the different responses of monocyte-derived dendritic cells from normal and asthmatic subjects to allergic mediators

By comparing the phenotype and function of Mo-DCs from normal and asthmatic subjects, differences in responsiveness to TNF-α, IFN-γ, IL-3, IL-5 and IgE were evident. Based on these differences it was suggested that Mo-DCs from asthmatic subjects may be prone to perpetuate the allergic asthmatic response (Chapter 4).

Mo-DCs from asthmatic subjects treated with IgE expressed significantly higher levels of CD80 and CD86. This increased capacity for costimulation could act together with FcεRI-mediated antigen presentation to further facilitate allergen presentation and activation of allergen-specific T cells. Mo-DCs from asthmatic subjects also presented a generally less mature phenotype than Mo-DCs from normal subjects when exposed to TNF-α, IFN-γ, IL-3 or IL-5. As discussed in Chapter 4 (4.5.4), it is difficult to predict what effect this phenotype may have on T cell differentiation. It will therefore be important to further extend this study to include the T cell differentiation assay and the IL-12 p70 assay (as suggested in 7.3.1.1) to reveal whether Mo-DCs from asthmatic subjects indeed are more prone to drive a Th2 response when exposed to an allergic environment. In the context, it will also be appropriate to investigate the effects of simultaneously exposing Mo-DCs from normal and asthmatic subjects to in particular, TNF-α, IgE, IL-3 and IL-5.

7.4 Chapter 5: The effects of interferon-γ on monocyte-derived dendritic cells

In Chapter 4, IFN-γ was shown to induce potent maturation of Mo-DCs as assessed by expression of phenotypic markers. However, these IFN-γ treated Mo-DCs did not significantly enhance T cell proliferation. This disparity between the phenotype and function of IFN-γ treated Mo-DCs was investigated further in Chapter 5 with the aim of

identifying possible mechanisms underlying the impaired T cell proliferation seen. From the results presented in Chapter 5, it was suggested that IFN-γ treated Mo-DCs impair T cell proliferation due to;

1) a lower number of viable Mo-DCs surviving during the proliferation assay.
2) induction of TGF-β1 in co-cultures of T cell and IFN-γ treated Mo-DCs which might suppress T cell proliferation.

The interpretations of these findings, in the context of the current literature, are discussed in section 7.4.1. Further experiments that could be conducted to address proposed mechanisms are presented in section 7.4.2.

7.4.1 IFN-γ treated monocyte-derived dendritic cells in the regulation of T cell responses

Although significantly enhancing T cell proliferation above that found for control Mo-DCs, seemingly mature IFN-γ treated Mo-DCs were not as potent at driving T cell proliferation as mature TNF-α treated Mo-DCs. This impaired T cell proliferation may be due to the reduced number of IFN-γ treated Mo-DCs surviving to drive T cell proliferation. As already discussed in Chapter 5 (5.4.3.3), the reduced number of viable IFN-γ treated Mo-DCs present shortly after setting up Mo-DC : T cell co-cultures may be due to ATP- and P2X7-mediated lysis of the Mo-DCs, as has been demonstrated in several other cell types [402-404]. This is supported by the presence of plasma membrane blebbing in the cultures of IFN-γ treated Mo-DCs (data not shown), a typical sign of P2X7 activation. It is also supported by literature showing increased expression of mRNA for P2X7 in response to treatment with IFN-γ, that resulted in increased lysis of macrophages[355] and the monocytic cell line THP-1[354]. Increased concentrations of extracellular ATP may result from cell damage induced by physical handling of the Mo-DCs during harvest at day 8. It is therefore also interesting to note that a reduced number of viable IFN-γ treated Mo-DCs was only noted after re-culture of the cells (Figure 5.8a). Since ATP also has been known to induce apoptosis of thymocytes[405,406], ATP-induced apoptosis may also explain the increased proportion of late apoptotic IFN-γ treated Mo-DCs observed on day 8 + 1 (Figure 5.6b). In summary, IFN-γ may increase the surface expression of P2X7 on Mo-DCs and thereby augment the effects of ATP present at higher concentrations following cell harvest (Figure 7.1). The lower number of viable Mo-DCs present to drive T cell proliferation in co-cultures of T cells and IFN-γ treated Mo-DCs might consequently affect the extent of T cell

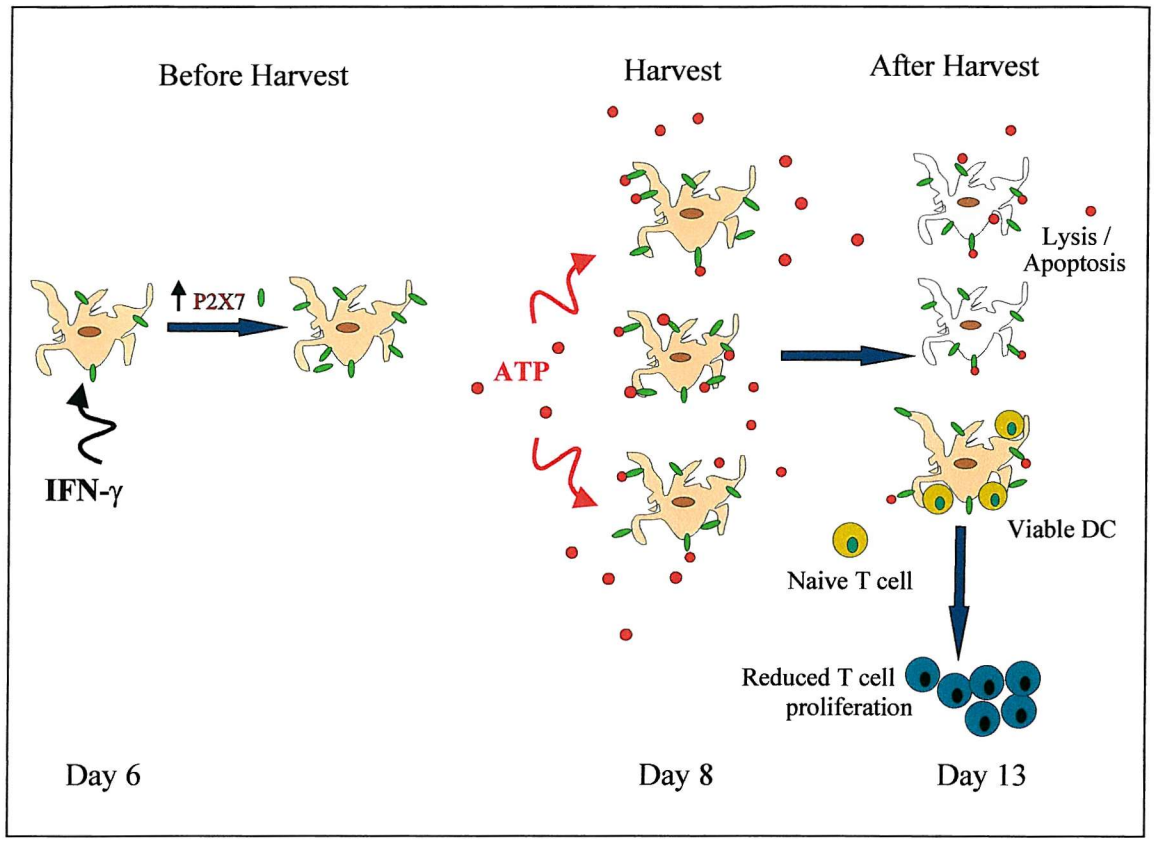


Figure 7.1 Augmentation of ATP-dependent induction of lysis / apoptosis by IFN-γ

proliferation. The proportion of Mo-DCs that survive and drive T cell proliferation may be insensitive to the effects of ATP due to lower surface expression of P2X7. This is supported by the finding that ATP-resistant murine DC clones lack P2X7 expression[402].

The other possible explanation for the impaired T cell proliferation seen in co-cultures of T cells and IFN-γ treated Mo-DCs is the presence of elevated levels of the immunosupporessive cytokine, TGF-β1. As discussed in Chapter 5 (5.4.4.2), the elevated levels of TGF-β1 found in these co-cultures may indicate that IFN-γ modifies Mo-DCs to promote the differentiation of a T cell population resembling Tr1 cells. This is also supported by the finding that the reduced level of proliferation in these co-cultures was not due to increased apoptosis of the T cells (Figure 5.4b) and therefore could be explained by the presence of a less proliferative T cell population, or a T cell population suppressing a second responding T cell population by the production of TGF-β, as has been reported both in mice[358] and man[359]. The production of TGF-β1 in these co-cultures may be regulated via CTLA-4 as shown in mice[358] but could also depend on the relative level of production of IL-12[360] by the Mo-DCs.

The impaired T cell proliferation, induced as a consequence of a reduction in the number of viable DCs or as a consequence of TGF-β1 production, may be viewed in the context of either restriction of an ongoing immune response or active induction of a state of immunological tolerance. The difference between these two mechanisms is not clear-cut and reports suggesting tolerance induction can often be reinterpreted as restriction of an immune response and vice versa. Whether it is by restricting an ongoing immune response or by active induction of a state of tolerance, other reports support the finding made here that IFN-γ treated Mo-DCs impair T cell responses. For example, spleen DCs treated with IFN-γ *ex vivo* were shown to delay the onset of IDDM when transferred into 1- or 4-week old NOD mice[329]. Further, murine DCs treated with IFN-γ [326] and human DCs derived in the presence of IFN-γ [317], were shown to drive impaired proliferation of allogeneic T cells *in vitro*, despite signs of maturation. Another way in which IFN-γ may act on DCs to downregulate T cell responses is by induction of PD-L1 or PD-L2, which was recently shown to occur on DCs[21] and monocytes[22] in response to IFN-γ. As reviewed in section 1.2.2.3.1, binding of PD-L1 or PD-L2 to PD-1, expressed on activated T cells, completely inhibits the proliferation of T cells at optimal CD3-stimulation [21]. Based on this and the fact that PD-1-deficient mice develop an autoimmune type of disease, PD-1 has been suggested to be involved in tolerance induction [22]. However, the PD-1-mediated effects on T cells may also be regarded as a route for restricting an ongoing T cell response.

If the effects of IFN-γ seen in the current study and those reviewed above are indeed associated with active induction of a state of T cell tolerance, this contrasts with recent reports suggesting a prominent role for immature, but not mature Mo-DCs in tolerance induction via the differentiation of regulatory T cells [328,407,408]. As no other studies have reported the effects of IFN-γ on monocytes that have already converted to Mo-DCs, it is possible that the mature IFN-γ treated Mo-DCs shown here are qualitatively different to DCs matured by LPS, TNF-α or IL-1β and have a distinct role in active tolerance induction. However, it is also possible that the length of exposure to IFN-γ is a critical factor determining the resulting T cell response. This is supported by the finding that "active" Mo-DCs matured with LPS or TNF-α for 8 hours produced IL-12 p70 and induced differentiation of Th1 cells whereas "exhausted" Mo-DCs matured for longer periods of time (24 to 48 hours) did not produce IL-12 p70 and induced differentiation of Th2 cells or non-polarised T cells[153]. Hence, shortly after initial exposure to IFN-γ, Mo-DCs may induce a different population of T helper cells than after prolonged exposure to IFN-γ. It is further interesting to note that it is only at the early stages of Mo-DC maturation that IFN-γ may enhance the capacity of the Mo-DC to produce IL-12 and drive Th1 differentiation when co-cultured with T cells[176,396]. DCs matured by IFN-γ may hence drive Th1 differentiation at early time points but restrict T cell responses at later time points, as seen here. During an anti-viral immune response, DCs exposed to IFN-γ may therefore initially promote Th1 development however following prolonged exposure to IFN-γ, DCs may serve to restrict the immune response in order to avoid fatal systemic inflammation.

7.4.2 Future Directions

Based on the observations made in Chapter 5 and ideas obtained from the literature, several lines for future experiments can be proposed which are summarised in Table 7.1.

Table 7.1 Summary of future directions leading on from Chapter 5

Aim	Justification	Approach
1. To determine the source of TGF-β1 in the co-cultures[+]	TGF-β1 can be produced by T cells with regulatory properties (Tr1)..	Intracellular staining for flow cytometry.
2. To determine if the impaired T cell proliferation is TGF-β1-dependent.	TGF-β-dependent suppression of T cells has been reported in mice[358]and human[359].	Blocking the effects of TGF-β1 using neutralising mAb (neut-mAb) in co-cultures[+].
3. Investigate the involvement of CTLA-4 in regulating the production of TGF-β in the co-cultures[+].	CTLA-4 has been found to regulate TGF-β production in mice[358].	Blocking the effects of CTLA-4 using neut-mAb in co-cultures[+].
4. To monitor the presence of IL-10 in the co-cultures[+].	Tr1-like population producing IL-10 exist [409].	ELISA.
5. To determine if the impaired T cell proliferation is IL-10-dependent.	Dependent on the result in 4.	Blocking the effects of IL-10 using neut-mAb.
6. To determine the level of PD-L1 and PD-L2 expressed on IFN-γ treated Mo-DCs.	If induced by IFN-γ, PD-L1 and PD-L2 may impairing T cell proliferation as suggested by literature[21,23].	Flow cytometry.
7. To determine if the impaired T cell proliferation is PD-L1- and PD-L2-dependent.	Depending on the results in 6.	Blocking PD-L1 and PD-L2 using neut-mAb.
8. To determine if IFN-γ affects the expression of P2X receptors in Mo-DCs.	Possible involvement of ATP and P2X7 in induction of lysis and apoptosis in DC cell lines[402].	TaqMan RT-PCR.
9. To determine if the impaired T cell proliferation is ATP-dependent.	As in 8.	Irreversibly blocking P2X receptors using oxidised ATP in co-cultures[+].[402].
10. Study the kinetics of IL-12 p70 production in response to IFN-γ	"Active" Mo-DCs produce IL-12 p70 and drive Th1, "exhausted" Mo-DCs do not produce IL-12 p70 drive Th2 cells or non-polarised T cells[153].	Measure IL-12 p70 in supernatants of Mo-DC and CD40L-transfected CHO cell co-cultures by ELISA.

[+] refers to the co-cultures of IFN-γ treated Mo-DCs and T cells.

7.5 Chapter 6: The mechanisms underlying the disappearance of CD23 from the surface of treated monocyte-derived dendritic cells

In Chapter 4, one of the most dramatic changes in Mo-DC phenotype was the significant reduction in surface expression of CD23 in response to TNF-α, IFN-γ and IgE at 100 nM. However, the mechanisms by which CD23 disappeared from the cell surface in response to these mediators were not clear. In Chapter 6, several possible mechanisms underlying this disappearance were explored. By investigating the kinetics of CD23 disappearance, CD23 internalisation, CD23 gene transcription, the cellular distribution of CD23 and shedding of sCD23, a picture emerged indicating a dynamic network of mechanisms that interact to regulate the rate of appearance and disappearance of CD23 on the surface of Mo-DCs and the effects of TNF-α, IFN-γ and 100 nM IgE on this balance.

7.5.1 Original observations and interpretation

Although surface expression of CD23 has been shown to be regulated by IL-4 in human peripheral blood DCs[367] and human LCs[375], this is the first detailed study of the mechanisms underlying surface expression of CD23 in DCs and the effects of TNF-α, IFN-γ and IgE on CD23 surface expression. Untreated Mo-DCs were shown to continually express new CD23 on their surface, however, kept a constant level of surface expressed CD23 by removing a proportion of the CD23 from the surface by internalisation as well as shedding of sCD23 into the culture supernatant. In response to TNF-α or IFN-γ, surface expressed CD23 was downregulated due to a reduction in synthesis of new CD23 and increased shedding of sCD23. However, following treatment with 100 nM IgE, CD23 was downregulated much more rapidly than was observed following exposure to TNF-α or IFN-γ. This was suggested to be due to rapid internalisation of surface expressed CD23 and efficient inhibition of exposure of new CD23 at the cell surface. Moreover, 100 nM IgE inhibited the release of sCD23.

Although few, $CD23^+$ dendritic cell populations have been reported *in vivo* [389,390], as already mentioned (7.2.1). It is therefore appropriate to consider the consequences of $CD23^+$ DCs being exposed to TNF-α, IFN-γ or high concentration of IgE *in vivo*. It can be assumed that following exposure to these mediators, DCs will express negligible levels of CD23 on their surface. This is however, part of the natural progress of DC-switching from an antigen capturing to an antigen presenting stage during their maturation, and may not significantly

affect the function of the DCs exposed to TNF-α or IFN-γ. In contrast, due to the rapid internalisation of CD23 on DCs exposed to high concentrations of IgE, the DC may be unable to take up allergens via allergen-specific IgE and CD23. It is possible that this inability is transient and that the DCs will regain their expression of CD23 after a period in the absence of IgE. However, it is very likely that, as a consequence of an ongoing allergic response, high concentrations of IgE *in vivo* will be present in combination with inflammatory stimuli, leading to simultaneous DC maturation and migration to the regional lymph nodes. The rapid internalisation of CD23 in response to IgE contrasts to FcεRI that may be stabilised at the cell surface by IgE. FcεRI is internalised first following cross-linking as a consequence of allergen capturing by IgE. Internalisation of IgE bound to CD23 expressed on DCs may hence have a role in decreasing the local concentration of IgE leading to restriction of the immediate-phase allergic response.

The exposure of $CD23^+$ DCs to TNF-α, IFN-γ or high concentrations of IgE *in vivo* may also affect surrounding cells. Soluble CD23 has been shown to activate monocytes[393,394], resulting in their production of pro-inflammatory mediators including TNF-α, GM-CSF, IL-1α, IL-1β, IL-6, IL-8 and PGE_2. By increasing the shedding of sCD23 from the surface of DCs and consequently activating monocytes, TNF-α and IFN-γ may boost the immune response in the inflamed tissue, such as asthmatic lung. Soluble CD23 may also participate in the regulation of production of IgE by B cells, as shown in mice[280 281] and man [281,282,410]. However, sCD23 released by DCs in the lung may not significantly affect IgE production by B cells, as lung DCs are a trace population that most likely only increase the sCD23 concentration locally without affecting the systemic concentration of sCD23. Further, as already mentioned (7.2.1), no B cells have been identified in the lung epithelium where most lung DCs reside[241,242]. In germinal centres, where regulation of B cell IgE production is more likely to occur, the DCs are furthermore unlikely to express CD23 or release sCD23 due to their maturation state.

It should also be noted that different size fragments of sCD23 have different activities. For example, the 29 kD fragment enhanced, whereas the 16kD fragment inhibited IgE production[112,411]. As cleavage of sCD23 is mediated via a yet unidentified metalloprotease or metalloproteases[364,412,413], it is possible that specific proteases produce sCD23 fragments of a definite size. Activation of different metalloproteases by TNF-α and IFN-γ could thus result in preferential production of a specific size of sCD23 fragment. Consequently, exposure of DCs to TNF-α and IFN-γ may lead to different biological outcomes.

7.5.2 Future Directions

In Chapter 6, experiments were undertaken to monitor CD23 internalisation. This internalisation assay will need to be optimised for further investigation of the effects of TNF-α, IFN-γ and 100 nM IgE on the regulation of CD23 in Mo-DCs. In particular, it is not clear if the reduction in over all BU38-FITC intensity over time is due to degradation or quenching in intracellular organelles, detachment of FITC from BU38 bound to surface expressed CD23 or a result of shedding of BU38-labelled CD23. It may therefore be better to detect internalised BU38-labelled CD23 by staining trypsin-treated Mo-DCs for the intracellular detection of BU38 using the PE conjugated secondary GAM antibody. To detect BU38-labelled CD23 by the secondary antibody, it may prove necessary to inhibit CD23 degradation using chloroquine. However, this may affect the rate of internalisation of CD23, as in preliminary experiments chloroquine activated Mo-DCs, evident by increased surface expression of HLA-DR (data not shown).

In light of the difference in CD23 surface expression between Mo-DCs from normal and asthmatic subjects observed in Chapter 3, and the potential role for sCD23 in monocyte activation and IgE production *in vivo*, it will further be interesting to compare the extent of sCD23 shedding in Mo-DCs from normal and asthmatic subjects in response to TNF-α, IFN-γ and 100 nM IgE. This may be done by measuring the sCD23 content in the culture supernatants by ELISA.

7.6 Modification of dendritic cell phenotype and function: consequences for allergic asthma

During this project the dendritic cell has been studied with one aim being to identify ways by which it may impact on allergic asthma. This possible contribution of DCs to allergic asthma was particularly relevant in the work conducted in Chapter 3, where Mo-DCs from normal and asthmatic subjects were compared, and in Chapter 4, in which the effects of allergic mediators on Mo-DCs were studied. Although the focus in subsequent chapters moved towards a more mechanistic analysis of how DCs respond to particular mediators in their environment, it is possible to interpret some of the observations made on Mo-DCs in the context of allergic asthma.

In Chapter 3, DCs derived from asthmatic subjects demonstrated a phenotype with significantly lower surface levels of CD23. If this reduction is found to result from increased release of sCD23, there is a possibility that DCs in the asthmatic lung are more

competent at perpetuating the allergic, asthmatic inflammation by activating monocytes and increasing the production of IgE, hence augmenting both the immediate- and late-phase allergic response (Figure 7.2a). By expressing lower levels of CD23, lung DCs in asthmatic subjects may also be less competent at clearing extracellular IgE than lung DCs in normal subjects, resulting in higher concentrations of IgE present in the asthmatic lung for binding to FcεRI expressing effector cells such as mast cells and basophils and lung DCs. In the presence of allergen, this would augment the immediate-phase allergic response and also the late-phase response via the lung DCs.

Similarly, high concentrations of IgE and TNF-α present at elevated levels in the allergic asthmatic lung, may affect the level of sCD23 released by lung DCs as suggested by findings in Chapter 6. Whereas high concentrations of IgE are predicted to reduce the levels of sCD23 released by lung DCs and possibly thereby restrict the immediate-phase response by reducing the production of IgE, lung DCs exposed to TNF-α may intensify the immediate-phase response by increasing the production of IgE but primarily perpetuate the late-phase allergic reaction by boosting the inflammatory response through activation of monocytes (Figure 7.2b). CD23 expressed on lung DCs may also serve as a route for antigen capturing in the presence of IgE. In this context the simultaneous presence of elevated levels of IgE and TNF-α in the asthmatic lung would enhance antigen presentation to allergen-specific T cells, thereby boosting the late-phase response.

The increased expression of CD80 and CD86 provoked by IgE in Mo-DCs from asthmatic subjects (Chapter 4) may suggest that DCs in the asthmatic lung are better suited for antigen presentation of allergens taken up via IgE (Figure 7.2c). These DCs would consequently intensify the late-phase response by activating allergen specific Th2 cells. Further lung DCs in asthmatic subjects exposed to elevated levels of IL-3 and IL-5, may not respond as much as DCs from normal individuals, giving a "semi-mature" phenotype that may drive the differentiation of T helper populations that support allergic inflammation. As discussed in section 1.5.1, all infants are born with Th2-skewed immunity. However, most infants develop a normal Th1 response to antigens postnatally. As atopic asthma is characterised by a continued Th2 response, it can be argued that the lung DCs of infants that later develop asthma are not able to switch to a phenotype capable of driving Th1 differentiation. In Chapter 4 it was found that Mo-DCs from asthmatic subjects treated with IFN-γ expressed a less mature phenotype (significantly lower levels of CD83) and did not enhance T cell proliferation to the same extent as Mo-DCs from normal subjects. It is therefore possible that lung DCs in infants that go on to develop asthma are prone to initiate

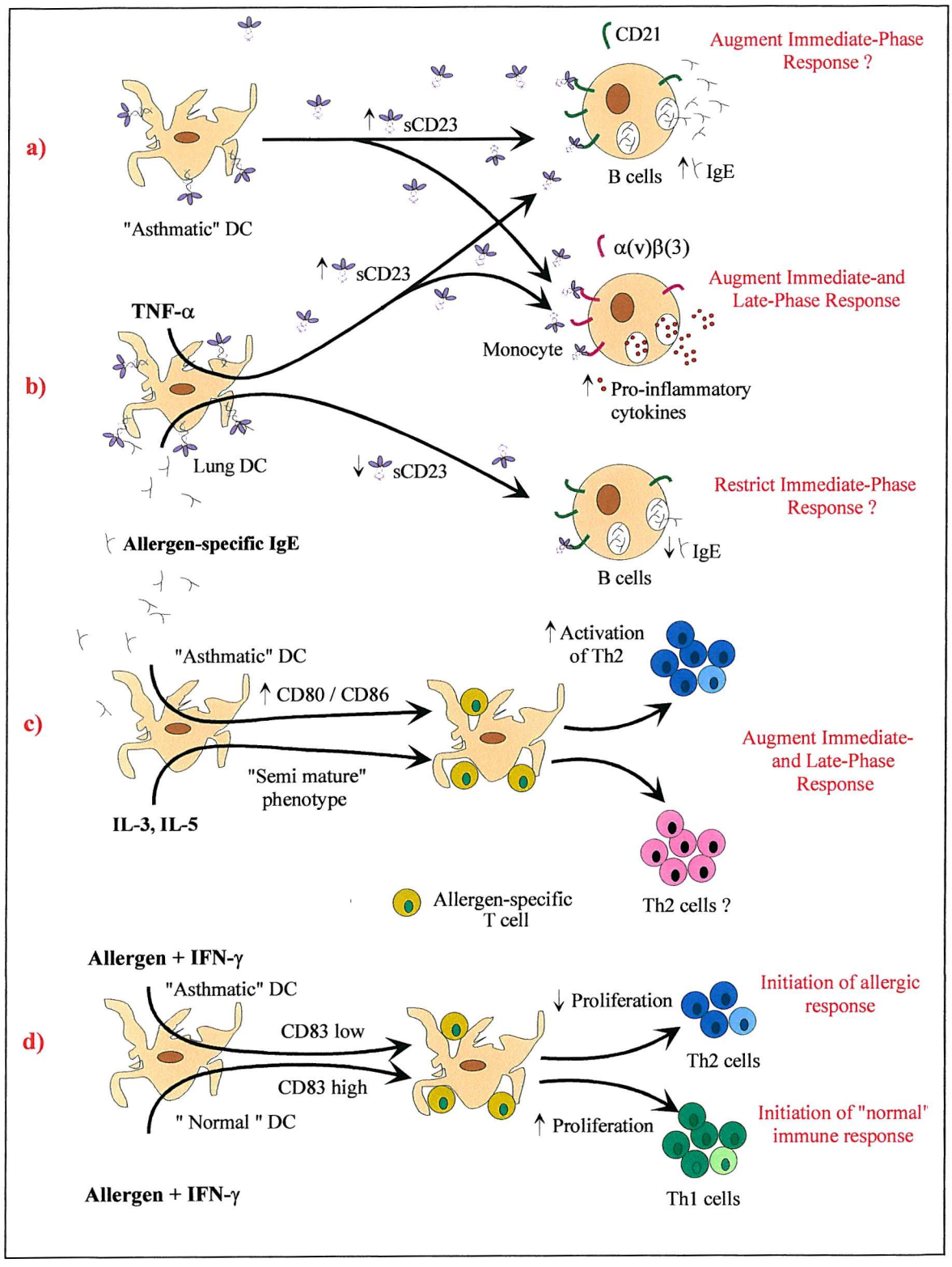


Figure 7.2 Some of the possible ways by which DCs may influence the allergic asthmatic response.

allergic inflammation by not having the capacity to switch Th2 response to a Th1 response in response to IFN-γ present during early-life infection (Figure 7.2d).

7.6.1 Conclusion to the project

The work presented in this thesis supports a role for the DC in allergic asthma. The work has demonstrated that the DC phenotype changes in response to mediators typically elevated at sites of inflammation in the asthmatic airways. In may cases, these changes act to increase T cell activation which may overcome the normal state of T cell non-responsiveness in the healthy lung, thus leading to the increased T cell activation seen in allergic asthmatic airways. Further work will be required to determine whether these phenotypic changes in DCs exposed to an allergic microenvironment result in preferential differentiation of Th2 cells. A second important finding of this study is that DCs from normal and asthmatic subjects were different with respect to their CD23 expression and responded differently to allergic mediators. Thus, DCs in asthmatic subjects may be more prone to perpetuate the allergic inflammation than DCs in normal subjects. This predicted nature of lung DCs in asthmatic subjects may partly be responsible for the well-established Th2 inflammation seen in the asthmatic lung. Future work may confirm these predictions by investigating the nature of the T cell response resulting from the interaction with mediator-treated DCs from both normal and asthmatic subjects. Hence, the work presented in this thesis may serve as a staring point for further investigations of the mechanisms underlying the proposed role of DCs in allergic asthma. As DCs have a central role in initiating and maintaining immune responses, the work presented in this thesis supports the DC as a target for future therapeutic inventions aimed at eliminating or reducing the symptoms associated with the immediate- and late-phase response as experienced by atopic asthmatic individuals.

Chapter Eight

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