MODULATION OF DENDRITIC CELLS BY HUMAN NEUTROPHIL ELASTASE AND ITS INHIBITORS IN PULMONARY INFLAMMATION

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DECLARATION

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree or professional qualification. All work presented in this thesis was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

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2007

ABSTRACT

Dendritic cells (DC) are sentinels of the immune system that display an extraordinary capacity to present antigen to naïve T cells and initiate immune responses. DCs are distributed throughout the lungs in the conducting airways of the tracheobronchial tree and in the parenchymal lung, and play a pivotal role in controlling the immune response to inhaled antigens. The respiratory surface is continually exposed to potentially injurious particulates and pathogenic organisms, to which tightly regulated innate and adaptive immunological responses are made. The airways are usually sterile in healthy individuals. However, patients with chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) have increased susceptibility to microbial infections and increased neutrophil elastase (NE) in lung secretions.

This thesis was designed to test the hypotheses that; (*i*) excess NE may result in a dysregulation of lung DCs function in pulmonary chronic diseases, and (*ii*) the natural NE inhibitors in the respiratory system are able to rescue the NE-mediated dysregulation of DCs and potentially enhance their antigen presenting activity.

The data in this thesis demonstrate that purified human NE down-regulated murine bone marrow (BM)-derived DC co-stimulatory molecules (CSM; CD40, CD80 and CD86), which was due to its proteolytic activity. NE-treated LPS-matured DCs were less efficient at presenting ovalbumin (OVA) peptide to naïve OVA-specific transgenic (D011.10) T cells. In addition, immature DCs (iDC) simultaneously treated with LPS and NE failed to mature fully and produced significantly less IL-12 and TNF-α than DCs matured in the presence of LPS alone. Similarly, treatment of mature DC (mDC) with pooled and individual COPD and CF sputum samples caused a reduction in CD80 and CD86 levels (but not CD40) which positively correlated with the NE concentration present in the samples.

The demonstration that NE could adversely affect DC phenotype and function suggested that augmentation of NE inhibitors could reverse this process and

preserve DC function in inflammatory microenvironments. Over-expression of an NE specific inhibitor (elafin) in the lungs of mice (using either replication-deficient adenovirus [Ad] or elafin transgenic [eTg] mice) increased the number (immunofluorescence) and activation status (flow cytometric measurement) of $CD11c^{+}/MHCII^{+}$ lung DCs in *in vivo* models. Replication-deficient Ad vectors encoding NE inhibitors, namely elafin, secretory leukocyte protease inhibitor (SLPI) and α1-protease inhibitor (α1-PI), were also used to infect DCs *in vitro*, to further study the effect of these NE-inhibitors on DCs in isolation.

These findings suggest that purified NE and NE-containing lung inflammatory secretions are powerful down-regulators of DC maturation, resulting in reduced capacity of these potent APCs to efficiently present antigens; whereas, NE inhibitors could boost immunity by increasing the activation state and/or number of DCs.

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DEDICATION

To my dear family,

And my fiancée Mina

ABBREVIATIONS

aa	Amino acid
Ab	Antibody
Ad	Adenovirus
Ad-α1PI	Adenovirus encoding murine α1-PI cDNA
Ad-elafin	Adenovirus encoding human elafin cDNA
Ad-eotaxin	Adenovirus encoding murine eotaxin cDNA
Ad-GFP	Adenovirus encoding green fluorescent protein gene
Ad-GMCSF	Adenovirus encoding murine GM-CSF cDNA
Ad-LacZ	Adenovirus encoding the lacZ gene
Ad-SLPI/GFP	Adenovirus encoding GFP gene and murine SLPI cDNA
ALI	Acute lung injury
ASL	Airway surface liquid
AM	Alveolar macrophage
APC	Antigen presenting cell
Arm. Ham.	Armenian hamster
BAL	Bronchoalveolar lavage
B. cenocepacia	*Burkholderia cenocepacia*
B.c.	*Burkholderia cepacia*
BSA	Bovine serum albumin
BM	Bone marrow
BM-DC	Bone marrow-derived dendritic cell
B. multivorans	*Burkholderia multivorans*
bp	Base pair
cALL	Common acute lymphoblastic leukaemia
CAR	Coxsackievirus Ad receptor
CD	Cluster of differentiation
CD40L	CD40 ligand
cDNA	Complementary deoxyribonucleic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator

CMV	Cytomegalovirus
ConA	Concanavalin A
COPD	Chronic obstructive pulmonary disease
CSM	Co-stimulatory molecules
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
E.coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
Erk	Extra-cellular signal receptor regulated kinase
eTg	Elafin transgenic
FACS	Fluorescence-activated cell sorter
FEV_1	Force expiratory $volume_1$
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Flt3L	Fms-like tyrosine kinase 3 ligand
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
FVC	Force vital capacity
g	Gravitational force
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
GSH	Glutathione
H. influenza	*Haemophilus influenza*
HBD	Human β-defensin
HD	Human defensin

HSP	Heat shock protein
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
IκB	Inhibitor of κB
ICAM	Inter-cellular adhesion molecule
ICOS	Inducible co-stimulator
ICOS-L	Inducible co-stimulator ligand
iDC	Immature DC
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IKK	Inhibitor of κB kinase
IL	Interleukin
IRAK	Interluekin-1 receptors-associated kinase
IRF-3	Interferon-regulatory factor-3
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
kDa	Kilodaltons
LC	Langerhans cell
LN	Lymph nodes
LPS	Lipopolysaccharide
LTB_4	Leukotrine B4
mAb	Monoclonal antibody
MACS	Magnetic cell sorting
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MCP	Monoctye chemotactic protein
M-CSF	Macrophage–colony stimulating factor
mDC	Mature DC
MDC	Monophage derived chemokine
MFI	Mean fluorescence intensity
mg	Milligram

MHC	Major histocompatibility complex
MIIC	MHC II–rich compartment
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inhibitory protein
ml	Millilitre
MOI	Multiplicity of infection
Mtb39	*Mycobacterium tuberculosis* antigen
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor κB
NK	Natural killer cell
ng	Nanogran
NTHI	Non-typeable *Haemophilus influenzae*
P.a.	*Pseudomonas aeroginosa*
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
pDC	Plasmacytoid DC
PD-L	Programmed death ligand
PE	Phycoerythrin
pg	Picogram
PGE2	Prostaglandin E_2
PIT	Protease inhibitor cocktail tablet
PI	Phosphoinositide
PMN	Polymorphonuclear leukocytes
PSR	Phosphatidylserine receptor
RANK	Receptor activator of NF-κB
RANTES	Regulated on activation normal T cell expressed and secreted
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
S.a.	*Staphylococcus aureus*
SAC	*Staphylococcus aureus* (Cowan strain)
SCF	Stem cell factor

SD	Standard deviation
SDF	Stromal derived factor
SDS	Sodium dodeocyl sulphate
Serpin	Serine protease inhibitor
SLAM	Signalling lymphocyte activation molecule
SLC	Secondary lymphoid-tissue chemokine
SLPI	Secretory leukocyte protease inhibitor
SOD	Superoxide dismutase
Sup.	Supernatant
TARC	Thymus and activation-regulated chemokine
TBS	Tris buffered saline
TBS-T	Tris buffered saline containing Tween-20
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRANCE	TNF-related activation induced cytokine
Treg	Regulator T cell
TRIF	Toll–IL-1 receptor domain-containing adaptor IFN-β
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor
WAP	Whey acid protein
WT	Wild-type
°C	Degrees centigrade
µg	Microgram
µm	Micromolar

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CHAPTER 1
INTRODUCTION

1.1. OVERVIEW

The lung possesses the largest surface area of the body which is in contact with the external environment in order to facilitate efficient gaseous exchange. As normal respiration occurs, the upper and lower airways are repeatedly exposed to a multitude of airborne particles and microorganisams. Since these agents are frequently deposited on the surface of the respiratory tract, an elaborate system of defence mechanisms is present to maintain the sterility of the lung. Innate defences are primarily responsible for the elimination of bacterial organisms from the respiratory tracts and alveoli. Early bacterial clearance is mediated by a dual phagocytic system involving both alveolar macrophages (AM) and DCs, and neutrophils. DCs are of particular importance, playing a central role in initiating protective T cell immune responses within the respiratory tract due to their potent ability to present antigen. The airways and lungs contain a rich network of DCs that are localised near the surface, so that they are ideally located to signal the entry of foreign substances that are inhaled. As a result of these highly co-ordinated defence systems, the airways are usually sterile and non-inflamed in healthy individuals. Conversely, continuous infection episodes are a feature of lung diseases such as COPD and CF. Frequent bacterial colonisations and cigarette smoking are instrumental in the generation of COPD and CF disease exacerbations. Consequently, the lungs of both COPD and CF patients are highly inflamed and are characterised by intense neutrophilic inflammation and the release of high levels of cytotoxic molecules, such as NE, that overwhelm the natural NE inhibitors and innate immune defences.

The work described in this thesis was therefore initiated by the following questions: Firstly, are cigarette smoke (CS) and COPD/CF lung inflammatory secretions (containing high concentrations of NE) able to influence the behaviour or phenotype of DCs in such a way as to inhibit their ability to present antigens and, thus, initiate protective immunity to pathogens? If so, can genetic augmentation of

natural NE inhibitors influence the activation status, function and/or number of lung DCs?

Against this background, the following section will provide a brief overview of DCs, particularly the respiratory tract DCs. This section will be followed by a discussion of the CF and COPD lung diseases, with special emphasis on the influence of infections on their development and pathologies, before going on to discuss the effects of CS on the innate and adaptive immune systems of the respiratory system. Finally, NE and endogenous NE inhibitors will be described in the latter part of this chapter.

1.2. DENDRITIC CELLS (DC)

1.2.1. DC overview

Optimal defence against the diverse types of microorganisams that invade our body requires specialised classes of non-specific and antigen-specific immune responses. The innate immune system includes phagocytic cells, natural killer (NK) cells, complement factors, and interferons (IFN). Cells of the innate immune system use a variety of pattern recognition receptors to recognise patterns shared between pathogens, for instance bacterial lipopolycaccharide (LPS), carbohydrates, and double-stranded viral RNA (Muzio *et al*., 1998; Brighthill *et al*., 1999; Aliprantis *et al*. 1999). As a result of evolutionary pressure, adaptive immunity has developed alongside the innate immune system. The key features of adaptive immune system are (*i*) the ability to rearrange genes of immunoglobulin (Ig) family, permitting creation of a large diversity of antigen-specific clones, and (*ii*) immunological memory. However this highly sophisticated and potent system needs to be instructed and regulated by antigen-presenting cells (APC). DCs are bone marrow (BM)-derived leukocytes and are the most potent type of APCs. They are responsible for the initiation of immune responses and hence function as the 'sentinels' of the immune system (Banchereau and Steinman, 1998). Paul Langerhans first described DCs in human skin but thought these were cutaneous nerve cells (Langerhans, 1868). Steinman and Cohn (1973) discovered these cells almost a century later in mouse spleen and applied the term 'dendritic cells' based on their unique morphology. DCs generally have a low buoyant density and are initially adherent to plastic but then readily detach (Steinman *et al*., 1979). In recent years, DCs have been increasingly studied for their role as critical adjuvants in vaccines for preventing microbial infection and allograft rejection and treatment of cancer and autoimmune diseases (see below).

Various methods have been employed for isolation of rare DC populations from tissues. Early methods used enzymes such as collagenases to obtain enriched populations of DCs from tissues. Selection of low-buoyant-density cells enriches for mononuclear cells, and the adherence step helps eliminate T cells and B cells from

the preparation. Further enrichment of DCs utilises various combinations of additional negative and positive selection steps. One negative selection step eliminates contaminating B cells, T cells, and NK cells from the preparation by a combination of immunophenotyping/immunomagnetic methods and cell sorting. Positive selection steps use various combinations of monoclonal antibodies (Ab) to isolate cells expressing important DC cell surface markers. With the use of multiple enrichment steps, pure populations of tissue-derived DCs have been obtained (Lipscomb and Masten, 2002). DCs can also be propagated *in vitro* from BM and blood using various combinations of growth factors, such as granulocyte macrophage-colony stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α), interleukin (IL)-4, stem cell factor (SCF), transforming growth factor-β (TGF-β), IL-3, and Fms-like tyrosine kinase 3 ligand (Flt3L) (Caux *et al*., 1992; Inaba *et al*., 1992; Strobl *et al*., 1997; Zhang *et al*., 1999). GM-CSF in combination with IL-4 or TNF-α and other cytokines provide important growth factors for interstitial DCs and Langerhans cells (LC). In addition, LCs also require TGF-β for their differentiation (Strobl *et al*., 1997). Flt3L has been used to stimulate the proliferation of stem cells and progenitor cells *in vitro* and expand and mobilise all DCs and their progenitors *in vivo* (Masten *et al*., 2004; Maraskovsky *et al*., 1996 and 2000).

Since DCs have numerous cytoplasmic processes giving rise to their stellate appearance, they have a high surface area permitting intimate contact with a large number of surrounding cells, *e.g.*, T cells, NK cells, neutrophils, epithelial cells etc. Consequently, *in vitro* DCs form large spherical aggregates with lymphocytes, and experimentally, only one mDC is required to stimulate 100-3000 T cells. DC precursors migrate form the BM through the blood stream to almost every tissue, where they eventually become resident iDCs. LCs in the epidermis are the best studied example of iDCs, which show a high ability for antigen uptake but a low capacity for antigen presentation. Immature DCs possess an array of mechanisms for sampling antigen. These include macropinocytosis, where fluid from the extra-cellular milieu is taken up into pinocytic vesicles and antigen is concentrated by expelling excess water via channels called aquaporins (Norbury *et al*., 1997). DCs also express a repertoire of receptors for efficient receptor-mediated antigen uptake. Additional receptors expressed by DCs include FcγRII (CD32), FcγRI (CD64)

(Fanger *et al*., 1996), FcεRI (Maurer *et al*., 1998) and the C3bi complement receptors (CD11b) (Green *et al*., 1980) which increase the efficiency of immune complex endocytosis.

During pathogen invasion, resident iDCs capture intruder antigens, and quickly leave the tissue (Macatonia *et al*., 1986). They crawl through the cells, cross the endothelium of lymphatic vessels and migrate to the draining lymph nodes (LN; Del Prete *et al*., 2006). During their migration from the peripheral tissues, DCs undergo phenotypical and functional maturation. Most remarkably, they stop capturing antigens while up-regulating the expression of co-stimulatory molecules (CSM). After reaching the subcapsular sinus of the LN, DCs move to the areas (T cell zones) through which T cells, recruited from the blood, percolate. Here, the interdigitating DCs are actively involved in the presentation of antigens to T cells (Figure 1.1). The presentation of antigen to the appropriate T cells seems to be the ultimate mission of the DCs recruited from the periphery, as most of them disappear in the T cell areas, most likely by apoptosis.

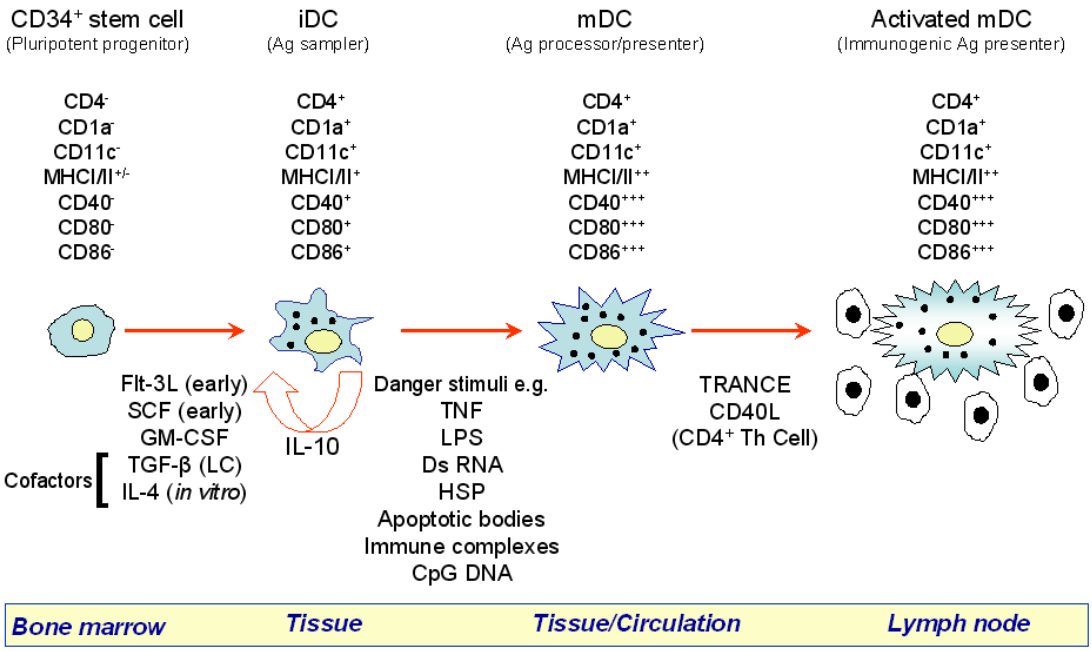


Figure 1.1. Dendritic cell life history

DC life history can be subdivided into a number of phases, all with discrete cellular functionality. Transition between phases is mediated by diverse signals and is accompanied by changes in expression patterns of many surface markers and secreted factors. SCF, stem cell factor; GM-CSF, granulocyte–macrophage colony stimulating factor; TGF, transforming growth factor; HSP, heat shock protein. TRANCE, tumour necrosis factor-related activation-induced cytokine. Adapted from Stockwin *et al.* (2000).

1.2.2. DCs innate receptor repertoire

The functional properties of DCs, as mentioned above, include their ability to sense the environment (*i.e.*, respond to environmental factors such as cytokines) and to sample microbes (*i.e.*, phagocytose microbes) at the mucosal sites. To exert these sampling and sensing functional activities, DCs have a broad innate receptor repertoire for the recognition of infectious non-self antigens (Janeway Jr and Medzhitov, 2002). In the mouse, this receptor repertoire includes the expression of Toll-like receptor (TLR) family members that bind a variety of microbial ligands (Takeda and Akira, 2005). Signalling through these receptors is mediated either via the myeloid differentiation factor-88 (MyD88) adaptor molecule, or via an independent pathway involving the Toll–IL-1 receptor domain-containing adaptor IFN-β (TRIF) molecule and the IFN regulatory factor-3 (IRF-3) transcription factor (Beutler, 2004). As a result of microbial activation through TLRs, the nuclear factor-kappa B (NF-κB) family members are activated and translocate to the nucleus (Hofer *et al*., 2001), where they induce the transcription of many NF-κB-dependent genes-mostly immune and inflammatory genes encoding cytokines and chemokines. In addition, microbial activation might lead to the transcription of the IFN-inducible genes via IRF-3 (Trottein *et al*., 2004). DCs can also display surface molecules such as the macrophage receptor with collagenous structure (MARCO) receptor, which has been shown to be involved in significant remodelling of the actin cytoskeleton (Granucci *et al*., 2003). Other receptors expressed by DCs are DC-specific ICAM (intra-cellular adhesion molecule)-3 grabbing non-integrin (DC-SIGN) and C-type lectin receptors, which are capable of binding a variety of microorganisams, including viruses, such as *HIV*, and pathogenic bacteria, such as *Mycobacterium tuberculosis* (van Kooyk and Geijtenbeek, 2003; Tailleux *et al*., 2003).

1.2.3. Antigen processing by DCs

Soluble and particulate antigens are efficiently captured by iDCs and targeted to major histocompatibility molecule class II (MHCII) compartments (Tan *et al*., 1997; Inaba *et al*., 1997 and 1998). Immature DCs constantly accumulate MHCII molecules in lysosome-related intra-cellular compartments identified as MHCII-rich

compartments (MIIC), with multivesicular and multilamelar structure (Nijman *et al*., 1995; Kleijmeer *et al*., 1995). Whereas, in iDCs, class II molecules are rapidly internalised and have a short half-life, maturation/inflammatory stimuli lead to a burst of MHCII synthesis and translocation of the MHCII-peptide complexes to the cell surface where they remain stable for days and are available for recognition by $CD4^+$ T cells (Inaba *et al*., 1997; Cella *et al*., 1997; Pierre *et al*., 1997). To generate $CD8^+$ cytotoxic killer T cells, DCs present antigenic peptides on MHC class I (MHCI) molecules, which can be loaded through both an endogenous and an exogenous pathway (Pamer *et al*., 1998; Rock *et al*., 1999).

1.2.4. DC-T cell interactions

The ability to prime naïve T cells constitutes a unique and critical function of DCs both *in vitro* and *in vivo*. Recognition of MHC-peptide complexes on DCs by antigen-specific T cell receptors (TCR) constitutes "signal one" in DC–T cell interaction. DC–T cell clustering is mediated by several adhesion molecules, like integrins β1 and β2 and members of the Ig superfamily (CD2, CD50, CD54, and CD58) (Hart, 1997). The crucial factor, that constitutes "signal two", required to sustain T cell activation, is the interaction between CSMs expressed by DCs and their ligands expressed by T cells. The B7 family members, CD80 and CD86, on DCs are so far the most critical molecule for amplification of T cell responses (Wang and Chen, 2004).

The TNF family of ligands and receptors, now totalling ~50, are also important co-stimulators in DC interactions with T cells. Particularly noteworthy is the interaction of CD40 on DCs with CD40L (CD154) on T cells. T cells can activate DCs via CD40 ligand (CD40-L)-CD40 signalling leading to increased expression of CD80/CD86 and cytokine release (IL-1, TNF, chemokines, and IL-12) (Schoenberger *et al*., 1998). Engagement of receptor activator of NF-κB (RANK), a member of the TNFR family, by its ligand (RANKL/TRANCE) expressed on activated T cells, stimulates the secretion of cytokines like IL-1, IL6, and IL-12 by DCs. This results in increased DC survival, by inhibition of DC apoptosis and, in

turn, in enhanced proliferative T cell responses in mixed lymphocyte reactions (Table 1.1).

During the development of an adaptive immune response, the phenotype and function of DCs play an important role in initiating tolerance, memory, and polarised T-helper 1 (Th1), Th2 and Th17 differentiation. A Th1 response is characterised by the production of IFN-γ and TNF-α by T cells. It is the normal outcome after DC exposure to viruses and is crucial for the control of intra-cellular bacteria such as *Mycobacterium* spp. Th2 differentiation usually occurs after contact with extra-cellular parasites (*e.g.*, helminths and schistosome worms) and involves production of cytokines such as IL-4, IL-5, IL-9, and IL-13, resulting in IgE production as well as accumulation of eosinophils and mast cells (Pearce and MacDonald, 2002). Allergens are non-pathogenic environmental antigens that elicit an inappropriate Th2 response. Secretion or lack of secretion of factors by DCs, particularly IL-12, are instrumental in the final differentiation of T cells into type 1 or type 2 effector T cells, respectively (*signal 3*, Figure 1.2). A third main outcome is the induction of tolerogenic or regulatory T cells (Treg) producing immunosuppressive cytokines, such as IL-10 or TGF-β. This is probably the most prevalent response in steady-state conditions. It forms a constant safeguard against the emergence of inappropriate inflammatory reactions to harmless antigen. In the absence of sufficient co-stimulation, T cells exhibit anergy or undergo apoptosis.

Dysregulated or uncontrolled effector T cell responses can lead to autoimmune diseases and allergies (Sakaguchi *et al*., 2000). Recent studies in this field have led to the identification of IL-17-producing (Th17) T cells (Figure 1.2 D). Th17 cells produce IL-17A (IL-17) and IL-17F and, to a lesser extent, TNF-α and IL-6 (Langrish *et al*., 2005). It was initially proposed that Th17 and Th1 cells might be related and might originate from a common 'pre-Th1' precursor cell (Bettelli and Kuchroo, 2005). Accumulating data, however, suggest that Th17 cells (induced by IL-23) represent a distinct subset unrelated to Th1 or Th2 cells (Veldhoen *et al*., 2006; Bettelli *et al*., 2006). The cytokine IL-23 is a heterodimeric molecule, sharing the p40 subunit with the Th1 cytokine IL-12 but differing from IL-12 because of its unique p19 subunit. While IL-12 plays a key role in the differentiation of naïve T

cells to Th1 cells, IL-23 promotes the expansion of Th17 cells (Langrish *et al*., 2005). In adoptive transfer experiments, T cells producing IL-17 induced experimental allergic encephalomyelitis (EAE), but T cells producing IFN-γ could not, although both types of T cells could cross the blood-brain barrier (Langrish *et al*., 2005). Moreover, EAE severity was shown to be greatly reduced, though the disease was not abrogated, upon treatment with a monoclonal Ab to IL-17 in mice that were actively immunised with myelin antigen and adjuvant. Likewise, mice treated with Abs to IL-23 failed to develop EAE (Langrish *et al*., 2005). A critical role for IL-17 in other autoimmune and allergic conditions has also been demonstrated: both collagen-induced arthritis and allergic airway hypersensitivity are suppressed in IL-17-deficient mice (Nakae *et al*., 2003; Hellings *et al*., 2003). Experimental models suggest that Th17 cells may also be important for neutrophilic inflammation in acute airway inflammation (Hoshino *et al*., 2000; Hashimoto *et al*., 2005). Neutrophil infiltration is also observed in acute asthma attacks, including neutrophilia in bronchoalveolar lavage (BAL) fluids. Interestingly, sputum IL-17 mRNA was shown to correlate with CXCL8 and neutrophil counts (Bullens *et al*., 2006).

Table 1.1. Co-stimulation molecules for DC-T cell signals

Adapted from Lipscomb and Masten (2002).

DC	T cell
	B7 family and receptors
B7-1 (CD80)	CD28, CTLA-4 (CD152)
B7-2 (CD86)	CD28, CTLA-4 (CD152)
B7RP-1	ICOS
PD-L1 (B7-H1)	PD-1
PD-L2	PD-1
B7-H3	Not known
	TNF family ligand and receptors
CD40	CD40L (CD154)
OX40L	OX40 (CD134)
TRANCE (RANK)	TRANCE (RANK-L)
CD27	CD27L (CD70)
CD30L (CD153)	CD30
	Miscellaneous
ICAM-1 (CD54)	LFA-1 (CD11a/CD18)
DC-SIGN (CD209)	ICAM-3 (CD50), ICAM-2 (CD102)
SLAM*(CD150)	SLAM (CD150)
CD58	CD2

TNF, tumour necrosis factor; ICOS, inducible co-stimulator; ICAM, PD-L, programmed death ligand; intra-cellular adhesion molecule; SLAM, signalling lymphocyte activation molecule.

* SLAM binds to itself.

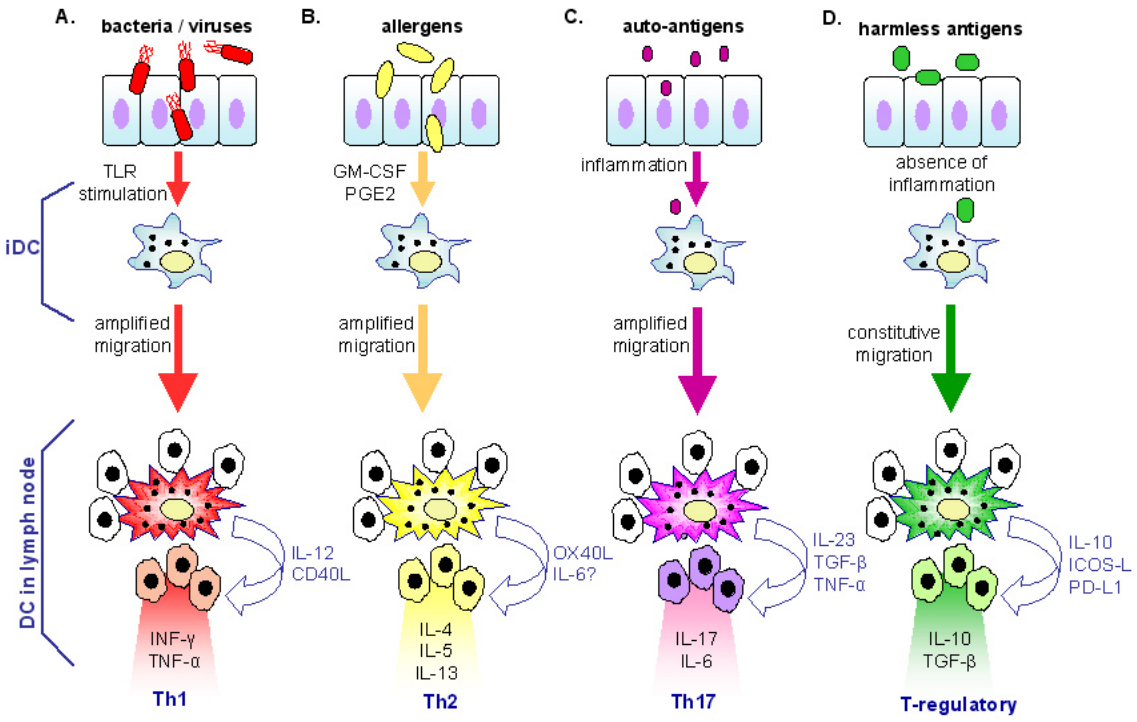


Figure 1.2. DC-T cell interactions

Schematic diagram showing how DCs translate stimuli detected in the inhaled air into a specific immune response. Inert antigen is continuously sampled by DCs, which go through a constitutive program of migration and maturation. **A**) Invasion by pathogens activates DCs through their TLRs. This leads to enhanced migration to the mediastinal LNs, where antigen is presented in the context of Th1-inducing signals. The Th1 lymphocytes thus generated are crucial for a resolution of the infection. **B**) Extra-cellular parasites and allergens can also cause DC activation, albeit through poorly defined molecular signals. Some allergens, such as house dust mite extracts, can proteolytically disrupt the integrity of the epithelial layer and activate underlying DCs. Alternatively, inert antigen can become allergenic because of the concomitant presence of a Th2-inducing environmental adjuvant such as diesel exhaust particles (not shown). **C**) Differentiation of Th17 cells from naïve precursors is induced by IL-1, TNF-α, IL-17 A/F and IL-6 in STAT1-, T-bet-, STAT4- and STAT6-independent manners. Rheumatoid arthritis and multiple sclerosis are emerging as models of IL-17-driven autoimmune inflammatory diseases. **D**) In the absence of inflammation, expression of tolerogenic receptors and soluble mediators is predominant. This leads to the generation of regulatory T lymphocytes capable of repressing any emerging immune response to this specific antigen. Activated DCs transport allergen to the draining LNs, where it is presented along with putative Th2-biasing signals. ICOS-L, inducible co-stimulator ligand; PD-L1, programmed death ligand-1; PGE2, prostaglandin E_2. Adapted from Vermaelen and Pauwels (2005).

1.2.5. DC trafficking

Each step of DC trafficking involved in either their steady-state distribution in peripheral and lymphoid organs, or in their recruitment upon inflammation/injury, is controlled by soluble chemotactic factors known as chemokines. *In vitro* iDCs have been demonstrated to respond to a large spectrum of chemokines such as macrophage inhibitory protein (MIP)-1α, MIP-1β, MIP-3α, MIP-3β, monocyte chemotactic protein (MCP)-3, MCP-4, MCP-5 and regulated on activation normal T cell expressed and secreted (RANTES/CCL5) (D'Amico *et al*., 1998; Dieu *et al*., 1998; Sozzani 2005 [Table 1.2 and Figure 1.3]). These chemokines transduce a chemotactic signal through CCR1, CCR2, CCR5, CCR6, CCR7 and CXCR4. In addition to chemokines, proteases, such as NE, which degrade several components of the extra-cellular matrix, have been shown to be highly expressed on DCs and may thus contribute to their migration through tissues (Banchereau *et al*., 2000). A comprehensive study by Hashimoto and co-workers (1999) analysed 17000 genes from a complementary-DNA library constructed from immature monocyte-derived DCs identified the expression of many genes presumably involved in cell migration, including a metalloprotease with elastolytic activity as well as a DC-specific *HAI-2* gene, a serine protease inhibitor of hepatocyte growth factor activator. Overall, the differentiation of monocytes to DCs was accompanied by significant changes in the expression of genes related to cell structure and motility.

Table 1.2. Expression of chemokine receptors by dendritic cells

MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T expressed and secreted; MCP, monocyte chemoattractant protein; TARC, thymus and activation-regulated chemokine; MDC, monophage derived chemokine; SDF, stromal derived factor; IL, interleukion; SLC, secondary lymphoid-tissue chemokine. Adapted from Banchereau *et al*. (2000).

Receptor	Ligands
Immature DC	
CCR1	MIP-1α, RANTES, MCP-3, MIP-5
CCR2	MCPs
CCR4	TARC, MDC
CCR5	MIP-1α, MIP-1β, RANTES
CCR6 (LC only)	MIP-3α
CXCR1	IL-8
CXCR4	SDF-1
Mature DC	
CCR7	MIP-3β, SLC (6Ckine)

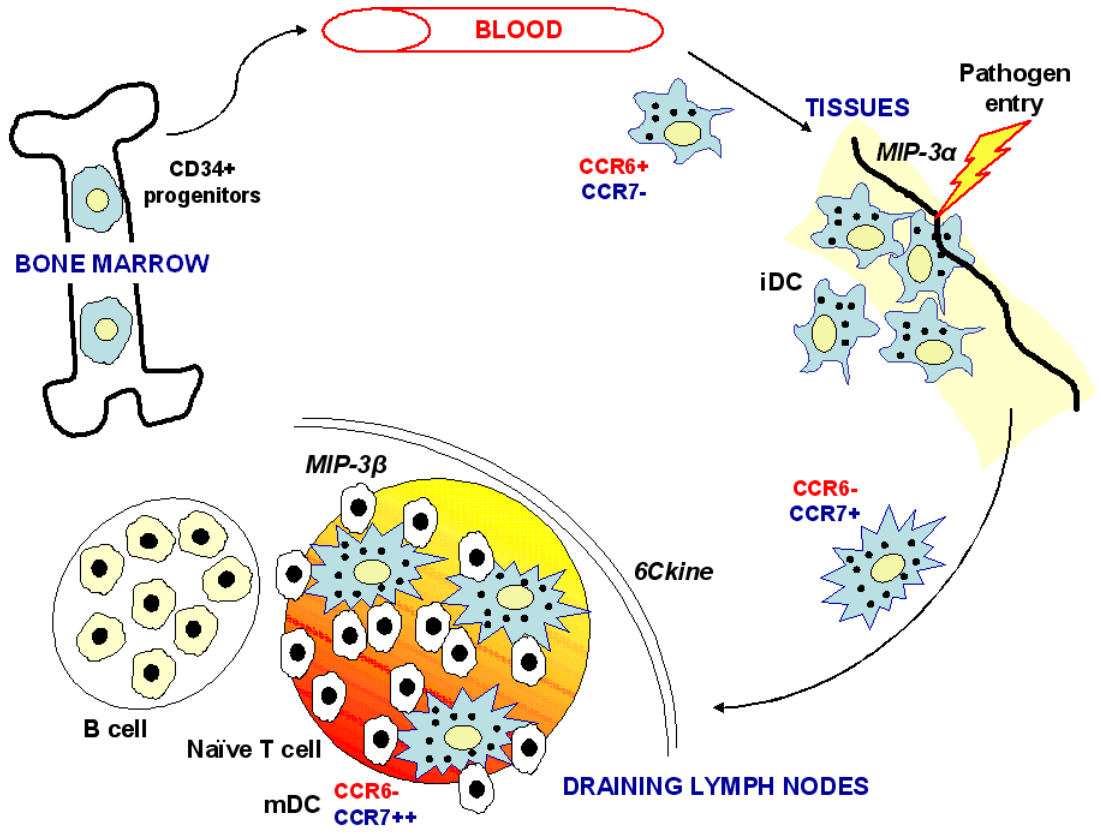


Figure 1.3. DC development and trafficking *in vivo*

Progenitor $CD34^+$ cells are released into the blood stream from the bone marrow where they can differentiate into effort cells such as DCs and macrophages under the influence of cell-type specific colony stimulating factors. Precursor and iDCs that display CCR6 are attracted by the epithelium that expresses the specific ligand MIP-3α. Upon antigen capture and activation, DCs CCR6 is replaced by CCR7, whose ligands are (*i*) 6Ckine, which is expressed on lymphatic vessel walls, and (*ii*) MIP-3β, which is expressed in the T cell areas of lymphoid organs. This may guide the maturing DCs to the T cell areas where they will start to produce chemokines that attract lymphocytes.

1.2.6. DCs exhibit plasticity

Although it is clear that distinct DC subpopulations exhibit distinct functions, there is also evidence that these DC functions can be altered by the cytokine environment. In particular, DCs exhibit considerable plasticity in their ability to skew Th responses, and DCs that normally induce Th1 profiles can be converted to Th2-skewing cells when treated with anti-inflammatory cytokines such as IL-10 and TGF-β or with steroids (Piemonti *et al*., 1999). In this context, DC subsets isolated from different organs differently affect Th responses. For instance, mouse and rat spleen DCs induce Th1 responses, whereas those from Peyers patches elicit Th2 responses (Iwasaki and Kelsall, 1999). Studies focusing on mucosal surfaces such as the gut (Mowat, 2003) and lung (Akbari *et al*., 2002; Dodge *et al*., 2003) clearly indicate that the baseline response to inert antigens at these sites displays a Th2 bias. These data indicate that resident lung DCs under steady-state conditions are programmed for induction of Th2-biased immunity (see section 1.2.10 for details).

DC plasticity is also reflected in their differentiation, which may determine the fate of antigen (*i.e.*, processing and presentation or degradation). This aspect is exemplified by the potential of macrophages to differentiate to DCs (Palucka *et al*., 1998; Zou *et al*., 2000), a pathway that may permit high antigen capture (macrophage) and presentation (DC) (Figure 1.4).

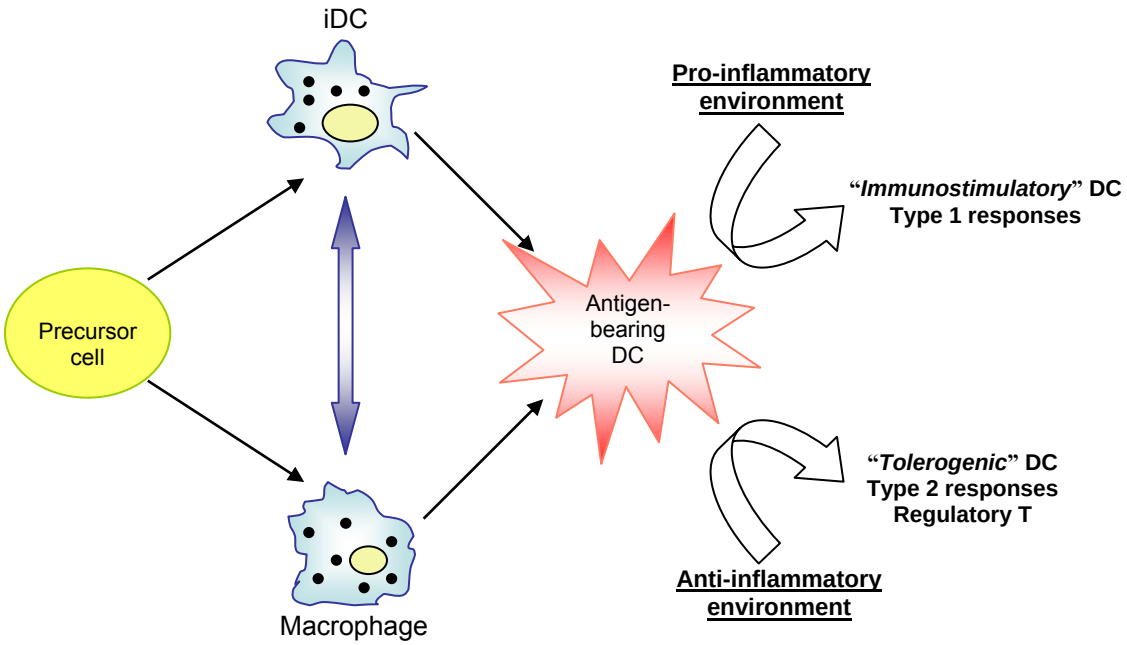


Figure 1.4. The plasticity of DCs

According to microenvironmental instructions (*e.g.*, cytokines), DC functions can be altered. For instance DCs can become macrophages with higher phagocytic functions in response to macrophage-colony stimulating factor (M-CSF). Furthermore, IL-12 secreting DCs that induce type 1 T cell differentiation can switch, in response to IL-10 and prostaglandin E2, to DCs inducing T cell anergy or T cell differentiation into type 2 cells or regulatory T cells. Adapted from Banchereau *et al*. (2000).

1.2.7. Heterogeneity of DCs

Both human and murine DCs are heterogeneous populations and depending on their precursors, and the local environment in which they differentiate and reside in, would have distinct morphology and function as will be briefly discussed below.

1.2.7.1. Human DCs

In humans, DCs are found as precursor populations in BM and blood and as more mature forms in lymphoid and non-lymphoid tissues. Three distinct subtypes of human DCs have been delineated based on studies of skin DCs, DCs generated *in vitro* from $CD34^+$ hematopoietic progenitors, and blood DC precursors (Shortman and Liu, 2002). Human skin contains two of the three DC subtypes in immature form: LCs and interstitial DCs. Both subtypes emerge in cultures from $CD34^+$ BM and $CD11c^+$ blood precursors in the presence of GM-CSF and either IL-4 or TNF-α (Caux *et al*., 1999). The $CD11c^+$ DC precursor expresses myeloid markers, including CD13 and CD33. Upon activation by CD40L, immature myeloid DCs undergo maturation and produce IL-12 (Cella *et al*., 1996). LCs and interstitial DCs also share the capacity to activate both CD4 and CD8 naïve T cells and secrete IL-12. One striking difference between LCs and interstitial DCs is the ability of interstitial DCs, but not LCs, to induce the differentiation of naïve B cells into Ig-secreting plasma cells (Caux *et al*., 1997).

Plasmacytoid DCs (pDC) are a third type of DC and are so named because at the ultra-structural level they resemble Ig-secreting plasma cells. These DCs are found in the T cell zones of lymphoid organs and in the thymus and blood and were previously described as plasmacytoid T cells or plasmacytoid monocytes (Facchetti *et al*., 1999; Sorg *et al*., 1999). pDCs are characterised by a unique phenotype, $CD11c^-CD4^+CD123^+CD45RA^+HLA\text{-}DR^+$, and possess the unique ability to secrete large amounts of IFN-α/β upon viral stimulation (Cella *et al*., 1999; Grouard *et al*., 1997). Unlike LCs and interstitial DCs, pDCs require IL-3 for their differentiation and are derived from a $CD11c^-$ blood precursor that has low expression of GM-CSF receptor, lacks the myeloid markers CD14, CD13, and CD33, lacks mannose

receptors, and expresses high amounts of CD123 (Grouard *et al*., 1997). pDCs do not express LPS-sensing receptors but typically express TLR7 and TLR9 (the receptor for unmethylated microbial DNA sequence) (Ito *et al*., 2005; Cao and Liu, 2006). Nevertheless, pDCs share a common function with LCs and interstitial DCs in having the capacity to activate CD4 and CD8 naïve T cells and secrete IL-12 upon CD40L activation (Cella *et al*., 2000).

1.2.7.2. Murine DCs

At least two distinct pathways of DC development have been identified in mice, myeloid and lymphoid. Evidence for the myeloid origin of DCs comes mainly from *in vitro* studies in which myeloid-committed precursors give rise to both granulocytes/monocytes and myeloid DCs under the influence of GM-CSF (Inaba *et al*., 1992). DCs can also arise from lymphoid-committed precursors (Wu *et al*., 1996). Lymphoid and myeloid DCs differ in phenotype, localisation, and function. Both subsets express high levels of CD11c, MHCII, and the CSMs CD80, CD86 and CD40. To date, the most reliable marker to distinguish these two subsets is CD8α, which is expressed as a homodimer on the lymphoid DC, but is absent from the myeloid subset. Other markers such as DEC-205 and CD1d are expressed at higher levels on lymphoid DCs, but they can be up-regulated on myeloid DCs by *in vitro* culture or LPS treatment. Lymphoid DCs are localised in the T cell-rich areas of the periarteriolar lymphatic sheaths in the spleen and LNs. In contrast, myeloid DCs are in the marginal zone bridging channels of the spleen (Wu *et al*., 1996; Pulendran *et al*., 1997).

The mouse equivalent of human pDCs has been identified in the recent years (Nakano *et al*., 2001; Bjorck, 2001). These cells have a unique surface phenotype: $CD45RA^{high}B220^{+}CD11c^{low}CD11b^{-}MHCII^{low}$. Although similar to their human counterparts, mouse pDCs showed notable differences in the expression of a few surface molecules. In contrast to human pDCs which express high levels of IL-3R and are negative for CD8, mouse pDCs lack of high expression of IL-3R and some express CD8α. Currently, the biological role of CD8α expression on mouse DCs and

pDCs is not yet clear. Similar to human pDCs, mouse pDCs express TLR7 and TLR9, and could produce large amounts of type I IFNs in response to viral stimulation or bacterial oligonucleotides containing CpG motifs (Hochrein and O'Keeffe; 2002).

1.2.7.3. Murine versus Human DCs

Murine DCs have been widely employed by researchers investigating the roles of DCs in the generation and regulation of specific immunity. Although it is clear that differences exist between murine and human DCs, it is evident that murine DCs are relevant to human DCs and provide an appropriate model for human cells in most cases. Like human DCs, murine DCs *1*) originate from $CD34^+$ BM stem cells, *2*) are found in blood and tissues, *3*) are able to take up and degrade antigen to antigenic peptides, *4*) express MHCII molecules complexed with antigenic peptide, *5*) express CSMs, *6*) mature and migrate in response to danger signals, and *7*) are responsive to the microenvironment with a controlled release of chemokines and cytokines.

1.2.8. DCs and disease

DCs have been studied in a number of common autoimmune conditions. Despite the diverse nature and tissue distribution of autoimmune diseases, DCs share certain common characteristics irrespective of the disease and sites from which they are recovered. These include increased numbers of tissue DCs, particularly in a perivascular distribution, DC infiltration at the earliest stage of disease and an altered DC phenotype. Early DC infiltration contributes to the recruitment of other immune cells.

The cell-mediated arm of the immune system plays an important role in the detection and elimination of malignant cells. T lymphocyte responses to tumour cells will require initial antigen presentation by professional APCs such as the DC. DCs infiltration into tumours has for many years been linked to increased patient survival and reduction in the number of metastases in a variety of malignancies (including endometrial, gastric and lung cancers) (Coppola *et al*., 1998; Tsujitani *et al*., 1993; Zeid and Muller, 1993). However this is tempered by the observation that DCs

isolated from many cancer patients show an impaired ability to present antigen as a product of decreased surface expression of the co-stimulators CD80 and CD86 (Chaux *et al*., 1996; Gabrilovich *et al*., 1997; Perrot *et al*., 2007). It also appears that many sporadic tumours actively exploit immunosuppressive pathways to interfere with antigen presentation. For example, tumour-derived IL-10 acts directly on tissue DCs to prevent maturation and therefore subsequent immunogenic presentation to T lymphocytes (Wittke *et al*., 1999). Increased expression of FasL on tumour cells may also induce apoptosis in DC and T cell effectors (Strand *et al*., 1999; Gratas *et al*., 1998). Vascular endothelial growth factor (VEGF) is produced by a majority of carcinomas and has been shown to inhibit maturation of DCs within the tumour and to impair differentiation of haemopoietic progenitors into DCs (Gabrilovich *et al*., 1996).

1.2.9. Therapeutic applications of DCs

The observation that DCs are the most potent APCs and play important roles in the progression and severity of many diseases has led many researchers to investigate how modulation of DC function could be therapeutically exploited. The influence of activated DCs could be used in cancer/intra-cellular parasitic infections to redirect an immune response towards defective (malignant)/infected cells that have evaded immunodetection. Alternatively, DCs could be suppressed to alleviate the symptoms of autoimmunity and allotransplant rejection. Some of these strategies are discussed in more details in this section.

There are currently two main procedures that exploit DCs as therapeutic agents in experimental and clinical trials worldwide, (*i*) gene transfer (see below for more details), and (*ii*) cellular immunotherapy. Both of these methodologies have proved to be extremely powerful in eradicating established infections as well as tumours (Moll, 2004; Reichardt *et al*., 2004; Wei *et al*., 2005). Adoptive transfer of pulsed DCs, presenting tumour antigens to resting naïve T cells, is currently of tremendous interest. Adoptive immunotherapy may ultimately have its greatest use in patients undergoing cellular rescue after ablative chemotherapy; the infusion of immunocompetent T cells, genetically modified stem cells, or programmed DCs may

offer the opportunity to direct a patient's immune response to eliminate residual microscopic diseases. Gene transfer techniques may offer more optimal ways to generate therapeutic DCs as will be discussed below.

1.2.9.1. Adenoviral gene therapy to modulate DCs

Viral vectors have provided effective *in vivo* gene delivery methods for therapeutic purposes. The ability of viruses to infect a wide variety of cell types *in vitro* and *in vivo* has been exploited for several applications. Adenoviruses (Ad) are non-enveloped DNA viruses with ~30 kb genomes. There are more than 50 human Ad serotypes. However, most of the recombinant Ad vectors used in gene therapy applications are based on serotypes 2 and 5 (Jooss and Chirmule, 2003). The viral capsid consists of 252 capsomeres (240 hexons and 12 penton molecules). The viral capsids interact with the coxsackievirus Ad receptor (CAR) and co-receptors like integrins α5β1, αvβ3, αvβ5, αMβ2 to infect permissive cells. Subsequent to entering the cells, the virus escapes endosomal lysis and enters the nucleus by traversing microtubules, binds to the nuclear envelope and inserts its genome through nuclear pore complexes. Various Ad strains have been associated with a number of infectious diseases in normal subjects, for example upper respiratory infections and in conjunctivitis. A wide range of cell types, for example, respiratory epithelial cells, endothelial cells, myoblasts, glial cells, DCs, macrophages and hepatocytes have been shown to be infectible with Ad vectors, albeit to different degrees (Liu and Muruve, 2003).

Ad vectors have the potential of activating both innate and adaptive immune systems both *in vitro* and *in vivo* (Jooss and Chirmule, 2003). The innate immune response to Ad represents the most significant hurdle in clinical application of Ad vectors for gene therapy, but it is an attractive feature for vaccine development. Incidentally, recent studies demonstrate that Ad vectors elicit innate immune responses through a MyD88/TLR9-dependent and/or -independent manner according to cell type (Zhu *et al*., 2007; Yamaguchi *et al*., 2007).

DCs pulsed *ex vivo* with antigens have the potential use as cell-based vaccines against tumours. Viral vectors derived from Ad have also been extensively used to pulse DCs *ex vivo* by delivering genes encoding immunomodulatory molecules and tumour antigens, since these vectors are relatively safe, effective in inducing the maturation of DCs, and can accommodate large expression cassettes encoding antigens (Miller *et al*., 2003). One of the hurdles for gene delivery to DCs by Ad vectors, however, is low transfection efficiency of DCs due to the paucity of Ad receptor on DCs. To overcome this obstacle, targeted Ad vectors have been made by modifying viral capsid proteins. These targeted Ad vectors not only enhance the gene delivery to DCs, but also allow *in vivo* gene delivery to DCs, thus avoiding *ex vivo* manipulation of DCs (Wu *et al*., 2005; Xia *et al*., 2006). On the other hand, a few methods have been developed in recent years which significantly enhance the Ad infection efficiency of DCs both *in vitro* and *in vivo*, such as centrifugal methods (Nishimura *et al*., 2001a and 2001b), and use of polycation complexes (Di Nicola *et al*., 2000).

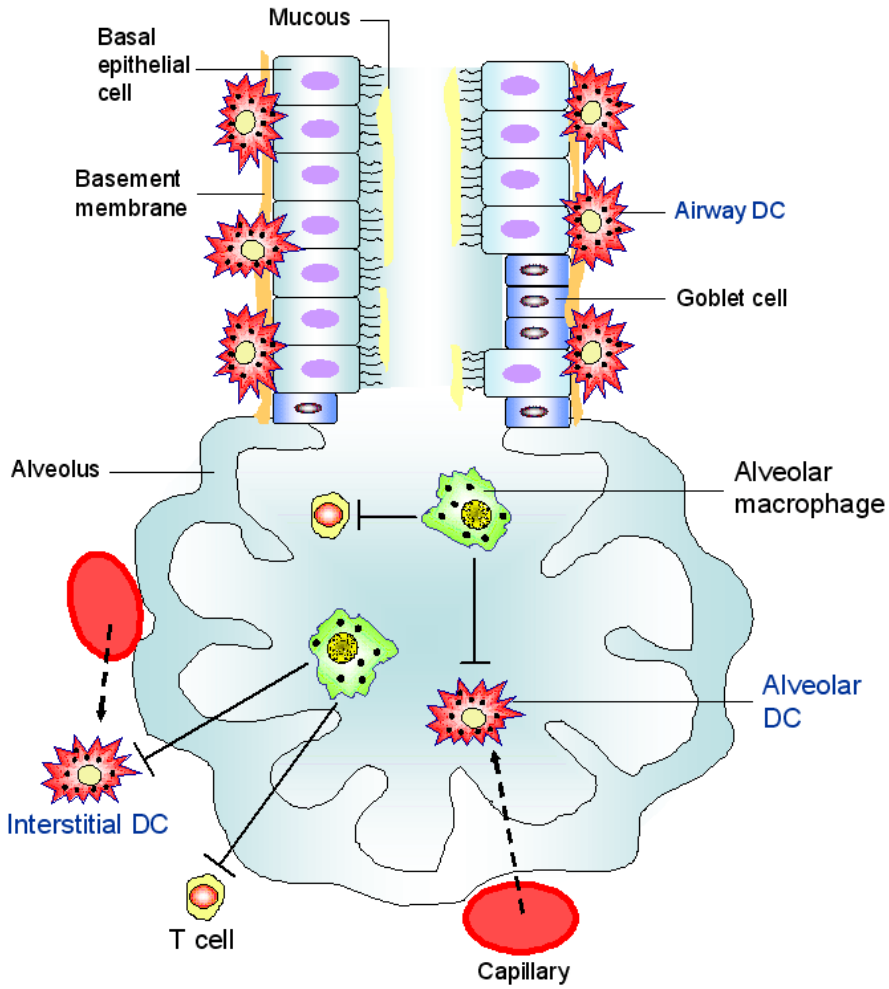
A considerable wealth of literature is available on the use of genetically engineered DCs in combating infections and established tumours in experimental animals. Brown and co-workers (2003) recently provided the first validation in a primate model for the use of Ad vectors to induce robust T cell immunity to a virus via DCs. These findings are in agreement with the findings of previous *in vitro* and *in vivo* studies (*e.g.*, murine models) showing the efficacy of this approach. Investigators from Crystal's laboratory have shown that genetic Ad modification of murine DCs to express CD40L accomplishes the goal of directly activating B cells to induce functionally relevant antigen-specific humoral immune responses that lead to protection against the lethal infection in a microbe-specific manner (Kikuchi *et al*., 2000). A recent study by Nakamura and co-workers (2005) showed that DCs transduced (using an Ad vector) with the natural tumour antigen provided a remarkably higher therapeutic efficacy in comparison to using antigen-pulsed DCs. Similarly, whole organ gene therapy using agents that are able to induce DC survival and differentiation have been employed to augment DC numbers *in vivo*. In this regard, Wang *et al*. (2000) investigated the role of GM-CSF on the differentiation of

DCs during pulmonary viral infection by using a mouse model of GM-CSF transgene expression (Ad-GMCSF). They concluded that GM-CSF promotes the differentiation and activation of a myeloid DC population primarily by acting on macrophages during pulmonary immune responses. In a similar study, Miller *et al*. (2002) used an Ad-GMCSF vector in mice and found that up to 24% of splenocytes became $CD11c^+$ and the number of DCs increased up to 260-fold. They demonstrated that following intravenous (i.v.) administration of Ad-GMCSF, the splenic DC numbers remained substantially elevated even 75 days after treatment. Relatedly, Ad vaccination protocols exploiting DC function have been successfully tested out in animal experiments (*e.g.*, Tuting *et al*., 1999; Murakami *et al*., 2004; Gyorffy *et al*., 2005; Malowany *et al*., 2006), in *in vitro* protocols (*e.g.*, Jonuleit *et al*., 2000; Yang *et al*., 2006; Tokunaga *et al*., 2005) and in human clinical trials (*e.g.*, Kusumoto *et al*., 2001; Trudel *et al*., 2003) which appear to have promising applications in both immunosuppressed and immunostimulatory scenarios.

1.2.10. Pulmonary DCs

The lung, representing the largest epithelial surface area of the body, is continuously exposed to a vast array of airborne particles and invading organisms during respiration. In order to protect the host against these recurrent challenges, a complex defence system has evolved. The normal host defence responses of the lungs, as in any other organ, include both the innate and acquired immune responses. Innate defences consist of the structural defences, the antimicrobial peptides generated in the airways (discussed in section 1.7), and the phagocyte defences provided by the resident macrophages and the neutrophils which are recruited to the lung in response to pulmonary infection/inflammation. Acquired immune defence is the antigen-specific mechanism of the lung host defence including cell-mediated and Ab-mediated immune responses, important for the eradication of infections caused by virulent encapsulated bacteria, viruses, and intra-cellular pathogens that survive within the resident macrophages and epithelial cells lining the respiratory tract.

A growing body of experimental evidence indicates that the 'sentinel' paradigm of DC function applies extremely well to the respiratory system. This implies (*i*) a constant recruitment of DCs into the lung, (*ii*) a capacity for these cells to sense and capture inhaled antigens, and (*iii*) an efficient transport of processed antigens to the draining pulmonary LNs, where DCs decide which type of immune response will ensue, as discussed in detail below. Sertl and colleagues (1986) were one of the first pioneer groups to describe the presence of DCs in the airway epithelium, lung parenchyma, and visceral pleura of human and mouse specimens. These cells displayed a typical dendritic morphology, expressed copious amounts of MHCII on their surface, and acted as potent T cell stimulators *in vitro*. Later studies have provided more detail into the features and localisation of DCs in the different pulmonary compartments. DCs form a tightly meshed network within antigen-exposed tissues of the upper airway, bronchial mucosa, lung interstitium and pleura (Sertl *et al*., 1986; Holt and Schon-Hegrad, 1987; von Garnier *et al*, 2005). In general, a few different phenotypes of DCs are found in the lungs, including conducting airway DCs, alveolar DCs, and interstitial DCs (Figure 1.5). Within the bronchial epithelium, DCs are found with typical features of LCs, such as the presence of Birbeck granules, the expression of CD1a, and Langerin (Demedts *et al*., 2005). Recently, pDCs have been identified in both human and murine lungs (Asselin-Pature *et al*., 2003; Demedts *et al*., 2005). Lung pDCs express low levels of CD11c, and are positive for Gr1, B220 and 120G8 as previously reported in lymphoid tissues (Asselin-Pature *et al*., 2003), and produce large amounts of IFN-α in response to virus infections.



1.5. Distribution of DCs in the lungs.

Airway DCs are located as a network immediately above and beneath the basement membrane, in between basal epithelial cells. Interstitial DCs (stained for CD11c) are composed of a $B220^{+}Gr1^{+}$ pDC subset and a $B220^{-}$ myeloid DC subset. Alveolar DCs can consistently be recovered by bronchoalveolar lavage (BAL) in humans, rats and mice, particularly when inflammation is induced. Adapted from Lambrecht and Hammad (2003).

The density of DCs in the airways is related to the extent of antigen exposure, being greatest in the proximal airways, and diminishing towards the periphery. A variety of pro-inflammatory stimuli induce a marked increase in the density of lung DCs, including exposure to bacterial and viral particles, IFN-γ, and allergen inhalation in previously sensitised animals (McWilliam *et al*., 1996). It has been estimated that, in the absence of inflammation, DCs are distributed at an average density of several hundred cells per millimeter squared in the large airways, decreasing to less than a hundred DCs per millimeter squared within smaller intrapulmonary airways (Schon-Hegrad *et al*., 1991). DCs in the lung parenchyma are present in larger numbers. They are mostly found within inter-alveolar septa (Holt *et al*., 1987). Following antigen uptake, some airway DCs are able to rapidly transport antigen to the T cell zones of regional LNs (Vermaelen *et al*., 2001), although it is clear that a significant proportion of antigen-loaded DCs also remain within the lung where they are able to activate T cells locally, long after antigen exposure (Julia *et al*., 2002). Pulmonary DCs, like immature monocyte-derived DCs, express both CCR1 and CCR5, whereas mDCs migrating towards lymphoid structure no longer express these two receptors, but acquire CCR7 (Cochand *et al*., 1999). Even in the absence of overt inflammation, DCs or their precursors are constantly recruited from the blood into the lung. Holt and co-workers (1994) were the first to demonstrate that respiratory tract DCs are continuously replenished by a steady-state BM output. Not surprisingly, inflammatory stimuli in the inhaled air have a profound impact on these steady-state dynamics. In a series of hallmark experiments, McWilliam and colleagues (1994) described how the inhalation of pathogenic material, such as bacteria or viral particles, induced a very rapid influx of DCs into the airways of rats, in some cases peaking already 2 hours after challenge. Remarkably, this DC recruitment was as fast or sometimes ahead of the prototypic neutrophil influx. This surprising discovery overthrew the classical theory which stated that neutrophils were the first leukocytes infiltrating the inflammatory tissues. This further confirms the view that DC recruitment is an integral part of the early phase of the innate immune response, with the potential to progress toward a powerful adaptive defence. Consequent resolution of the inflammation usually restores baseline numbers of pulmonary DCs.

One of the most crucial specialised functions of pulmonary DCs, as any other specialised resident DC, is the capture and delivery of antigens to local lymphoid tissues. Using a mouse model, Vermaelen and colleagues (2001) elegantly demonstrated that antigen uptake by airway DCs can proceed without any breach to the mucosal barrier, and in the absence of any inflammatory stimulus. By instilling inert fluorescent macromolecules into the airways they were able to track DCs migrating and transporting antigen from the airway mucosa toward pulmonary LNs. This transport was rapid, occurred constitutively (*i.e.*, in the absence of inflammation), and the migratory DCs were seen to specifically penetrate the T cell zones of the mediastinal LNs. Analogous conclusions have been reached using intra-tracheal (i.t.) transfer of *in vitro*-derived DCs (Havenith *et al.*, 1993; Lambrecht *et al.*, 2000), although these cultured DCs might not reflect the exact nature of the endogenous pulmonary DC populations in terms of phenotype, activation level, and antigen-uptake capacity.

As mentioned above, the current hypothesis states that to counter the potential inflammatory tissue injury of chronic Th1-mediated responses against antigens, mucosal sites may skew toward Th2 immune responses. In this regard, a number of mechanisms exist to regulate DC function within the lungs. Freshly isolated lung DCs exhibit a poor capacity for antigen presentation and express only low levels of CSMs (Cochand *et al*., 1999). However, culturing purified lung DCs for short periods in the absence of exogenous stimuli is sufficient to elicit spontaneous DC activation, suggesting that the tissue environment in the lung provides inhibitory signals that counteract DC activation *in vivo* (Holt *et al*., 1992). AMs are known to maintain lung DCs in an immature state through the release of nitric oxide and it is likely that airway epithelial cells also regulate DC function through the release of soluble mediators such as TGF-β and nitric oxide, and through adhesion molecules such as the cadherins and integrins (Holt *et al*., 1993). In the steady state, lung DCs do not synthesise IL-12 and preferentially stimulate Th2 rather than Th1 immunity (Stumbles *et al*., 1998; Constant *et al*., 2002). This phenomenon has also been shown to be associated with expression of both IL-10 and IL-6 by resident myeloid DCs within the lung. Recent studies by Akbari and co-workers (2002) showed that IL-10 production by DCs is critical for the induction of

tolerance, and that phenotypically mature pulmonary DCs mediate tolerance induced by respiratory exposure to antigen. Relatedly, Dodge and colleagues (2003) demonstrated that IL-6 negatively regulates IL-12 production, as pulmonary DCs from IL-$6^{-/-}$ mice produced significant levels of IL-12 and induced Th1 polarisation of naïve $CD4^+$ T cells. The most recent finding to explain this phenomenon is the intriguing observation showing that murine pulmonary interstitial $CD11c^+$ APCs suppressed T cell responses *in vitro* and *in vivo* by production of indoleamine 2,3-dioxygenase (IDO), an enzyme that catabolises tryptophan to its by-product, kynurenine (Swanson *et al*., 2004). The production of IDO was also shown to be unique to pulmonary APCs, as cells isolated from the liver or spleen did not produce IDO, constitutively, or even in response to INF-γ, the most potent inducer of IDO expression (Swanson *et al*., 2004).

Last but not least, an integrin expressed by gut DCs has been shown to give Treg cells the green light for suppression. CD103 has been reported to be essential for the adhesion of human and mouse intestinal lymphocytes to epithelial cells through the interactions with E-cadherin (Cepek *et al*., 1994). Annacker and colleagues (2005) showed that DCs expressing the integrin CD103 are required for Treg cells to subdue gut-attacking effector T cells in a mouse model of inflammatory bowel disease (IBD). CD103 has been shown to be expressed on 30-50% of the lung-derived $CD11c^+$ DCs migrating to the bronchial LNs (Hintzen *et al*., 2006). A recent report has identified the $CD103^+$ DC in mice as a major DC population residing in the lung mucosa (Sung *et al*., 2006). $CD103^+$ DCs in the lung do not express the typical plasmacytoid DC (B220, Gr-1) or lymphoid markers (CD8α), but express high amounts of CD11c, MHCII, and low levels of CD11b (Sung *et al*., 2006). Antigen-carrying $CD103^+$ bronchial LN DCs are specialised in cross-presenting innocuous inhaled antigen (Sung *et al*., 2006) and might also be responsible for subsequent down-regulation of lung immune responses, as previously demonstrated in the gut (Annacker *et al*., 2005).

1.3. CYSTIC FIBROSIS (CF)

Second only to sickle-cell anaemia, CF is the most common genetic disease, occurring once in every 3600 children born alive, causing early death (Grosse *et al.*, 2005). Although pulmonary disease causes most of the morbidity and mortality associated with CF, the lungs are thought to be anatomically normal at birth (Chow *et al.*, 1982). The first detectable evidence of lung disease in patients with CF is infection and/or inflammation in BAL fluid, denoted by elevated concentrations of IL-8 and neutrophils and the presence of microorganisams (Khan *et al*., 1995; Armstrong *et al*., 2005). Sufferers have an average life expectancy of 37 years, and current therapies treat the disease's symptoms rather than its cause. The defective gene was identified in 1989 and appeared to be located on chromosome 7. It encodes the CF transmembrane conductance regulator (CFTR), which acts as a chloride channel. The most common frequent mutation of this gene is the deletion of phenylalanine at position 508 (Δ508) (Renders *et al.*, 2001). Since that early discovery, more than 700 additional CFTR mutations related to CF have been identified (Schwiebert *et al*., 1998). The gene defect results in a combination of low or absent chloride secretion and increased sodium absorption in the airways. Consequently, the disease is characterised by the production of abnormally viscid bronchopulmonary secretions interfering with mucociliary clearance of bacteria. The recognition that CF lung disease reflects the failure of airways innate defence has focused much attention on normal defence mechanisms that protect this region from inhaled bacteria. The reason for the high prevalence of bacteria in the early stage of CF is currently unclear. However, a number of hypotheses have been postulated to account for innate airways defence, including those based on bacterial binding to airway epithelial surfaces (Imundo *et al*., 1995), or clearance of locally deposited bacteria by CFTR attachment-site mediated epithelial phagocytosis (Pier *et al*., 1996). Mutations of CFTR are also hypothesised to reduce airway surface liquid (ASL) glutathione levels, which may enhance oxidant injury (Noah *et al*., 1997; Hiatt *et al*., 1999). On the other hand, various components of the innate and adaptive immune system might be adversely affected in CF lungs leading to their immunocompromised status. Although this field has not been exploited in detail yet,

a very limited number of studies published to date are encouraging and indicate the need for more research in this area. For instance, an interesting study by Knight *et al*. (1997) demonstrated that APCs isolated from BAL fluid of CF patients were unable to present antigen and stimulate the allogeneic normal circulating lymphocytes. This was despite the fact that blood monocytes taken from the same patients were functional in both assays (Knight *et al*., 1997). Regardless of the mechanisms leading to airway infection, once the airways are colonised, a vicious cycle of infection, inflammation, and airway damage begins. Figure 1.6 summarises the pathways of CF lung disease pathogenesis supported by various *in vivo* and *in vitro* studies.

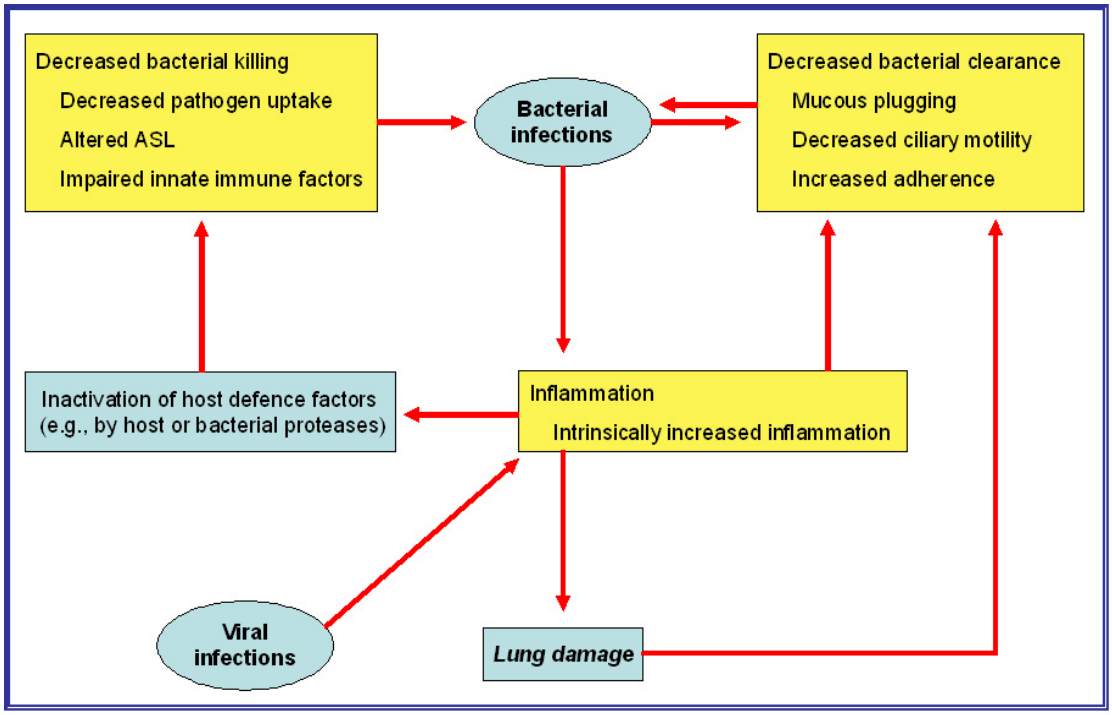


Figure 1.6. Pathogenesis of lung disease in patients with CF

Regardless of the initial inflammatory insult or defective process, once this cycle is started, it will lead to all elements being increasingly perturbed. Several interconnected cycles perpetuate and further impair host defence, leading to a persistent bacterial infections and progressive lung disease. ASL, airway surface liquid. Adapted from a review by Starner and colleagues (2005).

1.3.1. CF Pathogens

To date, the source of most pathogens in CF patients remain unknown. Potential reservoirs include the natural environment (*e.g.*, soil and water), the environment in health care settings (*e.g.*, sinks), contaminated equipment (*e.g.*, nebulisers), other contaminated objects, and other CF patients.

Staphylococcus aureus (*S.a.*) is often the first pathogen to infect the respiratory tract of CF patients. In the preantibiotic era, *S.a.* and non-typeable *Haemophilus influenzae* (*NTHI*) caused substantial morbidity and mortality in infants with CF, but with the advent of antibiotic therapy effective against these pathogens, the life expectancy of infants with CF has increased. Colonisation of the anterior nares with *S.a.* is an important risk factor for subsequent disease with the same genotype in CF and non-CF patients (Perl *et al.*, 1995; Goerke *et al.*, 2000; von Eiff *et al.*, 2001). CF patients without recent treatment with antibiotics had a higher prevalence of nasal colonisation with *S.a.* than did CF patients receiving recent treatment or healthy controls (Goerke *et al.*, 2000). *S.a.* strains spread within families, but loss or replacement of a strain was frequently observed in families with and without a family member with CF. In contrast, CF patients may be infected or colonised with the same strain of *S.a.* for at least 1 to 2 years (Branger *et al*., 1996).

Pseudomonas aeruginosa (*P.a.*) is considered the major pathogen in later stages of the disease. Eighty-two percent of patients have colonisation with *S.a.* and *NTHI* before they have colonisation with *P.a.* (Abman *et al*., 1991). Studies of BAL fluid of single-pathogen infections with *P.a.*, *S.a.*, and *NTHI* in CF patients did not reveal clinically significant differences in IL-8 or neutrophil levels (Muhlebach *et al.*, 1999), indicating that all of these early pathogens are associated with high levels of inflammation. Other studies of BAL fluid found similarly increased inflammatory cells and cytokines regardless of the pathogen (Rosenfeld *et al*., 2001).

Most of the patient-to-patient transmission studies performed to date have focused on *Burkholderia cepacia* (*B.c.*) complex. *B.c.* complex was first described as

a pathogen in CF patients in the late 1970s and early 1980s. Initial descriptions documented the marked virulence of this multidrug-resistant pathogen, the so-called '*cepacia* syndrome', *i.e.*, high fever, bacteraemia, and rapid pulmonary deterioration that led to the death of 62 to nearly 100% of patients (Isles *et al*., 1984; Tablan *et al*., 1987). Thus, infection with *B.c.* complex can cause a decline in lung function and a shortened median survival (Whiteford *et al.*, 1995; Liou *et al.*, 2001; Navarro *et al.*, 2001). Different species of the *B.c.* complex appear to occupy different niches in the natural environment. *B. cenocepacia* strains have been found in agricultural soil (Balandreau *et al*., 2001), the maize rhizosphere (Bevivino *et al*., 2002), onion fields (LiPuma *et al*., 2002), and occasionally soil in urban settings (Miller *et al*, 2000). To date, *B. multivorans* has only rarely been recovered from soil samples. The risk posed by strains in the natural environment remains uncertain.

Fungi and molds can also be detected in the lungs of CF patients. The annual prevalence of *Aspergillus* as reported in 2001 was 10.6%. Most isolates reported are *A. fumigatus*, although some CF patients harbour other *Aspergillus* spp. and, rarely, other molds (Brown *et al.*, 1999).

In addition to bacteria, fungi and molds, respiratory viruses are important pathogens in patients with CF. Viruses such as respiratory syncytial virus (RSV), influenza virus, parainfluenza virus, Ad, and rhinoviruses have relatively short incubation periods (less than 1 week), and transmission occurs primarily via direct contact with infected persons or indirect contact by touching items handled by infected persons. Droplet transmission of infectious respiratory secretions occurs with influenza virus and Ad. As with non-CF patients, viral particles enter the mucous membranes of the eyes and nose of susceptible patients and multiply in the respiratory epithelium, subsequently decreasing ciliary movement and increasing mucous production (Garner *et al*., 1996). Viral respiratory infections may predispose CF patients to bacterial infection (Smyth *et al.*, 1995; Armstrong *et al.*, 1998). Ad infection has been shown to lead to worsening lung function and increased airway obstruction after the acute phase of illness (Hiatt *et al.*, 1999).

1.4. CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

COPD is a major global health problem. Epidemiological studies by Murray and Lopez (1997a and 1997b) in late 90's found that that COPD was the sixth commonest cause of death worldwide in 1990, but made the alarming prediction that it would become the third commonest cause of death by 2020 irrespective of public health interventions. Furthermore, COPD was recognised as being the twelfth greatest cause of chronic morbidity, with a predicted increase to become the fourth most important disability-producing illness by 2020. Data from WHO are in agreement with these predictions and state that by the turn of the last century COPD had become the fourth leading cause of mortality worldwide (Murray *et al.*, 2001). COPD is rare before the age of 40, but after that age symptoms of hypersecretion occur with increasing frequency. Persistent airflow obstruction becomes more common at around age 60. The prevalence of COPD then increases progressively until 60-70 year of age when, in large part due to mortality, it becomes more stable. The greater prevalence of COPD in men has diminished recently because of increased smoking by women (Feinleib *et al.*, 1989). Smoking cessation early in the natural history of COPD not only returns the subsequent rate of declining function towards normal, but also reduces future mortality (Calverley and Walker, 2003).

COPD, like CF, is characterised by persistent inflammation throughout the airways and lung parenchyma, the intensity and cellular characteristics of which vary as disease progresses. The inflammation involves a multitude of cells, mediators, and inflammatory effectors. Activated inflammatory cells release mediators that are capable of damaging lung structures. These mediators include a spectrum of potent proteases, oxidants, and toxic peptides. The damage induced by these moieties may further potentiate inflammation by releasing chemotactic factors from the extra-cellular matrix (Shapiro and Ingenito, 2005). Assessment of forced expiratory volume$_1$ (FEV_1) / forced vital capacity (FVC) is considered as the most sensitive indicator for early airway obstruction, and an accelerated decline in pulmonary function can be readily detected from repeated yearly measurements.

There are several risk factors as to how and why COPD progresses which include α1-protease inhibitor (α1-PI) deficiency, recurrent bronchopulmonary infection, air pollution, socioeconomic status, lower birth weight, and a history of severe childhood respiratory infections (Edelman *et al.*, 1992; Snider, 1989). However, the most striking relationship is between cigarette smoking and COPD. Nearly 90% of all COPD patients are either current or ex-smokers (Snider, 1989; Peto *et al.*, 1992). Figure 1.7 summarises the pathways by which cigarette smoke (CS) components both directly and indirectly could lead to the development of COPD in smokers and passive smokers. However, it should be acknowledged that, there is great variability in lung function among smokers, with only a minority (only about 20%) of smokers developing COPD.

In healthy non-smokers, macrophages comprise the major host defence cell in the lower airspace. Cigarette smoking is associated with more than 5 fold increase in total cells recovered by BAL, with macrophages comprising 95-90% (Shapiro, 1999). The inflammatory response in the airways and lung parenchyma in COPD is characterised by an increase in macrophages, T lymphocytes (predominantly $CD8^+$ T cells), and neutrophils. There may also be an increase in eosonophils in some patients, particularly during exacerbations. Many studies have revealed a correlation between the number of inflammatory cells of various types in the lung and the severity of COPD (Finkelstein *et al.*, 1995; O'Shaughnessy *et al.*, 1997; Di Stefano *et al.*, 1998; Keatings *et al.*, 1996). The role of neutrophils in COPD is shown by the fact that neutrophils, but not macrophages, are increased in the BAL fluid of COPD patients who have never smoked (Lusuardi *et al.*, 1994). However, the role of neutrophils in COPD is not yet clear to date. Neutrophils have the capacity to induce tissue damage through the release of serine proteases and oxidants (Figure 1.8). Priming is a prerequisite for degranulation and superoxide anion generation in neutrophils (Condliffe *et al.*, 1998). Neutrophils in the peripheral circulation show evidence of priming in COPD (Noguera *et al.*, 2001), but this may result from, rather than contribute to, lung pathophysiology. There are several chemotactic signals that have the potential for neutrophil recruitment in COPD, including leukotrine B4 (LTB_4), IL-8 and related CXC chemokines, including growth-related oncoprotein-α and

epithelial neutrophil activating protein of 78kDa which are increased in COPD airways (Traves *et al.*, 2002; Tanino *et al.*, 2002). These mediators may be derived from AMs and epithelial cells, but the neutrophil itself may be a major source of IL-8 (Bazzoni *et al.*, 1991).

There are two central hypotheses as to why and how COPD could develop in individuals affected, which are briefly outlined below:

1- *The oxidant:antioxidant imbalance hypothesis*- Pulmonary antioxidant defences are widely distributed and include both enzymatic and non-enzymatic systems (Halliwell, 1996). The major enzymatic antioxidants are superoxide dismutase (SOD), which degrades $O_2^{\cdot -}$ and catalase, and the glutathione (GSH) redox system, which inactivates H_2O_2 and hydroperoxidases. Vitamin E, β-carotene, vitamin C, uric acid, flavonoids, and billirubin are some of the non-enzymatic factors that may function as antioxidants (Halliwell, 1996). There is compelling evidence for increased oxidative stress in patients with COPD as a number of studies have shown an increased oxidant burden and consequently increased markers of oxidative stress in the airspaces, breath, blood, and urine in smokers and patients with COPD (Repine *et al.*, 1997). The lungs are exposed to oxidants generated either endogenously or exogenously. Cigarette smoke (see below) and inflammatory cells have the capacity to produce reactive oxygen species, and they have been postulated to play a variety of roles in the pathogenesis of emphysema and COPD (Rahman, 2005). Oxidants cannot degrade extra-cellular matrix but might modify elastin, making it more susceptible to proteolytic cleavage. Moreover, Drost and co-workers (2006) recently demonstrated that inhibitor of NF-κB-α (IκBα) levels were significantly decreased in healthy smokers and current and ex-smoking patients with COPD when compared with non-smokers, with an associated increase in NF-κB

DNA binding in current smokers, resulting in transcription of pro-inflammatory genes such as IL-8 and TNF-α. Finally, reactive oxygen species may also promote apoptosis or necrosis of structural cells, a recent concept for imitation of emphysema.

2- *The elastase:antielastase imbalance hypothesis*- Just over 40 years ago, two lines of evidence, one experimental and one clinical, suggested that emphysema is caused by destruction of elastic fibres by elastase. This concept started with the observation by Laurell and Eriksson (1963) that showed individuals deficient in the serum protein α1-PI are at risk of developing emphysema. The second evidence was produced by Gross and co-workers (1965) which demonstrated papain instillation into the lungs of rodents induced emphysema. Subsequently, to further prove the truth in this hypothesis, investigators instilled a variety of proteases into animal lungs. Kuhn and colleagues (1976), Senior and co-workers (1977), Janoff and associates (1977), and Snider and colleagues (1984) were among the group of investigators who subsequently demonstrated that only elastolytic proteases, including pancreatic elastase and the more relevant human NE-caused emphysema. Hoidal's group showed that proteinase 3 (1988) also caused destructive lung disease. More recently, Shapiro and colleagues (2003) generated an $NE^{-/-}$ mouse line, and to address the role of NE in pulmonary emphysema they exposed NE-deficient mice and wild-type (WT) littermate controls to long-term cigarette smoke. They found that compared to WT littermates, mice that were deficient in NE were significantly protected (59%) from the development of emphysema. It has become clear that the elastase:antielastase imbalance can have several causes including inflammation, genetic factors (*e.g.*, α1-PI gene mutation, the most common of which is the Z mutation that converts 342Glu to Lys; see below), and oxidative stress, and also by direct cigarette

smoking. To this day, the elastase:antielastase hypthesis remains a prevailing concept in emphysema, although as one can acknowledge, the real is picture is far more complex.

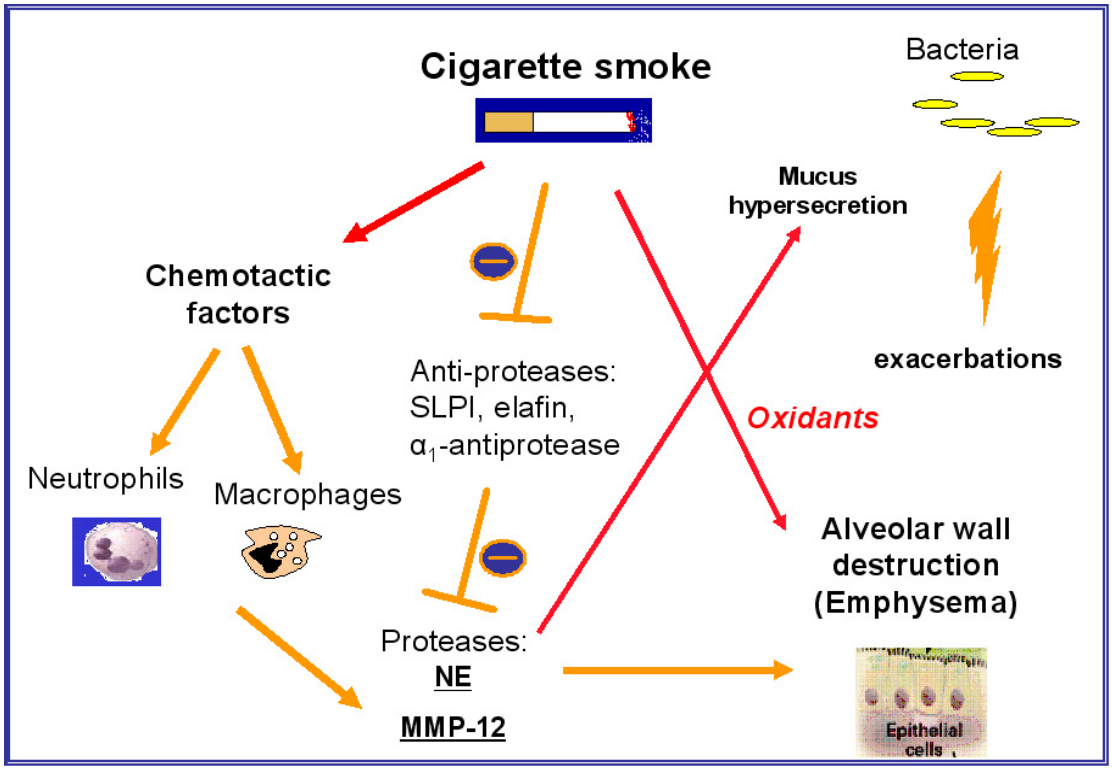


Figure 1.7. Cigarette smoke, oxidative stress, proteolytic activity of enzymes and emphysema/COPD

Numerous processes increase lung oxidative stress and protease:antiprotease imbalance and contribute to a variety of abnormalities that contribute to COPD. Adapted from Repine *et al*. (1997). Red arrows are indicative of direct effects of the danger, and the orange arrows are indicative of the indirect consequence of the danger signals present in the tissues.

1.4.1. Infections in COPD

It is well known that the lower airways of healthy individuals are not colonised by microorganisams (van Alphen *et al.*, 1995), whereas patients with COPD are frequently infected for many known and unknown reasons, one obvious reason being the possible impairment of the mucociliary system to prevent infection/colonisation (Wilson *et al.*, 1996). Microorganisams that eventually enter respiratory airways, via inhaled air or following micro-aspiration of the pharyngeal contents, impact with mucous of the airways, and then are trapped by mucin macromolecules. Consequently, the mucociliary drainage is able to carry these microorganisams up to the oropharynx, which can last up to 6 hours (Knowles and Boucher, 2002). Some patients with COPD are prone to frequent exacerbations which are a major cause of morbidity and mortality and an important determinant of health related quality of life (Seemungal *et al*., 1998). COPD exacerbations have been associated with a number of etiological factors, including infection and pollution episodes. COPD exacerbations are frequently triggered by upper respiratory tract infections, and these are commoner in the winter months, when there are more respiratory viral infections in the community (Donaldson *et al.*, 1999).

Respiratory viral infections occurring during COPD exacerbations are more likely to lead to hospitalisation. Viral infections are an important trigger for COPD exacerbations. Recent studies have shown that around half of COPD exacerbations are associated with viral infections that the majority of these were due to rhinovirus (Wedzicha, 2004). The major viruses that cause upper and lower respiratory tract infections and that are thus associated with exacerbations are listed in Table 1.3.

Between 25 and 100% of stable COPD patients have been shown to have lower respiratory colonisation by bacteria including *NTHI*, *Streptococcus pneumoniae*, and *Branhamella catarrhalis* (Palmer, 1987; Elborn and Shale; 1992). The presence of bacteria in the lower airways of COPD patients implies a breach of host defence mechanisms, which set up a vicious cycle of epithelial damage, impaired mucociliary clearance, mucous hypersecretion, increased submucosal

vascular leakage, and inflammatory cell infiltration. The airway bacterial load in the stable state is associated with airway markers, and thus, airway inflammation (Hill *et al.*, 2000). Several recent studies by Wedzicha and co-workers (2005) have shown higher sputum IL-8 levels are associated with higher bacterial load and faster decline in FEV_1 and thus, the bacteria increase airway inflammation in the stable state and this in turn may increase disease progression.

However, despite all these observations confirming the immunocompromised COPD lungs, the role of DCs in COPD has not been thoroughly investigated and still awaits further more in-depth studies (see Section 1.5.2 for details).

Table 1.3. Respiratory viruses associated with COPD exacerbations

Table taken from a review by Wedzicha (2004).

Viruses	Rhinovirus (common cold)
	Coronavirus
	Influenza A and B
	Parainfluenza
	Adenovirus (Ad)
	Respiratory syncytial virus (RSV)
Atypical organisms	*Chlamydia pneumoniae*
	Mycoplasma pneumoniae

To summarise, five potential pathways by which bacteria could contribute to the course and pathogenesis of COPD are as follows: (*i*) childhood lower respiratory tract infection impairs lung growth, reflected in smaller lung volumes in adulthood, (*ii*) bacteria cause a substantial proportion of acute exacerbations of chronic bronchitis which cause considerable morbidity and mortality, (*iii*) chronic colonisation of the lower respiratory tract by bacterial pathogens amplifies the chronic inflammatory response present in COPD and leads to progressive airway obstruction (vicious circle hypothesis), (*iv*) bacterial pathogens invade and persist in the respiratory tissues, alter the host response to cigarette smoke, or induce a chronic inflammatory response and thus contribute to the pathogenesis of COPD, and (*v*) bacterial antigens in the lower airway induce hypersensitivity that enhances airway hyperreactivity and induces eosinophilic inflammation.

1.5. CIGARETTE SMOKE (CS)

As described earlier, CS and other risk factors induce tissue destruction in the lung leading to emphysema and COPD in individuals. Cigarette smoking is a worldwide epidemic, and it is one of the main preventive causes of death and disability worldwide (Table 1.4). CS can be separated into two phases, a gas phase that passes through a filter and a tar that collects on the filter. Both phases of CS contain very high concentrations of free radicals and other chemical compounds. Cigarette tar contains about 10^{17} free radicals per gram and gas-phase CS contains more than 10^{17} free radicals per puff (Church and Pryor, 1985). The gas phase contains more than 4500 compounds which comprise of five known human carcinogens and many toxic agents, including carbon monoxide, ammonia, acrolein, acetone, nicotine, benzopyrenes, hydroquinone and nitrogen oxides. Therefore, it would not be surprising to see that chronic inhalation of CS alters a wide range of immunological functions, including innate and adaptive immune responses. Indeed it has been speculated that many of the health consequences of chronic inhalation of CS might be due to its adverse effects on the immune system (Sopori, 2002). Many studies have linked smoking to higher incidence of infections, in particular those

infections of the respiratory system (see above). All in all, as explained above, these findings indicate that CS, at least in part, is directly responsible for the chronic infections in smokers and patients suffering from emphysema and COPD. In this section, the known effects of CS on various components of the immune system, with a special emphasis on DCs and antigen presentation, will be reviewed.

Table 1.4. World Health Organisation (WHO) Smoke Statistics

Taken from the WHO website (http://www.who.int), data published on 28 May 2002.

Adults

- About a third of the male adult global population smokes.
- Smoking related-diseases kill one in 10 adults globally, or cause four million deaths. By 2030, if current trends continue, smoking will kill one in six people.
- Every eight seconds, someone dies from tobacco use.
- Smoking is on the rise in the developing world but falling in developed nations. Among Americans, smoking rates shrunk by nearly half in three decades (from the mid-1960s to mid-1990s), falling to 23% of adults by 1997. In the developing world, tobacco consumption is rising by 3.4% per year.
- About 15 billion cigarettes are sold daily - or 10 million every minute.
- About 12 times more British people have died from smoking than from World War II.
- Cigarettes cause more than one in five American deaths.
- Among WHO Regions, the Western Pacific Region - which covers East Asia and the Pacific - has the highest smoking rate, with nearly two-thirds of men smoking.
- The tobacco market is controlled by just a few corporations - namely American, British and Japanese multinational conglomerates.

Youths

- Among young teens (aged 13 to 15), about one in five smokes worldwide.
- Between 80,000 and 100,000 children worldwide start smoking every day - roughly half of whom live in Asia.
- Evidence shows that around 50% of those who start smoking in adolescent years go on to smoke for 15 to 20 years.

1.5.1. CS and the innate immune system

There is an endless body of evidence in the literature describing the adverse effects of CS in both humans and experimental animals as well as cell/tissue culture experiments *in vitro* and *ex vivo.* AMs and monocytes are the most important part of the innate immune system in the lungs. CS increases the number of AMs by several folds, and these express increased levels of lysosomal enzymes and secrete elastase (Barnes, 2004). In addition, AMs from smokers produce significantly higher levels of oxygen radicals and have higher myeloperoxidase activity. However, despite this the AMs from smokers have a reduced ability to phagocytose and/or kill bacteria. King and colleagues (1988) tested whether AMs from normal non-smokers versus normal smokers differed in their ability to phagocytose and to kill the facultative intra-cellular bacterium *Listeria monocytogenes*. Although they found no differences in phagocytosis, the AMs from non-smokers, however, killed *Listeria*, whereas those from smokers had no bactericidal or bacteriostatic activity. Further, McCrea and colleagues (1994) showed that even in young healthy smokers the pulmonary microenvironment is markedly abnormal, characterised by depressed levels of IL-6, macrophages that have a markedly depressed capacity for LPS-induced cytokine release and, in some smokers, increased concentrations of IL-8. These cytokines are crucial for early responses to pathogens and the up-regulation of local host defences. This defect in bacterial clearance has also been shown in animal models of infection following chronic exposure to CS. The most recent study on this was performed by Drannik and co-workers (2004) in which they observed a delayed rate of *P.a.* clearance in smoke-exposed compared with sham-exposed mice.

In addition to AMs, other cells involved in innate immunity are affected by CS. Because CS is associated with increased risk of various cancers, several investigators have evaluated its effects on the function of NK cells. Ferson and colleagues (1979) showed that activity of NK cells against melanomas and other cancer cells was reduced significantly in smokers compared to non-smokers. These data have also been confirmed and extended in animal models, in which resistance to the growth of implanted tumour cells was significantly lower in smoke-exposed mice

(Holt, 1997). Further, a more recent study has shown that male smokers have a decreased NK-cells absolute number compared to their control group (Moszczynski *et al*., 2001).

In addition to the cells described above, CS components are also able to directly influence the epithelial cells of the oral cavity and the respiratory tract, as well as endothelial cells and fibroblasts. In the presence of LPS, CS has been shown to inhibit IL-8 mRNA expression and release of all chemokines by primary human alveolar epithelial type II cells (Witherden *et al*., 2004). Glutathione protected the cells against the effects of CS, suggesting involvement of the oxidative mechanisms. In a similar study by Laan and co-workers (2004), it was demonstrated that the pre-treatment with CS extract concentration dependently inhibited the LPS-induced GM-CSF and IL-8 protein release, which was accompanied by decreased expression of mRNA in a human bronchial epithelial cell line (Beas-2B). The increase of neutrophil chemotaxis induced by conditioned medium from LPS-treated Beas-2B cells was also suppressed by CS. In addition, they demonstrated that the activity of LPS-induced transcription factor activating protein-1, but not NF-κB, was down-regulated by CS.

Wickenden and colleagues (2003) showed that higher concentrations of CS condensate induced necrosis in alveolar epithelial cells and human umbilical vein endothelial cells and that CS was able to inhibit apoptosis induced by staurosporine or Fas ligation, with both effects prevented by the antioxidants glutathione and dithiothreitol. However, these observations are contradictory to the previous data in the literature suggesting that in fact CS could induce cell death via activating the apoptotic pathways (Tithof *et al*., 2002). CS extract has also been shown to induce oxidative stress and apoptosis in human lung fibroblasts which could be protected by *N*-acetylserine which is a potent antioxidant agent (Carnevali *et al*., 2003).

1.5.1.1. CS and antiproteases/antimicrobial proteins

Apart from its direct effects on cells involved in the innate immune response and the epithelial cells, CS has also been shown to inactivate antiproteases secreted in the lung. Many amino acid residues of proteins are susceptible to oxidation by reactive oxygen specious. Methionine, the most sensitive of amino acids to oxidation, is readily transformed into methionine sulfoxide. Of the three major NE inhibitors in lung, α1-PI was the first antiprotease described to be affected in late 1970s and early 1980s. Gadek *et al.* (1979) reported that α1-PI activity is decreased in cigarette smokers' lung fluid in comparison to similar fluid obtained from non-smokers. α1-PI was shown to be inactivated by both reactive oxygen specious (*e.g.*, hydrogen peroxide, superoxide anion, and hydroxyl radicals) derived from smoke-activated neutrophils, that accumulate in the lung following smoking, as well as exposure to direct CS (Carp and Janoff, 1979; Carp *et al.*, 1982). The inactivation of α1-PI is believed to involve the oxidation of a critical methionine residue. Further to this, secretory leukocyte protease inhibitor (SLPI) was also shown to loose its anti-NE activity following exposure to a variety of oxidants *in vitro* (Vogelmeier *et al*., 1997), and by direct CS exposure in an *in vivo* system (Cavarra *et al*., 2001). More recent data shows that, exposure of full length and C-terminus recombinant elafin molecules to oxidants also results in its poor protease inhibitory activity *in vitro*. At their physiological concentration, fully oxidised elafin and C-terminus elafin did not inhibit more than 30% of an equimolar concentration of NE or proteinase 3 (Nobar *et al*., 2005).

1.5.2. CS and the adaptive immune system

A well-documented effect of CS in humans is leukocytosis, however the function of these cells (predominantly macrophages and neutrophils) is greatly reduced. Smoking has been shown to predispose individuals to more incidents of infections. In similar animal models, mice chronically exposed to CS were more susceptible to influenza and murine sarcom virus (Sopori, 2002; Razani-Boroujerdi *et al*., 2004). This might result, in part, from lower titers and a decreased half-life of the antigen-specific Abs in smokers. On the other hand, a deficiency in DC function might explain these observations. Although to this date there are no comprehensive

studies on pulmonary DCs in healthy smokers or past/current COPD patients, this issue has been recently studied to an extent using both *in vitro* models and *in vivo* animal systems. Nevertheless, as will be discussed below, these data are very contradictory to each other and as yet do not give a clear picture of the effects of CS on DCs. In one of the first studies to address this issue, Zeid and Muller (1995) documented an increase in pulmonary Langerhans-like cells on tissue sections of CS-exposed mice compared to their controlled group. In line with these observations, D'hulst *et al*. (2005) documented a similar relentless increase in lung DCs in a murine model of CS-induced pulmonary emphysema. They showed that after 6 months of CS inhalation, DC numbers in the BAL fluid were 10-fold greater than those observed in control animals. In contrast, Robbins and co-workers (2004) reported a decrease in pulmonary DC numbers with dramatically reduced levels of CD86 CSM after CS inhalation (2-4 months). Furthermore, CS-smoked mice challenged with three consecutive doses (2×10^8 plaque-forming units [pfu]) of replication-deficient Ad had reduced expansion and maximal activation of CD4 and CD8 T-cells, as well as significantly decreased serum Ad-specific IgG1 and IgG2a levels (Robbins *et al*., 2004). In the most recent published study examining the effects of CS on pulmonary DCs, Tsoumakidou and co-wrokers (2007) demonstrated that the number (per square millimeter) of $CD83^+$ mDCs was significantly lower in smoker patients with asthma in comparison with never-smoker steroid-naïve and steroid-treated patients with asthma or control subjects. Moreover, they showed that B cells were fewer in smoker versus never-smoker steroid-naive and steroid-treated patients with asthma and in smoker steroid-naïve patients with asthma versus control subjects (Tsoumakidou *et al*., 2007). Relatedly, in agreement with these studies, unpublished data from Shapiro's group (Franco *et al*., personal communication) demonstrate that *in vivo* smoke-exposed lung DCs have impaired maturation and ability to present antigen to $CD4^+$ T cells in the LNs as compared to DCs in control mice. The reasons for these divergent observations could be attributed in part to the methodology used for exposure regiments (*e.g.*, different brands of cigarette, different doses of CS and experimental time points) and the cohort of patients used in the study. Similarly, the *in vitro* studies available are somehow contradictory. Recently, Vassallo and colleagues (2005) demonstrated that CS extract inhibited

human DC-mediated priming of T cells. CS also significantly inhibited IL-12p70 release by LPS-matured DCs, in contrast IL-10 secretion was increased by these cells. Conditioning of DCs with CS extract also suppressed LPS-mediated induction of CD40, CD80, and CD86, and suppressed maturation-associated CCR7 expression. These data provided evidence that soluble components extracted from CS suppress key DC functions and favour the development of Th2 immunity. Also, human monocyte-derived DCs cultured in the presence of nicotine have also been shown to manifest lower endocytic and phagocytic activities, and in the presence of LPS produced lower levels of pro-inflammatory cytokines, notably IL-12. Nicotine-treated DCs also revealed a reduced ability to stimulate DC-dependent T cell Th1 response, as evidenced by a decreased frequency of IFN-γ-producing effector cells (Nouri-Shirzai and Guint, 2003). In a follow up study, the same group showed that in a nicotinic environment, while human monocytes differentiate into DCs with a typical morphology, they display unique phenotype and cytokine profile that adversely affect their function. Furthermore, nicotinic DCs expressed lower levels of CD40, CD80 and CD86 CSMs and displayed profoundly reduced Th1 promoting capacity (Guinet *at al.*, 2004). On the other hand, oxidative stress and reactive oxygen species have been shown to activate human DCs and up-regulate IL-8 and TNF-α synthesis (Rutault *et al*., 1999; Verhasselt *et al*., 1998; Kantengwa *et al*., 2002).

CS has also been shown to suppress T cell proliferation. Chang and co-workers (1990) documented that chronic inhalation of CS in mice preferentially inhibited the antigen-specific T cell proliferative response of lung-associated LNs compared to anatomically distant LNs. These authors believe that T cell function in lung-associated lymphoid tissue may be inhibited in smokers, leading to a localised predisposition to respiratory tract infections. The effect of CS might be partly due to the direct effects of nicotine on T cells (Geng Y *et al.*, 1996; Razani-Broujerdi *et al.*, 2004). To determine the mechanism of CS-induced immunosuppression and to compare it with chronic nicotine exposure, Karla *et al*. (1999) presented evidence suggesting that T cells from long-term CS-exposed rats exhibited decreased antigen-mediated proliferation and constitutive activation of protein tyrosine kinase and

phospholipase C-γ1 activities. Moreover, spleen cells from CS-exposed and nicotine-treated animals had depleted inositol-1, 4,5-trisphosphate-sensitive Ca^{2+} stores and a decreased ability to raise intra-cellular Ca^{2+} levels in response to T cell antigen receptor ligation. These results suggested that chronic smoking causes T cell anergy by impairing the antigen receptor-mediated signal transduction pathways and depleting the inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores.

1.6. NEUTROPHIL ELASTASE (NE)

Polymorphonuclear leukocytes (PMN/neutrophils) play a crucial role in the first line of defence against invading bacteria, fungi and protozoa. Neutrophil infiltration is a common pathological feature in acute inflammatory disorders. The primary function of neutrophils is the phagocytosis and eradication of microorganisams. After being generated in the BM the neutrophils circulate in the blood stream. Upon infection/inflammation, they adhere to the endothelium and migrate to the infected site where they release oxygen radicals and their internal constituents, which are stored in different granules (Faurschou and Borregaard, 2003). Neutrophils contain at least four types of granules: azurophil granules, specific granules, gelatinase granules and secretory granules (Faurschou and Borregaard, 2003). The various subsets of granules contained within the neutrophil constitute an important reservoir not only of antimicrobial proteins, proteases, and components of the respiratory burst oxidase, but also of a wide range of membrane-bound receptors for endothelial adhesion molecules, extra-cellular matrix proteins, bacterial products, and soluble mediators of inflammation. It is the controlled mobilisation of these cytoplasmic organelles that permits transformation of the neutrophil from a passively circulating cell to a potent effector cell of innate immunity.

Human NE is a serine protease found in the azurophil granules of the neutrophil. The gene for NE, *ELA 2*, is located within a 50-kilobase segment in the terminal region of the short arm of chromosome 19 (Zimmer *et al.*, 1992). The

glycoprotein product contains 218 amino acids and four disulfide bridges, and is a member of the serine protease family (Bode *et al*., 1989). Neutrophils can be stimulated to release NE upon exposure to various cytokines and chemoattractants, including TNF-α, IL-8, C5a, LPS, and a tripeptide derived from bacterial wall (*N*-formyl-methionyl-leucyl-phenylalanine [fMLP]) (Lee and Downey, 2001).

The concentration of NE in neutrophils exceeds 5mM (Belaaouaj *et al*., 1998); each neutrophil contains approximately 400 NE-positive granules, and the total cellular concentration of NE has been estimated at 1 to 2 picograms (Vender *et al*., 1996). Although NE is most abundant in neutrophils, small amounts are expressed by monocytes and T cells (Ariel *et al*., 1998; Shapiro 1994). Other cells, including epithelial, endothelial, and fibroblasts, also synthesise proteases (Barnes *et al*., 2003). When neutrophils are activated, NE is rapidly released from cytoplasmic granules into the extra-cellular space, although some remains bound to the neutrophil plasma membrane (Owen *et al*., 1995).

Passively released or actively secreted NE from neutrophils has been linked to the pathologic processes of a variety of inflammatory diseases. Neutrophils which normally pass through the lung relatively unimpeded, are sequestered and activated in the pulmonary microvasculature during the genesis of an inflammatory response, such as the chronic inflammatory situations that exist in CF or COPD lungs (see previous sections). Neutrophils accumulating in the airways of patients with CF and COPD, as a response to chronic infection/inflammation, release high concentrations of NE (~ 0.5-5 μM) (Fujita *et al*., 1990; Doring *et al*., 1995). In the lung, NE functions either intra-cellularly or after secretion into the extra-cellular environment. Under normal circumstances, the extra-cellular NE activity is inhibited by NE inhibitors, such as the endogenous serine protease inhibitors (serpin) that form complexes with the NE. The major NE inhibitors in the extra-cellular milieu of the lung are elafin, SLPI, and α1-PI (also called α1-antitrypsin; see next section for details). In the normal lung, natural NE inhibitors prevent the deleterious effects of proteases, because they are present in higher concentrations than the proteases, providing a protective 'barrier'. However, when the protease concentration in the

local milieu overwhelms the local antiprotease protective barrier, such as the situation that occurs in chronic/infective lung disease, *e.g.*, CF/COPD, this results in excessive extra-cellular activity, leading to degradation of lung tissue (Figure 1.8).

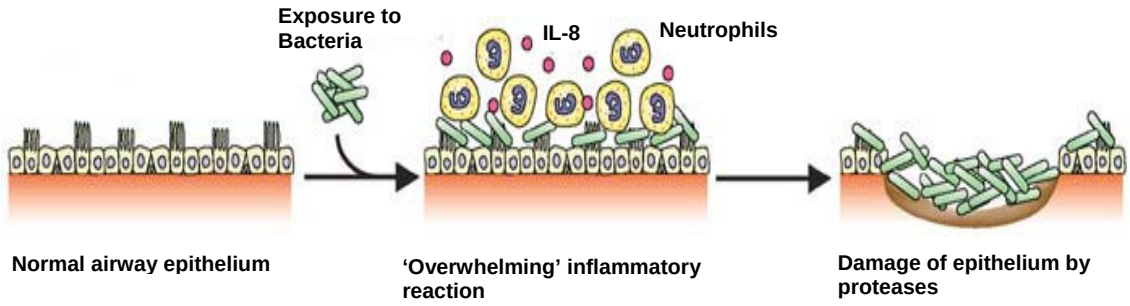


Figure 1.8. Proteolytic destruction of airway epithelium and bacterial colonisation

Neutrophils accumulate in lung inflammatory tissues (*e.g.*, COPD or CF lungs) and upon activation release high concentrations of oxidants and proteases such as NE in the microenvironment which in addition to killing bacteria overwhelms the natural local antiprotease barrier and damages the lung tissue by its potent proteolytic activity. Adapted from Takai *et al*. (2004).

1.6.1. NE proteolytic modification of proteins

Many biological molecules like cytokines and their receptors contain putative cleavage sites for neutrophil serine proteases. It is therefore not surprising that many receptors, cytokines and other molecules have been found to be natural substrates for NE (Table 1.5). For instance, free active NE is able to degrade the extra-cellular matrix molecules such as collagen, fibronectin, laminin, proteoglycans, and more importantly elastin, an abundant component of lung parenchyma. NE also affects the epithelium and endothelium by degrading vascular endothelial (VE)-cadherin (Ginzberg *et al*., 2001), and increasing cellular permeability (Peterson *et al*., 1995), and more dramatically, by inducing cell lysis (Nahori *et al*., 1992; Simpson *et al*., 2001b). More recently it has been shown to induce epithelial cell apoptosis (Ginzberg *et al*., 2004). In addition, NE may potentially be involved in modulating

inflammation through cleavage of a number of cell surface molecules. For instance, NE has been shown to reduce the surface expression of different receptors such as CD2, CD4 and CD8 on lymphocytes (Doring *et al*., 1995), CD16 (Tosi *et al*., 1992), CD43 (Remold-O'Donnell *et al*., 1995), and the 75-kDa from of the TNF-α receptor (Porteu *et al*., 1991) on neutrophils.

The regulation of an intense inflammatory response in the lung depends on the rapid removal by phagocytosis of dying (apoptotic) inflammatory cells prior to their necrotic degeneration. The recognition mechanisms involved in apoptotic cell removal include CD36 and other scavenger receptors, CD14, integrins, the phosphatidylserine receptor (PSR), and soluble tethering proteins such as collectins and thrombospondin. NE has recently been demonstrated to impair the apoptotic cell recognition by cleaving various receptors involved in the removal process of apoptotic cells. These include CD14 and PSR expressed on surface of macrophages (Le-Barillec *et al*., 1999; Vandivier *et al*., 2002; Henriksen *et al*., 2004). Hence, it is speculated that severe and sustained inflammation in the airways of CF and COPD patients may, due to the proteolytic activity of NE, in part, be enhanced by the defective clearance of dying inflammatory cells.

Chemokines and cytokines have also been reported to be processed and cleaved by NE. For instance, Valenzuela-Fernandez and colleagues (2002) recently demonstrated that limited degradation by NE, restricted to the amino-terminal domains of both SDF-1α and CXCR4, abrogates their interaction and function. Last but not least, a number of cytokines including IL-2 (Ariel *et al*., 1998), G-CSF (Hunter *et al*., 2003) and TNF-α (van Kessel *et al*., 1991) are also proteolytically processed and degraded by NE, leading to their inactivation.

1.6.2. NE activation of cellular receptors

NE might not only regulate the activity of chemokines and cytokines by proteolysis, but might also modulate host immune responses through cellular activation. NE appears to be the most important factor present in the CF lung

responsible for IL-8 expression because inhibition of NE activity in CF BAL fluid almost completely prevents IL-8 message in bronchial epithelium (Nakamura *et al*., 1992). Interestingly, NE has been shown to act at least in part via an IL-1 receptor-associated kinase-1/MyD88/NF-κB-dependent pathway in bronchial epithelial cells; this can be inhibited by a dominant negative variant of MyD88 (Walsh *et al*., 2001). More recent data by the same group suggests that the up-regulation of IL-8 by NE occurs in part through the cell surface membrane bound TLR4, which can be readily abrogated by using a TLR4 neutralising Ab (Devaney *et al*., 2003). Clearly, as discussed in the previous sections, expression of IL-8 and LTB_4 has repercussions for neutrophil migration to the lung, and given the high neutrophil and NE burden present in the CF/COPD lungs this has lead to the 'vicious cycle' hypothesis whereby NE is the driving force behind IL-8 production and neutrophil influx into the CF/COPD lung.

Studies also indicate that NE contributes to the chronic inflammatory airway disease by inducing mucin production in airway epithelial cells. Mucins are involved in binding and removing bacteria via the mucociliary ladder, a process by which bacteria are transported along the respiratory tract and then ingested (Rose *et al*., 2001). For instance, NE increases the mRNA expression of a major respiratory tract mucin, MUC5AC, by enhancing mRNA stability (Zheng *et al*., 2006). In normal human bronchial epithelial cells, NE also increased MUC4 mRNA levels in both a concentration- and time-dependent manner (Fischer *et al*., 2003). The role of NE in mucin production is also most relevant in CF patients where there is an NE burden and mucus hypersecretion but may also be important in other chronic lung diseases, such as COPD and asthma, where mucus hypersecretion is also a common feature.

In addition, NE has been shown to increase the secretion of elafin and SLPI by lung epithelial cell lines *in vitro* (Sallenave *et al*., 1994). NE is also able to up-regulate human beta-defensin-2 (HBD-2, a small cationic peptide with a broad range of antimicrobial activity) gene expression in bronchial epithelial cells (Griffin *et al*., 2003). However, the mechanisms of elafin, SLPI and HBD-2 up-regulation by NE are currently unknown.

Table 1.5. Overview of different targets of NE

Target	*Hypothetical biological function*	*Reference*
Receptors		
PAR-1	Inactivation, modulation of response	Loew *et al*., 2000
PAR-2	Inactivation, modulation of response	Loew *et al*., 2000
PAR-3	Inactivation, modulation of response	Cumashi *et al*., 2001
IL-2Rα	Inhibiting cellular response and prolongation of cytokine half-life time	Bank *et al*., 1999b
TNF-RII	Inhibiting cellular response and prolongation of cytokine half-life time	Porteu *et al*., 1991
C5aR (CD88)	Inhibition of chemotaxis, feedback mechanism	Tralau *et al*., 2004
C3b/C4b R (CD35)	Inhibition of complement signalling	Sadallah *et al*., 1999
urokinase R (CD87)	Regulation of cell migration	Beaufort *et al*., 2004
G-CSF-R	Growth inhibition	Hunter *et al*., 2003
CD43 (sialophorin)	Regulation of adhesion	Hunter *et al*., 2003
CD16 (low-affinity IgG Fc receptor)		Galon *et al*., 1998
CD14	Inhibition of LPS-mediated cell activation / apoptotic cell recognition	Le-Barillec *et al*., 1999
CD2, CD4 and CD8	Impairment of T lymphocytes	Doring *et al*., 1995
Cytokines/Chemokines		
TNF-α	Regulation of inflammation	van Kessel *et al*., 1991
IL-2	Regulation of inflammation	Ariel *et al*., 1998
IL-6	Regulation of inflammation	Bank *et al*., 1999b
IL-8	Regulation of inflammation	Leavell *et al*., 1997
G-CSF	Growth inhibition	Hunter *et al*., 2003
Integrins/others		
Intercellular adhesion molecule-1	Regulation of adhesion	Champagne *et al*., 1998 Robledo *et al*., 2003
Vascular endothelium cadherin	Regulation of adhesion	Hermant *et al*., 2003
Proepithelin	Wound healing	Zhu *et al*., 2002

1.6.3. Therapeutic interventions to inhibit NE

As neutrophils are often involved in inflammatory diseases (see above), it is temping to speculate that inhibitors of serine proteses, in particular NE, may provide a logical therapeutic approach in these diseases. Indeed, animal experiments have shown that elafin efficiently reduces posthypoxic damage in the lung (Zaidi *et al*., 2002), the heart (Cowan *et al*., 1996; Ohta *et al*., 2004) and a vein graft model (O'Blenes *et al*., 2000). Ad augmentation of elafin has also been demonstrated to protect murine lungs against acute injury mediated by activated neutrophils and bacterial infection (Simpson *et al*., 2001b; McMichael *et al*., 2001). Antiprotease clinical trials have been performed using α1-PI and SLPI, both of which have successfully inhibited NE activity *in vivo* in CF and α1-PI deficiency (Hubbard *et al*., 1989; McElvaney *et al*. 1991; McElvaney *et al*., 1992).

A small molecular weight synthetic NE inhibitor called 'sivelestat' has been shown to be effective in multiple animal models of acute lung injury (ALI) by the same investigators (Zeiher *et al*., 2002). In a similar setting, a phase 3 study in patients with ALI associated with systemic inflammatory response syndrome, demonstrated that sivelestat improved pulmonary function and slightly reduced the duration of intensive care unit stay (Tamakuma *et al*., 2004). In a more recent clinical trial Inoue and colleagues (2006) evaluated whether sivelestat improves leukocyte deformability and pulmonary function in patients with ALI, and concluded that sivelestat is able to reduce NE concentration and neutrophil stiffness and improves pulmonary oxygenation in patients with ALI. Similarly, sivelestat as well as the specific NE inhibitor EPI-hNE-4 have been shown to have beneficial effects on belomycin-induced lung fibrosis (Taooka *et al*., 1997; Honore *et al*., 2004). Three major naturally produced respiratory NE inhibitors (elafin, SLPI and α1-PI) are discussed in more details in the following section.

1.7. LUNG NE INHIBITORS

The natural inhibitors of NE constitute a pleiotropic group of molecules that are capable of limiting the function of potentially injurious pathogen and host-derived proteases. These molecules can be broadly classified into two groups, the alarm and the systemic antiproteases. Although some epithelial surfaces and cells of the immune system may produce small quantities of systemic protease inhibitors, such as α1-PI (Sallenave *et al*., 1997; Paakko *et al*., 1996), they are produced primarily by hepatocytes and constitute a circulatory source of antiprotease activity which acts as a protective shield against protease-induced injury. However, during infection and/or heightened NE release, this protective shield can be overcome and is supplemented by the activity of the alarm protease inhibitors such as elafin and SLPI. Produced by epithelial cells and cells of the immune system, alarm protease inhibitors are generated locally in areas of infection or neutrophil infiltration and are up-regulated by pathogen- and inflammation-associated factors, including NE itself (Sallenave *et al*., 1994). In addition to their antiprotease properties, and because of their biochemical characteristics (low molecular mass cationic peptides, heavily disulphide-bonded, present at mucosal sites), elafin and SLPI have recently been proposed to possess 'defensin [1] /cathelicidin [2] -like' properties. This section will describe the characteristics of the alarm protease inhibitors, elafin and SLPI, and the systemic protease inhibitor α1-PI, and discuss the evidence pertaining to their pleiotropic functions in inflammation and immunity.

[1] Defensins are 3- to 5-kDa peptide members of a widely distributed family with characteristic three-dimensional folding with six cysteine-three disulfide patterns. Defensins have broad cytotoxic activity against bacteria, fungi, parasites, viruses and even host cells (Bals and Hiemstra, 2004; Williams *et al*., 2006).

[2] Cathelicidins are a family of antimicrobial proteins found in neutrophil specific granules of which LL-37 is the only human member identified to date. LL-37 was identified based on its broad-spectrum antimicrobial activity and ability to neutralise LPS. Subsequent studies revealed other activities, such as chemotaxis of neutrophils, eosinophils, T cells and mast cells, modulation of DC function, induction of chemokine and cytokine synthesis in epithelial cells and monocytes, induction of angiogenesis and epithelial wound healing *in vitro* (Hiemstra, 2006).

1.7.1. Alarm protease inhibitors

1.7.1.1. Elafin

Elafin (previously known as elastase specific inhibitor [ESI], skin-derived antilukoprotease [SKALP] or trappin-2) was first isolated from the skin of psoriatic patients (Wiedow *et al*., 1991) and simultaneously from sputum of normal subjects and those with farmer's lung (Sallenave *et al*., 1991; Tremblay *et al*., 1996). The gene encoding elafin was cloned and sequenced by Saheki *et al*. in 1992 and subsequently by Sallenave and Silva in 1993, and shown to code for a 117 amino acids protein, of which the first 22 amino acids represent a hydrophobic signal peptide. Elafin is produced as a 9.9-kDa full-length non-glycosylated cationic protein composed of an N-terminal 'cementoin' domain which facilitates transglutaminase-mediated cross-linkage on to polymers or extra-cellular matrix components (Nara *et al*., 1994) and a globular C-terminus, containing the protease inhibitor moiety, the primary substrate of which is NE (Simpson *et al*., 1999). The C-terminus has also been identified separately in human lung secretions (Sallenave *et al*., 1991 and 1992), possibly generated from the full-length molecule by tryptase-mediated cleavage (Guyot *et al*., 2005). The C-terminal domain of elafin is often termed the whey acid protein (WAP) domain[3], leading Schalkwijk *et al*. (1999) to classify elafin as a member of the 'trappin' family, hence the name trappin-2. Elafin molecule shares ~40% homology with SLPI, although the scissile bond forming the active inhibitory site of elafin has been shown to be Ala-Met (compared with Leu-Met in SLPI), and this difference is thought to be important for the disparity in protease inhibitory spectra of these molecules (Sallenave and Ryle, 1991). Elafin has been shown to be a more specific inhibitor of proteases than SLPI (see below), since it inhibits NE, porcine pancreatic enzyme, and proteinase 3 (Wiedow *et al*., 1990; Sallenave and Ryle, 1991; Ying and Simon, 2001), but does not inhibit cathepsin G, trypsin or chymotrypsin (Wiedow *et al*., 1990; Sallenave *et al*., 1991).

[3] The 50-amino acid whey acidic protein (WAP) motif has 8 cysteines at defined positions that form 4 intra-cellular disulfide bonds. WAP motif-containing proteins, such as WFDC12, have antibacterial and/or antiprotease activities (Hagiwara *et al*., 2003). Elafin and SLPI are the WAP motif proteins with both 'antibacterial' activity and 'antiprotease' activity.

Elafin is produced in a variety of epithelial and mucosal sites, such as the skin, the oral cavity, the small intestine, and the lung (Molhuizen *et al*., 1993, Sallenave *et al*., 1993; Nara *et al*., 1994; Pfundt *et al*., 1996). Lung expression of elafin may be associated with alveolar epithelium, Clara (bronchial) cells, or tracheal epithelium (Sallenave *et al*., 1993; Nara *et al*., 1994; Suzuki *et al*., 2000). *In-vivo*, it is suggested that elafin contributes up to 20% of the antielastase capacity of the lung (Tremblay *et al*., 1996). Moreover, elafin production has also been described in inflammatory cells such as neutrophils and macrophages (Sallenave *et al*., 1997a; Mihaila and Tremblay, 2001; Simpson *et al*., 2001a). The regulation of elafin expression during inflammation has received much attention. *In vitro* bronchial and alveolar epithelial cells produce little elafin protein, but the quantity of elafin recovered from the supernatant can be greatly increased by addition of the inflammatory cytokines IL-1 and TNF-α (Sallenave *et al*., 1994). These cytokines produce similar increases of expression of elafin from keratinocytes *in vitro* (Tanaka *et al*., 2000). The c-jun, p38 MAP kinase and NF-κB pathways are thought to be implicated in the elafin response to inflammatory cytokines (Pfundt *et al*., 2000; Pfundt *et al*., 2001; Bingle *et al*., 2000). Of note, the cytokine-mediated increase in elafin production by epithelial cells is greater than the increase in SLPI production (Sallenave *et al*., 1994). Hence whereas SLPI has been described as providing a baseline antiprotease shield and can be isolated from bronchial lavage samples from healthy individuals (van Seuningen *et al*., 1995), elafin might be of greater significance during an inflammatory challenge to the lungs. In keeping with this notion, elafin mRNA expression in bronchial epithelial cells is increased by free NE, which is in abundance at times of inflammation (Reid *et al*., 1999; van Wetering *et al*., 2000).

Although inhibition of proteolytic activity has historically been considered to be the primary role of elafin, recent work has highlighted further properties of this cationic molecule which propose it as 'defensin/cathelicidin-like' entity. Simpson and colleagues (1999) in our laboratory demonstrated that elafin has antibacterial activity against Gram-negative *P.a.* and Gram-positive *S.a.*, and further established that, while antiprotease activity resides exclusively in the C-terminus, the majority of antimicrobial activity of elafin resides in its N-terminal domain (Simpson *et al*.,

1999). To confirm this, investigators from Wiedow's laboratory showed that supernatants of *P.a.* could induce elafin production in human keratinocytes, and that elafin was able to inhibit the growth of *P.a.*, but not *E.coli in vitro* (Meyer-Hoffert *et al*., 2003). Further, Simpson *et al*. (2001b) demonstrated that Ad augmentation of human elafin in murine lungs was able to protect the lungs against injury mediated by *P.a.*, and also reduced bacterial numbers. Similarly, McMichael *et al*. (2005a) using an Ad-mediated gene transfer strategy, showed that over-expression of elafin dramatically improved the clearance of *S.a. in vitro*. In addition, they demonstrated that intra-tracheal (i.t.) instillation of Ad-elafin significantly reduced the lung bacterial (*S.a.*) load and demonstrated concomitant anti-inflammatory activity by reducing neutrophil numbers and markers of lung inflammation, such as BAL levels of TNF and myeloperoxidase.

Elafin has also been demonstrated to possess immunomodulatory activities during inflammation/infection in various *in vitro* and *in vivo* experiments carried out both in our laboratory and elsewhere. Work carried out using transgenic mice expressing human elafin (eTg) has demonstrated that these mice exhibit lower serum-to-BAL fluid ratios of pro-inflammatory cytokines (TNF-α, MIP-2 and MCP-1) than WT mice, following LPS administration to the lungs, which was also associated with an increase in neutrophil and macrophage influx, suggesting priming of innate responses in the lung (Sallenave *at al*., 2003). Conversely, when LPS was given systematically, eTg mice had lower levels of serum TNF-α than WT mice, while peritoneal macrophages from eTg mice were hypo-responsive to LPS (Sallenave *at al*., 2003). In this eTg mouse model, elafin appears to function to promote local immunity, but reduces systemic inflammation. Relatedly, recent *in vitro* data by our group demonstrated that elafin is indeed able to bind both smooth and rough forms of LPS (McMicheal *et al*., 2005b). Moreover, the interaction of elafin with LPS resulted in an augmentation of the LPS-induced TNF-α response in a murine macrophage cell line (RAW 264.7) in serum-free conditions, whereas it caused a significant decrease in TNF-α in serum-positive conditions (McMicheal *et al*., 2005b). Furthermore, similar to other antimicrobial peptides, *e.g.*, defensins and LL-37 (Yang et al., 1999; Yang *et al*., 2000; Oppenheim and Yang, 2005; Tjabringa

et al., 2006), elafin may exhibit chemotactic activity for leukocytes. Ad-mediated expression of elafin enhanced neutrophil migration *in vitro*, which could be inhibited by neutralising Ab to elafin (Simpson *et al*., 2001a). In addition, infection of murine airways with Ad-elafin significantly augmented LPS-mediated neutrophil migration *in vivo*, and was shown to enhance LPS-induced levels of TNF-α and MIP-2 in BAL fluid (Simpson *et al*., 2001a). It is believed that elafin in this model provides local antimicrobial protection and enhances neutrophil influx, while protecting host tissues from subsequent NE-mediated damage (Simpson *et al*., 2001a). However, this is in contrast to the findings reported by Vachon *et al*. (2002) who showed that recombinant elafin could reduce influx of neutrophils, gelatinase activity, and levels of MIP-2 and TNF-α in lung following intranasal LPS administration. Another *in vivo* model has further indicated that exogenous human elafin can play an anti-inflammatory role in inhibiting NE-induced ALI in hamsters (Tremblay *et al*., 2002). These findings would suggest that elafin may play a role as a potent anti-inflammatory mediator in the lung. The discrepancies between this study and those described above may reflect differences in elafin concentration, dose of LPS administered, route of delivery, and duration of experiment. On the other hand, while viral-derived elafin gains access to intra-cellular compartments, administration of recombinant elafin is, at least originally, likely to be limited to extra-cellular contact with the cell membrane. In addition, studies using Ad to deliver elafin must also consider the 'danger signals' provided by the Ad vector.

In summary the alarm antiprotease elafin has been shown to have multiple bioactive functions including inhibition of proteolytic enzymes at times of inflammation, suppression of harmful inflammation and stimulation of innate immune defences. In this context further examination of the functions of elafin in innate and adaptive immunity is required.

1.7.1.2. Secretory leukocytes protease inhibitor (SLPI)

SLPI is an 11.7-kDa protein that was first isolated from human parotid gland secretions (Thompson and Ohlsson, 1986). SLPI orthologs have also been

demonstrated in mice, rats, pigs and sheep (Zitnik *et al*., 1997; Song *et al*., 1999; Brown *et al*., 2005). It is a non-glycosylated, highly basic, acid-stable, cysteine-rich, 107-amino acid, single-chain polypeptide (Thompson and Ohlsson, 1986). The tertiary structure of the SPLI molecule resembles a boomerang, with each arm carrying one domain (Grutter *et al*., 1988). The four-in-each-domain disulfide bridges formed between the cysteine residues, as well as the two-domain interaction, contribute to the conformation and efficacy of the molecule (Hiemstra *et al*., 1996). SLPI provides a significant component of the human antiprotease shield within the lung. Through its C-terminal domain SLPI is a serine protease inhibitor and provides significant protection against NE. Importantly, unlike α1-PI (see below) the interaction between SLPI and NE is reversible. SLPI also inhibits the serine protease cathepsin G as part of its antiprotease effects in the lung (Gauthier *et al*., 1982). Its N-terminal domain has no such properties, but it may aid in stabilising the protease:antiprotease complex (Grutter *et al*., 1988). SLPI is constitutively expressed at most of mucosal sites by a variety of epithelial cells. In the lung it is produced in the central airways by serous glandular cells and in the lower respiratory tract by Clara cells and goblet cells (Hiemstra *et al*., 1996). It has also been shown to be produced by various inflammatory cells, such as neutrophils (Sallenave *et al*., 1997a), mast cells (Westin *et al*., 1999), and macrophages (Mihaila and Tremblay, 2001). It is estimated that SLPI is present at concentrations of 0.1-2 μg/ml in airway lavage fluid (Vogelmeier *et al*., 1991; Kouchi *et al*., 1993) and 2.5μg/ml in nasal secretions (Lee *et al*., 1993).

It is believed that SLPI also shields the tissues against inflammatory products by down-regulating the macrophage responses against bacterial LPS. Patients with sepsis have been shown to have elevated circulating SLPI levels and LPS is the key mediator in bacterial endotoxic shock (Grobmyer *et al*., 2000; Duits *et al*., 2003). LPS seems to induce SLPI production by macrophages directly or by way of IL-1β, TNF-α, IL-6, and IL-10 (Jin *et al*., 1997; Gipson *et al*., 1999). SLPI in turn inhibits the downstream components of the NF-κB pathway by protecting I-κβ from degradation by the ubiquitin-proteosome pathway. Indeed SLPI has recently been shown to enter cells, becoming rapidly localised to the cytoplasm and nucleus where

it affects NF-κB activation by binding directly to NF-κB binding sites in a site-specific manner (Taggart *et al*., 2005). Thus, SLPI renders macrophages unable to release pro-inflammatory cytokines and nitric oxide (Jin *et al*., 1997). These data are also confirmed by *in vivo* studies demonstrating that SLPI knockout ($SLPI^{-/-}$) mice show increased susceptibility to endotoxic shock and macrophages and B lymphocytes from the same mice show increased activation after administration of LPS when compared to control ($SLPI^{+/+}$) mice (Nakamura *et al*., 2003). Ding *et al*. (1999) point out that the inhibitory effect of SLPI on macrophage responses may be due to its blockade of LPS transfer to soluble CD14 and its interference with the uptake of LPS from LPS-soluble CD14 complexes by macrophages. In contrast, Sano and co-workers (2003) recently found that SLPI may up-regulate the LPS-induced activation of NF-κB in terms of transcriptional function.

In addition to its NE inhibitory and immunomodulatory activities, SLPI has broad-spectrum antibiotic activity that includes bactericidal, antiviral and antifungal properties. Fahey and Wira (2002) examined the production of antibacterial factor(s) by uterine epithelial cells from pre- and postmenopausal women. Apical rinse fluids from polarised epithelial cells recovered from women at the proliferative and secretory stages of the menstrual cycle were equally effective in killing *S.a.* and *E. coli*, but those from postmenopausal women were not. SLPI production correlated with bactericidal activity with respect to menstrual status and culture time. Anti-SLPI significantly decreased bactericidal activity of premenopausal epithelial cell rinse fluids. It seems that the N-terminal domain is responsible for the dose-dependent bactericidal properties of SLPI against both Gram-positive (*S.a.*) and Gram-negative (*E. coli*) bacteria. The antimicrobial actions of SLPI are likely to be particularly important in the protection of uterus from infection during implantation and early pregnancy. Hiemstra *et al*. (1996) showed that the activity of this domain is not as efficient as the one of the intact molecule. Hence, they speculated that a conformational change in the N-terminal domain is induced by the cleavage of the native protein. In addition, Miller and colleagues (1989) suggested that the mechanism of the SLPI-mediated bactericidal activity may include binding of the

protease inhibitor to the bacterial mRNA and DNA, but Hiemstra *et al.*'s findings proved that this binding is not significant to explain the antibacterial activity of SLPI.

Tomee and co-workers (1997) showed that SLPI has activity (50% fungicidal activity) against human isolates of the pathogenic fungi *A. fumigatus* and *Candida albicans*. They also found partial inhibition of fungal protease activity by recombinant SLPI, a putative virulence factor of *A. fumigatus*, and subsequent inhibition of the inductive pro-inflammatory cytokine response in cultured human airway epithelial cell lines. In a recent study, Chattopadhyay and co-workers (2004) showed that the increase of salivary SLPI levels (to >2.1 μg/ml) along with other factors, such as low levels of CD4, antiretroviral monotherapy, and smoking, is a key predictor of oral candidiasis in human immunodeficiency virus type 1 (HIV-1)-infected persons. A possible biological explanation for this association is that SLPI is up-regulated in response to the infection in order to kill the pathogen and resolve the disease. An individual threshold limit to SLPI production and secretion may be reached. As with the antibacterial-bactericidal activity, the antifungal activity was mainly localised in the N-terminal domain. It is believed that killing of fungus protects the epithelia from the fungal proteases (Tomee *et al*., 1997). Probably the antibacterial and antifungal activities are related to the cationic nature of SLPI (Tomee *et al*., 1998). In addition, SLPI is likely to be a major deterrent of HIV-1 transmission through oral secretions (Chattopadhyay *et al*., 2004). SLPI was found to be the most potent anti-HIV-1 factor among several innate inhibitory molecules, namely, virus-specific Abs, mucins, and thrombospondins, identified and purified from saliva (Tomee *et al*., 1998). In 1995, McNeely and co-workers showed that infection of adherent primary monocytes with HIV-1 was significantly suppressed in the presence of human saliva. Of the proteins present in the saliva, only SLPI was found to have antiretroviral activity at physiological concentrations (0.1-10 μg/ml) (McNeely *et al*., 1995). To further confirm these findings Jana *et al*. (2005) recently demonstrated that brief exposure of keratinocytes to HIV-1 leads to a significant dose and time dependent increase in SLPI mRNA and protein secretion that occurs rapidly after virus contact, and does not require productive cellular infection, which is elicited specifically by the external envelope glycoproteins of HIV-1. The

mechanism by which SLPI inhibits HIV-1 infection is still elusive, but it appears to involve the host cell target rather than binding to the virus (McNeely *et al*., 1995; Hocini *et al*., 2002). Moreover, the antiviral activity of SLPI appears to be distinct from its known antiprotease activity (McNeely *et al*., 1997). Evidence suggests that SLPI blocks HIV-1 internalisation in a dose-dependent manner rather than binding to CD4 (McNeely *et al*., 1997). McNeely *et al*. (1997) found that SLPI most likely inhibits a step of viral infection that occurs after virus binding but before reverse transcription. They also succeeded in preventing HIV infection of macrophages after pre-treatment with SLPI. Moderate activity is also exerted against HIV-2 strains, but only when the concentration of SLPI exceeds the norm. Saliva is rich in SLPI and it is felt that this accounts for the low viral transmission rates via saliva (Wahl *et al*., 1997; McNeely *et al*., 1997).

Last but not least, SLPI and elafin expression have also been noted in response to cutaneous injury in humans (van Bergen *et al*., 1996; Wingens *et al*., 1998). Further, Ashcroft *et al*. (2000) generated mice null for the gene encoding SLPI, which showed impaired cutaneous wound healing with increased inflammation and NE activity. This altered inflammatory profile was shown to involve enhanced activation of local TGF-β in $SLPI^{-/-}$ mice. Therefore, in addition to their roles in innate immunity, these low molecular mass NE inhibitors seem to be involved in resolution and subsequent tissue regeneration.

1.7.2. The systemic proteases inhibitor α1-PI

α1-PI is a 52-kDa secreted glycoprotein and is the prototypic member of serpin superfamily of proteins, which have a major role in inactivating NE and other proteases to maintain protease:antiprotease balance. α1-PI is predominantly produced in the liver and reaches the lungs by diffusion from the circulation and by local production in macrophages and bronchial epithelial cell (Lomas and Mahadeva, 2002; Stecenko and Brigham, 2003). Normal serum concentrations of α1-PI range from 20-53 μM, and levels in the lung are about 2-6.5 μM. Despite its early name (α1-antitrypsin), it actually reacts with NE much more readily than with trypsin (Beatty

et al., 1980), and represents a major defence against the elastolytic burden in the lower airways posed by NE, which is produced by neutrophils (see previous section, 1.6).

Serpins have been likened to mousetraps complete with bait, a loaded high-energy but unstable state, and a swinging arm. In the case of α1-PI (Figure 1.9), the bait is a methionine amine acid side chain in the reactive centre of the serpin. Docking of the NE on the residue cleaves the reactive centre, releases α1-PI from its metastable high energy state, and allows the cleaved reactive loop to snap back, with the protease in tow (Huntington *et al*., 2000). While this process is mutually suicidal and ensures the destruction of both molecules, there is normally an excess of α1-PI in the lung, thereby providing an adequate protective screen against the elastolytic burden of NE (Eriksson, 1999).

Quantitative studies show that α1-PI is the predominant NE inhibitor in CF sputum (Suter *et al*., 1986). However, the majority of endogenous α1-PI in the sputum of COPD and CF patients is in an inactive form. This suggests the existence of a mechanism for inactivating α1-PI within COPD and CF lungs. It is believed that excess concentrations of proteases and/or oxidants, such as CS and myeloperoxidase released from primed neutrophils, may be responsible for this inactivation of α1-PI. Indeed, as mentioned above, a critical methionine residue at position 358, which determines its specificity toward NE, is very sensitive to inactivation by oxidants or matrix metalloproteases released from primed activated neutrophils (Ossanna *et al*., 1986; Vissers *et al*, 1988).

α1-PI deficiency is a genetic disorder that affects about 1 in 2000-5000 individuals. The most frequent mutation that causes α1-PI deficiency gives rise to the Z allele. α1-PI deficiency is characterised by a decrease in levels of α1-PI, which results in early-onset of emphysema in affected individuals. It was originally believed that this emphysema was caused by decreased α1-PI in the respiratory tract, leading to unopposed and prolonged NE activity (Campbell *et al*., 1999). However, recent evidence suggests that the Z variant of α1-PI, when polymerised, may be pro-

inflammatory, acting as an important chemoattractant for neutrophils in the α1-PI – deficient lung and adding to the excessive neutrophil and NE burden (Parmar *et al*., 2002). Indeed, a substitution of lysine for glutamic acid at position 342 in the Z protein widens β-sheet A and allows polymerisation in an irreversible process (Figure 1.9; Lomas and Mahadeva, 2002). Polymerisation, and retention of polymers in the endoplasmic reticulum of liver cells, causes a decrease in the amount of plasma α1-PI that is available to protect the lung against proteolytic cleavage.

In addition to its role as an antiprotease, α1-PI has important anti-inflammatory properties (Stockley *et al*., 2002). These effects are independent of the protease inhibition and include blocking of the pro-inflammatory effects of human NE (Spencer *et al*., 2004), and regulating expression of pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, MCP-1, and IL-1β by monocytes (Aldonyte *et al*., 2004; Janciauskiene *et al*., 2004). In a recent study Petrache *et al*. (2006b) noted that α1-PI could also inhibit alveolar cell apoptosis *in vivo*. A follow-up study by the same group suggested that direct inhibition of active caspase-3 by α1-PI may represent a novel anti-apoptotic mechanism relevant to disease processes characterised by excessive structural cell apoptosis, oxidative stress, and inflammation, such as pulmonary emphysema (Petrache *et al*., 2006a).

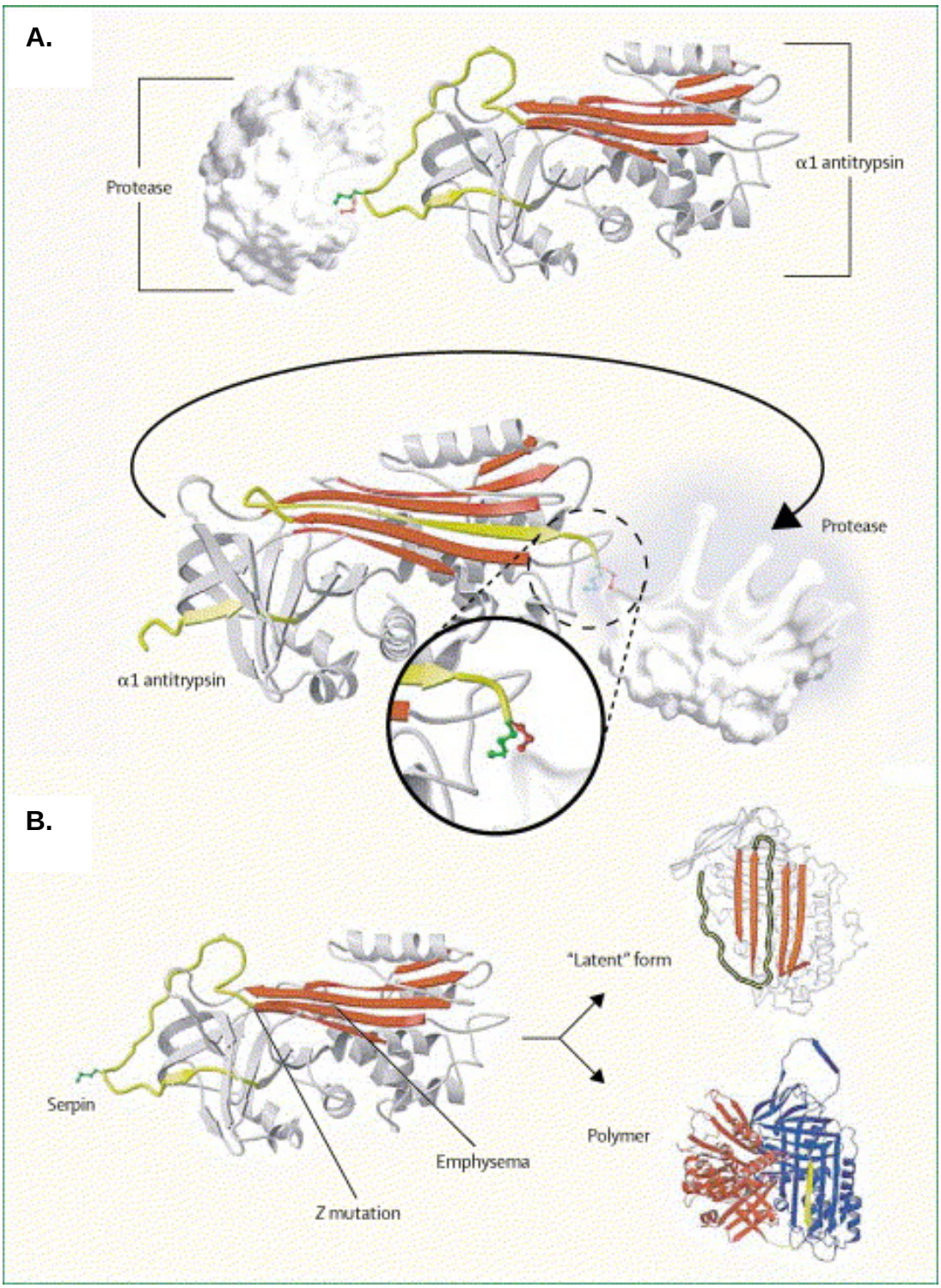


Figure 1.9. Mechanism of inhibition of proteases by α1-PI and polymerisation of α1-PI

A) Docking of protease to the reactive centre loop of α1-PI (upper). The protease has cleaved the reactive loop, releasing it from its metastable high energy state. This process distorts and alters the structure of the protease. **B**) Mutation of serpins, *e.g.*, α1-PI, can result in several diseases such as emphysema. Adapted from Lomas and Mahadeva, 2002.

1.8. CENTRAL HYPOTHESES AND AIMS

This introduction has outlined the adverse effects of high NE concentration in the lungs, its involvement in CF and COPD pathologies and increased disease severity and acute exacerbation in these patients. The colonisation of microorganisams in lower airways of CF and COPD patients implies a breach of the host defence mechanisms. Since DCs are the most potent APCs and bridge the innate and adaptive arms of the immune system in the respiratory system, the following hypotheses were investigated:

1- ***Cigarette smoke (CS) and lung inflammatory secretions from COPD and CF patients adversely affect the DC phenotype and function, hence making patients immunocompromised to frequent opportunistic respiratory infections.***

2- ***NE inhibitors are able to interact with DCs and the local augmentation of these NE inhibitors is able to activate DCs and increase their numbers in the lung tissue.***

The following experimental aims were developed to address these hypotheses. These form the basis of subsequent results chapters and as such, give a brief insight into the basis of the work presented herein.

AIMS

Inhibition of DC maturation and function by CS and NE-containing inflammatory lung secretions/purified NE *(Chapter 3)*

- Culture murine BM cells in the presence of GM-CSF to generate a highly pure and dense iDC population

- Expose DCs to fresh CS condensate (CSC) and assess their phenotype
- Collect sputum samples from CF and COPD patients and measure their active NE concentrations
- Expose DCs to sputum supernatants collected from CF and COPD patients as well as purified human NE and analyse following treatment
- Co-culture treated DCs with ovalbumin (OVA) specific D011.10 transgenic lymphocytes to assess their antigen presenting function
- Characterise the cytokine profile of lymphocytes stimulated with treated DCs

Characterisation of lung DCs following *in vivo* augmentation of elafin *(Chapter 4)*

- Develop protocols for isolation of murine lung APCs
- Analyse WT and elafin transgenic (eTg) lung APCs following Ad infection by flow cytometry and related techniques
- Analyse the inflammatory cell differentials and profile of cytokines in BAL fluid of Ad treated mice
- Develop vaccination strategies by means of NE inhibitor gene therapy

***In vitro* effects of NE inhibitors on DC maturation, function and chemotaxis** *(Chapter 5)*

- Develop protocols for efficient infection of DCs by Ad constructs *in vitro*
- Examine the effects of lung natural NE inhibitors, namely elafin, SLPI and α1-PI, on DC phenotype and maturation in *in vitro* systems
- Investigate the chemotactic activity of elafin on DCs

CHAPTER 2
MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Source of chemicals and reagents

Unless stated, chemicals and reagents were obtained from Sigma (Poole, Dorset, UK).

2.1.2. Polyclonal rabbit anti-human elafin antibody (Ab)

The polyclonal rabbit anti-human elafin IgG, prepared from antiserum raised against COOH-terminal 2.5-kDa fraction of human elafin (Sallenave *et al.*, 1992), was available in-house from the outset of this work. Biotinylated rabbit anti-human elafin IgG was also available in-house.

2.1.3. Adenoviral (Ad) constructs

Five E1-, partially E3-deleted Ad vector constructs of serotype 5 were used, and these vectors were available in-house from the outset of this work. The vectors used were: Ad-elafin, encoding the cDNA for human elafin; Ad-SLPI/GFP, encoding the cDNA for murine SLPI; Ad-null, an empty vector encoding no transgene; Ad-α1PI, encoding murine α1-PI 2; Ad-GFP, encoding the cDNA for green fluorescent protein, Ad-eotaxin, encoding the cDNA for murine CC chemokine eotaxin, and Ad-GMCSF, encoding the cDNA for murine GM-CSF, which is a member of the heamapoetic cytokine family. The techniques used to generate these Ad vectors have been described in detail elsewhere (Hitt *et al*., 1995; Addison *et al*., 1997; Jobin *et al*., 1998; Sallenave *et al*., 1998; Wang and Huang, 2000). The transgene in each vector is located downstream of a fragment of the murine cytomegalovirus (CMV) immediate early promoter (Dorsch-Hasler *et al*., 1985). While the Ad-elafin, Ad-

α1PI, and Ad-eotaxin vectors encode a single transgene driven by a 1.4-kb fraction of the murine CMV promoter, Ad-SLPI/GFP is a bicisteronic vector encoding both murine SLPI (followed by an internal ribosomal entry site) and green fluorescent protein (GFP), under the control of a 531 base pair fraction of the murine CMV promoter.

The Ad-elafin vector was originally constructed by Professor Jean-Michel Sallenave in Professor Jack Gauldie's laboratory at McMaster University, Hamilton, Ontario, Canada (Sallenave *et al*., 1998). Ad-SLPI/GFP was constructed in-house in our laboratory by other investigators. Ad-null was a kind gift from Professor Jack Gauldie and Dr Mary Hitt (McMaster University). Ad-α1PI, Ad-eotaxin and Ad-GMCSF were a kind gift from Professor Zhou Xing (McMaster University). I, Ali Roghanian, have not been involved in the constructions or amplification of any Ad vectors mentioned herein.

2.1.4. Mice

Female BALB/c and C57BL/6J mice aged between 4 and 8 weeks were obtained from Charles River UK, Margate, UK.

Human elafin transgenic (eTg) mice were previously derived from C57BL/6J × CBA mice in our laboratory and were bread in-house (Sallenave *et al*., 2003). They express the elafin gene under the murine CMV promoter, which has previously been shown to be inducible by inflammatory mediators by our group (Simpson *et al*., 2001). DO11.10 CD4-T cell receptor (TCR) transgenic mice were maintained as an in-house breeding colony, housed in isolators.

All mice were housed in the animal facilities of the University of Edinburgh and all animal experiments were conducted in accordance with the specifications of the local regulatory committee (The University of Edinburgh) and the Home Office guidelines.

2.2. METHODS

2.2.1. Cell culture

2.2.1.1. GM-CSF secreting cell line

Murine GM-CSF secreting cells were a gift from Professor David Gray, The University of Edinburgh (Svensson *et al*., 1997). The cells were grown in a selection medium containing RPMI 1640, 5% foetal calf serum (FCS; heat-inactivated at 56°C for 1 hour prior to use), 2-mercaptoethanol (ME) (50μM), 1mg/ml G418/Geneticin (Invitrogen, Paisley, UK) and 1% L-glutamate until they reached a stage of confluency. The cells were then cultured in a medium without G418/Geneticin for 2-4 days after which they were harvested and the GM-CSF concentration was estimated by titration and/or murine GM-CSF ELISA kit (R&D Systems, Oxon, UK). Normally the optimum concentrations of GM-CSF for generating BM-derived DCs (BM-DC) cultures were 5-10% v/v depending on the preparation.

2.2.1.2. Raw 264.7 cells

The RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Easgle's Medium (DMEM) supplemented with 0-10% (v/v) FCS (Labtech, Ringmer, UK), 100 U/ml penicillin, 100μg/ml streptomycin, 4mM L-glutaminutese, 4.5g/l glucose and 1.5g/l sodium bicarbonate. For simplicity throughout this thesis 'DMEM' is used to describe this culture medium, unless otherwise indicated.

In experiments where RAW 264.7 cells were used to assess possible contaminating endotoxin presence in Ad constructs, the cells were plated at a density of ~2×10^5 cells/ml in DMEM and incubated overnight at 37°C to settle down. They were then stimulated with increasing concentrations of LPS (*Escherichia coli*

serotype 026:B6) as well as Ad constructs for 4 hours. The supernatants were then analysed for TNF-α as described below.

2.2.1.3. Clara cells

The murine Clara (bronchial epithelial) cell line DJS2-2 was a kind gift from Dr. Franco DeMayo (Baylor College of Medicine, Houston, TX, USA). Cells were cultured in DMEM supplemented with 0-10% (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM L-glutamine.

All cells were maintained at 37°C in a humidified incubator containing 5% CO_2, and used for assays at the lowest possible passage number. Estimation of the number of cells in each monolayer was performed by washing cells with phosphate buffer saline (PBS), and counting of cells using a haemocytometer. Cell viability was assessed by staining with trypan blue, with exclusion indicating viability.

2.2.1.4. Bone marrow-derived DC culture and maturation

BM-DCs were generated in two ways as described previously (Inaba *et al.,* 1992; Lutz *et al*., 1999). The first method (method 1) was based on the 'Inaba method'. BM from BALB/c mice was extracted from cleaned femurs and tibiae (surrounding tissue was removed by rubbing with antibacterial disinfectant wipes). Intact bones were rinsed in 70% ethanol and washed with PBS. The bone ends were then cut using a disposable scalpel and the marrow flushed through with DC complete medium (RPMI 1640 supplemented with 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 10% heat-inactivated FCS), containing ~10% of conditioned supernatant from an hybridoma expressing GM-CSF (at a final concentration of 20–30ng/ml), using a syringe with 0.45mm (26-gauge) diameter needle. Cells were centrifuged for 5 minutes, 300 g. The pellet was then resuspended in red blood cell (RBC) lysis buffer (1mM NH_4HCO_3 and 114mM NH_4Cl; 1ml per mouse) and incubated on ice for 2 minutes. Cells were then washed in DC complete medium and resuspended in 10ml for counting. Cells were seeded at 3.75×10^5

cells/well of a 24-well plate in 1ml DC complete medium and incubated at 37°C, 5% CO_2. Culture medium was aspirated after 3 and 6 days of culture. This involved gently swirling the plate before aspiration so as to remove non-adherent granulocytes and B cells without dislodging loosely adherent clusters of developing DCs. Medium was replaced with fresh DC complete medium. On day 7, loose DC aggregates were removed by pipetting, washed and counted. On day 7, the cells were heterogeneous, with 60-85% having a morphology and a cell surface receptor phenotype characteristics of iDC (see Chapter 3). Where matured DCs were required, day 7 cells were stimulated with LPS (*E. coli* serotype 026:B6) for 24 hours.

In method 2, adopted from the 'Lutz method', briefly, femurs from BALB/c mice were removed, dipped in 70% ethanol for 1-2 minutes, and then placed in DC complete medium, and ~5% of conditioned supernatant from a hybridoma expressing GM-CSF (at a final concentration of 10-20ng/ml). After flushing of the femur's content, 10ml of a single-cell suspension (2×10^5/ml) was plated in non-tissue culture grade 100mm Petri dishes. On day 3 an additional 10 ml of fresh medium was added to the cultures. On days 6 and 8, half of the medium was removed, and 10 ml fresh medium was added to the Petri dishes. On day 10 of the culture, non-adherent cells were removed, leaving strongly adherent macrophages on the plate. The non-adherent cells were centrifuged (300×g, 5 minutes), resuspended in DC medium, and counted before use. On day 10, the cells were heterogeneous, with 85-95% having a morphology and a cell surface receptor phenotype characteristics of iDC (see Chapter 3).

Unless otherwise stated, mature DCs (mDCs) were obtained after a further incubation of iDCs with 100ng/ml LPS (*E. coli* serotype 026:B6) in 10% heat-inactivated FCS for 24 hours. When maturation was performed in serum-free medium, LPS was used at a concentration of 2μg/ml for 4 hours.

2.2.2. Flow cytometry

2.2.2.1. Fluorescence-activated cell sorter (FACS) analysis of DC surface markers

Cells were immediately washed post each treatment with PBS without Ca^{2+}/Mg^{2+} (PBS w/o) containing 0.1% bovine serum albumin (BSA) (FACS wash). Blocking was performed using 10% mouse serum (Serotec, Oxford, UK) for 15 minutes; subsequently, cells were stained with relevant monoclonal Abs (mAb) at 4°C in the dark for 30 minutes. Cells were then washed and resuspended in 200µl of FACS wash and analysed using FACSCalibur and Cellquest softwares (BD Biosciences, CA). All stained samples were compared with appropriate isotype controls. Unless otherwise stated, the expression of molecules on DCs was either expressed as percentage or geometric mean fluorescence intensity (MFI) and was compared to that of cells treated with isotype-matched controls. The MFI of a population of cells labelled with a fluorochrome conjugated to a ligand or mAb is linearly related to the mean number of receptors/cell or mean number of mAb biding sites/cell.

2.2.2.2. Assessing cell viability by FACS

To assess the viability of DCs in the experiments performed in this project, DCs were immediately stained with propidium iodide (PI; Invitrogen Ltd., Paisley, UK) and FITC-annexin V (Caltag Laboratories, CA) after each specific treatment. Staining cells simultaneously with FITC-annexin V (green fluorescence) and the non-vital dye PI (red fluorescence) allows (bivariate analysis) the discrimination of intact cells (annexin V^- PI^-), early apoptotic (annexin V^+ PI^-) and late apoptotic or necrotic cells (annexin V^+ PI^+). Hence cells were suspended in 10mM HEPES buffer contacting 4mM $CaCl_4$, and were stained with FITC-annexin V according to the manufacturer's protocol for 15 minutes in the dark at room temperature. Immediately prior to running the samples on FACS, PI was added and cells were analysed by flow cytometry.

2.2.3. CSC/CF/COPD/NE studies

2.2.3.1. Preparation of CSC

Cigarette smoke condensate (CSC) was produced fresh by exposing PBS to whole cigarette smoke obtained from 'Regal king-size' cigarettes containing 0.9mg nicotine, 10mg tar and 10mg carbon monoxide per cigarette (Figure 2.1). One ml of PBS w/o per cigarette was pipetted into a tonometer and the smoke from the cigarette was drawn up into a glass syringe and passed into a tonometer agitating PBS (Rahman *et al*., 1995). Each puff consists of ~35ml of CS, with 20 puffs being achieved from each cigarette. The condensate obtained was filtered thorough a 0.2μM Minutesisart® microfilter (Sartorius, Goettingen, Germany) and used at pathophysiological concentrations of 10% or less.

Figure 2.1. Smoke machine

The apparatus shown above was manufactured at the University of Edinburgh and was used to generate fresh samples of CSC for *in vitro* and *in vivo* experiments. CSC was prepared by drawing 35 ml of cigarette smoke into the syringe and then slowly bubbling the smoke into sterile PBS w/o. The cigarette was smoked up to 1-cm butt length. One cigarette was smoked per 1ml of PBS. Following preparation of CSC, the solution was filtered through 0.2-μm filters and used immediately.

2.2.3.2. Exposing DCs to CSC

Immature DCs (iDC) as well as LPS (100ng/ml)-matured DCs (mDC) were seeded in 6 well cell culture plates (Corning Incorporated, Corning, NY) at a density of 2×10^6 cells/well supplemented with 2ml of serum^{+ve} or serum^{-ve} RPMI medium. Freshly prepared CSC was added at different doses of 1%, 5% and 10%, and PBS w/o was also added to the control wells. Cells were incubated for 4 hours, after which, their co-stimulatory molecules (CSMs) and MHC class II (MHCII) expression levels as well as their viability were assessed by FACS.

2.2.3.3. Sputum samples from COPD patients

COPD sputum samples were obtained from patients attending a COPD clinic in the Edinburgh Royal Infirmary. A total of 27 COPD patients (11 F and 16 M, see subjects' demographics in Chapter 3, Appendix I) with an average age of 69 ± 8.29 (SD) (SEM=1.6) were studied. FEV1 % was 39 ± 12.9 (SEM=2.6) predicted and FEV1/FVC % was 47 ± 12.9 (SEM=2.6) predicted. Pack year history was 55 ± 17 (SEM=3.4). All the sputum samples were processed within 2 hours of production as described by Popov *et al*. (1994). CF sputum samples were collected from a total of 18 CF patients (9 F and 9 M, see subjects' demographics in Chapter 3, Appendix II) with an average age of 31.5 ± 12.44 (SD) attending a CF clinic at the Western General Hospital (Edinburgh). Subsequently, the samples were re-suspended in Sputolysin reagent (Calbiochem, San Diego, USA) at a ratio of 1:1 (v/v) and were treated as for COPD samples. The sputum samples were either induced or spontaneous, but most were induced as described by Drost *et al*. (2005). All subjects were informed of the nature and purpose of the study and gave their consent. The study had the approval of the Lothian Ethics Committee.

2.2.3.4. Neutrophil elastase (NE) assay

The concentration of NE in COPD and CF sputum samples was determined as described before (Sallenave *et al*., 1998). Briefly, samples were diluted in NE

reagent buffer (50mM Tris, 0.5M NaCl, 0.1% Triton X-100, pH 8) and NE activity was measured by adding the NE substrate MeOsucc-Ala-Ala-Pro-Val-p-nitroanilide (0.2mg/ml) and measuring the optical density of the released product at 450 nm on a microplate spectrophotometer (Dynax Technologies, West Sussex, UK). Serial dilutions of purified NE (Elastin products, Missouri) were performed, adding dithiothreitol (DTT)/Sputolysin to the same final concentration as previously added to patient's samples, and were used as standards in the assay.

2.2.3.5. Treatment of DCs with either NE or sputum samples from patients with COPD/CF

LPS-matured DCs (mDCs) were washed with PBS and checked for viability by Trypan blue staining. Cells were resuspended in 300µl PBS containing 0.1% (w/v) BSA, with active NE (0.5-3µM) or with sputum and incubated at 37°C for 90 minutes on a shaker, as described previously (Nemoto *et al*., 2002). The percentage reduction in CSMs expression was determined by flow cytometry and calculated as:

$$100 - [(\text{MFI NE/sputum-treated cells}) / (\text{MFI PBS-treated cells}) \times 100]$$

, and plotted against the concentration of active NE in sputa. When NE inhibition was assessed, purified active NE (2µM) or a pool of COPD or CF sputa (containing 0.2µM and 3.39µM active NE respectively) were pre-incubated at 37°C for 90 minutes on a shaker either with excess concentrations (8µM) of recombinant murine secretory leukocyte protease inhibitor (SLPI, a kind gift from Dr C. Wright, Amgen, CA, USA) or with a 1:25 dilution of an EDTA-containing broad spectrum protease inhibitor cocktail tablet (PIT), inhibiting serine, cysteine, metalloproteases as well as calpains (Roche Diagnostics GmbH, Mannheim, Germany).

2.2.3.6. Assessment of CSMs re-expression by DC post-NE treatment

In some experiments, mDCs (10^6/ml) were incubated at 37^{o}C in 24-well Costar plates in serum-free medium with or without 2µM purified active NE for 90

minutes. FCS, which is a rich source of antiproteases, was then added (final concentration of 15%) to inhibit the NE activity. Cells were then taken at various time-points (2, 4, 6 and 8 hours post NE inhibition) and tested for expression of surface markers by FACS (all done in triplicates).

2.2.3.7. Stimulation of iDC ± NE and ± LPS

Immature DC were seeded at a density of 1×10^6 cells/well in a 12 well plate in serum-free medium. They were either stimulated with PBS, 2µg/ml LPS, 2µM NE, or 2µg/ml LPS and 2µM NE simultaneously. The cells were incubated for 5 hours and then the DCs were collected for FACS analysis and their supernatant was frozen at -20°C for cytokine analysis.

2.2.3.8. Effects of NE on recombinant IL-12p40 subunit protein

One microgram/ml of recombinant IL-12p40 protein (R&D Systems, Oxon, UK) was incubated with 2µM NE at 37°C for 5 hours. The samples were treated with excess concentrations of SLPI (~4µM) to inhibit remaining NE activity and samples were run on IL-12p40 ELISA plates (see 2.2.3.11).

2.2.3.9. Murine CD86 Western blot analysis

a) mDC cell culture supernatants

Post NE treatment, mDC culture supernatants were concentrated 10 times (with cold methanol); pellets were resuspended in reducing sample buffer and were immediately heat-denatured at 70°C for 10 minutes prior to electrophoresis on a NuPAGE 4-12% Bis-Tris polyacrylamide gel (NuPAGE Invitrogen Ltd., Paisley, UK) at 200 Volts (constant voltage) for 35 minutes.

b) mDC lysates

mDCs were washed with PBS and lysed (10^6 cells/ml) in 1:1 ratio of PBS:RIPA buffer (150mM NaCl, 50mM Tris-HCl, 0.5% DOC, 0.1% SDS and 1% NP40) .The

cell lysate was heated at 95°C for 10 minutes and centrifuged at 10000 × g at room temperature for 5 minutes. Supernatants were concentrated by methanol precipitation as described above; pellets were resuspended in PBS and frozen at -20^{o}C until further use. For CD86 digestion assays, the mDC lysate was incubated with various concentrations of purified NE on a shaker at 37°C for various times. For NE inhibition studies, SLPI was pre-incubated with NE at 37°C for 30 minutes. mDC lysates were added to electrophoresis reducing sample buffer and SDS-PAGE gels run as above.

c) Western blotting

Samples were prepared and run on 4-12% Bis-Tris NuPAGE SDS-PAGE gels as described above. Transfer to Hybond ECL nitrocellulose membranes (in a X Cell II Blot module) was performed according to the manufacturer's (InVitrogen Ltd, Paisley, UK) instructions. Membranes were then blocked in Tris buffered saline containing 0.05% Tween-20 (TBS-T) and 5% dry skimmed milk for 2 hours. After blocking, membranes were probed overnight at 4°C with 0.1μg/ml goat polyclonal anti-CD86 (B7-2) (R&D Systems, Oxon, UK) in TBS-T. Membranes were washed trice for 5 minutes with TBS-T and incubated with rabbit anti-goat IgG horse radish peroxidase (HRP)-conjugated secondary Ab (1:50,000; DAKO, Cambridgeshire, UK) for 1 hour at room temperature. Membranes were incubated with Hybond ECL Plus reagent and exposed to Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK).

2.2.3.10. Lymphocyte proliferation assays

Single cell splenocyte suspensions were made from spleens of 6-8 week old female ovalbumin (OVA) CD4-T cell TCR transgenic DO11.10 mice (BALB/c background, 2-4 animals per experiment) by passing the spleen through a 40-μm pore size cell strainer (BD Biosciences, San Jose, USA). Debris and red cells were removed by density gradient sedimentation through Lympholyte-M (Cedarlane Laboratories, Ontario, Canada) according to the manufacturer's instructions. Day 10 WT BALB/c BM-DCs were matured with 100ng/ml LPS overnight, washed with

PBS and incubated with or without 3μM NE for 90 minutes as described above. The treated mDCs were then pulsed with 0.1μM OVA peptide ($OVA_{323–339}$; Peptides International, Kentucky, USA) for 90 minutes at 37°C and washed thoroughly. 10^4 OVA-pulsed mDCs were added to 10^5 DO11.10 lymphocytes in 96-well tissue culture plates in a final volume of 200μl and incubated in a humidified 5% CO_2 incubator. Activation of the transgenic DO11.10 CD4 T cells was assessed after 48 hours by removing 100μl of the supernatants (for cytokine analysis) and proliferation was determined by adding AlamarBlue™ (Serotec, Oxford, UK) to the cells, to yield a final concentration of 20% in the incubation mixture. AlamarBlue™ is soluble, stable in culture medium and is non-toxic. As with tetrazolium salts, AlamarBlue™ monitors the reducing environment of the proliferating cell and allows continuous monitoring of cells in culture. Proliferation measurements with AlamarBlue™ may be made either spectrophotometrically by monitoring the absorption of AlamarBlue™ supplemented cell culture media at two wavelengths. Here, cell proliferation was measured by fluorimetry at 545nm excitation wavelength and 590nm emission wavelength, using a plate reader (Fluoroskan Ascent FL, LabSystems, Minnesota, USA).

2.2.3.11. Cytokine analysis

Cytokine secretion was assessed either by using the mouse Th1/Th2 Cytometric Bead Array (CBA) kit, (BD Biosciences, San Jose, USA) or by use of ELISA kits (R&D Systems, Oxon, UK) in accordance with the manufacturers' instructions. The detection limit for CBA kit cytokines was 20pg/ml; the detection limits for IL-12p40 and TNF-α were 15pg/ml for in the ELISA kits.

2.2.4. *In vitro* and *in vivo* Ad infection assays and DC analysis

2.2.4.1. *In vivo* analytical methods

2.2.4.1.1. Intra-tracheal (i.t.) Ad administration

C57BL/J6 mice received i.t. instillations of either PBS, Ad-null (2.5×10^8 pfu), or Ad-elafin (2.5×10^8 pfu). Elafin transgenic (eTg) and their WT control litter mates received either PBS, Ad-null (2.5×10^8 pfu). Jean-Michel Sallenave kindly performed the i.t. procedures in these experiments. On day 10, animals were anaesthetised and blood collected by cardiac puncture and sera and BAL fluid were kindly prepared by Lesley A. Farrell and stored at -30°C until use. Spleens were collected, and lungs, hearts and tracheae were removed *en bloc*.

The experiments described in the rest of the paragraph 2.2.4.1.1 were part of an 'honours project' for Mr. Steven Williams. They were propagated and put by Jean-Michel Sallenave, Steven Williams and Tara A. Sheldrake. My contribution was limited to the histological analysis of the lung sections to examine lung $CD11c^+$ cell numbers following Ad vaccination (see Roghanian *et al*. [2006b] for details). In experiments to assess the secondary anti-Ad immune response and study further the potential of Ad-elafin, 4 groups of mice were used: at day 0, the first group received PBS alone; the second group received 2.5×10^8 pfu Ad-null (8.8×10^9 total virus particles); the third group received 0.3×10^8 pfu Ad-elafin and 2.2×10^8 pfu Ad-null (8.2×10^9 total virus particles). On day 14, all mice received Ad-LacZ (2.5×10^8 pfu; 2.04×10^{10} particles) i.t. and they were culled 3 days later. Sera, BAL fluid, lungs and spleens were obtained as explained elsewhere. For determination of Ad-derived β-galactosidase expression, lungs were snap-frozen in liquid nitrogen and stored at -80°C.

2.2.4.1.2. Retrieval of BAL fluid and lungs and flow cytometry

Tracheae were intubated and BAL fluid was obtained by instillation of 2 × 0.4 ml of sterile PBS. Typically, 0.4 to 0.6 ml was retrieved per animal. The cell pellet was used for cell differential analysis (performed after centrifugation of BAL fluid at 2000 × g for 5 minutes). Cytospinning (100 × g for 3 minutes at room temperature) was performed and slides were stained with Diff-Quick (Dade Diagnostika, GmbH, Germany). Supernatants were used for elafin and cytokine ELISA analysis (see below). In an experimental subset, mice were sacrificed, right heart catherization was performed and the pulmonary circulation was perfused with PBS. After carefully dissecting the thymus and all thoracic lymph nodes, the lungs were removed and collected in ice-cold PBS. They were minced using fine scissors and a scalpel and incubated in PBS containing 0.7 mg/ml collagenase A (Roche Diagnostics, GmbH, Germany) and 30 μg/ml DNase I in a humidified incubator at 37°C and 5% CO_2 for 45 minutes. These combinational doses of collagenase A and DNase I were chosen because they were found to yield the maximum number of APCs from perfused lung tissues by previous investigators in our centre (documented in Sharon Ahmed's PhD thesis, CIR; 2003). Subsequently, the digested tissue was mechanically disrupted and passed thorough a 40-μm cell strainer (Falcon). Finally cells were subjected to RBC lysis buffer, washed in complete medium (RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin/streptomycin [P/S]), and kept on ice for immunofluorescent labelling.

mAbs used to identify mouse DC populations were APC-CD11c (N418, eBiocience), and FITC-MHCII (2G9; BD Biosciences Pharminutesgen). Additional markers used for phenotyping were: PE-conjugated anti-CD11b/Mac-1α (M1/70), CD3, CD40 (1C10), CD80/B7-1 (16-10A1), CD86/B7-2 (GL1), Gr1, B220/CD45R (RA3-6B2) (all from eBiocience, CA) and F4/80 (CI-A3-1; Caltag, CA). Isotype-matched control Abs to each mAb were used.

All staining reactions were performed on ice in FACS buffer (PBS containing 0.2% BSA). Cells were pre-incubated with mouse serum (Serotec, Oxford, UK) to reduce non-specific binding of mAb. Cells were stained with mAbs for 30 minutes on ice and washed with FACS buffer before being analysed. Flow cytometry data

acquisition was performed on FACSVantage SE flow cytometer (Becton Dickinson, CA) and around 1×10^5 events were collected per sample. CellQuest™ software was used for data analysis (BD Biosciences, CA).

2.2.4.1.3. Sorting of lung antigen presenting cells (APC)

Mouse lungs were perfused and digested as described above and the lung single cell suspension from 2-4 mice were pooled and stained with mAbs to CD11c and MHCII (see above). The cells were then washed with FACS wash and resuspended in FACS wash buffer at a density of ~5×10^6 cells/ml. They were then passed thorough the FACSVantage SE flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and the $CD11c^+MHCII^+$ and $CD11c^+MHCII^-$ cells were collected in two separate tubes. The sorted cells were further stained with mAbs to CSMs and their isotype control and were analysed by FACS. CellQuest™ software was used for data analysis (BD Biosciences).

2.2.4.1.4. Magnetic separation of lung $CD11c^+$ cells

Mouse lung single cells were prepared as described above. They were then incubated with mouse serum for 10 minutes in the fridge (4-6°C) and were resuspended in 400µl of buffer (PBS w/o supplemented with 0.5% BSA, pH 7.2). The cells were stained with 100µl of monoclonal hamster anti-mouse CD11c MicroBeads (N418; Miltenyi Biotec Ltd., Bergisch Gladbach, Germany) and incubated for 15 minutes at 4-6°C (fridge). At this stage APC-conjugated CD11c mAb was also added to the cells for another 10 minutes in the fridge (4-6°C). Following staining, cells were washed by adding 10-20× volume of buffer and centrifuged at 200 × g for 10 minutes and the cell pellet was resuspended in 500µl of buffer in a 50-ml Falcon tube.

A positive selection column type MS^+/RS^+ was chosen on the magnetic cell separator MACS (Miltenyi Biotec Ltd.) and the column was washed with buffer. Cells labelled with CD11c MicroBeads were collected in a new 50ml Falcon tube in

the positive column, and the negative cells were flushed to thorough the negative column. $CD11c^+$ cells were further stained with MHCII mAbs and were analysed by FACS.

2.2.4.1.5. CD11c immunohistochemistry

Sections (15μm) were cut from OCT-inflated lungs using a microtome with a cabinet temperature of -30°C, mounted on polylysine-coated slides and fixed with 100% methanol. Sections were washed twice with PBS and incubated for 2 hours with either PE-conjugated hamster anti-mouse CD11c (clone N418) or isotype control (BD Biosciences, CA), diluted 1:20 in ChemMate™ Antidody Diluent (DakoCytomation, UK). Slides were washed with PBS and briefly counterstained with TO-PRO-3 (Molecular Probes Invitrogen, UK). Digital images were acquired with a confocal microscope (Leica TCSNT; Leica Microsystems, Germany). Cells stained with CD11c Ab were counted from three independent confocal fields taken at magnifications of 10× and 83×.

2.2.4.1.6. Enzyme-linked immunosorbent assays (ELISA)

ELISA analyses to detect murine GM-CSF, IL-12p40, IFN-γ and TNF-α were performed by using commercially available ELISA kits (R&D Systems, Oxon, UK) in accordance with the manufacturer's instructions. Human elafin was measured by using an ELISA developed in-house (Reid *et al*., 1999). IL-12p40 ELISA was performed on BAL fluid using paired mAb and cytokine standards (BD Biosciences, CA) as described (MacDonald *et al*., 2001).

2.2.4.2. *In vitro* analytical methods

2.2.4.2.1. Ad infection of BM-DCs *in vitro*

Immature DCs (2×10^5 cells/condition) were washed, checked for viability by Trypan blue staining, and suspended in fresh complete RPMI medium in 2ml Eppendorf tubes or 12-well Costar plates. Two infections methods were applied. In the first protocol, Ad was applied to cells cultured in plates at various multiplicity of infection (MOI) in a total volume of 500µl for 45-90 minutes at 37°C. Medium was carefully removed and DC were reconstituted with 2ml complete or serum-free RPMI and incubated overnight at 37°C.

In the second method, Ad was applied to cells suspended in Eppendorf tubes at various MOIs in a total volume of 500µl and the cells were spun at a speed of 2000 × g for 70 minutes at room temperature (Nishimura *et al.*, 2001). Following centrifugation the infection medium was removed and cells were washed with PBS × 2 and DC were resuspended in 2ml fresh complete or serum-free RPMI medium and cultured in 12-well plates and incubated overnight at 37°C. Then, cells were either treated with other reagents or were further analysed by FACS. The supernatants of treated DCs and their corresponding controls were kept at -20°C for further analysis.

2.2.4.2.2. Ultraviolet (U.V.) light-inactivation of Ad vectors

Ad vectors were exposed to direct U.V. light for a period of 45 minutes for complete inactivation and then incubated with DCs. To test the protocol of inactivation, DCs were infected with active and U.V.-inactivated Ad-GFP and GFP expression was assessed by flow cytometry 24 hours post infection.

2.2.4.2.5. DC trans-migration assay

Murine Clara cells were seeded at a density of $1x10^6$ in 1ml $serum^{+ve}$ DMEM medium in a 24-well culture plates (Costar, UK). After reaching confluency, the Clara cells were washed with PBS and infected with either Ad-elafin or Ad-null at an MOI of 100, for ~1 hour at 37°C, 5% CO_2. The infected Clara cells were then washed with PBS 3× to remove all FCS residues (as they contain chemotactic proteins that greatly interfere with the trans-migration assay), and were incubated for

a further period of 48 hours enabling them to produce and secrete the transgene into the medium. Supernatants were collected and centrifuged to remove the floating cells and debris and stored at -20°C.

On the day of the experiment, day 7 iDCs were harvested and washed with PBS, resuspended in serum^{-ve} DMEM medium and counted. At the same time, 600µl of the infected Clara cell supernatant (source of elafin) were pipetted into the lower chamber of the trans-well Costar plates (Costar, UK) and pre-incubated at 37°C. Supernatants taken from uninfected Clara cells and Clara supernatants containing 100ng/ml MIP-3α (Peprotech Ltd., London, UK) were also used as negative and positive controls, respectively. Next, 1×10^5 iDCs in 100µl were added to the top chamber and incubated for 90 minutes at 37°C. After the incubation period, the medium in both top and bottom chambers were collected and examined microscopically for the presence of iDCs.

Furthermore, the remaining media was removed from the upper side of the trans-well membranes with cotton buds, and they were submerged into absolute methanol for 10 minutes and air dried. They were then placed in Diff-Quick II (Dade, Germany), to fix the cells stuck onto the trans-well membranes, for 20 minutes at room temperature and cleaned thoroughly with cotton buds. Finally using an inverted microscope cells that migrated to the lower side of the trans-well membrane were counted, 5-10 fields were chosen systematically and the average number of iDCs stuck onto the membranes were calculated.

2.2.4.2.4. Western blotting for detection of murine α1-PI

To detect murine α1-PI in cell supernatants, it was first deemed necessary to optimise the system. Recombinant human α1-PI and rabbit anti-human α1-PI Ab (Sigma) were both available in house and hence used in the following experiments. Rabbit anti-human α1-PI Ab was shown to recognise the murine α1-PI protein too. Preliminary experiments were performed to choose the optimum concentrations of rabbit anti-human α1-PI Ab (with a stock concentration of 42mg/ml) which was

found to be 1:1000. Cells were infected with Ad-α1PI for 48 hours and supernatants were collected and concentrated in methanol as described above. Pellets were resuspended in reducing sample buffer and were immediately heat-denatured at 70°C for 10 minutes prior to electrophoresis on a NuPAGE 4-12% Bis-Tris polyacrylamide gel (NuPAGE Invitrogen Ltd., Paisley, UK) at 200 Volts (constant voltage) for 35 minutes. The blotted membranes were blocked in 8% milk-Tween solution for 1 hour, washed and incubated with rabbit anti-human α1-PI Ab diluted in 8% milk-Tween for 1 hour at room temperature. Goat anti-rabbit-HRP (DAKO, Cambridgeshire, UK) was used as a secondary Ab which was diluted in 8% milk-Tween at a final concentration of 1:4000, for 1 hour at room temperature. Membranes were incubated with Hybond ECL Plus reagent as recommended by the manufacturers and exposed to Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK).

2.2.6. Statistical analysis

Results are reported either (*i*) as pooled data from a series of *n* separate experiments, and presented as mean ± S.D., or (*ii*) as individual experiments, carried out in duplicate or triplicate, or (*iii*) single experiment using pooled cells from 2-4 mice.

Statistical significance was analysed by Student's unpaired *t*-test using the GraphPad Prism 4 statistical software (San Diego, CA). Statistical significance was assigned to data returning a 'P value' of less than 0.05.

CHAPTER 3

THE EFFECTS OF CIGARETTE SMOKE CONDENSATE AND LUNG INFLAMMATORY MEDIATORS ON DENDRITIC CELL PHENOTYPE AND FUNCTION

3.1. AIMS

The upper and lower airways are repeatedly exposed to a multitude of airborne particles and microorganisams. However, due to the presence of a tight defence network comprising various components of the innate and immune systems, the airways are usually sterile and non-inflamed in healthy individuals (Zalacain *et al*., 1999). On the other hand, lungs of chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) patients are frequently infected and colonised by microorganisms. The latter is associated with chronic infections with *Staphylococcus aureus (S.a.), Pseudomonas aeruginosa* (*P.a.*), and often in the worst cases with *Burkholderia cepacia* (*B.c.*). Although cigarette smoking is the accepted aetiologic factor in the pathogenesis of COPD in the huge majority of cases, recent evidence suggests that respiratory tract of COPD patients is often colonised in 50-100% of cases (Zalacain *et al*., 1999; Sethi *et al*., 2006; Tumkaya *et al*., 2006) and that infections with viruses and bacterial pathogens such as non-typeable *Haemophilus influenzae* (*NTHI*), *Streptococcus pneumoniae*, and *Branhamella catarrhalis* are instrumental in the generation of disease exacerbations (Patel *et al*., 2002; Sethi *et al*., 2006). COPD and CF are characterized by intense neutrophilic inflammation and the release of high levels of cytotoxic molecules such as NE that overwhelm and inactivate antiprotease and innate immune defences (Suter, 1989; Fujita *et al*., 1990; Birrer *et al*., 1994; Tumkaya *et al*., 2006; Sethi *et al*., 2006). NE is capable of modifying many cytokines and cell surface receptors through its proteolytic activity (see Table 1.5). For instance, it is able to cleave important receptors from the surface of macrophages which could potentially impair the clearing of apoptotic cells and perpetuate a pro-inflammatory phenotype (Le-Barillec *et al*., 1999; Vandivier *et al*.,

2002; Henriksen *et al*., 2004). On the other hand cigarette smoke (CS) has been shown to modulate immune responses (see section 1.5). As DCs are one of the first lines of defence to be present in the tracheobronchial tree (see Figure 1.5), it was hypothesised that CS and COPD/CF lung inflammatory secretions containing high concentrations of NE are able to influence the behaviour or phenotype of DCs in such a way as to inhibit their ability to present antigens and, thus, initiate protective immunity to pathogens. Therefore, the principal aim of the work described in this chapter was to investigate whether cigarette smoke condensate (CSC) and sputum secretions (containing NE) from COPD and CF patients as well as purified human NE are able to 'deactivate' DCs and impair their functional activity, thereby providing a mechanism by which microbes may escape immune surveillance. Hence, the effects of CSC and human lung secretions/purified human NE on the function of murine BM-DCs were investigated using a variety of parameters, namely the expression of DC CSMs 'CD40, CD80, and CD86', and pro-inflammatory cytokine such as IL-12p40 and TNF-α. BM-DCs, and not lung DCs, were used in this study because they could be generated at high numbers *in vitro*, thereby reducing the number of animals used. Nonetheless, progenitors of lung DCs also originate from $CD34^+$ BM stem cells.

In the event that upon treatment, DC phenotype and activation state was affected, a further aim was to investigate how this could influence the maturation of iDCs in the presence of LPS and to test functional activity of DCs, using lymphocytes derived from DO11.10 transgenic mice and OVA-induced mediated T cell proliferation as a read-out of effective antigen presentation.

Since the OVA transgenic mice used here were derived from a BALB/c background, the source of DCs used throughout this chapter was from BALB/c mice BM cells. The CSC was freshly produced for each experiment as described in Chapter 2 (section 2.2.3.1), chromotographically purified human NE from purulent sputum was purchased from Elastin Products (Missouri, USA); COPD and CF sputum samples were prepared as described in Chapter 2 (section 2.2.3.3).

3.2. RESULTS

3.2.1. Preliminary experiments to generate and optimise yields of BM-DCs *in vitro*

As DCs are rare populations in all organs, their generation from haematopoietic precursors in large quantities has proven critical to study their biology and their use in modulating the immune response in *in vivo* studies. The principal source of DCs used through out this project was murine BM-DC that as their name suggests were generated from murine haematopoietic BM cells, under the influence of GM-CSF over a period of several days (see Chapter 2 for more details). Given that this was the first project in the laboratory to use DCs, it was first essential to optimise an efficient protocol which could provide a ready supply of iDCs for routine *in vitro* studies. Currently, there are two widely used standard protocols of producing BM-DCs *in vitro* (Inaba *et al.,* 1992; Lutz *et al.*, 1999). At the start of this project, protocols derived from these two sources (referred to as method 1 and method 2 respectively) were applied to determine which is the most efficient method (both in terms of the final yield and DC purity) for generating BM-DCs, for day to day use in the laboratory. Each of these methods has advantages and disadvantages; Table 3.1 summarises and compares the conditions used in methods 1 and 2. As shown in Figure 3.1, microscopic analysis of cells revealed that method 1, generated cells with typical DC morphology as well as neutrophils, whereas method 2 contained an almost homogenous population of cells having DC morphology; the FACS data (Gr1 and F4/80 staining) were also in agreement with these observations (not shown). In general, when cells were stained with FACS mAbs to CD11c and MHCII (markers used to identify DCs), method 2 generated a more heterogeneous population of cells (which could be separated into iDCs and semi-mDCs) than method 1 (Figure 3.2 A, bottom dot plots). When compared like for like, cells generated by method 1 expressed slightly higher levels of the activation markers, CD40, CD80 and CD86 (Figure 3.2 B).

Since method 2 resulted in significantly higher number of cells and a far higher BM-DC purity than method 1 (despite its longer culture period) it was decided to use this method for the rest of this project. In the rest of this thesis, BM-DCs would be referred to as DCs for simplicity.

Table 3.1. Comparison of DC culture techniques
Adapted from Lutz *et al*., 1999.

Parameter	Method 1 (Inaba *et al.*)	Method 2 (Lutz *et al.*)
Depletion of erythrocytes within fresh BM	Yes	No
Culture plastic	Tissue culture quality 24-well plate	Bacterial quality 100mm petri dishes
Culture medium	RPMI + 10% FCS	RPMI + 10% FCS
GM-CSF dose	200-1000U/ml throughout (=5-25ng/ml)	200U/ml (=20ng/ml), from day 10 30-100U/ml
Feeding	Day 3 and day 6 aspirate and discard 50% of the culture supernatant, add fresh medium and GM-CSF	Day 3 add 100% medium + GM-CSF, day 6, 8 and 10 aspirate and discard 50% of the culture supernatant, add fresh medium and GM-CSF
Culture period before passage (expansion)	6 days	10 days
Cell treatment before passage	Clusters dislodged by vigorous pipetting	Plates rinsed, and cells transferred to cell culture plastic plates
DC harvest time point	Days 7-8	Days 10-12, maximally 14
DC purity with GM-CSF	Day 8: 60%	Days 10-12: 80-90%
DC yield/mouse	7×10^6	$1\text{-}3\times10^8$

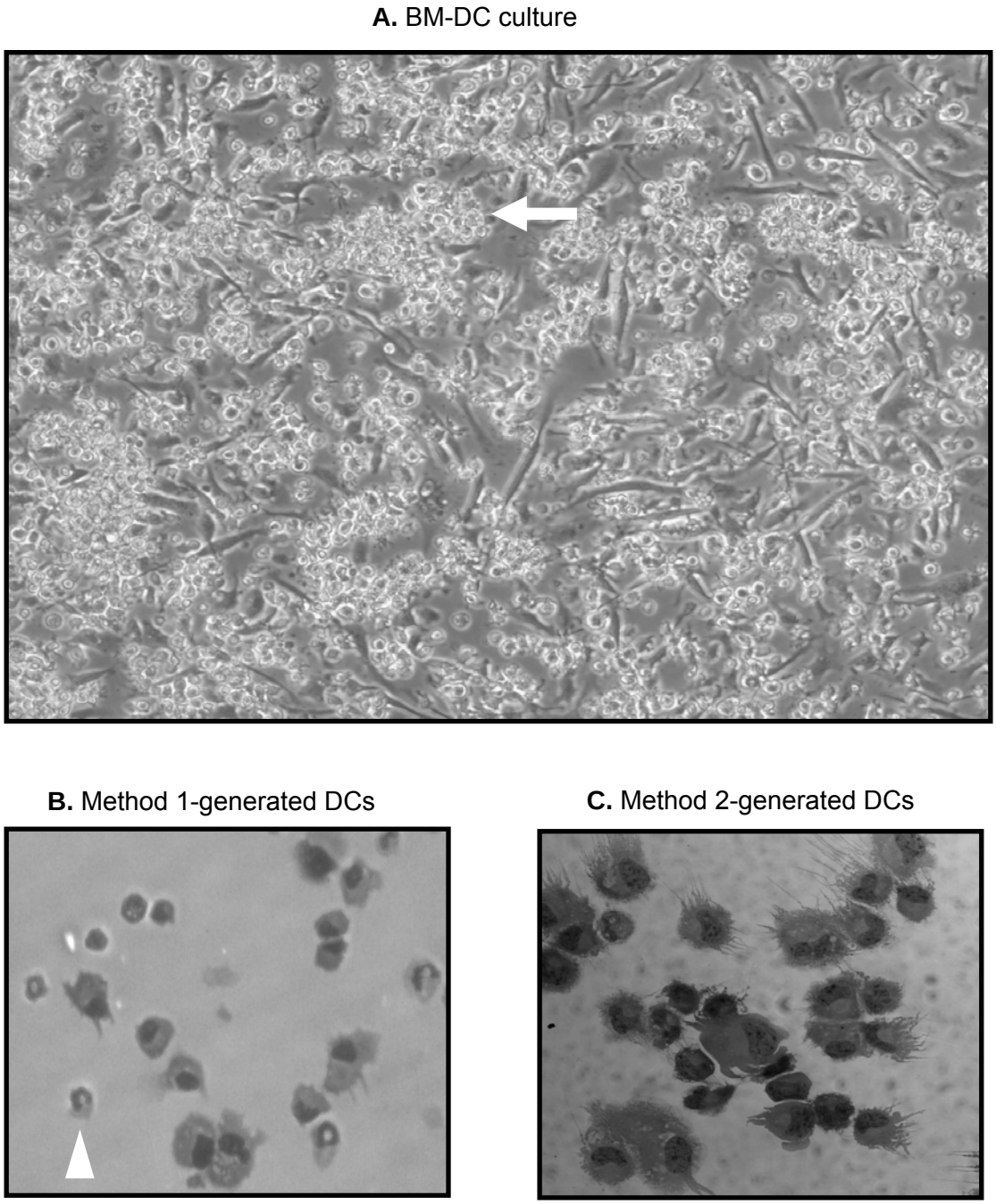


Figure 3.1. DC culture phenotypic assessments

On day 7 (method 1) or day 10 (method 2) of the DC cultures, loosely adherent cells were removed by pipetting and washed. **A)** Day 10 DC cultures were analysed by light microscopy. White arrow indicating GM-CSF-induced DC colonies in culture. **B & C)** Cytospinning (100 × g for 3 minutes at room temperature) of method 1 and method 2 DC cultures was performed and slides were stained with Diff-quick (Dade Diagnostika, GmbH, Germany) and fixed. Representative pictures are shown (n=6). White arrow head showing contaminant neutrophils in culture.

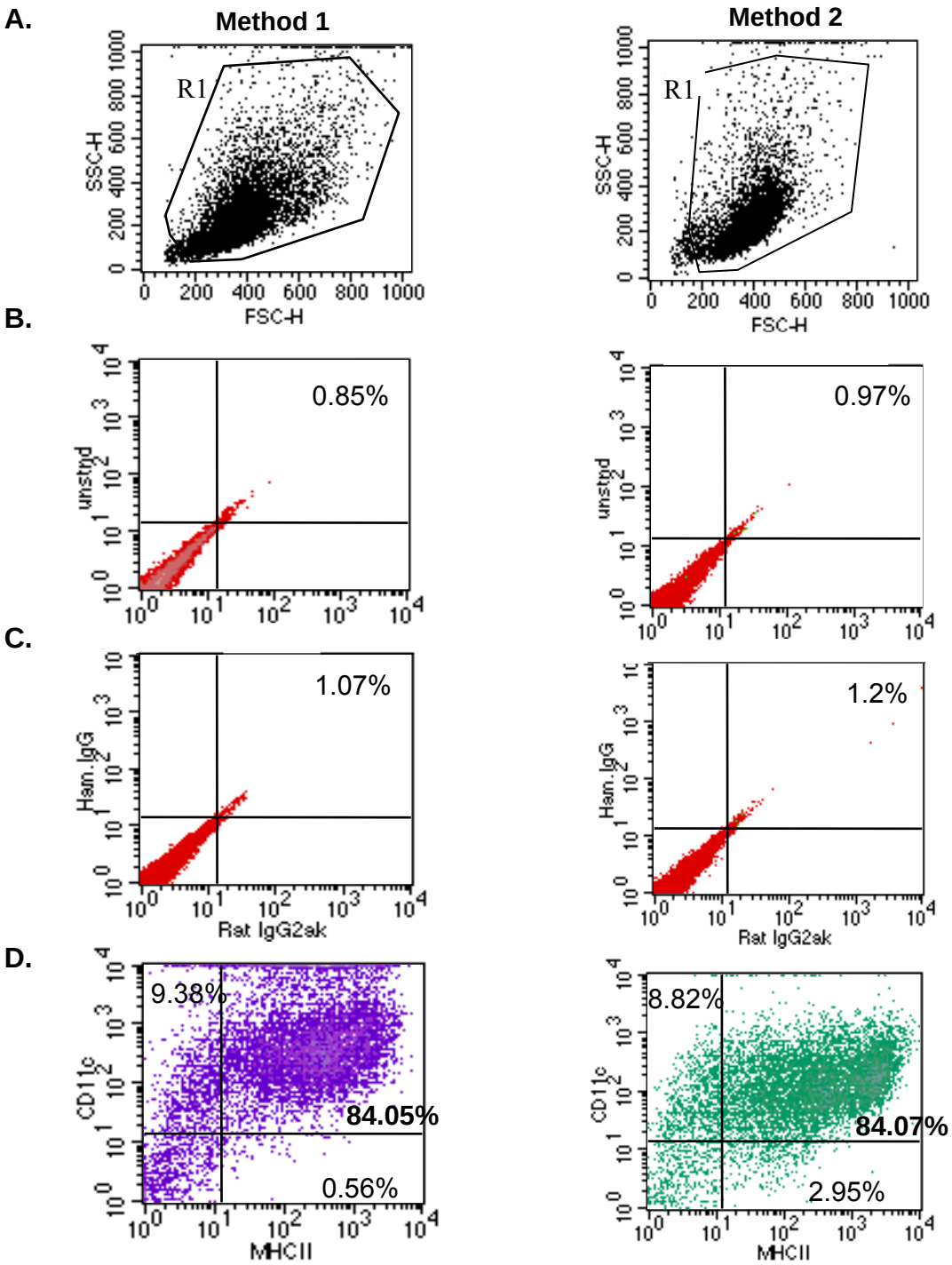


Figure 3.2 (Part A). Comparison of method 1- and method 2-derived DC phenotype and activation

BM-DC were generated using two widely methods for generating murine iDC *in vitro*; referred to as Method 1 (Inaba *et al*., 1992) or Method 2 (Lutz *et al*., 1999) (see Chapter 2, section 2.2.1.4). On day 7 or 10 of the cultures, respectively, cells were washed and stained with FACS Abs to assess final DC purity (n=3). **A**) Forward/side scatter plots of DCs, **B**) Unstained profiles of DCs, **C**) profiles of DCs stained with isotype-matched control Abs (Armenian hamster IgG-APC and Rat IgG2aκ-FITC, respectively), and **D**) profiles of DCs stained with CD11c-APC and MHCII-FITC Abs.

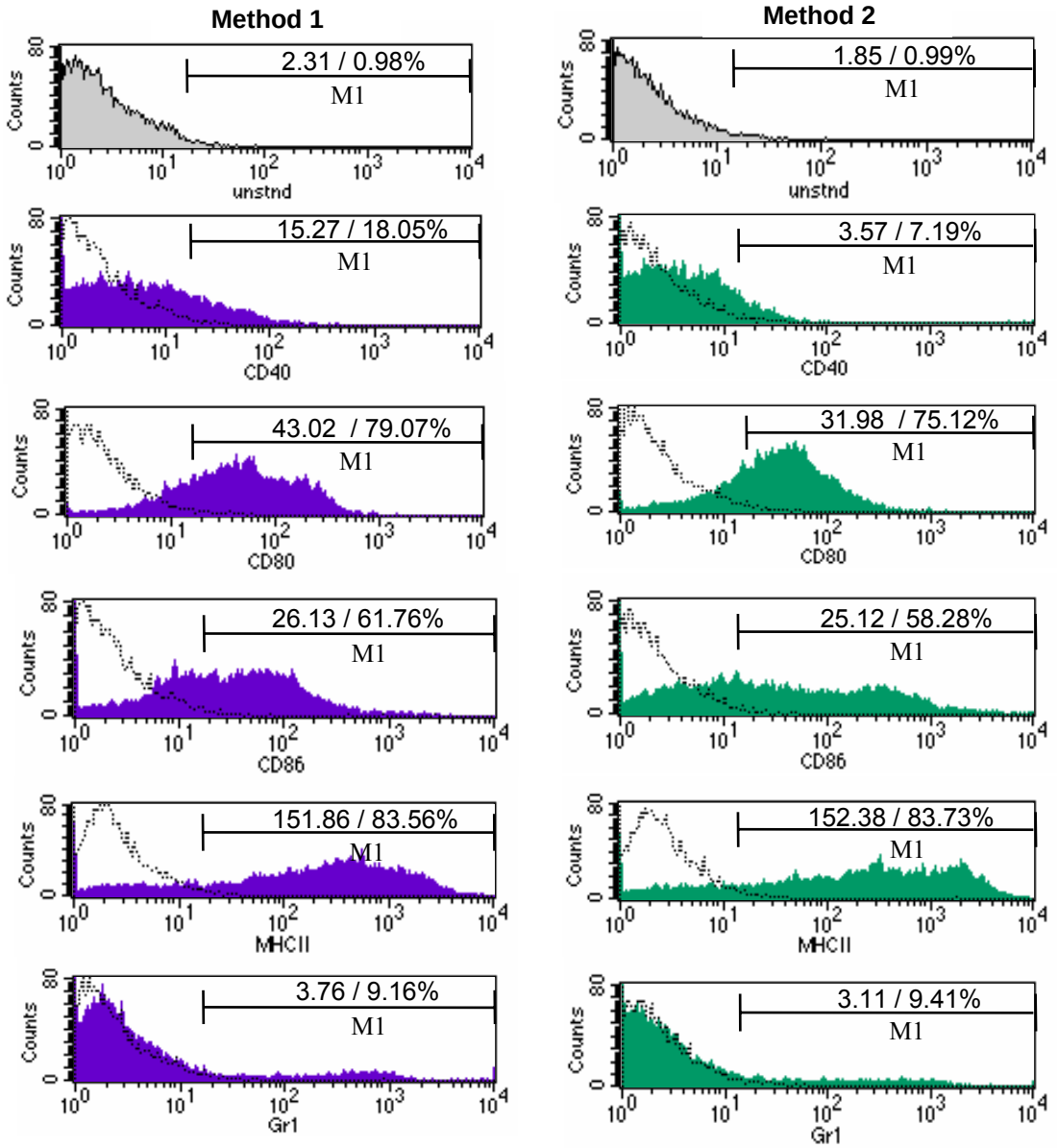


Figure 3.2 (Part B, continued). Comparison of method 1- and method 2-derived DC phenotype and activation

BM-DCs generated by Method 1 and Method 2 were stained with mAbs to CSMs (CD40, CD80 and CD86), MHCII and Gr1 and analysed by flow cytometry. Dotted lines indicate isotype-matched controls. Blue histograms (right) represent method 1-generated DCs and the green histograms represent method 2-generated DCs. Data shown is representative of three independent experiments, MFI and % positive cells shown for each marker. To enable direct comparisons, similar DC purity cultures from each method were chosen here.

3.2.2. Stimulation of immature DCs with lypopolysaccharides (LPS)

As explained in Chapter 1 (section 1.2), DCs exist in two main maturation states, referred to as immature DC (iDC) and mature DC (mDC). When DCs generated from BM are harvested at the end of the culture period, they predominantly have an immature phenotype which is indicated by very low expression levels of CD40, and moderate levels of CD80, CD86 and MHCII (Figure 3.2 B). A common way of inducing DC maturation is by stimulating them with Gram-negative bacterial outer surface antigenic components such as LPS. iDCs are strongly activated by LPS which signals via the conserved TLR2 and TLR4 receptors highly expressed on DCs and other phagocytic cells (Takeuchi *et al.*, 1999). The engagement of TLR2/TLR4 induces nuclear translocation of the NF-κB transcription factor via activation of an adaptor protein (MyD88) and a serine/threonine kinase (IRAK) (Medzhitov *et al.*, 1997; Sweet and Schorey, 2006). Also, microbial activation might lead to the transcription of the IFN-inducible genes via IRF-3 (Trottein *et al*., 2004). On day 10 of culture, iDCs were initially stimulated with LPS or 0.02% (w/v) *S.a.* (Cowan strain) (SAC) to assess cell activations by morphology and TNF-α secretion (Figure 3.3). An LPS titration of iDCs over a 24 hour period was also performed in serum^{+ve} (10% v/v) complete medium in order to optimise the LPS concentration for the experiments undertaken throughout this project (Figure 3.4). These experiments indicated that 100ng/ml LPS was the optimum concentration to use in serum^{+ve} medium over this period and further addition of LPS did not make a significant impact on DC maturation. Representative FACS data presented in Figure 3.5 compare the cell surface phenotypes of iDCs and LPS-matured DCs. Following LPS stimulation the CD40 levels are increased from just a few percent of CD40-expressing iDCs to more than 60% CD40-expressing mDCs. Whereas, ~40% of iDC express CD80 and CD86 molecules which increased upon stimulation to ~80%. On the other hand, the majority of iDCs express MHCII (~85%) which is slightly increased (~90%) upon LPS-stimulation. However, it should be acknowledged that the expression levels of MHCII on individual DCs was dramatically increased as assessed by MFI (iDC vs mDC; ~440 and ~934,

respectively. This is also the case for the other DC surface markers examined here (CD40, CD80 and CD86) (Figure 3.5 B).

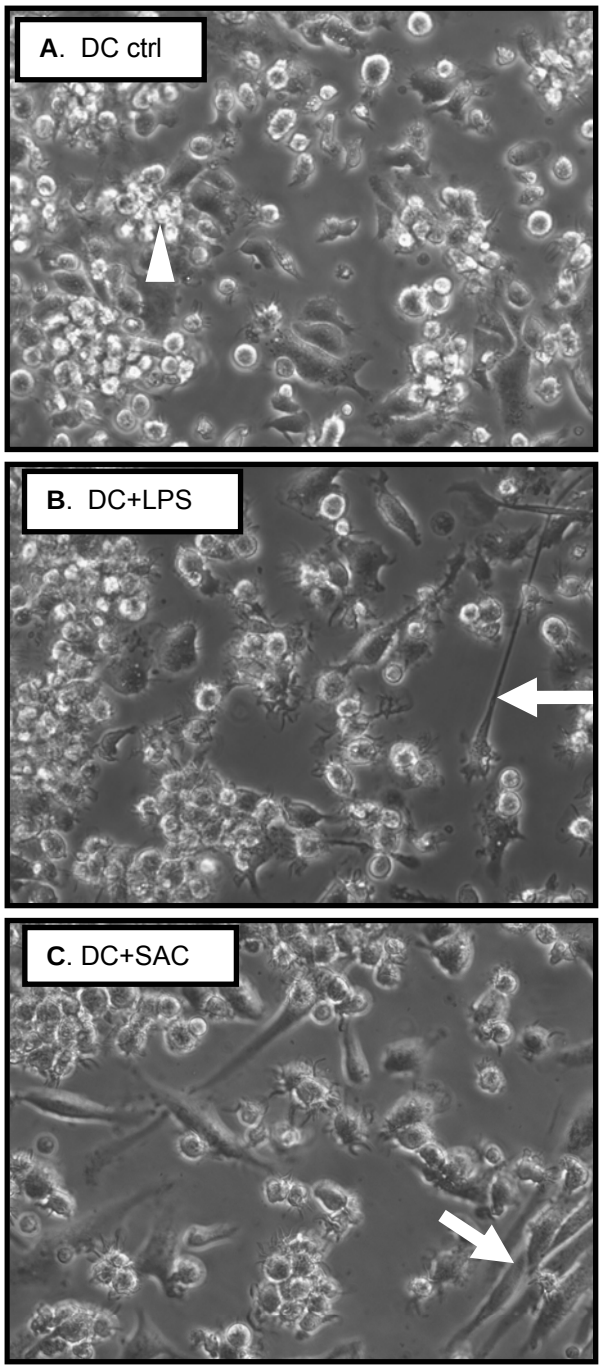


Figure 3.3 (Part A). Stimulation of DCs

iDCs were generated as described above. On day 10 of the culture, fully differentiated DCs were removed, washed and replated in 12-well Costar plates at a density of 1×10^6 cells/ml in complete RPMI medium. They were then stimulated with 100ng/ml LPS or SAC (0.02% w/v) and analysed by light microscopy 24 hours later. **A**) White arrowhead showing iDC colonies, **B**) white arrows indicating LPS-matured mDCs, and **C**) SAC-matured mDCs. Representative pictures are shown (n=5).

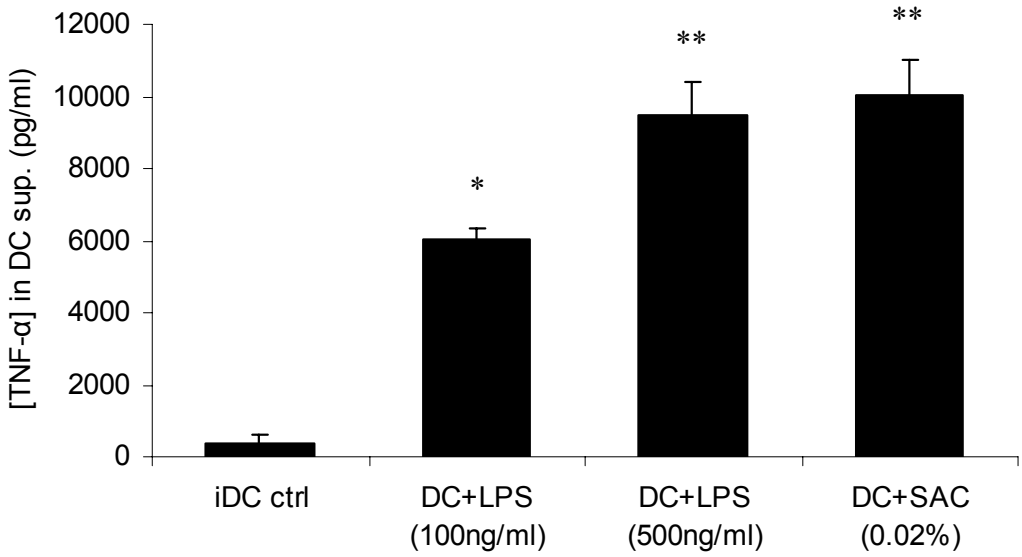
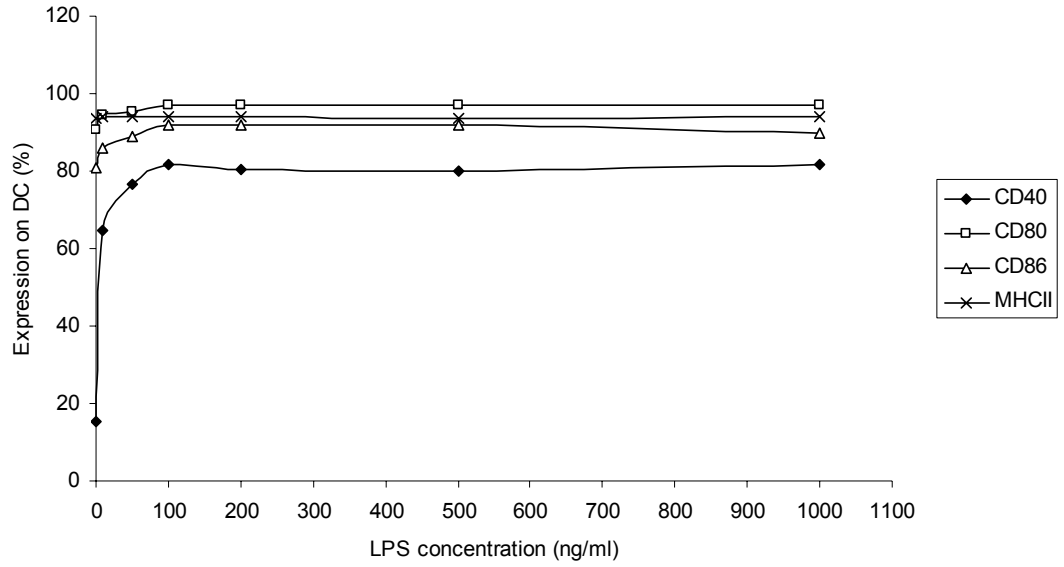


Figure 3.3 (Part B, continued) Stimulation of DCs

Immature DCs were generated and stimulated as described above. TNF-α output by activated DCs (24 hours post-stimulation) was analysed by ELISA. * indicates significance iDC vs LPS (100ng/ml)-matured DCs and ** indicates significance between matured cells; LPS (100ng/ml) vs LPS (500ng/ml)/SAC (0.02% w/v); $p<0.05$, $n=5$ independent experiments.

A.



B.

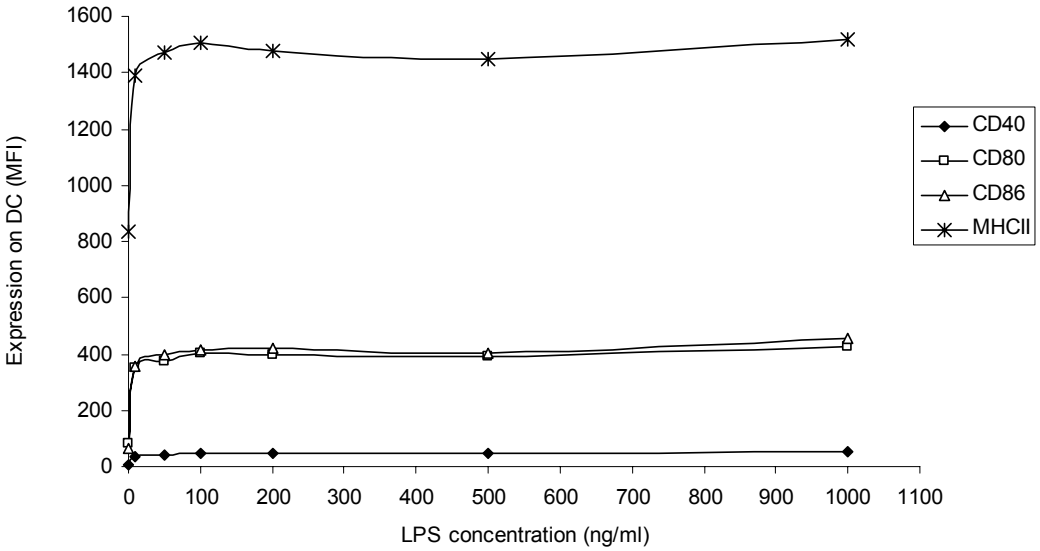


Figure 3.4. LPS titration of DC maturation

To optimise the concentration of LPS used in experiments where DC maturation was required, DCs were generated as described above (see legend of Figure 3.3) and stimulated with increasing concentrations of *E.coli* LPS for 24 hours in complete medium (serum^{+ve}). The cells were then washed and stained with mAbs to CSMs and MHCII and analysed by flow cytometry. **A)** Graph showing percentage of cells expressing CSMs and MHCII following LPS-stimulations. **B)** Graph showing mean fluorescence intensity (MFI) of CSMs and MHCII levels on DCs following LPS-stimulations; *n*=3 independent experiments.

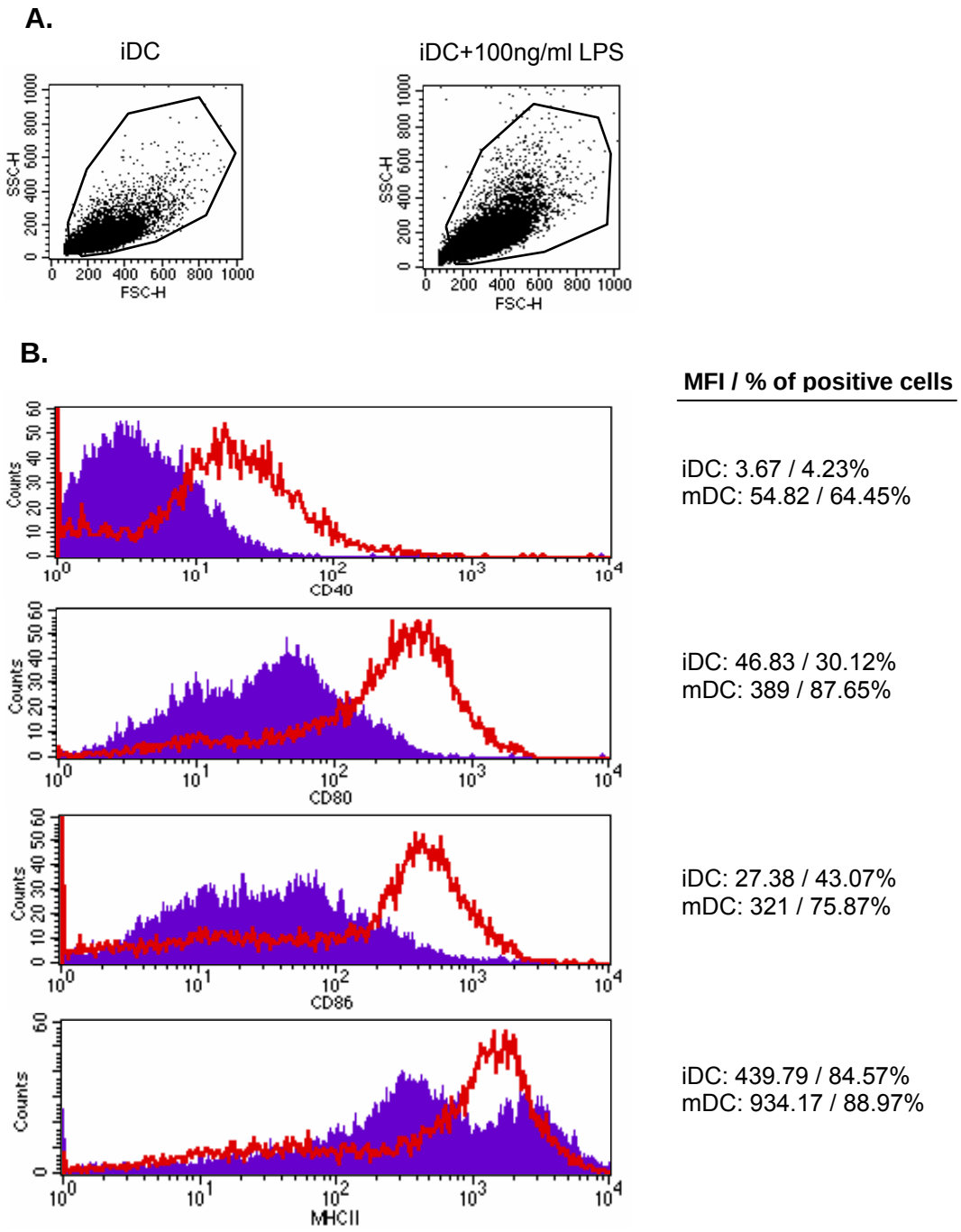


Figure 3.5. Comparison of surface expression levels of CSMs on iDCs versus mDCs

DCs were generated as described above and stimulated with 100ng/ml *E.coli* LPS for 24 hours in complete medium. They were then washed and analysed by flow cytometry for expression of CSMs and MHCII. **A)** Dot plots of iDCs and mDCs are shown, and **B)** histograms showing expression levels of CSMs and MHCII molecules on iDCs (filled blue) compared to LPS-matured DCs (mDCs; open red lines). Unstained cells, isotype-matched controls and gates are not shown for simplicity; *n*=10 independent experiments.

3.2.3. Effects of cigarette smoke condensate (CSC) on DCs

In order to investigate the effects of CSC on mDCs, iDCs were matured with 100ng/ml *E.coli* LPS for 24 hours, as described above, prior to the addition of freshly produced CSC. mDCs (as well as iDCs, not shown) were exposed to CSC (1%, 5% and 10%) or PBS (control) for 4 hours in complete medium. The cells were subsequently stained for CSMs as well as MHCII and analysed by flow cytometry. As Figure 3.6 demonstrates, CSC at higher doses (5% and 10%) caused a decrease in CSMs and MHCII expression on mDCs as indicated by a dose-dependent peak shift to the left. The reduction was more profound in the case of CD80 and CD86 as compared to CD40. This could be explained by the fact that iDCs hardly express CD40 and when stimulated to mature not all the cells are responsive, as shown in Figure 3.5 B.

3.2.3.1. CSC induces DC death in dose-dependent manner

Cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose phosphatidylserine (PS) due to its translocation to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. This PS exposure may represent a hallmark (early and widespread) for the detection of dying cells. Annexin V belongs to a recently discovered family of proteins, the annexins, which could be used as a useful marker for detection of apoptotic cells. This is because annexin V preferentially binds to negatively charged phospholipids like PS on apoptotic cells in the presence of Ca^{2+}. Changes in PS asymmetry, which is analyzed by measuring annexin V binding to the cell membrane, could be detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. By conjugating a fluorochrome such as FITC to annexin V it is possible to identify and quantify apoptotic cells on a single-cell basis by flow cytometry. Staining cells simultaneously with FITC-annexin V (green fluorescence) and the non-vital dye propidium iodide (PI; red fluorescence) allows the discrimination of

intact cells (annexin V^- PI^-), early apoptotic (annexin V^+ PI^-) and late apoptotic or necrotic cells (annexin V^+ PI^+).

To investigate the reason for the down-regulation of DC CSMs by CSC and to assess cell viability post CSC exposure (Figure 3.6), DCs treated with either PBS or 1%, 5% or 10% fresh CSC for 4 hours were stained with PI and annexin V and analysed by flow cytometry immediately after treatment. CSC caused a dose-dependent death in of DCs as demonstrated in Figure 3.7. This seemed to be mainly due to cell necrosis and little apoptosis, as majority of the cells were positive for PI.

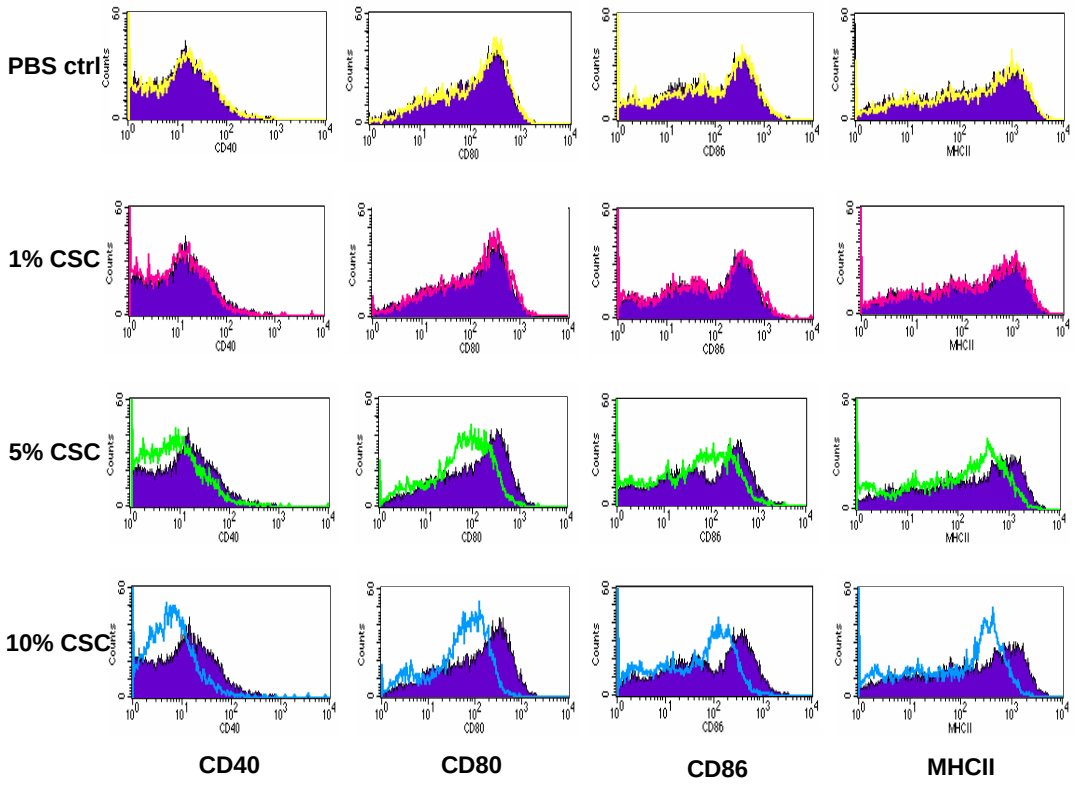


Figure 3.6. CSC causes down-regulation of DC surface molecules

DCs were generated as described above and stimulated with 100ng/ml *E.coli* LPS for 24 hours in complete medium. The cells were then exposed to either PBS or 1-10% freshly prepared CSC) and incubated for 4 hours at 37°C. They were then washed and analysed by flow cytometry for expression of CSMs and MHCII. Blue histograms represent untreated mDCs and coloured lins (overlays) represent expression levels of DC markers following PBS/CSC treatments. Representative data of at least three independent experiments are shown. Unstained cells, isotype-matched controls and gates are not shown for simplicity.

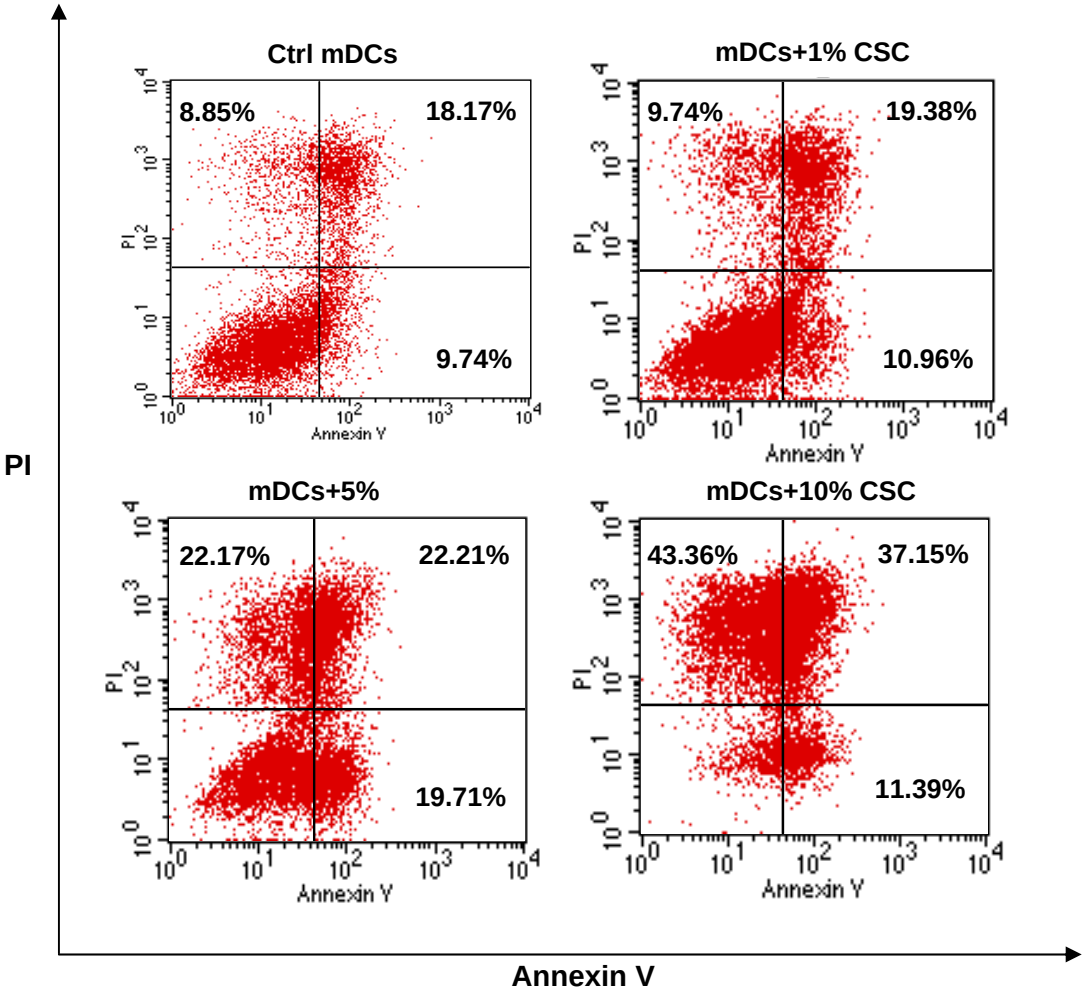


Figure 3.7. CSC exposure induces DC death in culture

DCs were generated as described above and stimulated with 100ng/ml *E.coli* LPS for 24 hours in complete medium. The cells were then exposed to either PBS or 1-10% freshly prepared CSC) and incubated for 4 hours at 37°C. They were then washed and analysed by flow cytometry for annexin V and propidium iodide (PI) positivity. Gates were chosen using unstained cells which fell in the bottom right quadrant (not shown), as before. Representative data of three independent experiments are shown.

3.2.4. Comparison of COPD and CF patients' sputum samples

NE concentrations in solubilised sputum samples prepared from samples collected from COPD/CF patients were measured as described in Chapter 2. As Figure 3.8 shows, the mean NE concentration in COPD sputum samples was 0.1143μM ± 0.04850 (*n*=27, 11 F and 16 M), whereas the mean NE concentration in CF sputum samples was more than 20 times higher (2.3245μM ± 1.5069; *n*=18, 9 F and 9 M) (COPD [NE] vs CF [NE], $p<0.0001$). Microbiological analyses showed that the majority of the COPD and all of the CF sputum samples contained one or more kind of pathogens. See Appendix (Tables A3.1 and A3.2) for comprehensive demographics of the COPD/CF subjects used in these experiments.

3.2.4.1. Effects of COPD and CF sputum samples on DC CSMs

Upon DC maturation by LPS (100ng/ml), the levels of the CSMs, CD40, CD80 and CD86, were typically increased 3-4 folds in an overnight culture (Figure 3.5 B). To examine the effects of sputum on DC morphology and phenotype, mDCs were incubated with COPD and CF sputum samples in 1.5ml Eppendorf tubes on a shaker for 90 minutes at 37°C. In preliminary experiments, using 7 of the COPD sputum samples and all of the 18 CF sputum samples, with the last marker (CD86) as shown in Figure 3.9, the levels of mDCs CD86 CSM were lower after treatment with CF sputum samples (mean NE concentration: 4.7μM ± 0.73, *n*=18, $p<0.0001$) than after treatment with COPD sputum samples (mean NE concentration: 0.11μM ± 0.05, *n*=7, p=0.0015). In addition, for each type of sputum, there was a strong, statistically significant correlation between the NE concentration in individual sputum samples and reduction of CD86 levels (CF: *r*=0.94 and COPD: *r*=0.76). A more comprehensive experiment using pooled sputum samples showed that mDCs incubated with the COPD/CF sputa expressed lower levels of CD80 and CD86, whereas the expression levels (expressed as MFI) of CD40 were increased (Figure 3.10), with no or very little change in expression levels of MHCII (not shown). Of note, even though mDCs were chosen because of their higher starting initial levels of CSMs expression, sputa had a similar effect on the expression of CSMs on iDCs (see

below, Figure 3.19 A). In parallel control experiments mDCs were incubated with the same concentrations of the Sputolysin reagent as in sputum samples; mDC CSMs were shown to be unaffected in the presence of Sputolysin reagent alone (data not shown).

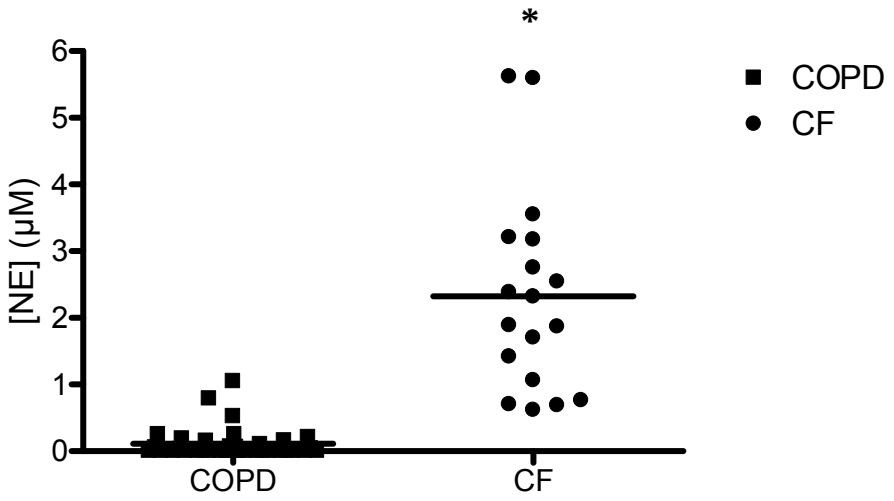


Figure 3.8. NE levels in COPD and CF sputum samples

COPD and CF sputum samples were prepared, and NE concentrations were measured as described in Chapter 2. Graph showing the concentrations of NE in COPD (n=27) and CF (n=18) sputum samples (* indicates statistical significance, p<0.0001).

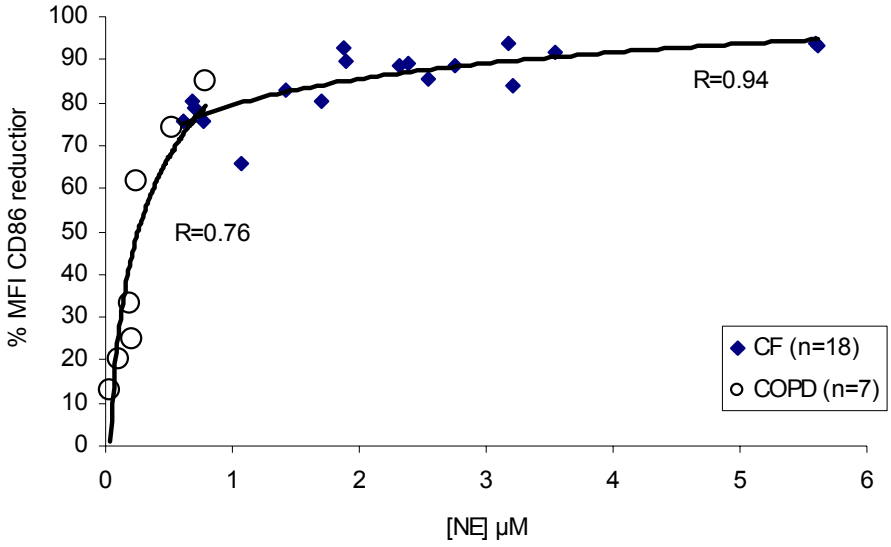


Figure 3.9. Reduction of CD86 by sputum samples

mDCs (matured with LPS in serum-containing medium for 24 hours) were washed to remove serum and incubated with individual sputa from COPD (*n*=7; Patients 1, 5, 6, 8, 12, 13 and 14) and CF patients (*n*=18; Patients 1-18) or an equivalent volume of PBS for 90 minutes on a shaker at 37°C, and cells were stained with CD86 mAbs and analysed by FACS. *R*=equation of straight line.

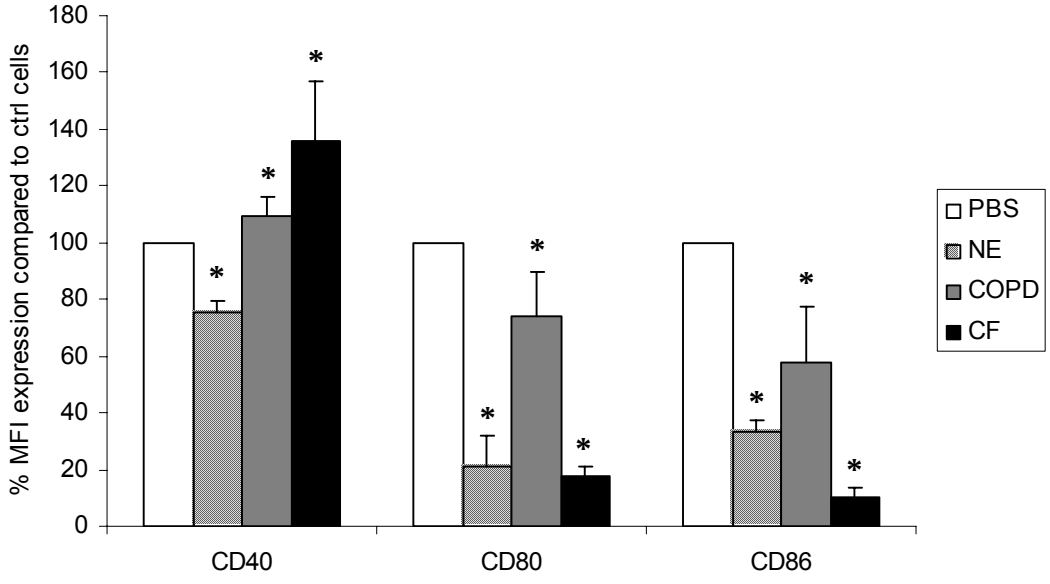


Figure 3.10. Effects of COPD and CF sputum samples on mDC CSMs

mDCs were washed to remove serum and incubated with either purified human NE (2µM), individual pooled sputa samples from COPD and CF patients or an equivalent volume of PBS for 90 minutes on a shaker at 37°C. Graph pooled from 2 independent experiments (triplicate wells). * indicates significant difference ($p<0.05$) compared to uninhibited PBS-treated mDCs (control).

3.2.5. Effects of purified human NE on DC CSMs

The data presented above indicate that patients' sputum samples are able to induce down-regulation of the CSMs (CD80 and CD86), and that the down-regulation of CD86 positively correlates with the concentration of NE in sputum samples. To test whether NE in isolation was also able to affect the levels of DC CSMs expression, mDCs were incubated with increasing pathophysiological concentrations of purified human NE (0.5-3 μM) and the levels of CSMs and MHCII expression were assessed by flow cytomerty. Purified NE caused a dose-dependent down-regulation in all CSMs under investigation (CD40, CD80 and CD86), but no or very little change in MHCII levels on mDCs (Figure 3.11; also see Figure 3.19 A). Importantly, the reduction in expression of CSMs on mDCs was not due to cell apoptosis and/or necrosis (measured by annexin V and propidium iodide staining) NE treatment (Figure 3.12).

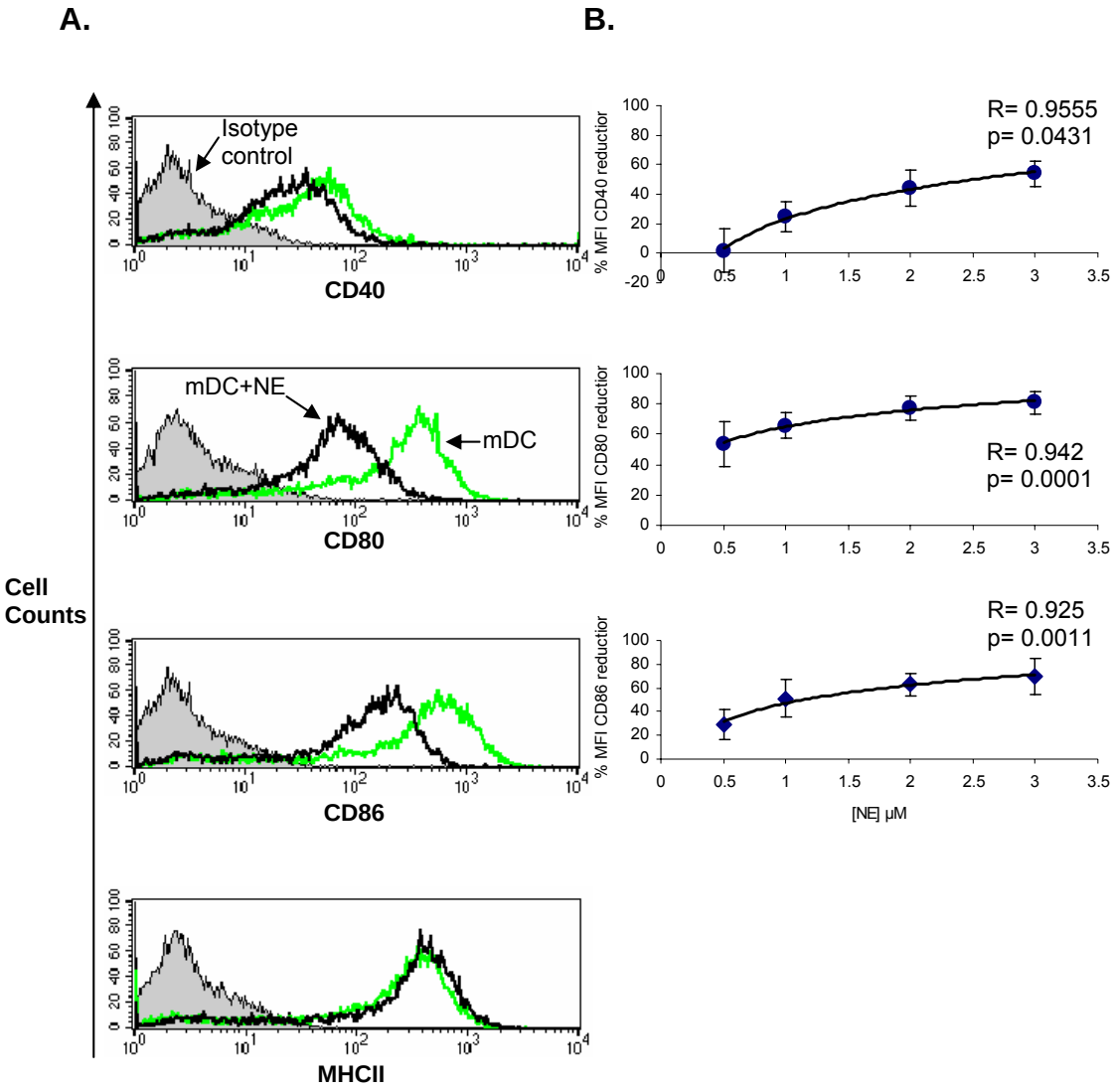


Figure 3.11. Reduction of CD40, CD80 and CD86 CSMs but not MHCII by purified NE

A) mDCs were washed, resuspended in PBS containing 0.1% BSA and exposed to NE (2µM) for 90 minutes at 37°C. Treated mDCs were then washed, stained with antibodies to CD40, CD80, CD86 and MHCII and analyzed by flow cytometry (green line: mDCs; dark line: NE-treated mDCs, filled grey line: isotype control). **B)** mDCs were exposed to a range of NE concentrations (0.5-3 µM) and expression of CD40, CD80, CD86 was analyzed. The % reduction was calculated as follows:

$$100 - \left[\frac{\text{MFI NE- treated cells}}{\text{MFI PBS treated cells}} \times 100 \right]$$

Values were pooled from 3-6 independent experiments and represent mean ± SD compared to untreated controls. *R*=equation of straight line.

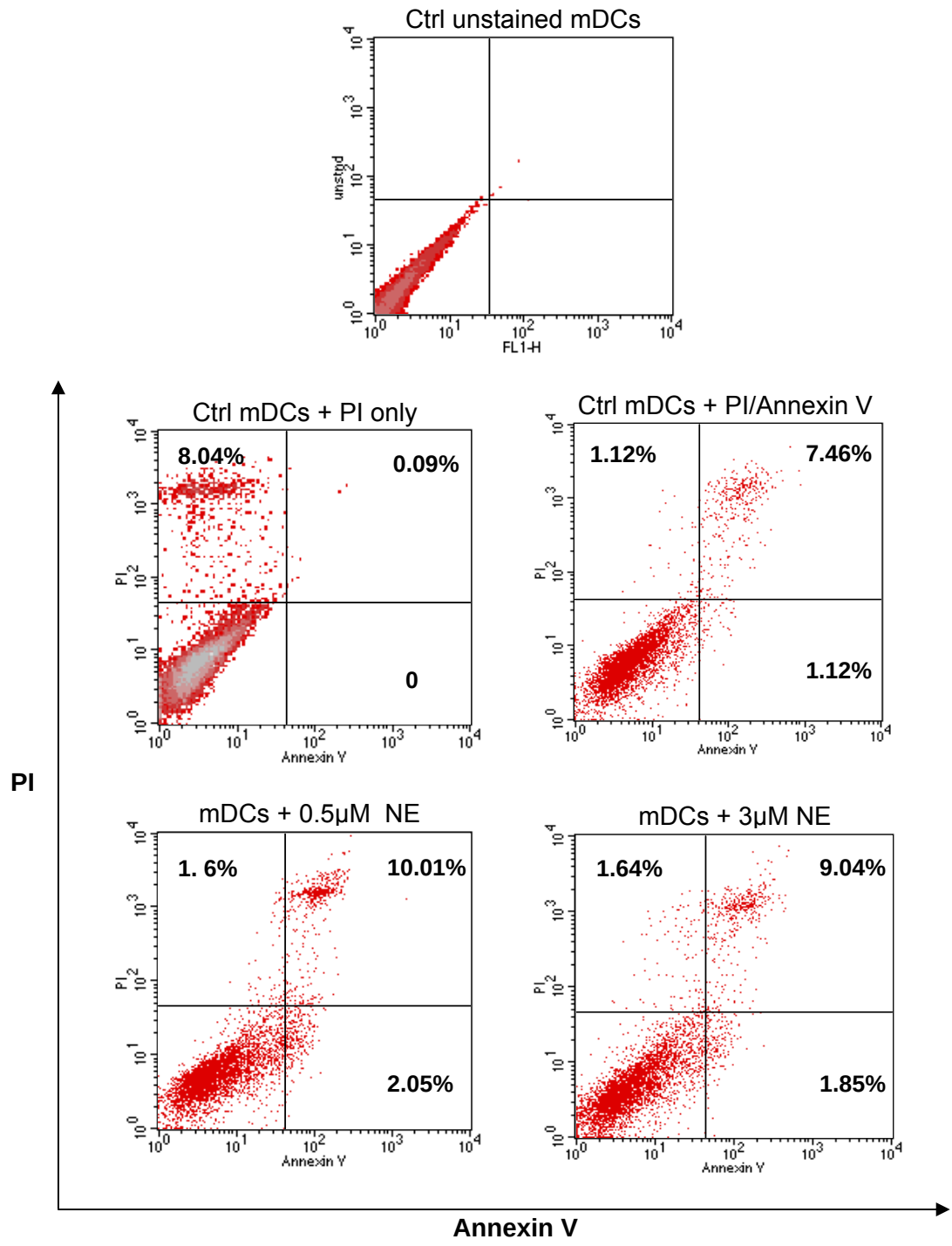


Figure 3.12. NE does not induce DC death

mDCs were washed, resuspended in PBS containing 0.1% BSA and exposed to NE (1 and 3 µM) on a shaker for 90 minutes at 37°C. Treated mDCs were then washed and resuspended in HEPES containing 4mM $CaCl_4$ buffer and stained with PI and annexin V and examined by FACS. Representative dot blot analysis of 3 independent experiments shown.

3.2.6. NE inhibitors prevent NE-induced reduction of CSMs

To prove that the reduction in CSMs levels was due to the proteolytic activity of NE in the sputum samples, recombinant murine SLPI (8μM)**,** a well characterized inhibitor of both murine and human NE (Wiersner *et al*., 2005; Junger *et al.*, 1992), and a 1:25 dilution of protease inhibitors tablet (PIT; Roche Diagnostics Ltd., East Sussex, UK) were incubated with either purified NE (2μM) or with pooled COPD (18 samples) or CF (9 samples) sputum samples (containing 0.2μM and 3.39μM NE respectively), prior to treatment of mDCs. Although SLPI and PIT significantly rescued the reduction of mDCs CD86 by purified NE (74% and 83% expression, respectively) or COPD sputa (100% and 85% expression, respectively), the rescue was more modest (but significant) after treatment with CF sputum samples (36% expression in both cases) (Figure 3.13). To investigate this effect, cells were assessed for viability by PI and annexin V assessments using FACS, following treatment of mDCs with NE and sputum samples ± inhibitors. Figure 3.14 shows data from a single representative experiment of four. As demonstrated, when SLPI and PIT were used to inhibit the free proteases (NE and sputa), despite full/partial rescuing of CD86 reduction, surprisingly there was increased cell death (PI^+) following the treatments. Necrotic cell death was more apparent when protease-inhibited mDCs were incubated with CF sputum samples.

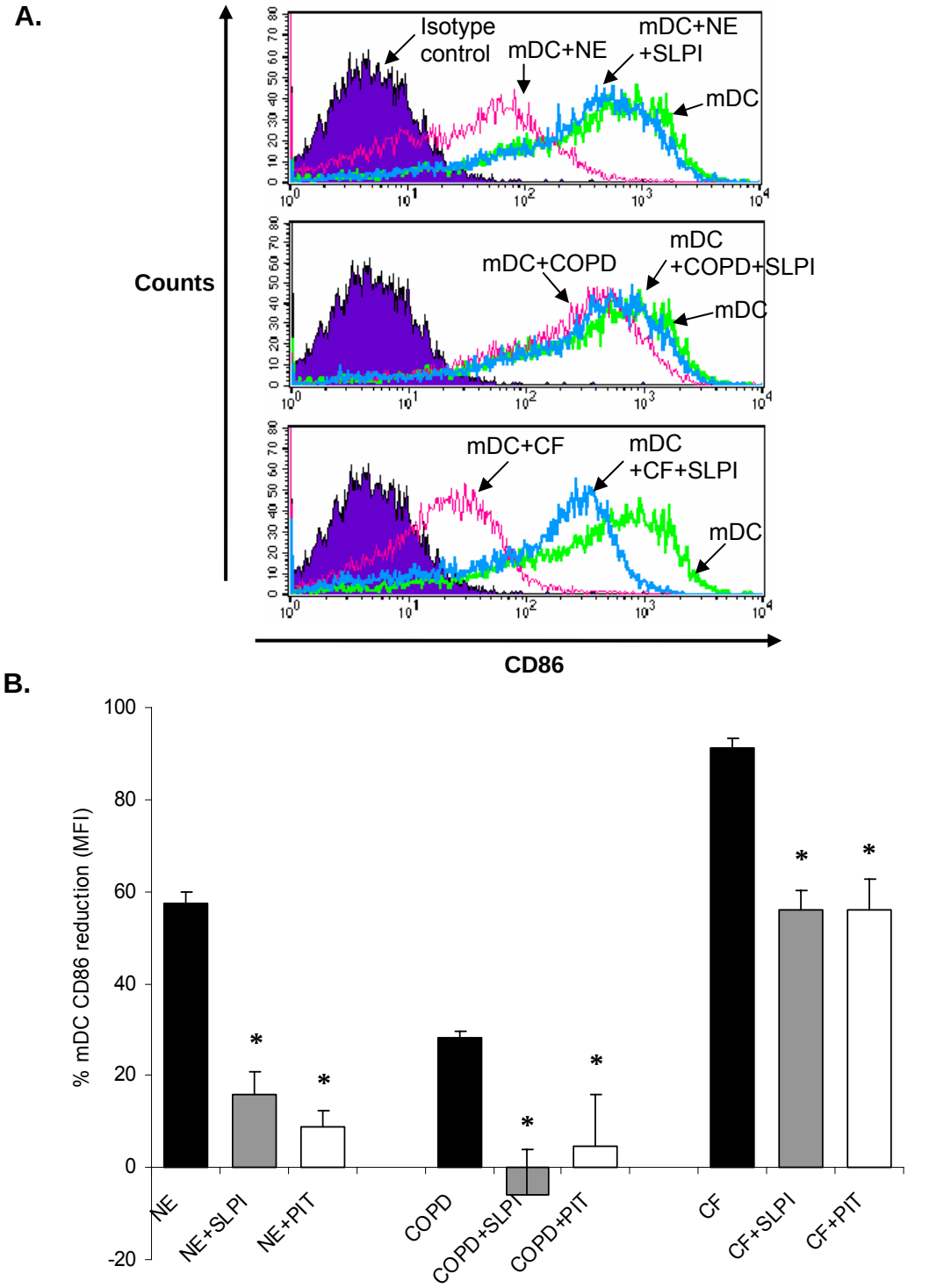


Figure 3.13. The reduction of mDC CD86 by purified NE is prevented by SLPI and PIT

Purified NE (2μM) and pooled COPD (n=18) and CF (n=9) sputa (containing 0.2μM and 3.39μM active NE respectively) were pre-incubated with either SLPI (8μM) or a cocktail of protease inhibitors tablet (PIT), before incubation with mDCs for 90 minutes at 37°C. **A)** Representative flow cytometry histograms shown. **B)** Pooled data from 3 experiments ± inhibitors presented. Values represent mean ± SD [* indicates significant difference (p<0.001) compared to uninhibited NE/sputa-treated mDCs].

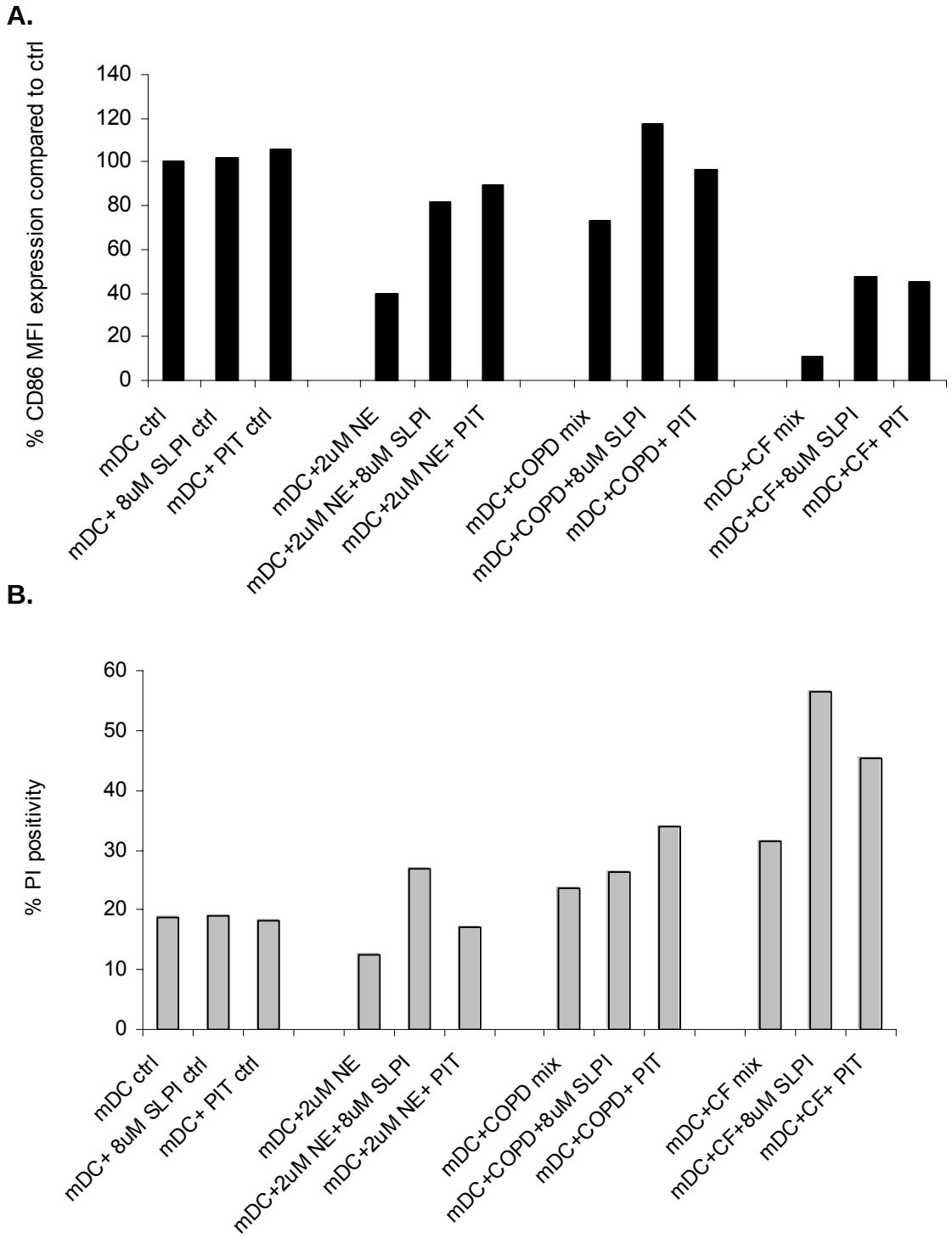


Figure 3.14. NE inhibition induces DC necrosis

Purified NE (2μM) and pooled COPD (*n*=18) and CF (*n*=9) sputa (containing 0.2μM and 3.39μM NE respectively) were pre-incubated with either SLPI (8μM) or a cocktail of protease inhibitors tablet (PIT), before incubation with mDCs for 90 minutes at 37°C. Subsequently the cells were analysed by flow cytometry. **A)** Representative treated-mDCs CD86 expression data from one of 3 experiments shown. **B)** A sample of treated mDCs in each group was also stained with PI and annexin V and analysed by flow cytometry. As annexin V was negative in all cases, only PI data is shown.

3.2.7. Western blot analysis confirmation of enzymatic cleavage of murine CD86 by NE

Following the observations that NE and sputa were able to affect the expression of CSMs and that protease inhibitors were able to alleviate this effect, it was deemed necessary to determine whether NE enzymatic activity was responsible for their shedding. For practical reasons (commercially available/reliable polyclonal Ab for Western blot analysis) CD86 was chosen for this part of the study. The Ab to CD86 had been raised in goat (R and D Systems, Oxon, UK) and hence a rabbit anti-goat immunoglobulin (Ig) G secondary Ab (DAKO, Cambridgeshire, UK) was used to detect it.

The CD86 cDNA is comprised of 1183 nucleotides, and exhibits a single large open reading frame of 927 nucleotides. The encoded polypeptide is 309 amino acids (aa) long and exhibits many features common to type I Ig superfamily membrane proteins. The mature protein consists of an approximately 222 aa extra-cellular region, and a hydrophobic trans-membrane domain of about 20 aa, and a cytoplasmic tail of approximately 44 aa (Freeman *et al.*, 1993). The CD86 is heavily glycosylated and its extra-cellular domain contains 9 potential N-linked glycosylation sites. The CD86 glycosylated protein has a MW of 60-100 kDa, which reduces to 34kDa after *N*-glucanase treatment (Hathcock *et al.*, 1993). Western Blot analysis for CD86 (Figure 3.15 A, lane 1 and 2) showed that mDC lysate solutions contained a protein detected by the goat anti-murine CD86 Ab, which had a molecular weight consistent with that of the CD86 protein (Freeman *et al.*, 1993; Freeman *et al.*, 1993). Indeed, the band around 38kDa may represent the unmodified protein while proteins migrating between 38 and 50kDa may represent glycosylation intermediates. In addition, mDCs incubated with NE released few proteins in the supernatant that cross-reacted with anti-CD86 Ab, the main one having a similar molecular weight than the one found in lysates (60kDa), suggesting that indeed this is a product of CD86 cleavage by NE. The slight difference between the lysate and the supernatant-derived molecule (lanes 1-2 and 4 respectively) likely reflects the presence of the intra-cellular portion (7kDa) in the lysate-derived molecule absent in

the species cleaved from the cell surface. As expected the supernatant analysed from mDCs control group did not contain any CD86 fragments (lane 3). To ensure that the secondary rabbit anti-goat Ab does not react with the CD86 protein, a second blot prepared in the same way was probed only with the secondary Ab, which when developed was blank (Figure 3.15 B).

NE treatment of mDC lysates induced a loss of CD86 immunoreactivity in a time- (Figure 3.16 A) and dose-dependent (Figure 3.16 B) manner. The latter is in agreement with the FACS data obtained in Figure 3.11 B (bottom graph). The finding that few fragments were released (Figure 3.16 A), compared to the almost total absence of CD86-reactive material in NE-treated mDC lysates (Figure 3.15 A) is probably best explained by the fact that released CD86 is in its 'native state' in mDCs supernatants whereas the RIPA buffer used for solubilisation of mDCs lysates has likely denatured the CD86, hence exposing the NE-sensitive cryptic sites.

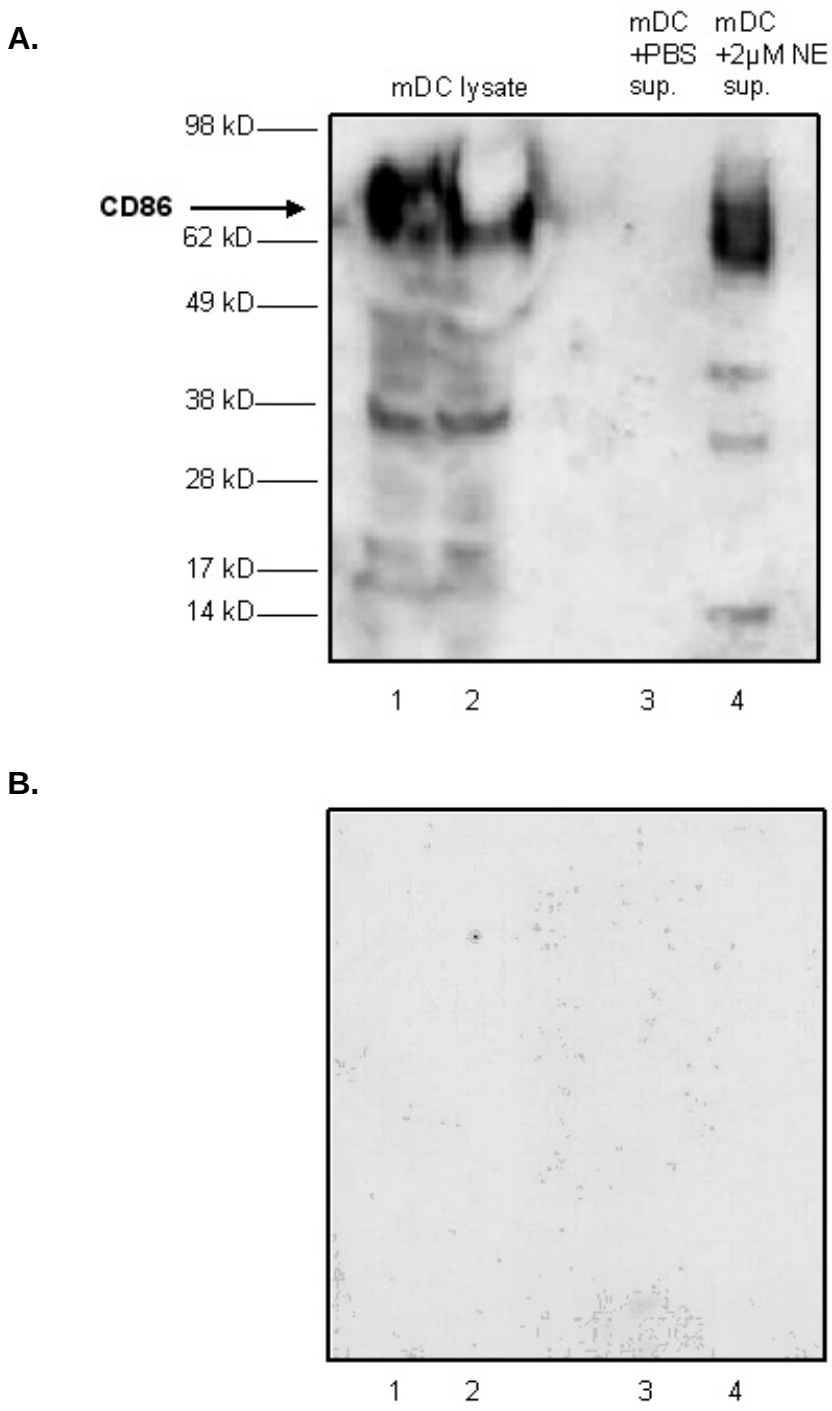


Figure 3.15. NE cleaves CD86 molecules on mDCs

mDC cell lysate were resuspended in PBS and exposed to 2μM NE or PBS for 90 minutes at 37°C. Supernatants were concentrated 10× and lysates were prepared and ran on a NuPAGE 4-12% Bis-Tris polyacrylamide gel, transferred onto a nitrocellulose membrane. **A)** Membrane was probed with anti-CD86 polyclonal Ab. Lanes 1 and 2 are samples of the same mDC lysate loaded neat (lane 1) or at 50% in PBS (lane 2), showing strong staining with the anti-CD86 polyclonal Ab. Lane 3 is 10× concentrated supernatant from untreated mDCs showing no evidence of CD86, and lane 4 represents supernatant from NE-treated mDCs showing staining with the anti-CD86 Ab. **B)** A second gel prepared as above was only probed with the secondary rabbit anti-goat Ab. Representative (n=2) Western blot data are shown.

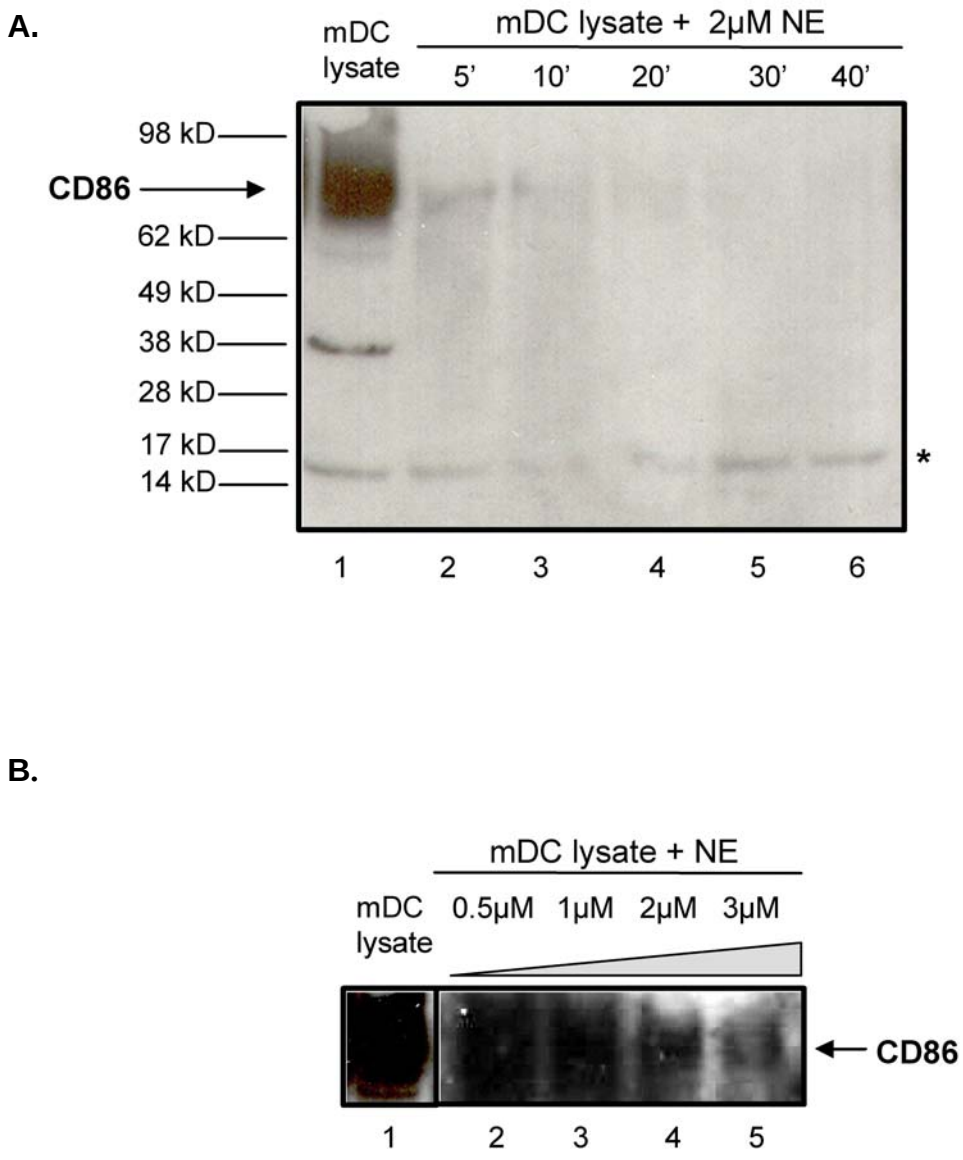


Figure 3.16. NE cleaves CD86 in mDC lysates

A) mDC cell lysate was incubated with 2μM NE at 37°C for 5-40 minutes. Samples were 10× concentrated and ran (10μl) on a NuPAGE 4-12% Bis-Tris polyacrylamide gel, transferred onto a nitrocellulose membrane and probed with anti-mCD86 polyclonal Ab. Lane 1 represents untreated mDC lysates showing CD86 staining, lanes 2-6 indicate that majority of the protein is degraded within 5 minutes of NE-treatment. * denotes non-specific labeling. **B)** mDC cell lysate (20μl) was incubated with 0.5-3μM NE as above for 10 minutes at 37°C. Lane 1 represents untreated mDC lysates showing CD86 staining, Lanes 2-5 show a dose dependent degradation of CD86 by NE. Representative (*n*=3) Western blot data are shown.

3.2.7.1. SLPI prevents enzymatic cleavage of CD86 by NE in cell lysate

Purified NE (2μM) was pre-incubated with SLPI (8μM) for ~30 minutes prior to its addition to the mDC lysate containing the CD86 protein. In agreement with data presented in Figure 3.13, the loss of CD86 was largely prevented (Figure 3.17 A, lane 2 versus lane 3). However, in lane 3, where SLPI was present in the sample, several extra bands were detected which were not previously seen (Figures 3.15 and 3.16) and were not present in the absence of SLPI in lanes 1 and 2. In addition, the strongest band was around 12kDa suggesting that it could be due to cross-reactivity of the anti-CD86 Ab with murine SLPI protein in the sample. To test this, the same membrane was stripped by submerging it in stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.2M Tris-HCl, pH 6.7) and incubated at 50°C for 30 minutes with occasional agitation. The membrane was then washed, stripped of the Abs and reprobed with primary rabbit Ab to SLPI and secondary goat anti-rabbit HRP-conjugated Ab. As predicted, the extra bands visualized in Figure 3.17 A were of SLPI origin and were readily picked up by specific Ab to SLPI (Figure 3.17 B, lane 3).

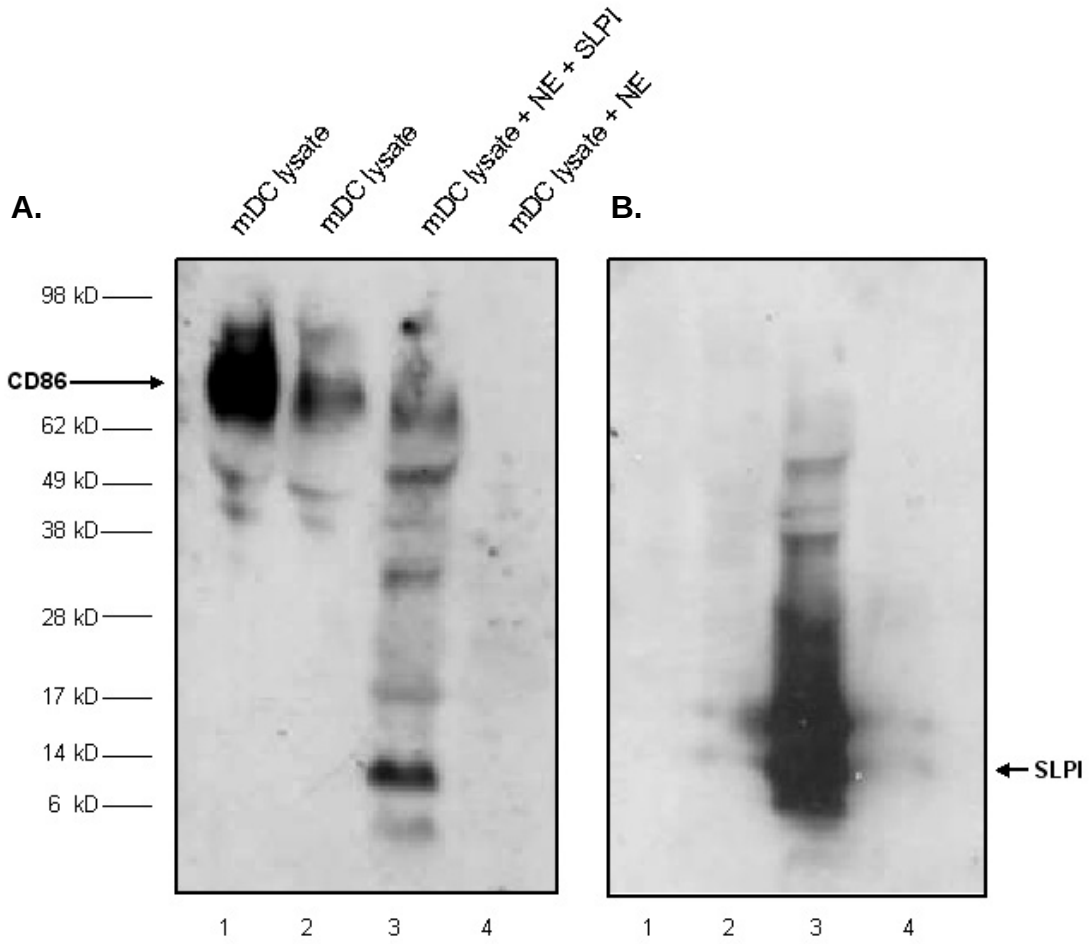


Figure 3.17. SLPI prevents NE-cleavage of CD86

mDC cell lysates were resuspended in PBS and exposed to 2μM NE preincubated ± SLPI (8μM) for 90 minutes at 37°C. Samples were then run on a NuPAGE 4-12% Bis-Tris polyacrylamide gel, transferred onto a nitrocellulose membrane. **A)** Membrane was probed with anti-CD86 polyclonal Ab. Lanes 1 and 2 are samples of the same mDC lysate loaded neat (lane 1) or at 50% in PBS (lane 2), showing strong staining with the anti-CD86 polyclonal Ab. Lane 3 is lysate treated with SLPI-inhibited NE, and lane 4 represents lysate treated with NE alone. **B)** The same membrane was stripped and probed with anti-murine SLPI Ab. Representative (*n*=2) Western blot data are shown.

3.2.8. Kinetics of CD40, CD80 and CD86 re-expression after NE treatment of mDCs

Migration of mDCs towards T cell area of regional LNs is a crucial component of DC's physiology, and hence, of the initiation of an immune response. Kinetic experiments using FITC-conjugated molecules to label airway DCs have shown that FITC-carrying airway-derived DCs appear in the thoratic LNs as soon as 6 hours after instillation of FITC-OVA (Vermaelen *et al*., 2001). To determine whether NE treatments induced a durable change in the mDC phenotype, mDCs were allowed to recover after 90 minutes of stimulus treatment (2μM NE) by supplementing the culture medium with ~15% FCS. FCS is known to contain a rich source of protease inhibitors (Johansson *et al*., 2001) which are able to inactivate NE. Cells from duplicate/triplicate wells were washed and stained for FACS at 2 hour intervals. Figure 3.18 shows the levels of CSMs re-expression over an 8 hour time period. The expression of CD80 and CD86 on mDCs increased gradually over the 8 hour period, although their levels did not reach pre-NE levels and were still significantly lower 8 hours post NE inhibition by FCS. Interestingly, CD40 levels did not recover at all during this period.

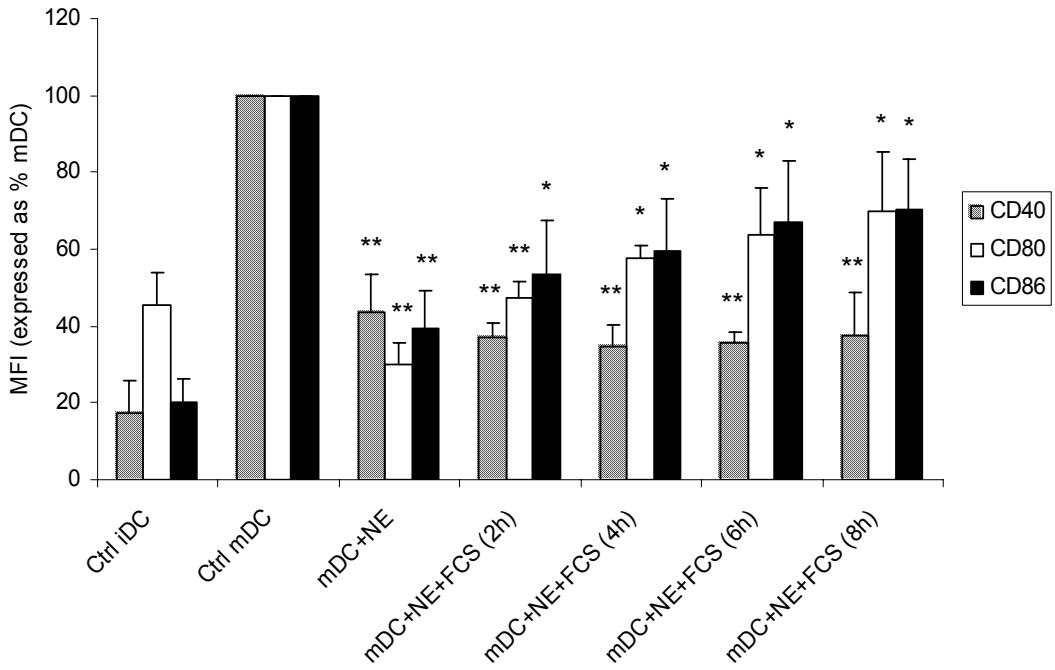


Figure 3.18. Re-expression of CD40, CD80 and CD86 after NE treatment of mDCs

mDCs were plated in serum-free medium and treated with NE (2μM) for 90 minutes at 37°C. FCS (15%) was then added to the wells (t 0) to inhibit NE and re-expression of CD40, CD80 and CD86 analysed every 2 hours up to 8 hours post inhibition. Expression on iDCs is given for comparison. Values represent mean ± SD of three independent experiments, each performed in triplicate (* and ** indicate significant difference, $p<0.01$ and $p<0.001$ respectively, compared to corresponding control mDCs).

3.2.9. NE interferes with normal LPS-induced maturation of iDCs

Since iDCs are exposed to bacterial or host-derived maturation signals in the context of high NE concentration in inflammatory conditions such as COPD and CF, it was decided to expose iDC to LPS (2μg/ml) and NE (2μM) simultaneously for 4 hours in serum-free conditions. In this way the inflammatory environment of the lung could be mimicked *in vitro*. Figure 3.19 A shows that the iDC + NE treatment resulted in lower expression of CSMs, indicating that (as mentioned above) not only mDCs, but iDCs are also susceptible to the enzymatic activity of NE, and in addition NE interferes with normal DC maturation upon LPS treatment. Even more significantly, it was shown that compared to iDCs + LPS alone, iDCs incubated with both LPS and NE had reduced levels of CSMs.

Interestingly, in these conditions, LPS- and LPS/NE-stimulated iDCs were still able to up-regulate MHCII on their surface compared to PBS-treated iDCs ($p=0.003$ and $p=0.01$ respectively). iDCs exposed to NE alone had also slightly increased levels of MHCII (although not statistically significant).

Concomitantly, the levels of the DC activation cytokines IL-12p40 and TNF-α were drastically reduced in iDCs simultaneously submitted to LPS and NE, compared with iDCs treated with LPS alone (Figure 3.19 B). Previously cytokines such as IL-2 (Ariel *et al.*, 1998) and IL-6 (Bank *et al.*, 1999), as well as TNF-α (van Kessel *et al.*, 1991) have been shown to be cleaved as a result of NE enzymatic activity, although there is no evidence in the literature showing that NE could directly affect IL-12. To test this hypothesis, recombinant murine IL-12p40 (1ng/ml) was co-incubated with NE (2μM) for 4 hours at 37°C. Then SLPI was immediately added to stop the NE activity and the concentration of IL-12p40 was measured by a specific ELISA kit (R and D Systems, Oxon, UK). As shown in Figure 3.20, co-incubation of IL-12p40 protein with active NE resulted in total loss of the protein in the mixture, and it could no longer be detected by the ELISA assay.

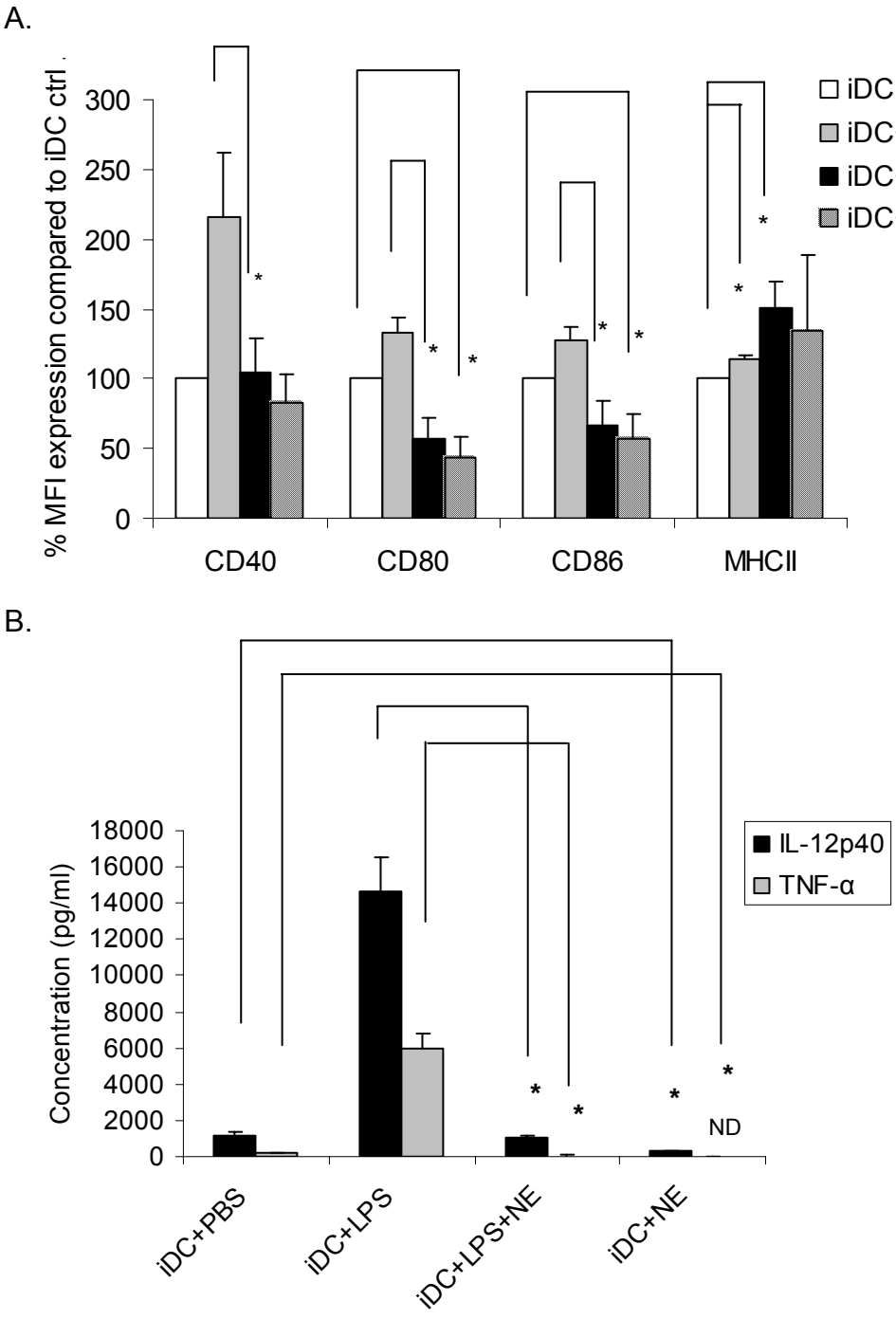


Figure 3.19. iDCs fail to mature normally when simultaneously stimulated with LPS and NE

iDCs were plated in serum-free medium and treated with either PBS (ctrl), LPS (2μg/ml) alone, NE (2μM) alone or LPS and NE together, for 4 hours at 37°C. Following treatment, supernatants were collected and cells were washed, stained and fixed for FACS analysis. **A)** Flow cytometry data was analysed and expressed as % change in MFI relative to iDC + PBS Controls (mean ± SD, *n*=3). **B)** Supernatants were analysed for pro-inflammatory cytokines IL-12p40 and TNF-α by ELISA. Values represent mean ± SD of *n*=3 independent experiments performed in triplicate wells (* indicates statistical significant difference, $p<0.01$ compared to corresponding controls; ND=non-detected).

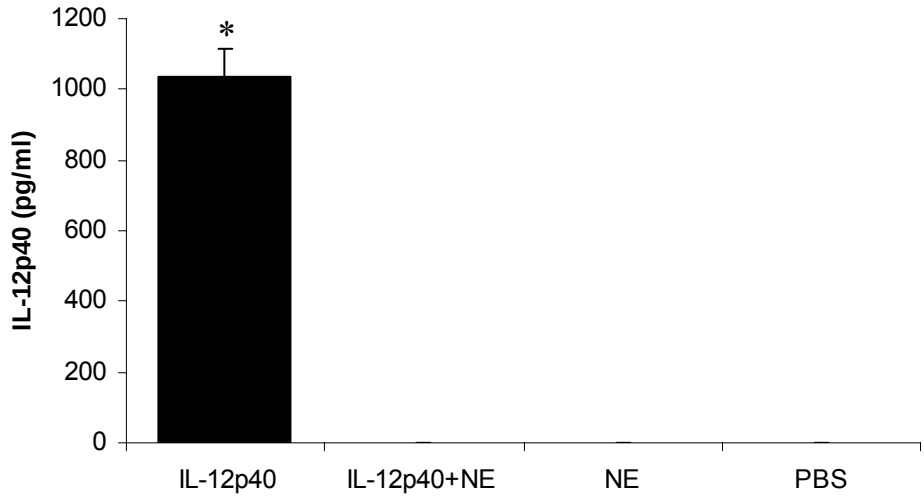


Figure 3.20. Proteolytic processing of IL-12p40 by human NE

Murine recombinant IL-12p40 (1000pg/ml) protein was incubated ± 2μM NE for 5 hours at 37°C. The samples were then treated with SLPI to inhibit free NE and were analysed by murine IL-12p40 ELISA (n=3 independent experiments, * indicates statistical significance compared to control samples (p<0.0001).

3.2.10. NE impairs the function of DCs: reduction in lymphocyte proliferation following OVA pulsing of DCs

To assess whether NE impaired the function of DCs, a DC-T cell system was adapted to address this issue as will be described below. Firstly, to optimise this system the mDCs were pulsed with various concentrations of chicken egg $OVA_{323–339}$ peptide (Peptides International, Kentucky, USA) for 90 minutes at 37^{o}C and co-cultured with D011.10-derived transgenic spleen lymphocytes. Activation of the transgenic DO11.10 $CD4^{+}$ T cells was assessed after 48 hours by cytokine analysis of 100µl of the supernatants and determining proliferation by adding Alamarblue™ (Serotec, Oxford, UK) to the cells. These preliminary experiments indicated that the optimum concentration of OVA was 0.1µM, since it allowed measurement of higher and lower proliferative responses in culture.

Consequently OVA-pulsed control mDCs or NE-treated mDCs (10^4) were co-incubated with DO11.10 lymphocytes (10^5). Figure 3.21 shows that pulsed DCs induced the proliferation of splenocytes to the same extent as Concanavalin A (ConA), a non-specific mitogen. By contrast, mDCs pre-treated with NE were less effective at inducing splenocyte proliferation ($p=0.042$), In parallel, when the cytokine profile of supernatants was assessed 48 hours post DC:T cell co-culture, TNF-α, IFN-γ and IL-2 levels were drastically reduced in supernatants arising from NE-treated mDCs. Whether NE affected type 2 cytokines such as IL-4 and IL-5 is difficult to ascertain using this protocol as their levels were very low.

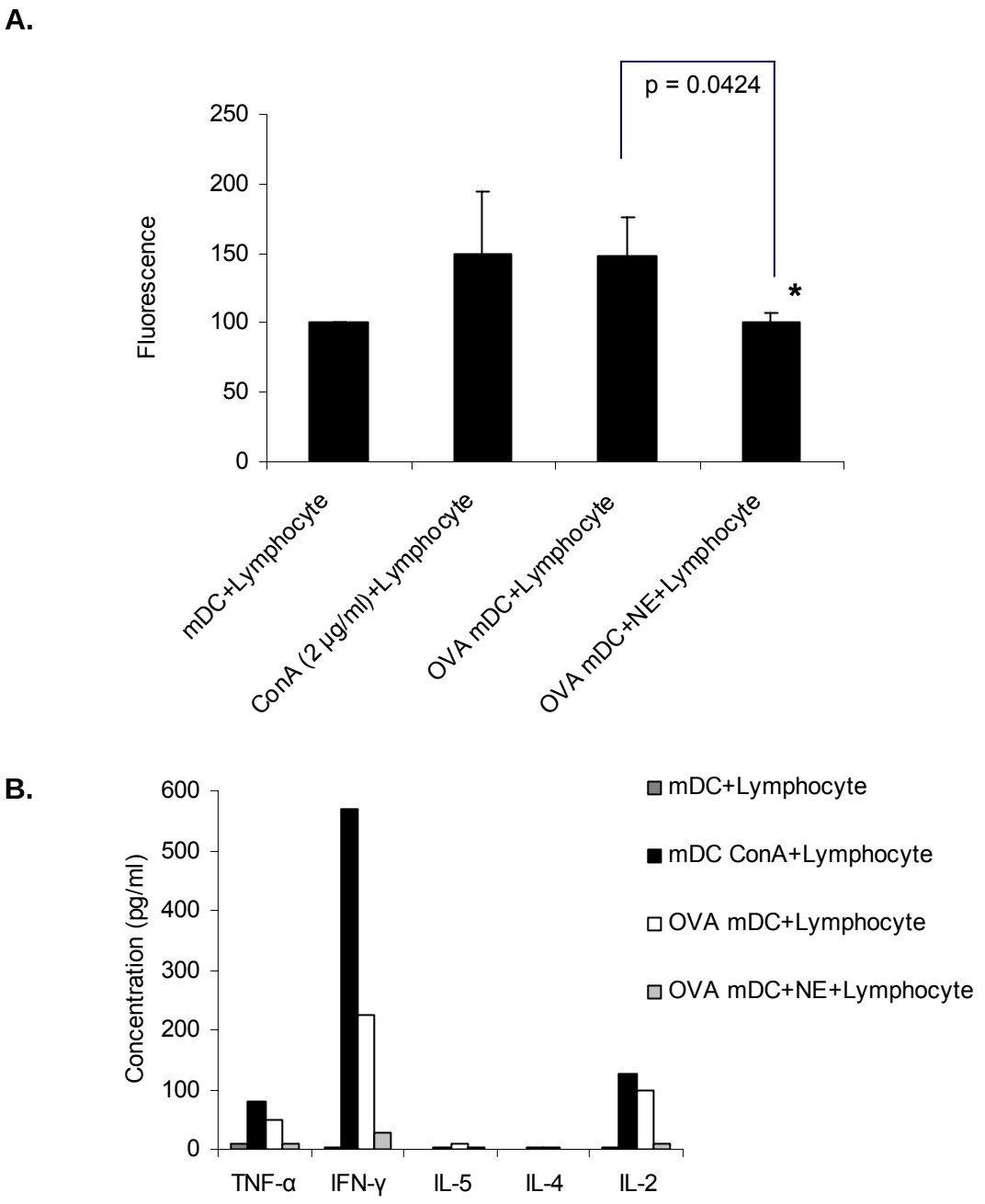


Figure 3.21. NE-treated mDCs have impaired antigen presenting ability

mDCs (from BALB/c wild-type mice) were treated with PBS or NE (3µM), washed, pulsed with OVA peptide (0.1µM) for 90 minutes at 37°C, and washed 3× with PBS. Spleen lymphocytes were purified from 2-4 D011.10 transgenic mice and incubated with NE-treated, OVA-pulsed mDCs at a ratio of (1:10, mDC [10^4/well] : lymphoctye [10^5/well]) in a 96-well plate and incubated at 37°C. ConA was used at a concentration of 2µg/ml as a positive control. Half of the supernatant (100 µl) was removed and saved for cytokine analysis after 24 hours and Alamarblue™ solution was added at a 20% final concentration to each well. **A)** Shows lymphocyte proliferation measured after 48 hours. Values represent mean ± SD of n=3 independent experiments. * indicates statistical significance (p< 0.05). **B)** Cytokine output following the co-culture was analyzed using a Th1/Th2 CBA kit (BD Biosciences, San Jose, USA). A representative experiment of two is shown.

3.3. DISCUSSION

The principal novel observations arising from the data in this chapter are that: (*i*) CSC kills DCs by inducing necrosis, (*ii*) NE-containing lung inflammatory secretions from patients with COPD or CF are able to disable DC function by reducing their cell surface CSMs, and (*iii*) purified NE also down-regulates DC CSMs, inhibiting their antigen presenting capability.

3.3.1. The effects of cigarette smoke condensate on DCs

A number of studies have implicated cigarette smoking as harmful to the innate and adaptive immune responses (see Chapter 1, section 1.5). Several lines of evidence suggest that cigarette smoking alters the respiratory tract's ability to defend itself from infection. Cigarette smoking is established as the main aetiological factor for COPD. Even individuals exposed passively to CS have higher rates of respiratory illnesses (Marcy and Merrill, 1987). However, the precise pathogenic mechanisms of the disease are still relatively poorly understood. APCs are an important component in the initiation and maintenance of adaptive immune responses. Interestingly, there is a paucity of data regarding the impact of CS on antigen presentation and the current data in the literature are very contradictory. In a recent study D'hulst and co-workers (2005) observed an infiltration of activated DCs in airways and lung parenchyma of CS-exposed mice. Indeed this observation was in line with two earlier studies that concluded cigarette smoking increases the number of human pulmonary DCs/LCs (Soler *et al*., 1989; Casolaro *et al*., 1988). On the contrary, two elegant studies by Robbins *et al*. (2004 and 2006) established that CS decreased the number and activation status of DCs in murine lung tissue. They also demonstrated that chronic CS exposure impairs the immune response against adenovirus and *Pseudomonas aeroginosa* (*P.a.*) (Robbins *et al*., 2004; Drannik *et al*., 2004). Likewise, CSC has been shown to suppress human DC function leading to preferential induction of Th2 priming *in vitro* (Vassallo *et al*., 2004). Nicotine has also been shown to affect the differentiation and functional maturation of human DCs *in vitro* (Nouri-Shirazi and Guinet, 2003; Guinet *et al*., 2004). Moreover, Chang

and co-workers (1990) have reported no change in the ability of APCs from lung-associated lymph nodes (LN) of CS-exposed mice to stimulate T cell proliferation. Overall, whether or not CS impacts APCs remains an unresolved issue.

Exposing animals to direct CS is illegal in UK universities according to Home Office guidelines. Therefore to further elucidate the effects of CS on DCs, attempts were made to administer fresh CSC into mouse lungs but it was very laborious and could not be done routinely due to the repeated anesthetisations and i.t. procedures involved in these experiments. Moreover, preliminary experiments showed mice receiving a single dose of 100% or 50% CSC did not have significant differences in their BAL fluid cell types/numbers and cytokines compared to control group receiving PBS alone, 24 hours post treatment (not shown). Therefore, the decision was taken to test the effects of CSC on murine DCs *in vitro*. Early experiments indicated that, as hypothesised, CSC had an adverse effect on the phenotype of DCs and was able to down-regulate their CSMs and MHCII in a dose-dependent fashion (Figure 3.6). However, upon PI/annexin V staining of the CSC-treated DCs the reduction of the cell surface markers examined was found to be due to the induction of necrosis (and partly apoptosis), and did not seem to be as a result of CSC-induced intra-cellular signalling (Figure 3.7). The *in vitro* effects of CSC observed here were irrespective of the maturation state of DCs and the presence or absence of serum (which contains antioxidants) in the culture media.

Oxidative stress is responsible for many of the effects of cigarette smoking seen in biological systems. Oxidants such as hydrogen peroxide have been shown to inhibit apoptosis (*e.g.*, via inhibition of caspases) and induce necrosis (Hampton and Orrenius, 1997; Samali *et al*., 1999; Lee and Shacter, 1999). Our preliminary data presented here is in agreement with these findings, and with the previous studies where CSC was shown to prevent apoptosis (through inhibition of caspase activation) and induced necrosis (Wickenden *et al*., 2003; Piperi *et al*., 2003). These observations may provide a possible mechanism as to why Robbins *et al*. (2004) observed a reduction in lung DCs of CS-exposed mice. Furthermore, antioxidants such as GSH and DTT have been shown to protect the cells against CSC-induced

necrosis, by preventing the inhibitory effect of CSC on caspase activation in an alveolar epithelial type II cell line (A549), primary human umbilical vein endothelial cells (HUVEC) and Jurkat T cells (Wickenden *et al*., 2003). Although these data might be also relevant to the current study, nonetheless as it stands now, better controlled *in vitro* and *in vivo* systems examining the effects of CS ± antioxidants on DCs are required to fully address this issue.

3.3.1. The effects of NE and sputum samples on DCs

COPD and CF affect millions of people worldwide. These conditions are characterised by an activation of the innate immune system, involving mostly neutrophils in CF and both neutrophils and macrophages in COPD. Because of an excessive and maladaptive activation of neutrophils, very high levels of NE are released into the lung fluid of patients with COPD and CF (Birrer *et al*., 1994; Rees *et al*., 1997 and 1999; Suter, 1989), and in combination with the pathogens lead to severe exacerbations in affected individuals. As DCs bridge the innate and acquired immune systems and because of the abundance of proteases, *e.g.*, NE, in COPD and CF patients' sputa, it was hypothesised that inflammatory lung secretions from these patients might interfere with the initiation of acquired immunity by inactivating or biasing DC function.

The data presented in the latter part of this chapter, demonstrate the novel finding that NE concentration correlated with the ability of sputa to reduce DC CD86 CSM expression and that CF sputa were more potent than COPD sputum samples (Figure 3.9). This difference could be explained by the fact that CF sputum samples contained far higher concentrations of active NE than the COPD sputum samples (Figure 3.8), which is well documented in the literature (Fujita *et al*., 1990; Schuster *et al*., 1995; Liu *et al*., 1999; Sagel *et al*., 2001; Tumkaya *et al*., 2006). The reason COPD and CF lung secretions contain high concentrations of NE is that although natural NE inhibitors are normally found in lung fluids and their levels even increase in the COPD and CF lungs, protection is inadequate against the overwhelming burden of NE and other proteases present as a result of neutrophil-dominated

inflammation (Khan *et al*., 1995; Birrer *et al*., 1994; O'Connor CM *et al*., 1993). On the other hand, as mentioned in Chapter 1 (1.5.1.1.), in chronic inflammatory tissues most of the secreted elafin as well as other natural protease inhibitors are inactivated by oxidation or proteolytic cleavage, and can not efficiently inhibit the free proteases in such pathologies (Nobar *et al*., 2005; Greene *et al*., 2003; Vogelmeier *et al*., 1997; Carp *et al.*, 1982; Taggart *et al.*, 2001).

Amongst the three CSMs examined following the brief treatment of DCs with the sputum samples (Figure 3.10), despite significant down-regulation of both CD80 and CD86, CD40 levels were up-regulated (as assessed by total MFI expression on cell surface). This observation was quite intriguing since in parallel experiments, where purified human NE was used (discussed below) there was a general down-regulation in all of the three CSMs examined, including CD40, although with different kinetics. Several arguments could explain this phenomenon. The most relevant argument is that, human NE used here has been chromotographically purified and is dissolved in a buffer (50mM Tris, 0.5M NaCl, 0.1% Triton X-100, pH 8), whereas the sputum samples came from COPD and CF lungs and hence contain various human and pathogen-derived molecules such as cytokines, LPS and bacterial unmethylated CpG DNA. Therefore it is likely that other factors present in these sputum samples could compete with and over-ride the NE-mediated CD40-down-regulation process, possibly by activating the specific signalling pathways responsible for CD40 (but not CD80 and CD86) transcription and/or relocation to the cell surface. At least three families of mitogen-activated protein kinases (MAPK), that is, extra-cellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38 MAPK, as well as the NF-κB family of transcription factors (composed of 5 members; p65 [Rel-A], Rel-B, c-Rel, p50 and p52), are involved in the DC maturation process. To support this argument, the general consensus in the literature is that CD40 signalling takes place predominantly via the IFN-γ-activated signal transducer and activator of transcription 1α (STAT-1α) and NF-κB signalling pathways (Qin *et al*., 2005; Benveniste *et al*., 2004; Nguyen and Benveniste, 2000), but not the MAPKs (Nakahara *et al*., 2004; Sun and Fernandes, 2003; Ardeshna *et al*., 2000). On the other hand, the transcription of CD80 and CD86 has been shown to be

controlled via the JNK and p38 MAPK signalling pathways (Nakahara *et al*., 2004; Ardeshna *et al*., 2000). Therefore, as mentioned above, it could be argued that certain factors present in the sputum samples at high concentrations, such as oxidants (*e.g.*, reactive oxygen species), might be responsible for activating the STAT-1α and NF-κB signalling pathways leading to rapid replacement of 'cleaved' CD40 on treated mDCs. Indeed, the number of $CD4^+$, $CD8^+$ cells and macrophages expressing NF-κB, STAT-4 and IFN-γ proteins is increased in mild/moderate COPD disease (Di Stefano *et al*., 2004). Oxidative stress activates NF-κB-mediated transcription of pro-inflammatory mediators either through activation of its activating IκB-α kinase or the enhanced recruitment and activation of transcriptional co-activators. Enhanced NF-κB-co-activator complex formation results in targeted increases in histone modifications, such as acetylation leading to inflammatory gene (*e.g.,* CD40) expression (Rahman, 2005).

The sputum data prompted the need to determine whether NE in isolation could also influence mDC CSM expression levels. It was found that NE-treatment of mDCs did not affect MHCII expression but significantly reduced CD40, CD80 and CD86 expression (Figure 3.11). Comparison of Figures 3.9 and 3.11 B (bottom graph) indicate that for the same concentration of NE, sputa were slightly more efficient at down-regulating CD86 expression than purified NE, suggesting that other factors may also be operative. Most importantly, the reduction in expression of CSMs on mDCs was not due to cell apoptosis and/or necrosis following treatment of DCs with pathophysiological concentrations of NE (1-3 μM), as measured by annexin V and PI staining (Figure 3.12).

Furthermore, the reduction in CD86 caused by the COPD sputum samples and NE was abrogated by the use of both the NE inhibitor SLPI, and that of a broad spectrum protease inhibitor cocktail (PIT) (Figure 3.13). In contrast, SLPI and PIT were not fully effective at reversing the effect of CF sputum samples on CSM expression (Figure 3.13, ~36% inhibition in both cases). This may reflect the fact that other proteases (not inhibited by SLPI and the protease inhibitors present in the PIT cocktail) could also be involved in affecting the CSM levels in sputum

secretions or that the protease inhibitors may be less active in the complex mix of inflammatory sputa, or due to possible cell death (see below). Incidentally, Rees *et al*. (1999) found that 90% inhibition of pure NE required half the concentration of a natural NE inhibitor (human monocyte/neutrophil elastase inhibitor) required to inhibit 90% equivalent NE activity in CF samples. Equally these protease inhibitors might partially lose their activities when co-incubated with the CF sputum samples prior to mDC treatment. Indeed, as explained above, although present in high amounts in lung inflammatory secretions, it has been shown that endogenous SLPI, elafin and α1-PI are inactivated in these secretions by proteolytic cleavage and oxidation (Nobar *et al*., 2005; Greene *et al*., 2003; Vogelmeier *et al*., 1997; Carp *et al*., 1982; Taggart *et al*., 2001; Suter, 1989). Possible candidates, explaining the partial inhibition of proteases in CF sputum samples by PIT (Figure 3.13), could include the degradative enzymes secreted by bacteria frequently colonising the CF airways, such as *P.a* (Doring *et al*., 1983 and 1984) and *Staphylococcus aureus* (*S.a.*) (Prokesova *et al*., 1988 and 1991). *P.a.* secretes several proteases (*e.g.*, elastase and alkaline protease) considered as important virulence factors, which are able to cleave cytokines (such as IL-6 and IL-8; Matheson *et al*., 2006), chemotactic proteins (such as RANTES and MCP-1; Leidal *et al*., 2003) and other secreted (*e.g.*, surfactants) and cell surface proteins (*e.g.*, proteinase-activated receptor 2) that have important roles in the innate immune responses to *P.a.* (Mariencheck *et al*., 2003; Dulon *et al*., 2005). Additionally, metalloproteases and serine proteases secreted by *S.a.* have been shown to modulate lymphocyte responses (Prokesova *et al*., 1988 and 1991), and similar to *P.a.* elastase and metalloproteases, are able to degrade proteins involved in host defense such as human Ig (Prokesova *et al*., 1995) and human LL-37 (Sieprawska-Lupa *et al*., 2004).

To our surprise, upon the treatment of mDCs with SLPI/PIT-inactivated-NE/sputum samples (Figure 3.14 B) there was increased staining with PI (but not annexin V) compared to mDCs treated with active NE/sputa. The PI positivity was the highest when mDCs were treated with SLPI/PIT-inactivated CF sputum samples compared to inactivated COPD sputa/NE (Figure 3.14 B). This is a very intriguing phenomenon which seems to come into effect only when NE is complexed with its

inhibitors. The NE-NE inhibitor complexes appear to be cytotoxic to DCs and lead to the destruction of these cells as observed here (Figure 3.14 B). This finding requires more in-depth examination in future studies.

In an attempt to investigate the mechanism by which NE was able to down-regulate the CSMs, a polyclonal Ab to murine CD86 was employed to detect any possible cleaved fragments of this CSM in supernatants of NE-treated mDCs and in NE-treated mDC lysates. As hypothesised, NE was able to release the extra-cellular portion of CD86 in mDC supernatants (Figure 3.15 A). NE was also able to degrade CD86 protein in mDC lysates and this was in a time- and dose-dependent manner (Figure 3.16 A and 3.16 B). To further prove that the proteolytic activity of NE was responsible for this down-regulation, NE was treated with SLPI prior to addition of mDC lysate. The degradation of CD86 in lysate was prevented by SLPI-inactivated NE (Figure 3.17 A), which was in agreement with the earlier FACS data (Figure 3.13). These observations are further strengthened by recent data by Lofdahl and colleagues (2006) showing that alveolar macrophages isolated from BAL fluid of COPD patients had a lower expression of CD86 molecule compared with 'healthy' smokers. Similarly, Droemann *et al*. (2005) have demonstrated that TLR2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients, which might also be as a result of increased proteolytic activity of NE in these subjects.

The decision was then taken to examine whether the effect of NE on CSMs expression was short-lived or whether it may impair the function of mDCs more durably. Figure 3.18 shows that up to 8 hours post NE treatment, a time-frame relevant to DC activation and migration to local LNs (Vermaelen and Pauwels, 2003), CSM re-expression was only partial; in particular, CD40, an essential molecule for providing an activation/anti-apoptotic signal to the cells (Bjorck *et al*., 1997; Koppi *et al*., 1997; Ludewig *et al*., 1995), did not show any increased level of expression over the 8 hour time period. This suggests that chronic NE over-expression in the lung, as in COPD or CF, may have a durable effect on DC antigen presenting ability.

In addition to reducing mDC CSM expression, NE also prevented the LPS-induced maturation of iDCs (Figure 3.19 A). In these experiments, NE could potentially be acting by either reducing the levels of LPS-induced CSMs or by interfering with the LPS signalling pathway, *e.g.*, by cleaving CD14 receptor molecules (Henriksen *et al*., 2004; Le-Barillec *et al*., 1999). DCs simultaneously treated with LPS and NE up-regulated MHCII levels equally which indicates that LPS was able to signal intra-cellularly and that NE is unlikely, in this model, to interfere with the LPS transduction machinery. The logical explanation is that NE acts by cleaving susceptible CSMs when they arrive at the cell surface, out-competing the LPS-induced transcription of them. The long term (4 hours) effect of NE on MHCII expression on DCs is interesting: NE alone slightly up-regulated MHCII levels on iDCs, but this did not reach significance (Figure 3.19 A), demonstrating that, as shown by our group and others previously in other cell types (Sallenave *et al*., 1994; Devaney *et al*., 2003; Nakamura *et al*., 1992)**,** NE can signal through receptors at cellular surfaces. Recent data suggests that NE signals via the cell surface membrane-bound TLR4 (Devaney *et al*., 2003), by activating the NF-κB signalling pathway (Lee *et al*., 2006; Walsh *et al*., 2001). Indeed, the up-regulation of MHCII on DCs and macrophages is dependent on NF-κB transcription factor (Sun and Fernandes, 2005; Rescigno *et al*., 1998; Qin *et al*., 2005), and consequently, could explain the observation that MHCII is up-regulated upon NE treatment of iDCs. However, whether this differential effect of NE on iDCs (up-regulating MHCII while down-regulating CSMs levels) is physiologically important is unclear.

Relatedly, iDCs simultaneously treated with LPS and NE concomitantly released significantly lower levels of IL-12p40 and TNF-α in culture (Figure 3.19 B). It could be argued that the reduction in these cytokines is also due to the proteolytic activity of NE; since NE released from activated neutrophils has been shown to cleave recombinant TNF-α (van Kessel *et al*., 1991). However, to date there is no data suggesting that IL-12 is also affected by NE. Therefore to test this, recombinant murine IL-12p40 subunit was co-incubated with NE and analysed by ELISA. As shown in Figure 3.20, IL-12p40 was fully degraded in the presence of NE and could not be detected anymore. Both IL-12 and TNF-α are essential for efficient DC

function and induction of Th1 responses (Romagnani, 1995). These observations further confirm the immunomodulatory effects of NE on DC function and maturation.

Ultimately, it was important to demonstrate whether the NE-differential effect on CSM levels on DCs had functional consequences on DC-lymphocyte interactions. Figure 3.21 shows that indeed, NE treatment of mDCs followed by OVA-pulsing significantly reduced lymphocyte proliferation and TNF-α, IFN-γ and IL-2 cytokine output. The output of type 2 cytokines (IL-4 and IL-5) by stimulated lymphocytes was very low, making it impossible to interpret the effects of NE on Th2 responses, if any. An interesting follow up study to these experiments would be to pulse DCs with an antigen (*e.g.*, Ad) and expose them to either PBS or NE *in vitro*, before adoptively transferring them into murine lungs. Consequently, the immune responses of mice towards i.t. Ad infection could be analysed as previously described by us (Roghanian *et al*., 2006b). Alternatively, NE/sputum samples (containing high NE concentrations) could be instilled into mouse lungs, and the lung DCs could be analysed (for expression of CSMs and their antigen-presenting capability to naïve lymphocytes) as described above. These experiments would shed further light into the effects of these NE-rich samples on DCs phenotype/function and the subsequent immune responses *in vivo*. The observations that NE-treated DCs were less efficient at stimulating lymphocytes are supported by earlier limited data published by Abe *et al*. (2002) and Knight *et al*. (1997). Abe and co-workers showed that patients with COPD who had exacerbations due to *NTHI* had a decreased lymphocyte proliferative response to P6 (a 16-kD lipoprotein that makes up ~1-5% of the total outer membrane proteins of the *NITH*) compared with patients with COPD with no exacerbations due to *NITH*, and compared with healthy control subjects. On the other hand, Knight and colleagues (1997) isolated macrophages separated from lavage samples obtained from lungs removed at transplantation from patients with CF and other lung disease, and compared them with circulating monocytes from the same patients for their ability to stimulate allogeneic normal circulating lymphocytes. They elegantly demonstrated that, compared to control non-CF patients, macrophages separated from CF patients were unable to stimulate allogeneic lymphocytes and to present antigen, although monocytes from the same patients were functional in both

assays. Although the role of NE was not studied by these groups, NE-cleavage of CSMs might be responsible for the defect in antigen presentation by lung APCs in COPD and CF patients, as demonstrated in the current project (Roghanian *et al*., 2006a).

The experiments performed in this part utilised murine DCs in conjunction with human NE/sputum samples. However, it should be noted that the murine and human CD40, CD80 and CD86 proteins share considerable homologies at protein levels (~54%, 46% and 50% identity, respectively; see Appendices III-V). Most importantly a recent study by Wiesner *et al*. (2005) concluded that substrate specificity and efficiencies of substrate hydrolysis of murine NE are very similar to human NE, thus supporting the validity of conclusions drawn from the data presented in this chapter, and similar studies using human proteases and protease inhibitors in animal models (Churg *et al*., 2003; Delacourt *et al*., 2002; Dhami R *et al*., 2000; Rees *et al*., 1999; Belaaouaj *et al*., 1998; Rees and Brain, 1995).

Although the characteristics and functional properties of lung DCs are extensively studied in animal models, very few data concerning their human counterparts are available. This is mainly due to the poor availability of tissue (especially when compared with animal models), the labour-intensive and time-consuming experimental procedures, and the lack of specific markers for DCs in the past (Vermaelen and Pauwels, 2005). The ability to access and characterise DCs and their precursors in COPD and CF is critical to the understanding of immune modulation in these pathologies. A recent study by Donnenberg and Donnenberg (2003) demonstrated that mDCs and their monocytic and plasmacytoid precursors can be sampled in the lung by the minimal invasive procedure of BAL, despite their relative scarcity and can be detected by rare event multi-parameter flow cytometry. Demedts and colleagues (2005), by using recently developed DC specific surface markers, also managed to identify different respiratory DC subsets in lung digests of patients who underwent lobectomia or pneumectomia for various reasons (mostly lung cancer). In a more recent study, McCarthy *et al*. (2007) investigated the respiratory tract DC surface markers, which comprised approximately 0.5% of viable

sputum cells, in induced sputum samples taken from steroid-naïve, allergen-challenged and allergen-naïve subjects. Therefore, these newly established methodologies could ideally be employed in future studies to elucidate the subtypes of DCs and their activation status in COPD and CF lung tissue sections and BAL fluids. These studies would further help investigators to confirm the *in vitro* findings (showing the adverse effects of COPD and CF patients on DCs) reported in the current study.

In conclusion, the data presented here show that NE and NE-containing secretions from patients with COPD or CF are able to disable DC function by interfering both with the ability of iDCs to mature in response to bacterial LPS stimulation, and by reducing the antigen presenting capability of mDCs. However, the *in vivo* effects of these changes still remain to be investigated and also these experiments should be further reassessed using human and murine lung DCs. The NE-induced inhibition of DC activation and secretion of pro-inflammatory Th1 cytokines (*e.g.,* IL-12) may be instrumental in the inability of these patients to clear semi-facultative intra-cellular pathogens in the lung, such as *P.a., B.c.* and *NTHI*, as suggested recently by a variety of animal and human studies (King *et al*., 2003; Evans *et al*., 2002; Moser *et al*., 2002; Martin and Mohr, 2000; Moser *et al*., 1997). In that context, therapeutic interventions to over-express the NE inhibitors such as SLPI and elafin could be dually advantageous (see Chapters 4 and 5; Roghanian *et al*., 2006b), firstly by inhibiting NE, and secondly as shown recently, by providing an antibacterial shield in the lungs (Gibbons *et al*., 2006; Fitch *et al*., 2006; Williams *et al*., 2006; McMichael *et al*., 2005a; Hiemstra *et al*., 2004; Simpson *et al*., 2001).

3.4. SUMMARY

The work described in this chapter demonstrates that exposure of DCs to mainstream soluble components of the CS result in cell death (primarily necrosis). On the other hand NE and NE-containing lung inflammatory secretions from patients with COPD or CF are able to disable DC function by reducing their cell surface CSMs important in antigen presentation. Furthermore, NE could interfere both with the ability of iDCs to mature in response to bacterial LPS stimulation and by reducing the allostimulatory activity of mDCs when co-cultured with D011.10-derived OVA transgenic spleen lymphocytes.

Chronic inhalation of CS and the presence of high concentrations of NE in the respiratory tract may each play important roles in pulmonary adaptive immune system by modulating host responses (via down-regulating DC activity) to invading respiratory pathogens, and specific therapeutic interventions are required to revert this immunosuppression state.

APPENDIX I

Table A3.1. COPD patients' characteristics

COPD sputum samples were obtained as described in Chapter 2. Individual patients' characteristics are listed, including FEV1, FEV1/FVC and smoking status. Data produced by collaborators at Edinburgh Royal Infirmary.

PATIENT'S NO.	SEX	AGE	FEV1	FEV1 PRED	FEV1/FVC RATIO	SMOKER-CURRENT	SMOKER-EX	PACK/YEAR
1	F	76	0.8	57	70	Yes	-	60
2	F	79	0.6	38	48	-	Yes	42
3	M	78	0.51	20	35	-	Yes	50
4	M	72	0.8	25	43	Yes	-	75
5	M	83	0.9	31	45	-	Yes	64
6	M	53	1.55	42	57	-	Yes	54
7	F	78	0.4	29	42	-	Yes	64
8	M	60	1.3	35	39	Yes	-	38
9	F	79	0.6	46	44	Yes	-	60
10	M	59	1.62	46	53	Yes	-	65
11	M	64	0.95	30	44	Yes	-	55
12	M	61	2	57	44	Yes	-	60
13	F	72	0.7	43	45	-	Yes	60
14	M	61	2.15	61	57	Yes	-	80
15	F	77	0.45	32	38	Yes	-	50
16	F	73	0.75	47	48	Yes	-	30
17	F	56	0.6	32	46	-	Yes	76
18	M	73	0.8	-	-	-	Yes	76
19	M	59	0.45	15	32	Yes	-	20
20	F	73	0.75	38	50	Yes	-	30
21	M	67	1.3	38	43	-	Yes	60
22	M	60	2.4	63	73	-	Yes	80
23	F	70	0.95	53	79	Yes	-	40
24	F	71	0.7	41	52	-	Yes	50
25	M	76	-	-	-	-	Yes	20
26	M	63	0.72	65	33	No	No	-
27	M	73	0.8	25	29	Yes	-	30

APPENDIX II

Table A3.2. CF patients' microbiological data

CF sputum samples were obtained as described in Chapter 2. Sputum samples were assessed for the presence of microorganisams shown in the table. Microbiological analyses were kindly performed by Dr. Catherine J. Doherty from the CF laboratory, Medical Microbiology, The University of Edinburgh.
P.a., *Pseudomonas aeroginosa*; B.c., *Burkholderia cepacia*; S.a., *Staphylococcus aureus*.

Patient's No.	Age (yr) / Sex	*P.a.*	*B.c.*	*S.a.*	*Haemophilus parainfluenza*	*β-hemolytic Streptococcus*	Yeasts	[NE] μM
1	57 / M	+						5.59
2	31 / F						++	1.7
3	21 / M			++			+	0.69
4	45 / M			++	+			1.87
5	20 / F		+++	++		+		0.70
6	19 / F	+	++	+				3.55
7	36 / M		+++					0.76
8	29 / M		++	+				1.89
9	38 / F	++			++			3.21
10	40 / M	++						1.42
11	21 / F			+++	+			2.54
12	26 / M	+						3.17
13	57 / M	+						5.62
14	23 / F	++						2.39
15	21 / F	+++						0.62
16	21 / M	++	++					1.06
17	22 / F	+		+				2.32
18	24 / F	+						2.75

APPENDIX III

CD40 Protein FASTA (Human vs Murine):

Human CD40 - AAH64518 (151 aa)

mvrlplqcvl wgclltavhp epptacrekq ylinsqccsl cqpgqklvsd cteftetecl pcgesefldt wnrethfhqh kycdpnlglr vqqkgtsetd tictceegwh ctseacescv lhrscspgfg vkqidicqph fpkdrglnll m

Murine CD40 - AAH29254 (289 aa)

mvslprlcal wgclltavhl gqcvtcsdkq ylhdgqccdl cqpgsrltsh ctalektqch pcdsgefsaq wnreirchqh rhcepnqglr vkkegtaesd tvctckegqh ctskdceaca qhtpcipgfg vmematettd tvchpcpvgf fsnqsslfek cypwtscedk nlevlqkgts qtnvicglks rmrallvipv vmgilitifg vflyikkvvk kpkdnemlpp aarrqdpqem edypghntaa pvqetlhgcq pvtqedgkes risvqerqvt dsialrplv

Human CD40 (top sequence) vs Murine CD40 (bottom sequence),

```
   >>QUERY sequence                                                          (289 aa)
 initn: 618 init1: 618 opt: 623  Z-score: 767.2  bits: 149.0 E(): 6.3e-41
Smith-Waterman score: 623; 53.793% identity (75.862% similar) in 145 aa overlap (1-139:1-145)

>QUERY     1- 139:------------------------------------------------------------     :

               10        20        30        40        50        60        70        80
QUERY  MVRLPLQCVLWGCLLTAVHPEPPTACREKQYLINSQCCSLCQPGQKLVSDCTEFTETECLPCGESEFLDTWNRETHFHQH
       :: ::  :.:::::::::    ..: .:::: ..:::.:::::..:.: :: . .:.: ::  .::   :::: . :::
QUERY  MVSLPRLCALWGCLLTAVHLGQCVTCSDKQYLHDGQCCDLCQPGSRLTSHCTALEKTQCHPCDSGEFSAQWNREIRCHQH
               10        20        30        40        50        60        70        80

               90       100       110       120       130             140       150
QUERY  KYCDPNLGLRVQQKGTSETDTICTCEEGWHCTSEACESCVLHRSCSPGFGVKQI------DICQPHFPKDRGLNLLM
       ..:.:: :::::..:.:.:.:.:::.:: :::::. ::.:. :  : ::::: ..       .:.:
QUERY  RHCEPNQGLRVKKEGTAESDTVCTCKEGQHCTSKDCEACAQHTPCIPGFGVMEMATETTDTVCHPCPVGFFSNQSSLFEK
               90       100       110       120       130       140       150       160

QUERY  CYPWTSCEDKNLEVLQKGTSQTNVICGLKSRMRALLVIPVVMGILITIFGVFLYIKKVVKKPKDNEMLPPAARRQDPQEM
              170       180       190       200       210       220       230       240
```

APPENDIX IV

CD80 Protein FASTA (Human vs Murine):

Human CD80 (B7-1)- AAH42665 (288 aa)

mghtrrqgts pskcpylnff qllvlaglsh fcsgvihvtk evkevatlsc ghnvsveela qtriywqkek kmvltmmsgd mniwpeyknr tifditnnls ivilalrpsd egtyecvvlk yekdafkreh laevtlsvka dfptpsisdf eiptsnirri icstsggfpe phlswlenge elnainttvs qdpetelyav sskldfnmtt nhsfmcliky ghlrvnqtfn wnttkqehfp dnllpswait lisvngifvi ccltycfapr crerrrnerl rresvrpv

Murine CD80 (B7-2) - NP_033985 (306 aa)

macncqlmqd tpllkfpcpr lillfvllir lsqvssdvde qlsksvkdkv llpcrynsph edesedriyw qkhdkvvlsv iagklkvwpe yknrtlydnt tysliilglv lsdrgtyscv vqkkergtye vkhlalvkls ikadfstpni tesgnpsadt kritcfasgg fpkprfswle ngrelpgint tisqdpesel ytissqldfn ttrnhtikcl ikygdahvse dftwekpped ppdskntlvl fgagfgavit vvvivviikc fckhrscfrr neasretnns ltfgpeeala eqtvfl

Human CD80 (top sequence) vs Murine CD80 (bottom sequence),

```
>>QUERY sequence                                                          (306 aa)
 initn: 529 initl: 529 opt: 783  Z-score: 956.7  bits: 185.0 E(): 1.8e-51
Smith-Waterman score: 783;  45.985% identity (72.263% similar) in 274 aa overlap (14-281:18-285)

>QUERY    14- 281:   ------------------------------------------------------------------ :

                  10        20        30        40        50        60        70
QUERY      MGHTRRQGTSPSKCPYLNFFQLLVLAGLSHFCSGVI-HVTKEVKEVATLSCGHNVSVEELAQTRIYWQKEKKMVLT
                       :: : .. ...:  ::.  : :  ...: ::. . : : .:   :. .. :::::: :.::.
QUERY  MACNCQLMQDTPLLKFPCPRL-ILLFVLLIRLSQVSSDVDEQLSKSVKDKVLLPCRYNSPHEDESEDRIYWQKHDKVVLS
            10        20        30        40        50        60        70

          80        90       100       110       120       130       140       150
QUERY  MMSGDMNIWPEYKNRTIFDITNNLSIVILALRPSDEGTYECVVLKYEKDAFKREHLAEVTLSVKADFPTPSISDFEIPTS
       ...: ...:::::::::..: :.  :..::.:  ::.::: ::: : :. ... .::: : ::.:::: ::.:..   :..
QUERY  VIAGKLKVWPEYKNRTLYDNTT-YSLIILGLVLSDRGTYSCVVQKKERGTYEVKHLALVKLSIKADFSTPNITESGNPSA
      80        90       100       110       120       130       140       150

         160       170       180       190       200       210       220       230
QUERY  NIRRIICSTSGGFPEPHLSWLENGEELNAINTTVSQDPETELYAVSSKLDFNMTTNHSFMCLIKYGHLRVNQTFNWNTTK
       . .:: : .:::::.:..:::::::.:: .::::.:::::.::::..::.:::: : ::.. :::::: .:.. :.:.
QUERY  DTKRITCFASGGFPKPRFSWLENGRELPGINTTISQDPESELYTISSQLDFNTTRNHTIKCLIKYGDAHVSEDFTWEKPP
      160       170       180       190       200       210       220       230

         240         250       260       270         280
QUERY  QEHFPD--NLLPSWAITLISVNGIFVICCLTYCFAPRCRER---RRNERLRRESVRPV
       ..  ::  : :  ..  . .:  . ::  .  ::   :..:   :::: :
QUERY  EDF-PDSKNTLVLFGAGFGAVITVVVIVVIIKCF---CKHRSCFRRNEASRETNNSLTFGPEEALAEQTVFL
      240       250       260       270         280       290       300
```

APPENDIX V

CD86 Protein FASTA (Human vs Murine):

Human CD86 (B7-2)- CAG46642 (323 aa)

mglsnilfvm afllsgaapl kiqayfneta dlpcqfansq nqslselvvf wqdqenlvln evylgkekfd svhskymgrt sfdsdswtlr lhnlqikdkg lyqciihhkk ptgmirihqm nselsvlanf sqpeivpisn itenvyinlt cssihgypep kkmsvllrtk nstieydgim qksqdnvtel ydvsislsvs fpdvtsnmti fciletdktr llsspfsiel edpqpppdhi pwitavlptv iicvmvfcli lwkwkkkkrp rnsykcgtnt mereeseqtk krekihiper sdeaqrvfks sktsscdksd tcf

Murine CD86 (B7-2) - NP_062261 (309 aa)

mdprctmgla ilifvtvlli sdavsvetqa yfngtaylpc pftkaqnisl selvvfwqdq qklvlyehyl gtekldsvna kylgrtsfdr nnwtlrlhnv qikdmgsydc fiqkkpptgs iilqqtltel svianfsepe ikldqnvtgn sginltcmsk qghpkpkkmy flitnstney gdnmqisqdn vtelfsisns lslsfpdgvw hmtvvcvlet esmkissklp lnftqefps pfpdhipwit avlptviicv mvfclilwkw kkkkrprnsy kcgtntmere eseqtkkrek ihiperstea qrvfkssskt sa---qtywk eitasvtvall lvmlliivch kkpnqpsrp----sntaskler dsnadretin lkelepqias akpnae scdksdtcf

Human CD86 (top sequence) vs Murine CD86 (bottom sequence),

```
>>QUERY sequence                                                    (309 aa)
 initn: 874 initl: 658 opt: 877  Z-score: 1071.3  bits: 206.4 E(): 7.3e-58
Smith-Waterman score: 892;  49.498% identity (73.244% similar) in 299 aa overlap (1-299:7-296)

>QUERY     1- 299:------------------------------------------------------------  :

                    10        20        30        40        50        60        70
QUERY         MGLSNILFVMAFLLSGAAPLKIQAYFNETADLPCQFANSQNQSLSELVVFWQDQENLVLNEVYLGKEKFDSVHS
              :::. ..::: ..:.: :. .. ::::: :: ::: :...:: :::::::::::::..::: : ::: ::.::::.
QUERY  MDPRCTMGLAILIFVTVLLISDAVSVETQAYFNGTAYLPCPFTKAQNISLSELVVFWQDQQKLVLYEHYLGTEKLDSVNA
            10        20        30        40        50        60        70        80

           80        90       100       110       120       130       140       150
QUERY  KYMGRTSFDSDSWTLRLHNLQIKDKGLYQCIIHHKKPTGMIRIHQMNSELSVLANFSQPEIVPISNITENVYINLTCSSI
       ::.::::::: ..::::::::.:::: : :.:.:..: ::: : ..:  .::::.::::.:::   .:.: :  :::::.:
QUERY  KYLGRTSFDRNNWTLRLHNVQIKDMGSYDCFIQKKPPTGSIILQQTLTELSVIANFSEPEIKLDQNVTGNSGINLTCMSK
              90        100       110       120       130       140       150       160

          160       170       180       190       200       210       220       230
QUERY  HGYPEPKKMSVLLRTKNSTIEYDGIMQKSQDNVTELYDVSISLSVSFPDVTSNMTIFCILETDKTRLLSSPFSIELEDPQ
       .:.:.::::  :.   ::: ::   :: ::::::::::...: :::.:::: . .::. :.:::.. .. :.:...  : :.
QUERY  QGHPKPKKMYFLI--TNSTNEYGDNMQISQDNVTELFSISNSLSLSFPDGVWHMTVVCVLETESMKISSKPLNFTQEFPS
              170         180       190       200       210       220       230

          240       250       260       270       280       290       300       310
QUERY  PFPDHIPWITAVLPTVIICVMVFCLILWKWKKKKRPRNSYKCGTNTMEREESEQTKKREKIHIPERSDEAQRVFKSSKTS
         .  :   .  ...  ..:. ::.   :: ..:       .::  . : ...  :: :.. :
QUERY  A---QTYWKEITASVTVALLLVMLLIIVCHKKPNQPSRP----SNTASKLERDSNADRETINLKELEPQIASAKPNAE
         240       250       260       270           280       290       300

          320
QUERY  SCDKSDTCF
```

Function used to analyse above sequences was FASTA [version 3.4t26 July 7, 2006]. Source: http://fasta.bioch.virginia.edu/fasta_www/cgi/search_frm2.cgi.

CHAPTER 4

IN VIVO MODULATION OF DENDRITIC CELLS BY ELASTASE SPECIFIC INHIBITOR (ELAFIN)

4.1. AIMS AND BACKGROUND

Having established in the preceding results chapter that NE could potentially down-regulate the DC CSMs and adversely affect their allostimulatory function (Roghanian *et al*., 2006a), it also seemed logical to study the effects of NE inhibitors in that context.

The primary aim of the work described in this chapter was to investigate the effects of augmentation of respiratory system NE-inhibitor molecules on the function and phenotype of DCs. The work described in this chapter was primarily performed using an adenovirus (Ad)-based strategy (also see Chapter 5) to over-express the genes of interest. This approach may allow the evaluation of these constructs for gene therapy strategies. Recombinant Ad gene transfer represents an effective method for the transfection of non-dividing cells, such as DCs (Miller *et al*., 2002). On the other hand, Ad DNA is episomal, reducing the risk of insertional mutagenesis associated with retroviral gene transfer. The Ad gene transfer approach uses replication-deficient Ad vectors which are both E1 and E3 deleted, encoding the cDNA for the genes of interest which are under the control of the powerful murine CMV promoter, to facilitate cellular expression of the transgene (Sallenave *et al*., 1998).

The decision was taken to use elafin in this part of the project, primarily because previous studies by investigators in our laboratory and other groups had demonstrated that elafin has various immunomodulatory properties, depending on the cells and tissues in which it is expressed (see Chapter 1, section 1.7.1.1). In addition, elafin transgenic (eTg) mice were available in house. The eTg mouse line had previously been developed using a 6.3-Kb fragment containing the human elafin

cDNA under control of the murine CMV promoter (the same as the promoter used in the Ad systems), which encodes full length elafin molecule (Sallenave *et al.*, 2003). Therefore, in addition to the WT mice, eTg mice were also used in the *in vivo* experiments. It should be noted that the rest of the experiments described herein were performed using C57BL/6J WT mice. The decision to use this strain of mouse, rather than the BALB/c strain used in previous sections, was made due to the background of our in-house eTg mice which were derived from C57BL/6J × CBA strains, enabling direct extrapolation and comparison of the data generated in this study.

Since its characterisation in early 90's (Wiedow *et al*., 1990; Sallenave and Ryle, 1991; Sallenave *et al*., 1992), elafin has been intensively studied in our laboratory, and previous studies by our group (Simpson *et at*. 2001b) have used the Ad gene transfer technology to demonstrate that Ad augmentation of elafin protects human alveolar epithelial (A549) cells against the injurious effects of both NE and whole activated neutrophils *in vitro*, and protects murine lungs against acute inflammatory injury by *Pseudomonas aeroginosa* (*P.a.*) *in vivo*. Relatedly, McMichael *et al.* (2005a) also demonstrated that i.t. instillation of Ad-elafin significantly reduced the lung *Staphylococcus aureus* (*S.a.*) load. Moreover, elafin over-expression has been shown to enhance human neutrophil migration *in vitro*, and to augment LPS-mediated neutrophil migration into WT and eTg murine airways *in vivo* (Simpson *et al*., 2001a; Sallenave *et al.*, 2003).

The source of DCs used in this study was from murine pulmonary DCs. As mentioned above, data presented in Chapter 3 demonstrated that both purified NE and lung sputa with detectable NE activity could down-regulate DC CSMs and consequently affect their T cell-stimulatory capacity. Additionally, NE interfered with normal maturation of iDCs in the presence of LPS and reduced the output of the pro-inflammatory cytokines IL-12 and TNF-α. The experiments described herein were undertaken to investigate whether *in vivo* genetic augmentation of an NE specific inhibitor (elafin) could modulate DC phenotype and function.

4.2. RESULTS

4.2.1. Effects of elafin gene augmentation on pulmonary DCs

While studying the effects of NE on DCs *in vitro*, parallel experiments, performed by other members of the laboratory, using two independent systems of elafin over-expression *in vivo* (recombinant Ad [Ad-elafin] and an eTg mouse line) indicated that these animals had an enhanced primary immune response towards Ad infections (Roghanian *et al.*, 2006b). These intriguing observations led us to hypothesise that at least in part some of these effects are due to the influence of elafin expression on pulmonary DCs. As mentioned, data presented in Chapter 3 indicated that NE could be down-regulating DC function and hence expression of a specific NE inhibitor such as elafin during infection/inflammation could be an ideal strategy to reverse these effects. With regards to these observations, it was deemed necessary to study the effects of elafin over-expression on DCs *in vivo*, as described below.

In the lung, DCs reside within and beneath airway epithelium, in alveolar septa, in the connective tissue surrounding pulmonary veins and airway vessels, and in the lung vascular compartment (Holt *et al.*, 1990). Various cell surface markers, including products encoded by the MHC class I and II genes, CSMs, adhesion molecules, and FcRs, can be used to discriminate lung DCs from other cell types, and to characterise their phenotype and function (Masten *et al*., 1997; von Garnier *et al*., 2005; de Heer *et al.*, 2005). Murine DCs are identified by their expression of CD11c and have been divided into three major subsets, *i.e.*, myeloid, lymphoid, and plasmacytoid, based on the differential expression of CD11b and CD45R/B220. Murine myeloid DCs co-express CD11c and CD11b, but lack CD45R/B220. Lymphoid DCs do not express CD11b or CD45R/B220. Plasmacytoid DCs (pDCs) co-express CD11c and CD45R/B220, but do not express CD11b (Maraskovsky *et al.*, 1996; Pulendran *et al.*, 1999; Nakano *et al.*, 2001). In addition, as mentioned, different subsets of lung DCs are differentially distributed and can be classed into: *1*) conducting airway DCs, *2*) interstitial DCs, and *3*) alveolar DCs (see Figure 1.5).

As DCs are a rare population, representing only around 1% of total lung cells in the absence of ongoing inflammation and immune responses (Robbins *et al.*, 2003; Masten *et al.*, 2004), their isolation is extremely laborious. Hence, at the start of this part of the project it was indispensable to choose a reliable, economical and efficient method to analyse lung DCs. Of the few lung DC analysis strategies available at the time of this project three were chosen and carried out in order to find an ideal protocol for our experiments (see below). In all the following experiments, lungs were perfused to remove blood cells and hence minimise contamination of the cell preparations with blood monocytes and circulating APCs (Figure 4.1). Enzymatic digestion of the lung tissue to extract viable interstitial lung cells was accomplished with collagenase A. Also DNase was included in the enzyme solution to minimize cell loss due to DNA-induced clumping. The cells were then subjected to red cell lysis treatment. In the final step, freshly isolated cells were stained with a range of cell surface markers (see below). The detailed procedure for preparation of lung single cell suspensions has been described in detail in Chapter 2 (section 2.2.4.1.2).

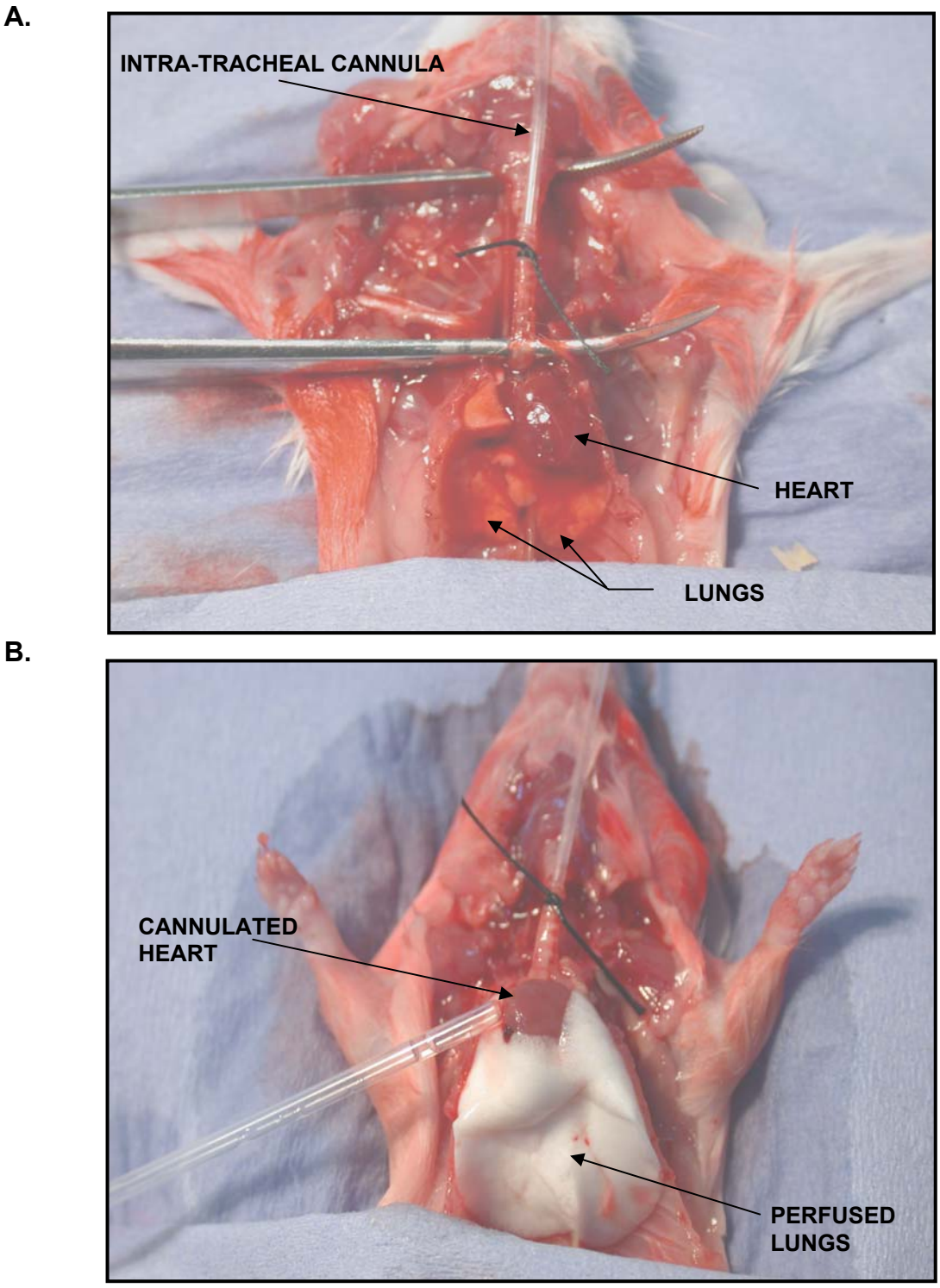


Figure 4.1. Perfusion of murine lungs

A) Uninflated murine lungs cannulated *in situ*, before perfusion. **B**) After perfusion, showing partial inflation of lungs, and complete clearing of blood. Pictures taken from Dr Sharon Ahmed's Ph.D. thesis (CIR, Edinburgh).

4.2.1.1. Isolation of lung APCs by fluorescence-activated cell sorter (FACS)

The FACS Vantage SE flow cytometer (Becton Dickinson, CA) could be applied to rapidly separate the cells in a suspension on the basis of size and the colour of the fluorescence conjugated to the Abs used to label cell surface markers of interest. The cells are not damaged by the process. In fact, because the machine can be set to ignore dead cells, the percent viability of the sorted cells can be higher than that in the original suspension. The lung single cell suspensions, pooled from lungs of 4 naïve C56Bl/6J mice, were blocked with mouse serum prior to being stained with mAbs raised against mouse CD11c (clone N418), and MHCII (clone 2G9), as described in detail in Chapter 2 (section 2.2.4.1.2). They were then washed and resuspended at ~5×10^6 cells/ml (~30×10^6 total cells from 4 pooled mouse lungs) of PBS containing 0.1% BSA, and put through the cell sorter. In this way, two major populations of APCs could be selected according to their expression levels of CD11c and MHCII. Figure 4.2 shows the FACS data analysing the cells before and after sorting of lung cells. The first population (P1) was highly positive for CD11c and had no/low expression levels of MHCII, which was considered to consist of macrophages/monocytic cells. The second population (P2) was highly positive for both CD11c and MHCII and was considered to represent 'bona fide' DCs (see below for rationale). The corresponding representative cytospin figures of the two isolated populations are demonstrated in Figure 4.3 A. The $CD11c^{+high}$ $MHCII^{+low}$ (P1) and $CD11c^{+high}$ $MHCII^{+high}$ (P2) cell population purities were relatively high (around 90 and 70%, respectively), although there were still contaminant cells (as observed in the case of CD11c magnetically-purified cells described in the following section). Despite this high purity, there were limitations with the final cell numbers obtained, as it was only possible to get around 2×10^5 cells/mouse in P1 and ~1×10^5 cells/mouse in P2. For this reason it was just possible to stain the cells for the three CSMs (CD40, CD80 and CD86). As shown in Figure 4.3 B P1 population was almost negative for CD40 and CD86, but strongly positive for CD80 (31.8% vs 7.6% in P2). On the other hand, the P2 population had a higher expression of CD40 (3.2% vs 1% in P1) and CD86 (12.8% vs 1.4% in P1) and around 8% were also stained positively with CD80.

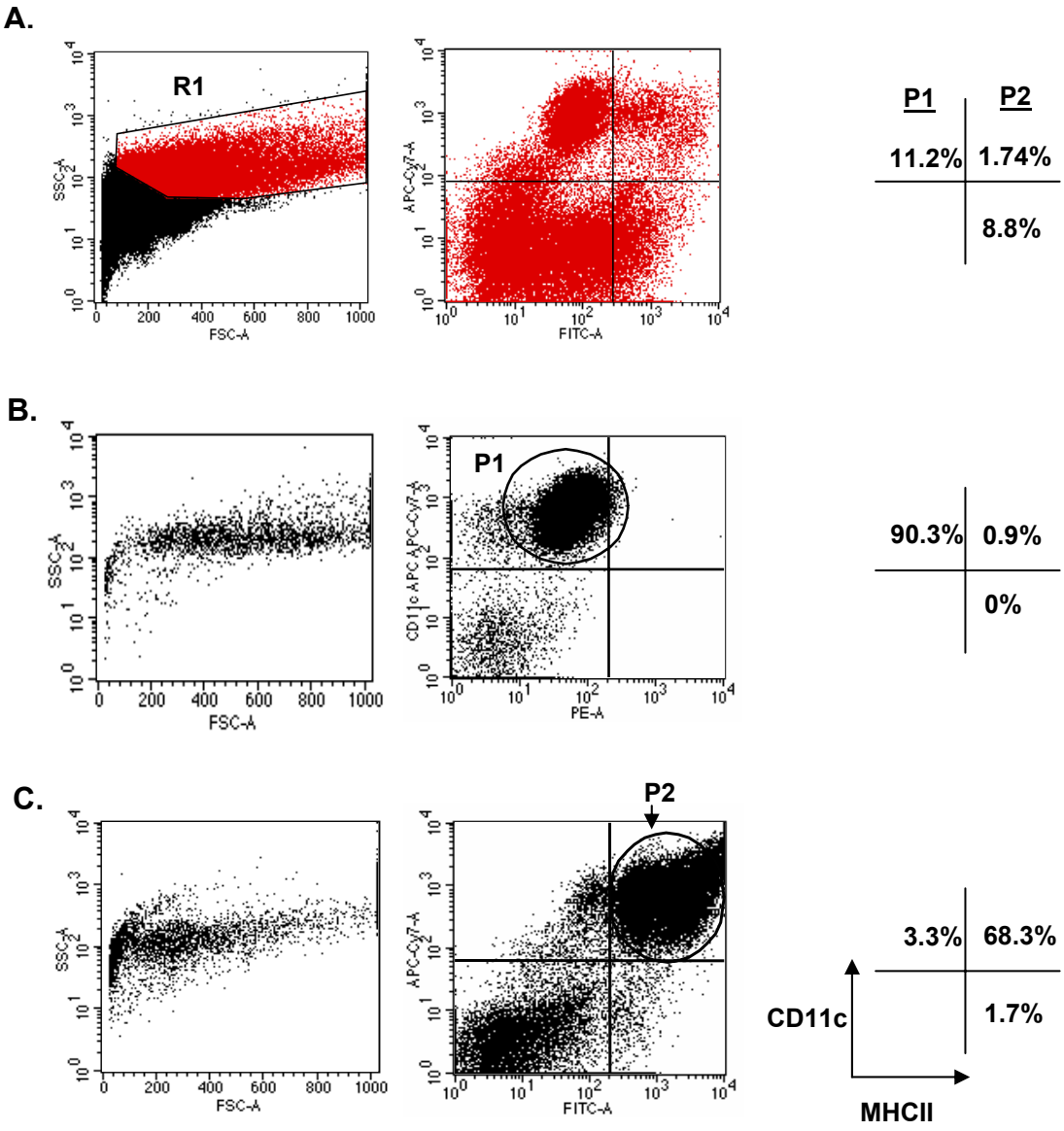


Figure 4.2. Sorting of lung APCs

Lungs of WT mice (*n*=4) were perfused and pooled cells suspensions were prepared as described in Materials and Methods (Chapter 2). They were then stained with FACS mAb to CD11c and MHCII, resuspended at ~5×10^6 cells/ml in PBS containing 0.1% BSA and passed through the fluorescence-activated cell sorter (FACS Vantage SE flow cytometer; Becton Dickinson, CA). **A)** Unsorted naïve lung cells. **B)** $CD11c^{+high}$ $MHCII^{+low}$ (P1) selected by sorting. **C)** $CD11c^{+high}$ $MHCII^{+high}$ (P2) selected by sorting. Data are representative of two independent experiments.

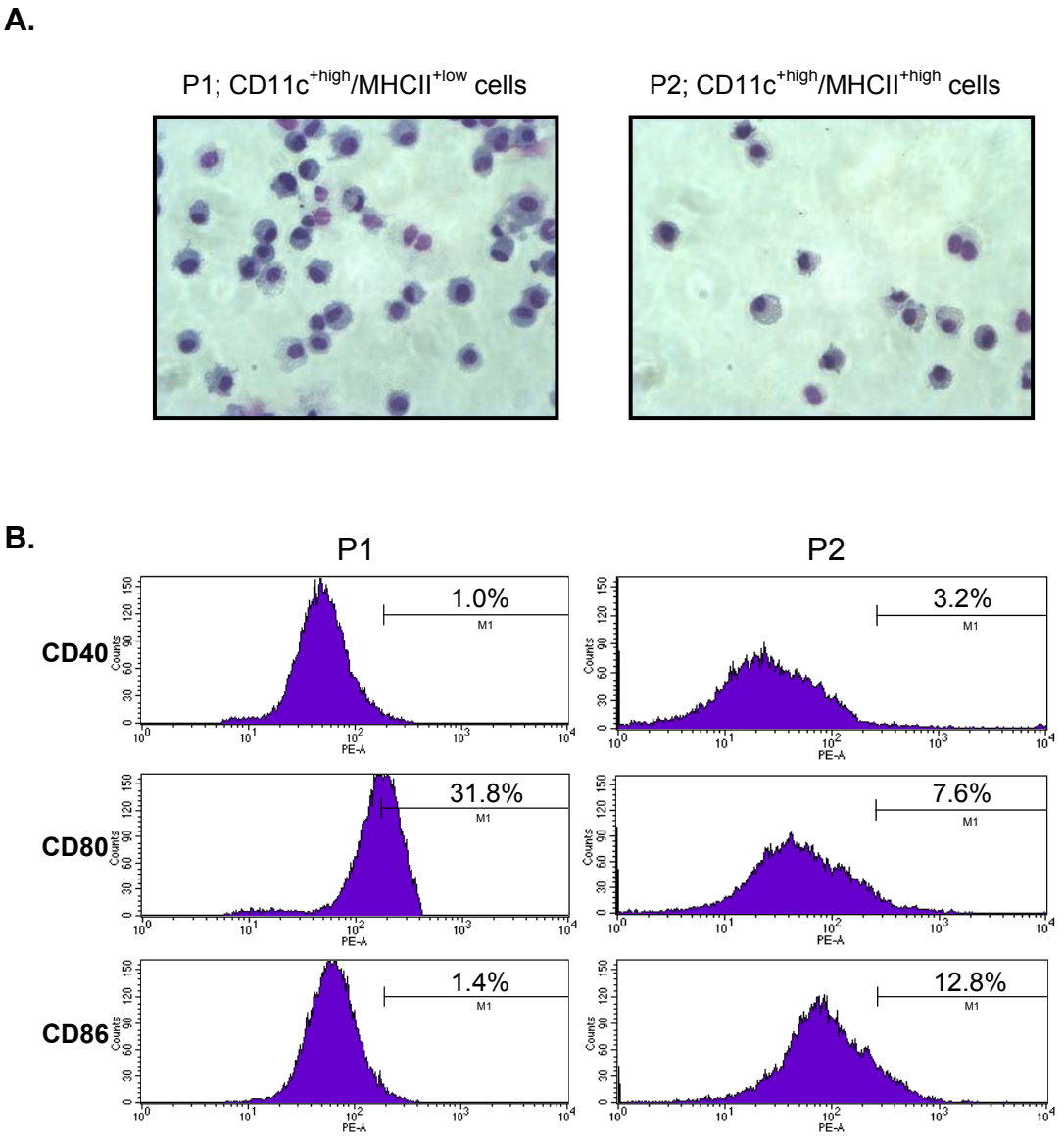


Figure 4.3. Analysis of APCs isolated by lung cell sorting

See legend of Figure 4.2 for details. **A)** Cytospin pictures of $CD11c^{+high}$ $MHCII^{+low}$ (P1) and $CD11c^{+high}$ $MHCII^{+high}$ (P2) isolated by FACS. **B)** Surface CSM phenotype of P1 (left panel) and P2 (right panel) of cells shown. Sorted P1 and P2 populations were washed and stained with PE-conjugated mAbs to CD40, CD80 and CD86 and analysed by flow cytometry. Percentage values of positive cells compared to isotype-matched control Ab-stained cells are shown in M1 regions.

4.2.1.2. Isolation of lung APCs by CD11c MicroBeads

CD11c MicroBeads (Miltenyi Biotec Ltd., Bergisch Gladbach, Germany) were available for the isolation of mouse DCs from single cell suspensions of lymphoid and non-lymphoid tissues. Unlike in humans, in mice CD11c is expressed on all defined DC subsets making them easier to isolate. The lung single cell suspensions, pooled from the lungs of 4 naïve C56Bl/6 mice, were washed once and resuspended in ice cold MACS buffer (PBS containing 1% BSA and 2mM EDTA) and Miltenyi CD11c MicroBeads as per the manufacturer's protocol. The cells were incubated for 15 minutes at 4-6^{o}C, then washed in 10-20× the volume of MACS buffer. As the cell count at this stage was consistently less than 10^7 per tube, the cells in each tube, were resuspended in 500µl MACS buffer ready for magnetic cell separation with an AutoMACS system (Miltenyi Biotec Ltd.). This method yielded around 1×10^5 CD11c$^+$-enriched APCs per mouse, which showed >95% viability as determined by trypan blue dye exclusion. Due to very low yield of cells it was not possible to analyse cells for CSM expression and other cell surface markers, apart from the standard markers (CD11c and MHCII) used throughout this project. Figure 4.4 shows the results of the lung CD11c$^+$ cell enrichment using AutoMACS system. The top panel represents the forward and side scatter of the cell profiles from left to right for: whole lung cells before separation, and the negative and positive cell fractions following the CD11c$^+$ cell enrichment. The bottom panel shows the subpopulations of cells according to their expression of CD11c and MHCII. Following the CD11c selection, approximately 50% of the cells were positive for CD11c (Figure 4.4 B), compared to only 6% in the whole lung cell suspension (left panel) and ~4% in the negative fraction (middle panel). In addition, the cytospin picture of CD11c$^+$-enriched cells shows cells typical macrophage/DC characteristics and phenotype.

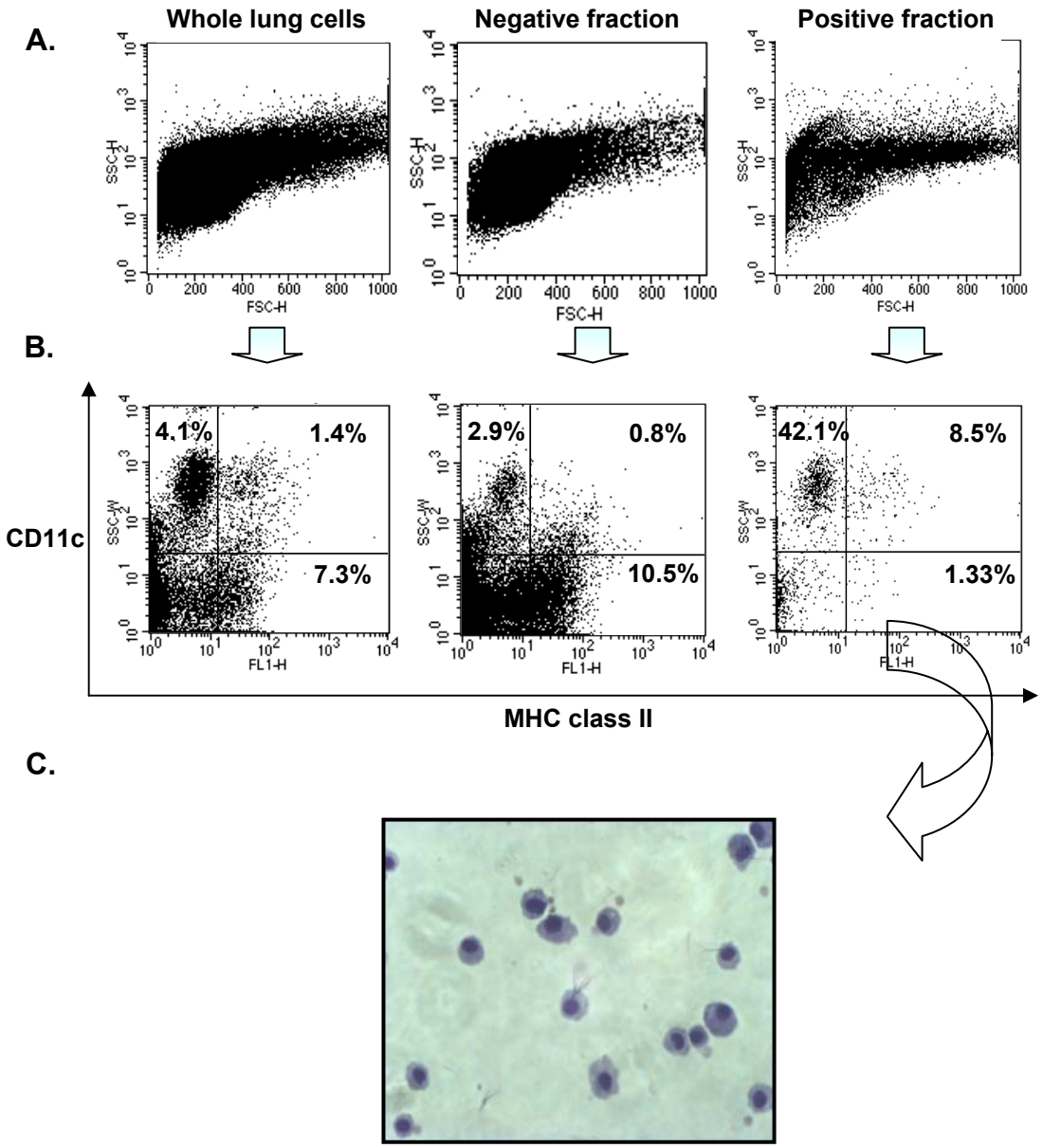


Figure 4.4. Lung CD11c$^+$ cell magnetic sorting

The lung single cell suspensions, pooled from lungs of 4 naïve C56Bl/6J mice, were washed once and resuspended in 400 µl ice cold MACS buffer and 100 µl Miltenyi CD11c MicroBeads (Miltenyi Biotec Ltd., Bergisch Gladbach, Germany) as per the manufacturer's protocol. The cells were incubated for 15 minutes at 4-6^{o}C, then washed in 10-20× the volume of MACS buffer. As the cell count at this stage was consistently less than 10^7 per tube, the cells in each tube, were resuspended in 500 µl MACS buffer ready for magnetic cell separation with an AutoMACS system (Miltenyi Biotec Ltd.). Positive (CD11c$^+$ enriched population) and negative fractions were stained with mAbs to CD11c and MHCII and cell were analysed by flow cytometry. **A)** FCS/SSC plots, **B)** Double stained cells, and **C)** cytospin picture of CD11c$^+$ enriched cells (positive fraction).

4.2.1.3. Analysis of lung APCs using whole lung cells

Since the two methods described above yielded very low number of cells and did not allow simultaneous analysis of other cell types such as T cells and neutrophils in the lung tissue, it was decided to analyse the whole lung cell suspension. This was done by using multicolour flow cytometric analysis of the cells. The mAb to mouse CD11c used was conjugated to APC fluorochrome, MHCII was conjugated to FITC fluorochrome, and the rest of the cell surface markers used here were conjugated to PE fluorochrome. Isotype- and fluorochrome-matched control Abs to each mAb were also used to take into account for any non-specific staining.

4.2.1.3.1. The effect of elafin over-expression (Ad-elafin) on lung APCs

To examine the effects of elafin over-expression on pulmonary APCs of WT C57BL/6J mice, 3-4 animals per group received i.t. instillations of either PBS, Ad-null (2.5×10^8 plaque-forming units [pfu]), or Ad-elafin (2.5×10^8 pfu). To investigate which cell types could be transfected and express the transgene in mouse lungs, Lei *et al.* (1998) delivered i.t. an Ad-LacZ vector into mouse lungs, and lung tissues were processed for β-galactosidase histochemical staining 24 hours post-gene transfer. Microscopic examination demonstrated that β-galactosidase was mainly localized to bronchial and alveolar epithelial cells and to a lesser degree, alveolar macrophages. As expected, they found no staining in lungs from mice treated with the control vector Ad-null. These findings are consistent with those of our groups and the published data in the literature (Xing *et al*, 1994). It should be mentioned that the preparation of mouse lungs and staining of the cells for flow cytometry were kindly performed by Miss Tara A. Sheldrake in this part of the project and Prof. Jean-Michel Sallenave performed all the i.t. instillations.

In the first instance, to examine the effect of elafin expression on lung APCs, the experimental animals were anaesthetised on day 4 and their lungs and tracheae were perfused with PBS and removed *en bloc*. Dual labelling of cells with CD11c and MHCII allowed identification of 2 main $CD11c^+$ 'high' cell populations (G1), one being $MHCII^+$ 'low' (G2) and the other $MHCII^+$ 'high' (G3) (Figure 4.5; see

below for details). At this early time point there were no detectable levels of elafin in the BAL fluid of the animals that received a dose of Ad-elafin; hardly if any increase in the number of neutrophils and lymphocytes in the lungs of mice treated with the Ad vectors compared to control PBS-treated group of mice (data not shown). However, mice receiving either Ad-null or Ad-elafin had a slight increase in the percentage of $CD11c^{+high}$ $MHCII^{+high}$ cells in G3 on day 4 of the experiment (Figure 4.5 C), which could be as a result of early immune responses due to the presence of the Ad particles in the lungs. There were also no major differences in expression of cell surface markers analysed (data not shown).

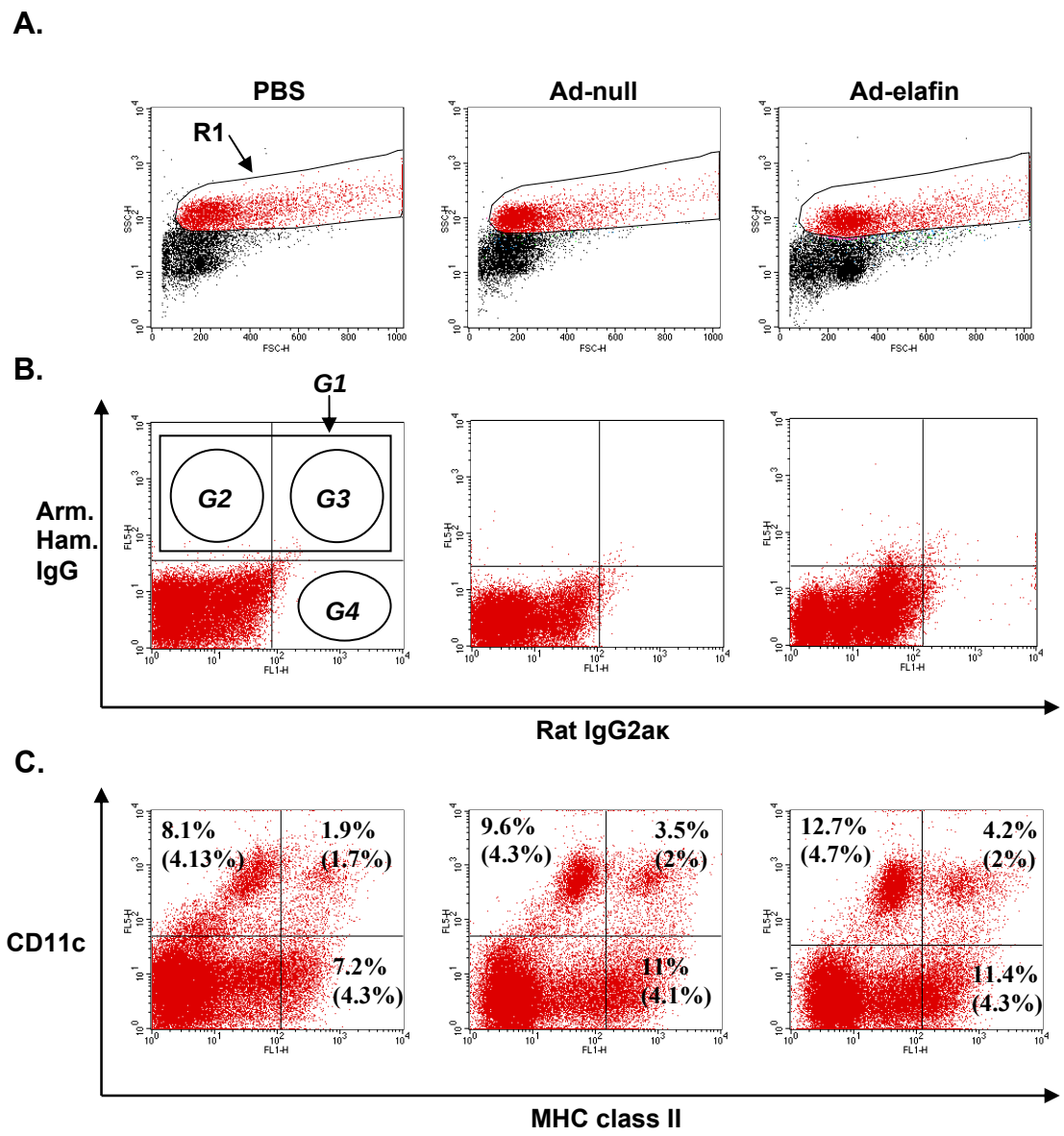


Figure 4.5. Lung myeloid cell population analysis 4 days post Ad i.t. instillation

Lungs of WT mice (4 mice per group) treated at day 0 with either PBS, Ad-null or Ad-elafin (2.5×10^8 pfu) were perfused on day 4 and pooled cells suspensions were prepared as described in Materials and Methods. **A)** A gate (R1) was created by FCS/SSC analysis. This gate was analysed for $CD11c^+$ cells and contained more than 90% of total lung $CD11c^+$ cells. **B and C)** These cells were analysed for CD11c and MHCII expression and their corresponding isotype-matched control mAbs (Armenian hamster [Arm. Ham.] IgG-PE and Rat IgG2aκ-FITC, respectively). The cells were subdivided in 4 gates CD11c low (G4); $CD11c^{+high}$ $MHCII^{+high}$ (G3); $CD11c^{+high}$ $MHCII^{+low}$ (G2) and total CD11c (G1). Numbers in brackets indicates total % of cells (ungated). Cells were then analysed within G1 and G3 for expression of CD3, CD40, CD80, CD86, CD11b, B220, Gr1 and F4/80 (not shown).

The level of the elafin transgene in the lungs of mice given Ad vectors has been shown to peak at around a week post Ad-elafin i.t. infection by previous members in our laboratory (Simpson *et al*., 2001a). Hence, with regards to this and also to similar data published by our collaborators using these vectors (Wang *et al*., 2000), the decision was taken to examine the lungs of treated animals 10 days post infection. In this way, it was expected that the elafin transgene would have been given reasonable time to express in the lung, and that it would be possible to detect any resulting changes in the lungs within this time-frame. WT mice do not express elafin, and as expected elafin was undetectable in WT mice receiving either PBS or Ad-null. Ad-elafin treatment of WT mice resulted in detectable levels of human elafin (5598.05 pg/ml ± 857.08; n=4 mice/group, Figure 4.6 A) in BAL fluid by day 10 of the experiment, which were, expectedly, lower than those obtained in the 'day 5 acute models' by our group (Simpson *et al.*, 2001a; McMichael *et al.*, 2005a). When digested lung cells were cultured ± LPS (100ng/ml) *ex vivo* for 24 hours, cells from Ad-elafin-treated lungs actively secreted human elafin which was significantly increased in the presence of LPS (297 vs 665.5 pg/ml, respectively; Figure 4.6 B). This observation, as described above, is probably due to the ability of LPS to activate the elafin murine CMV promoter (which contains NF-κB sites) in Ad-elafin-infected cells, as shown before (Simpson *et al.*, 2001a).

As hypothesised, when BAL fluid differential counts were performed 10 days post treatment (due to experimental limitations the total cell counts were not recorded) it is clear from the cytospins that Ad-elafin treated mice had a higher number of leukocytes in their BAL fluid than the control groups (Figure 4.7). As shown in Table 4.1, Ad-elafin-treated mice showed a significant increase in percentage of lymphocytes compared to Ad-null and PBS controls (Ad-elafin vs Ad-null: p=0.0012). In addition, local elafin production induced an increase in BAL fluid neutrophils as observed previously (Simpson *et al*., 2001a), although it did not reach significance, whereas it caused a reduction in percentage of monocytes (Ad-elafin vs Ad-null: p=0.0011).

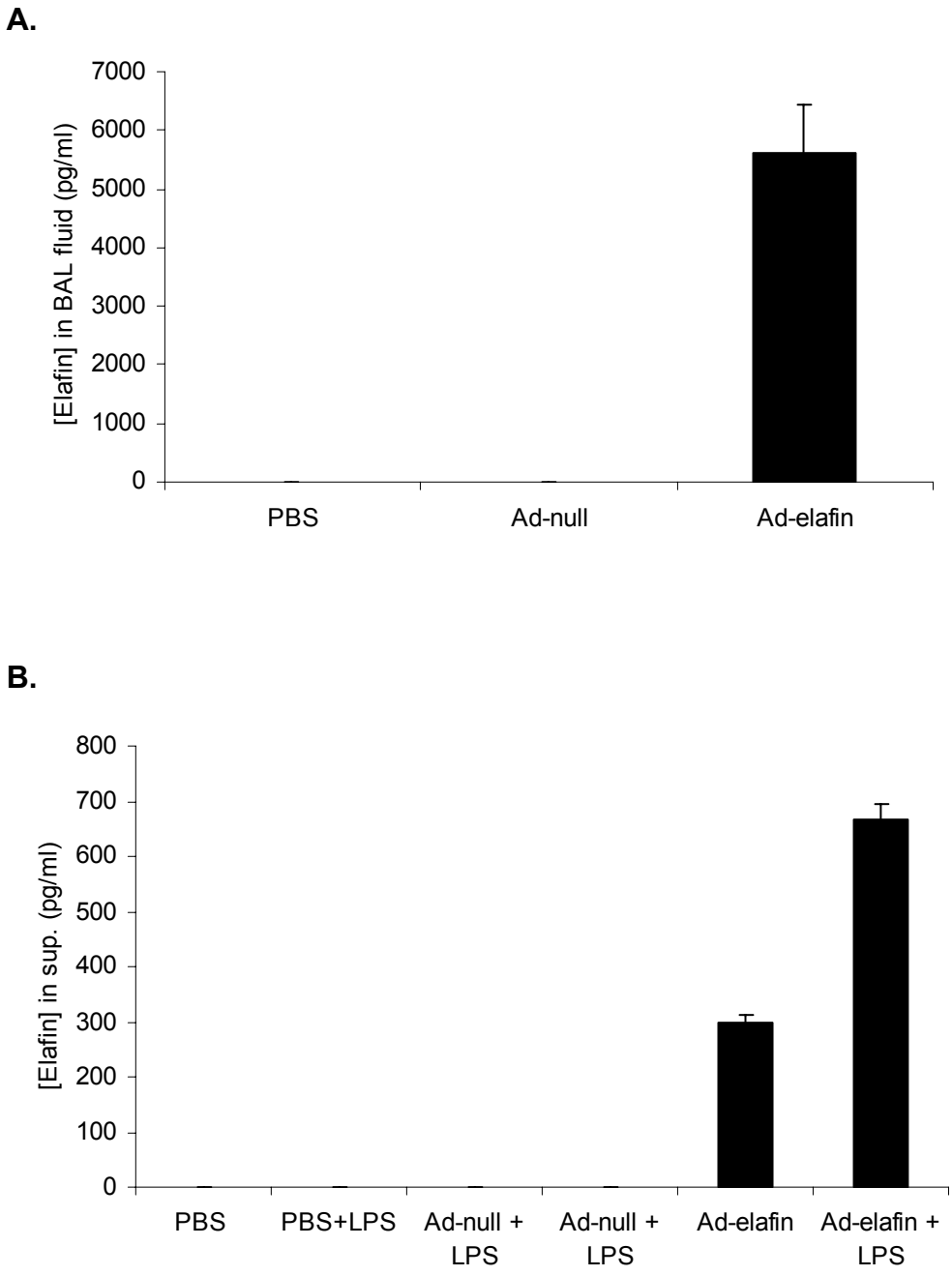


Figure 4.6. Elafin levels in BAL fluid (day 10) and in lung cell supernatants cultured *ex vivo*

On day 0, C57BL/6J WT mice (4 mice/group) were treated intra-tracheally with either PBS, Ad-null (2.5×10^8 pfu) or Ad-elafin (2.5×10^8 pfu). On day 10 of the experiment, mice were sacrificed, BAL was performed and; **A)** BAL fluid was analysed by elafin ELISA (4 mice/group). **B)** Four million digested whole lung cells pooled from 3 mice in each group were washed in PBS and plated in a 12-well cell culture plate in 2ml complete RPMI medium ±100ng/ml LPS and supernatant was collected 24 hours post culture and analysed for the presence human elafin by ELISA.

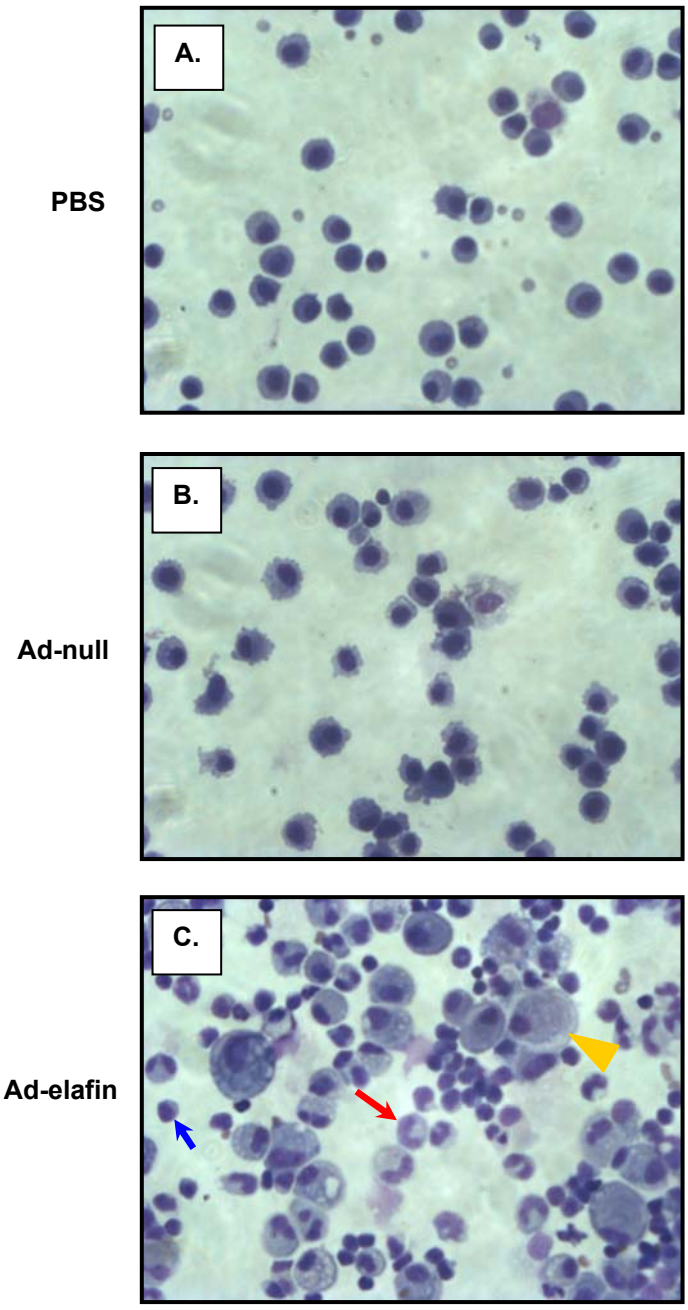


Figure 4.7. Inflammatory cells in BAL fluids (day 10)

On day 10 of the experiment, mice were sacrificed, BAL was performed and cell differential analysis was performed after centrifugation (2000 × g for 5 minutes). Cytospinning (100 × g for 3 minutes at room temperature) was performed and slides were stained with Diff-quick (Dade Diagnostika, GmbH, Germany) and fixed. Orange arrow head showing an APC, and red and blue arrows indicating neutrophil and lymphocyte respectively. Representative pictures are shown (n=4 mice/group).

Table 4.1. Bronchoalveolar lavage cell differential (WT mice)

On day 10, mice were sacrificed, BAL was carried out and cell differential analysis was blindly performed (in order to prevent biasing) by Prof. Sarah Howie after centrifugation (2000 × g for 5 minutes). Cytospinning (100 × g for 3 minutes at room temperature) was performed and slides were stained with Diff-quick. *n* represents the number of animals used in the experiment and results are expressed as % of total BAL cells (values represent mean and standard deviations [in brackets]). * indicates statistical significance, as compared to the Ad-null treatment (unpaired *t*-test analysis). p=0.0011 for myeloid cells and p=0.0012 for lymphocytes.

		n	Neutrophils	Myeloid cells	Lymphocytes
WT	PBS	4	0.13 (0.26)	97.31 (1.99)	2.69 (1.99)
	Ad-null	4	1.32 (1.12)	90.2 (4.15)	8.49 (4.41)
	Ad-elafin	4	4.01 (2.73)	59.59 (9.66)★	36.40 (8.62)★

The clear increase in percentage of lymphocytes in WT mice treated with Ad-elafin (Table 4.1) indicated a need to analyse the type of immunological response generated by this construct in the lungs of treated mice. IL-12, a heterodimeric cytokine consisting of two subunits (p35, and p40), is a pro-inflammatory cytokine secreted by activated DCs that has been recognised to play a role in pulmonary host defences (see discussions). As Figure 4.8 demonstrates, ELISA cytokine analysis revealed that BAL fluid IL-12p40 levels of Ad-elafin-treated mice were significantly higher compared to Ad-null-treated mice (p<0.001). In addition, supernatants of Ad-elafin treated lung cells cultured *ex vivo* secreted significantly higher levels of elafin compared to control groups (p<0.001; Figure 4.8B). Furthermore, cytokine analysis revealed that type 1 cytokines IFN-γ and TNF-α levels were also significantly higher in Ad-elafin-treated mice (p<0.01) compared to PBS and Ad-null-treated mice (Figure 4.9), although in general there were very low compared to IL-12p40 levels.

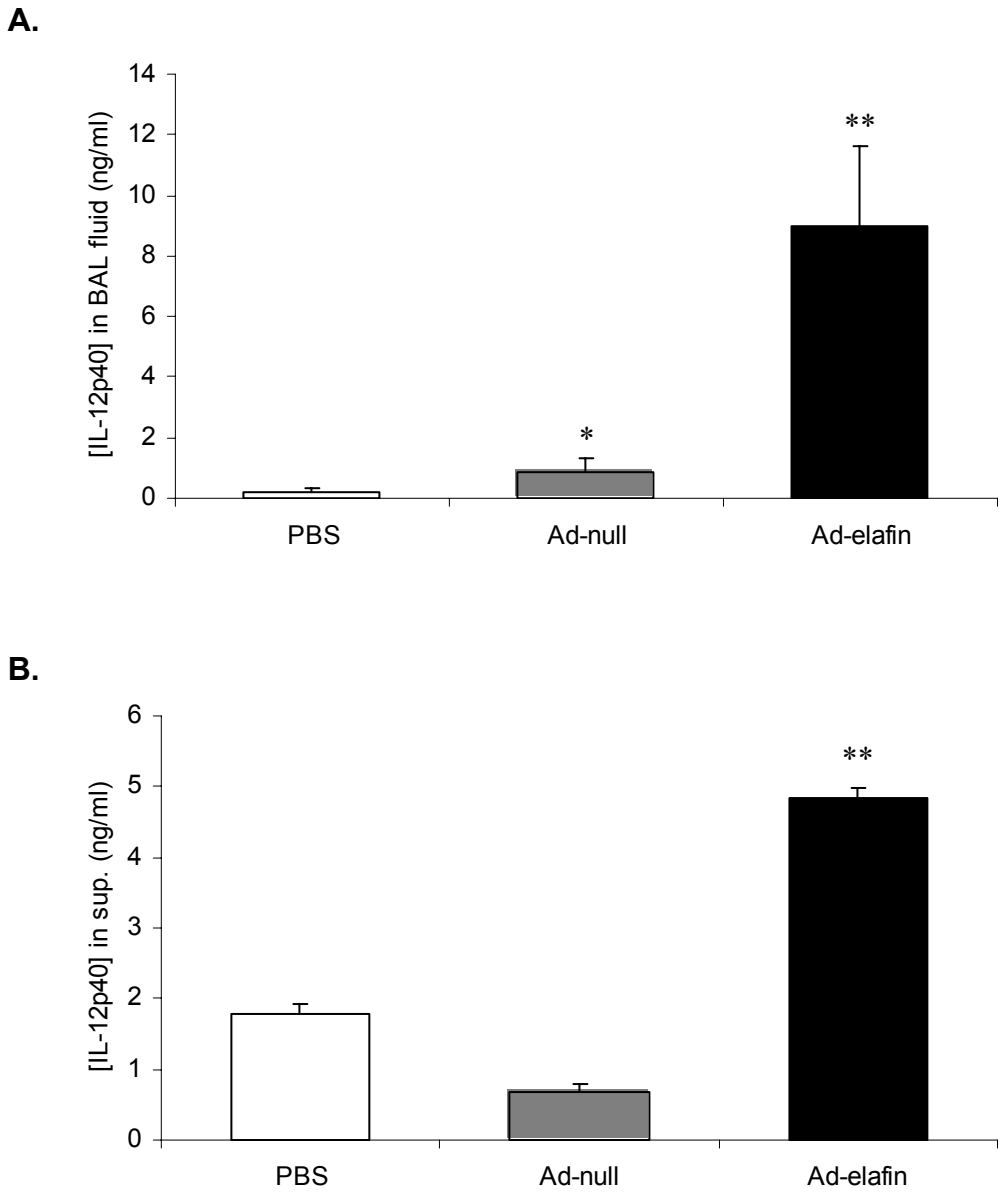


Figure 4.8. IL-12p40 levels in BAL fluid (day 10) and in lung cell supernatants cultured *ex vivo*

On day 0, C57BL/6J WT mice (4 mice/group) were treated i.t. with either PBS, Ad-null (2.5×10^8 pfu) or Ad-elafin (2.5×10^8 pfu). On day 10 of the experiment, mice were sacrificed, BAL was performed and; **A)** BAL fluid was analysed by IL-12p40 ELISA. **B)** Four million digested whole lung cells pooled from 4 mice in each group were washed in PBS and plated in a 12-well plate in 2ml complete RPMI medium and supernatant was collected 24 hours post culture and analysed for the presence IL-12p40 by ELISA. * indicates statistical significance between PBS and Ad-null-treated mice (p=0.0161), and ** indicates statistical significance (p<0.001) between Ad-null and Ad-elafin treatments (unpaired t-test analysis).

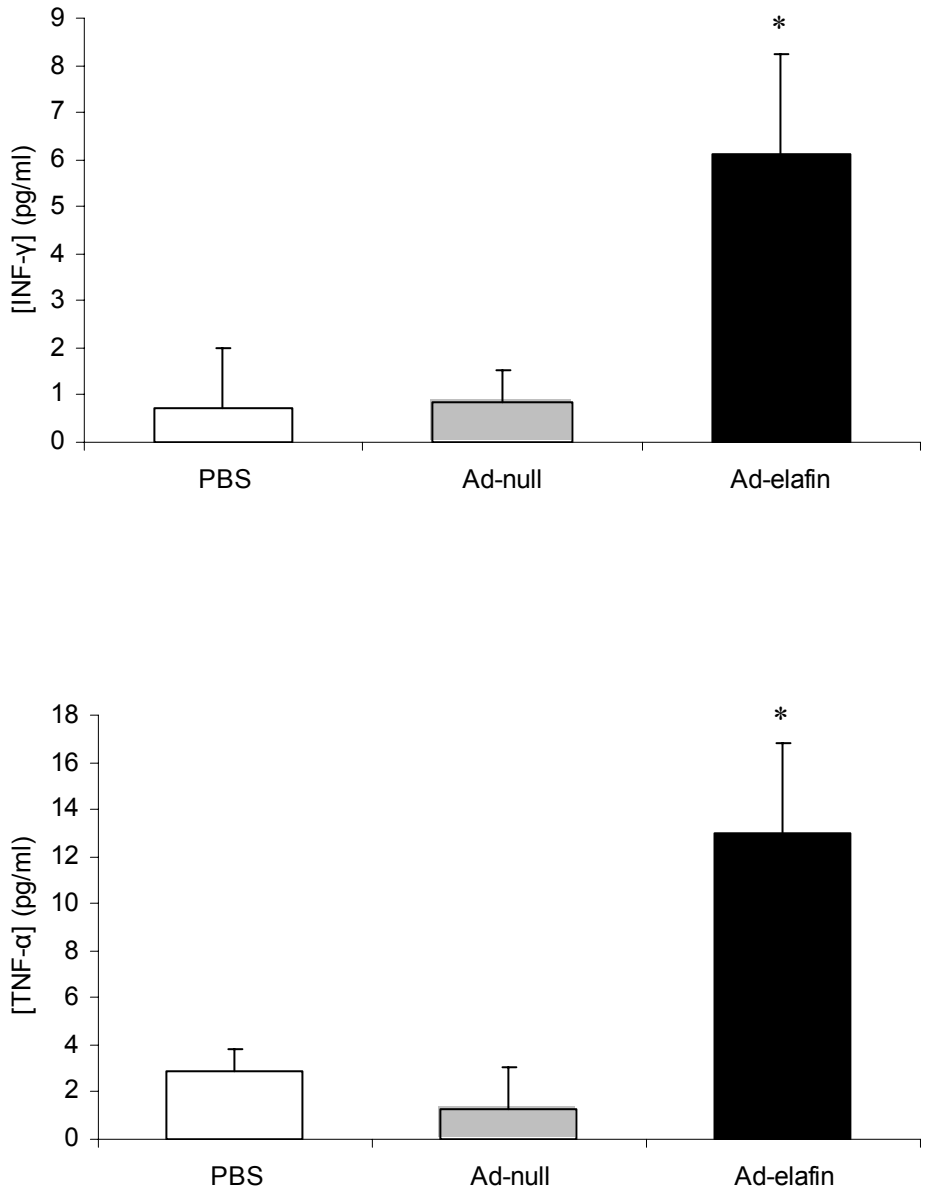


Figure 4.9. IFN-γ and TNF-α levels in BAL fluid

BAL fluid (see Figure 4.6 legend) was analysed for IFN-γ and TNF-α content by ELISA (see Materials and Methods, Chapter 2). BAL fluid samples collected from four mice were analysed per group and * indicates statistical significance ($p<0.01$), as compared to the Ad-null treatment (unpaired *t*-test analysis).

Whole lung single cell suspensions (*n*=3 mice per group; pooled samples) were stained with mAbs to CD11c and MHCII, as above. In addition to the data generated in the two other $CD11c^+$ cell enrichment methods (see above; Figure 4.2 B, 4.2 C and 4.4 A), back-gating analysis revealed that more than 90% of $CD11c^+$ cells were found within the R1 gate. Ad-elafin gene transfer increased the total number of $CD11c^+$ lung cells compared to those treated with Ad-null and PBS (Figure 4.10). The cells from the R1 region were further divided into four subpopulations (G1-G4) on the basis of CD11c and MHCII expression, as mentioned above. Dual labelling of cells with CD11c and MHCII allowed identification of 2 main $CD11c^+$ 'high' cell populations (G1), one being $MHCII^+$ 'low' (G2) and the other $MHCII^+$ 'high' (G3). G4 population consisted of $CD11c^+$ 'low' and $MHCII^+$ 'high' cells. In line with the literature, as mentioned above, the $CD11c^{+high}$ $MHCII^{+high}$ G3 gate were considered to represent 'bona fide' DCs and $CD11c^{+high}$ $MHCII^{+low}$ to be macrophages/monocytic cells for the following reasons: 'naïve' PBS-treated mice had a very low percentage of $CD11c^{+high}$ $MHCII^{+high}$ cells (around 1% of total lung cells), consistent with them being lung DCs (Gonzalez-Juarrero *et al*., 2001; Robbins *et al*., 2004; von Garnier *et al*., 2005). This population also expanded significantly upon Ad treatment (1.7% and 7.6% of total lung cells, after Ad-null and Ad-elafin treatments respectively, compared to 0.8% in PBS-treated mice, Figure 4.11). Furthermore, in PBS-treated mice, when cells were labelled with Abs to F4/80, a macrophage marker, about 32% of $CD11c^{+high}$ $MHCII^{+low}$ cells were labelled whereas only 15% of $CD11c^{+high}$ $MHCII^{+high}$ cells were positive for F4/80 (Figures 4.11 and 4.12). This analysis shows that the percentage of $CD11c^{+high}$ $MHCII^{+high}$ cells (G3) was markedly increased in WT mice treated i.t. with Ad-elafin. Interestingly, Ad-elafin also induced a small increase in the number of cells in G4 compared to Ad-null, which may be B lymphocytes (Figure 4.11). When cell surface markers such as the CSMs were analysed on these selected population (G1, G2 and G3), increased levels of CD80 and CD86 were observed in Ad-elafin treated mice (Figure 4.11, and Figure 4.12), particularly in the double positive 'DC G3'. Of note, the level of CD40 was low in PBS-treated mice, compared to CD80 and CD86 and was not induced by Ad treatments. In addition Ad-elafin-treated mice had higher expression levels of CD11b on these populations, compared to control groups (see Figure 4.13 below for details).

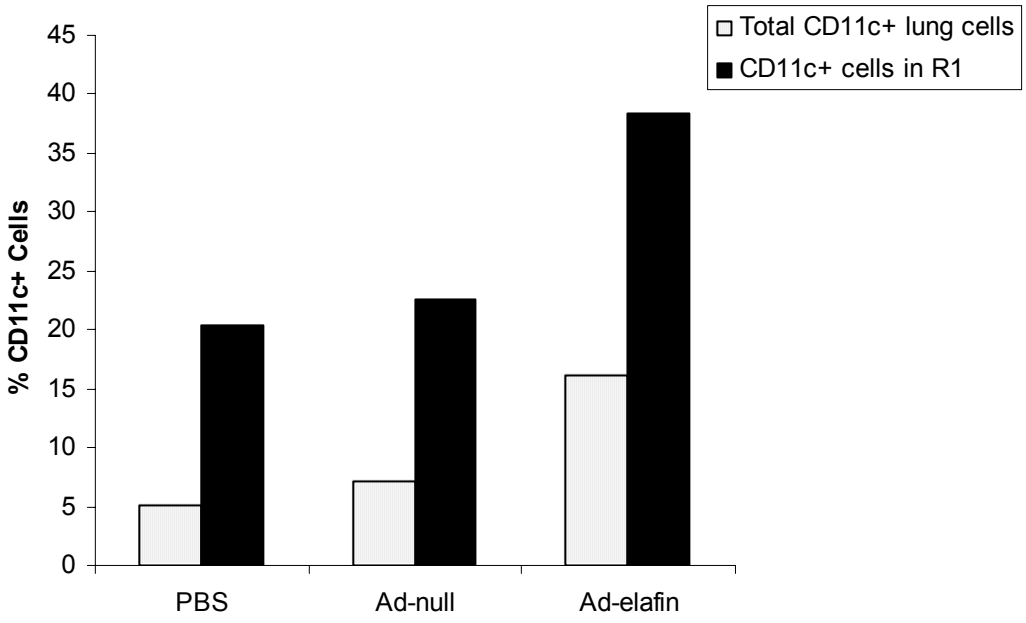


Figure 4.10. Total lung CD11c$^+$ cells 10 days post Ad i.t. instillation

See legend of Figure 4.6 for details. Graph showing % of CD11c$^+$ cells in total lung single cell digest taken from treated mouse lungs (dotted bars), as well as % of CD11c$^+$ cells in R1 selected on FCS/SSC plot (black bars). See Figure 4.11 for dot plots. *n*=3 mice/group.

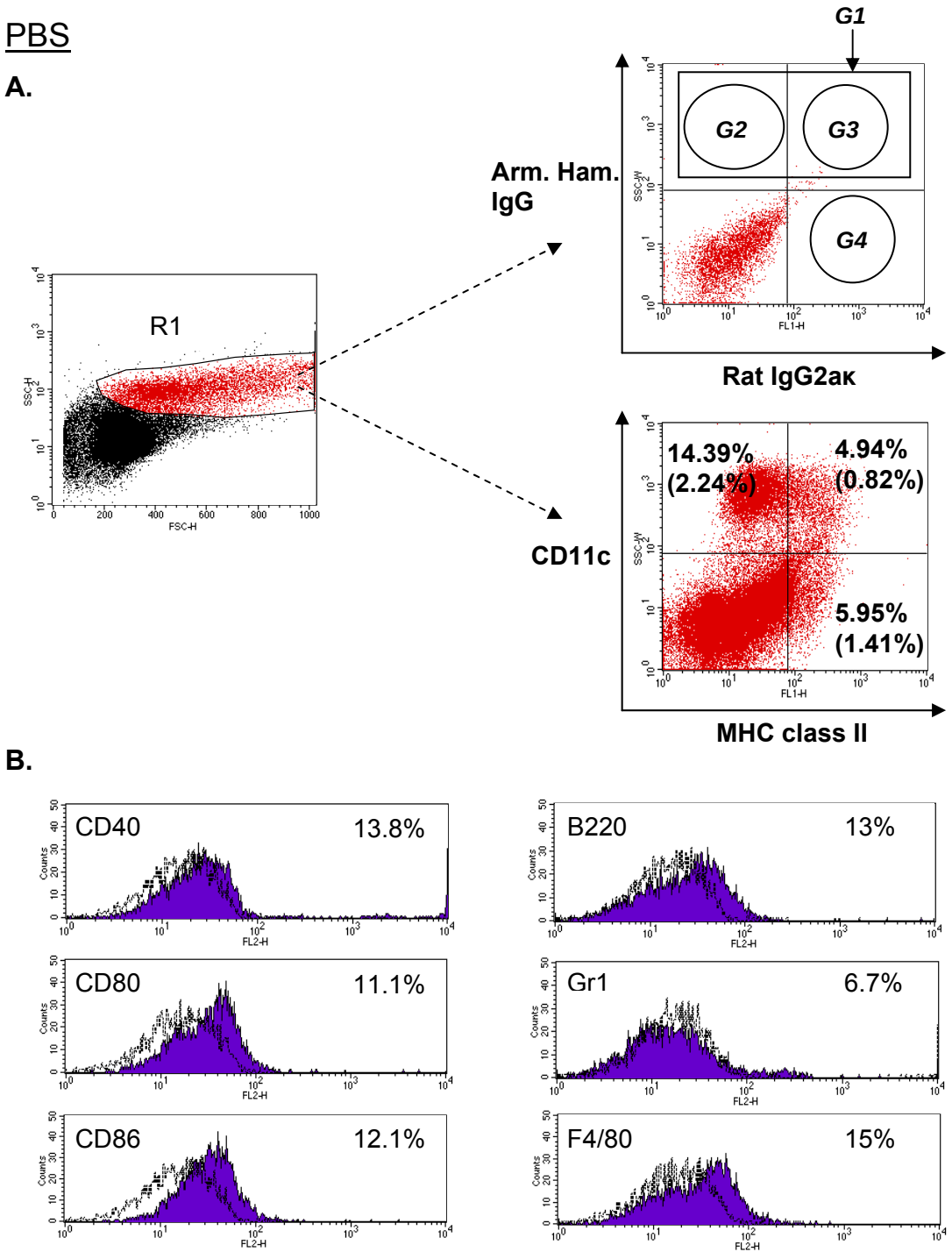


Figure 4.11 (Part A). Lung myeloid cell population analysis 10 days post PBS i.t. instillation

See legend of Figures 4.5 and 4.6. **A)** R1 gate was selected on FCS/SSC plot, analysed for $CD11c^{+}$ cells and was shown to contain more than 90% of total lung $CD11c^{+}$ cells. The cells were analysed for CD11c and MHCII expression and subdivided in 4 gates: $CD11c^{+low}$ (G4); $CD11c^{+high}$ $MHCII^{+high}$ (G3); $CD11c^{+high}$ $MHCII^{+low}$ (G2) and total CD11c (G1). Corresponding isotype-matched control Abs (Armenian hamster [Arm. Ham.] IgG-PE and Rat IgG2aκ-FITC, respectively) to CD11c and MHCII were also used to account for unspecific staining. Numbers in brackets indicates total % of cells (ungated). **B)** Cells were then analysed within G3 for CD40, CD80, CD86, B220, Gr1 and F4/80 expression (blue histograms) and their corresponding isotype-matched Abs (black line); *n*=1 (3 mice/group). Percentage values of positive cells are indicated on top right of the histograms.

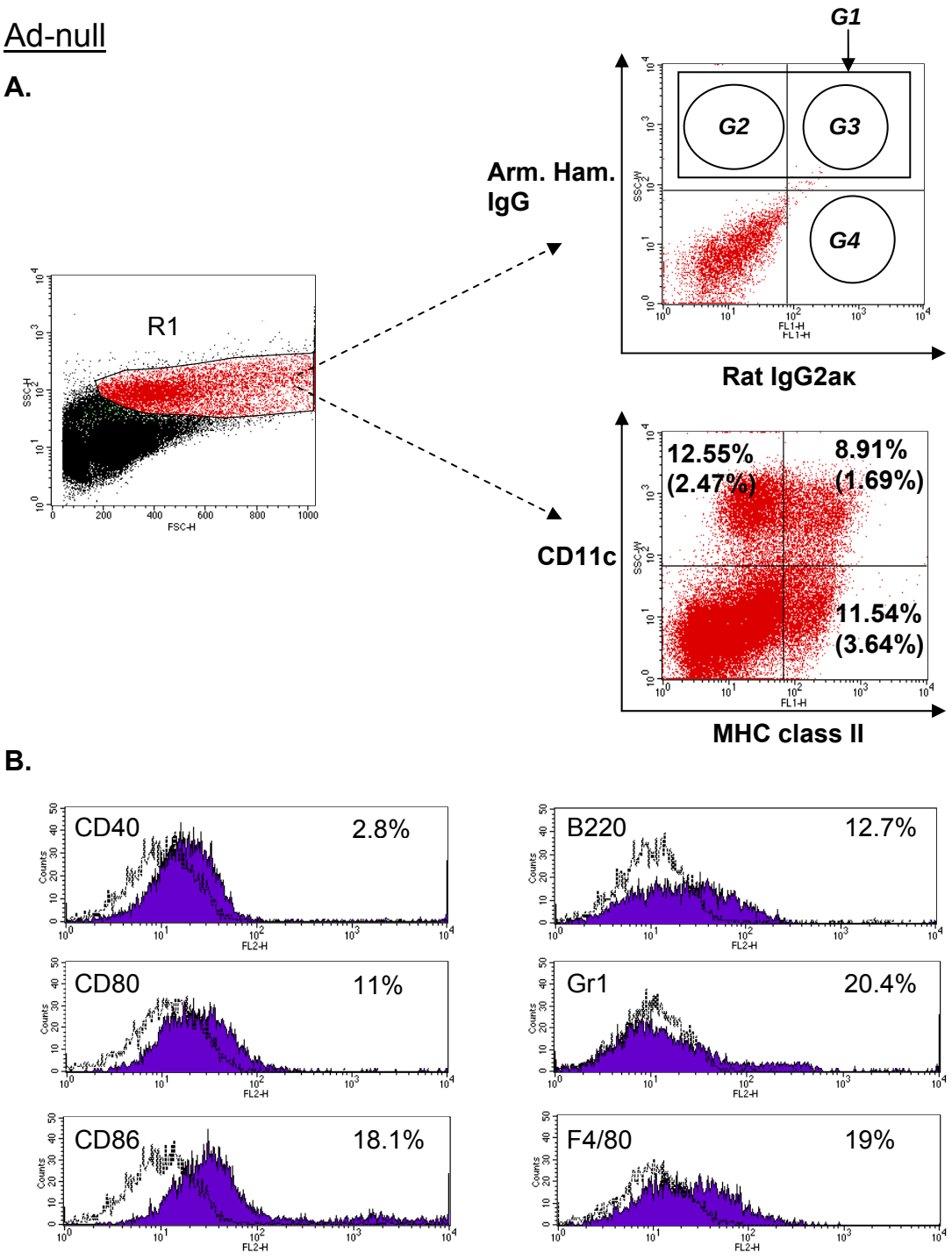


Figure 4.11 (Part B, continued). Lung myeloid cell population analysis 10 days post Ad-null i.t. instillation

See legend of Figures 4.11, Part A. **A)** R1 gate was selected on FCS/SSC plot, analysed for $CD11c^{+}$ cells. The cells were analysed for CD11c and MHCII expression and subdivided in 4 gates: $CD11c^{+low}$ (G4); $CD11c^{+high}$ $MHCII^{+high}$ (G3); $CD11c^{+high}$ $MHCII^{+low}$ (G2) and total CD11c (G1). Corresponding isotype-matched control Abs (Armenian hamster [Arm. Ham.] IgG-PE and Rat IgG2aκ-FITC, respectively) were also used. Numbers in brackets indicates total % of cells (ungated). **B)** Cells were then analysed within G3 for CD40, CD80, CD86, B220, Gr1 and F4/80 expression (blue histograms) and their corresponding isotype-matched Abs (black line); *n*=1 (3 mice/group). Percentage values of positive cells are indicated on top right of the histograms.

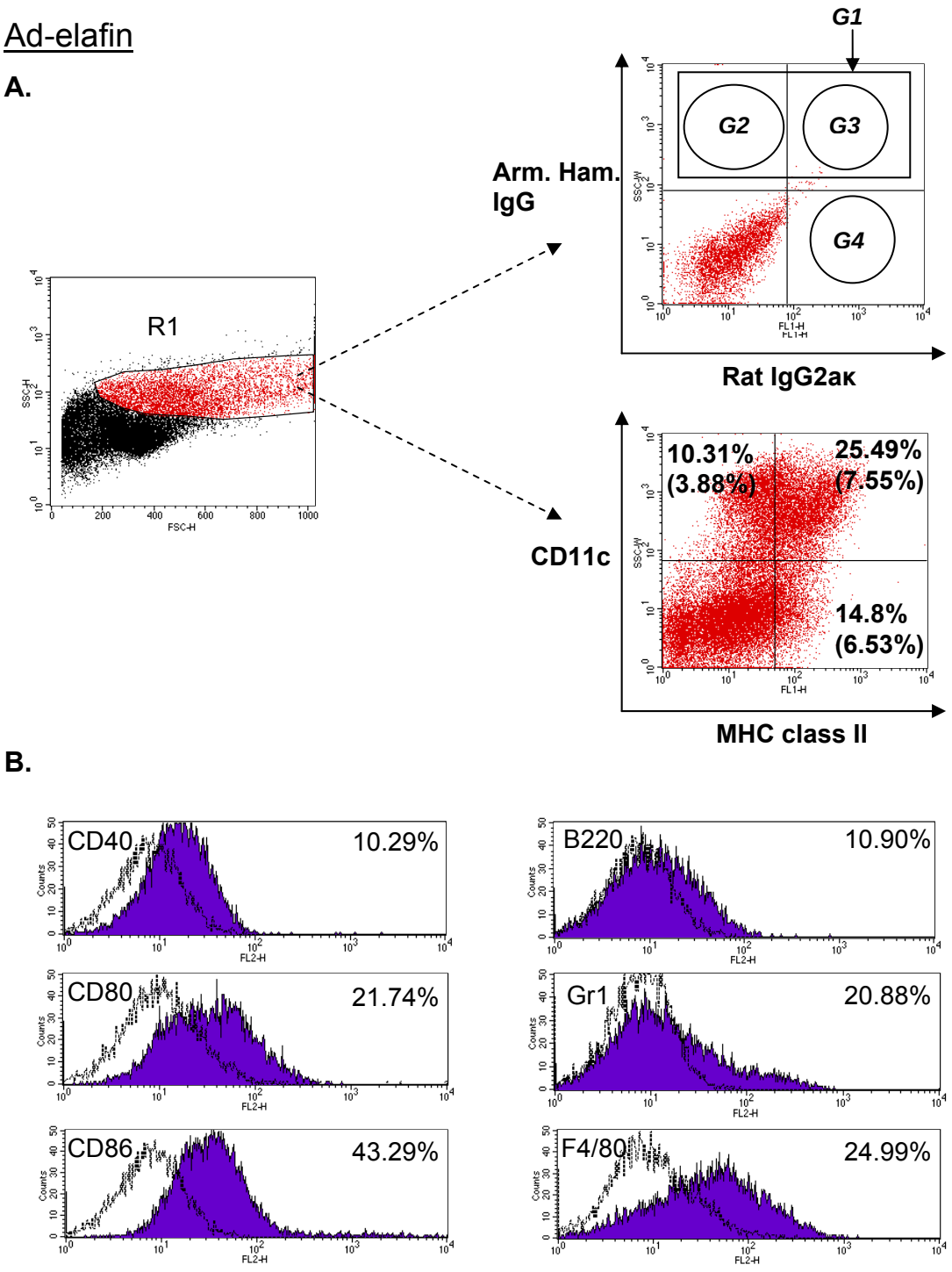


Figure 4.11 (Part C, continued). Lung myeloid cell population analysis after Ad-elafin i.t. instillation

See legend of Figures 4.11, Part A. **A)** R1 gate was selected on FCS/SSC plot, analysed for $CD11c^{+}$ cells. The cells were analysed for CD11c and MHCII expression and subdivided in 4 gates: $CD11c^{+low}$ (G4); $CD11c^{+high}$ $MHCII^{+high}$ (G3); $CD11c^{+high}$ $MHCII^{+low}$ (G2) and total CD11c (G1). Corresponding isotype-matched control Abs (Armenian hamster [Arm. Ham.] IgG-PE and Rat IgG2aκ-FITC, respectively) were also used. Numbers in brackets indicates total % of cells (ungated). **B)** Cells were then analysed within G3 for CD40, CD80, CD86, B220, Gr1 and F4/80 expression (blue histograms) and their corresponding isotype-matched Abs (black line); *n*=1 (3 mice/group). Percentage values of positive cells are indicated on top right of the histograms.

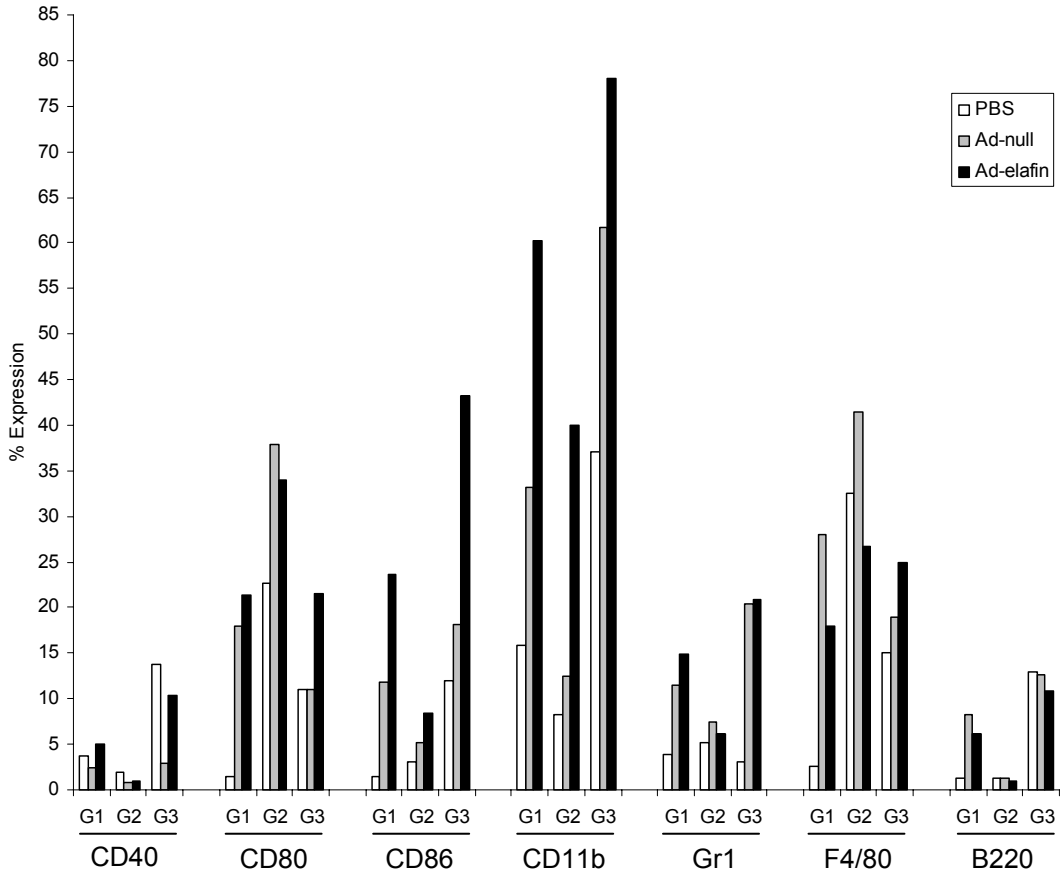


Figure 4.12. WT mouse lungs myeloid population analysis after Ad i.t. instillation

Lungs of WT mice (*n*=3 mice/group) treated at day 0 with PBS, Ad-null or Ad-elafin (2.5×10^8 pfu/mouse) were perfused on day 10 and pooled cells suspensions were prepared. These cells were analysed for CD11c and MHCII expression and were subdivided in 4 gates CD11c 'low' (G4); $CD11c^{+high}$ $MHCII^{+high}$ (G3); $CD11c^{+high}$ $MHCII^{+low}$ (G2) and total CD11c (G1). Cells were then analysed within G1, G2 and G3 for expression of CD40, CD80, CD86, CD11b, Gr1, F4/80 and BB20 compared to their corresponding isotype-matched control Abs.

CD11b (also known as Mac-1 or CR3) is a member of the β2-integrin family of cell surface adhesion molecules, and plays an important role in the initiation of leukocyte adherence to and subsequent migration through vascular endothelial cells. As shown in Figure 4.12, following both Ad-null and Ad-elafin treatment, the expression of this marker was up-regulated on cell populations in G1-G4 gates indicating that these cells originated from myeloid precursors and that these $CD11b^{+}$ cells form a large part of APC augmentation in the lungs. To analyse the expression of CD11b by the lung APCs in more detail, FACS plots of CD11c/CD11b were also produced (Figure 4.13 A). These indicated that Ad treatment on its own was able to change the profile of the CD11b expressing cells and there was an increase in the population of $CD11c^{+high}CD11b^{+high}$ (DCs; 1.55% vs 0.78% respectively) as well as $CD11c^{+low}CD11b^{+high}$ (monocytes/macrophages; 3.46% vs 2.23% respectively) compared to PBS-treated mice (Figure 4.13). However, as Figure 4.13 A demonstrates, compared to mice treated with Ad-null control construct, Ad-elafin-treated mouse lungs showed a dramatic rise in $CD11c^{+high}$ $CD11b^{+high}$ cells (11.76% vs 27.27%, respectively) which are thought to be myeloid DCs. Conversely there was a decrease in the $CD11c^{+low}$ $CD11b^{+high}$ population (monocytes/macrophages) compared to Ad-null group (Ad-null vs Ad-elafin; 26.66% vs 11.44%). Analysis of whole lung cell suspensions (ungated) indicated that compared to Ad-null group, Ad-elafin-treated mice had increased numbers of $CD11c^{+high}$ $CD11b^{+low}$ (DC precursors) and $CD11c^{+high}$ $CD11b^{+high}$ myeloid DCs (2.35% vs 4.89; 1.55 vs 7.16%), whereas there was not a considerable difference in $CD11c^{+low}$ $CD11b^{+high}$ populations (monocytes/macrophages) in these groups (3.46% vs 3.0%).

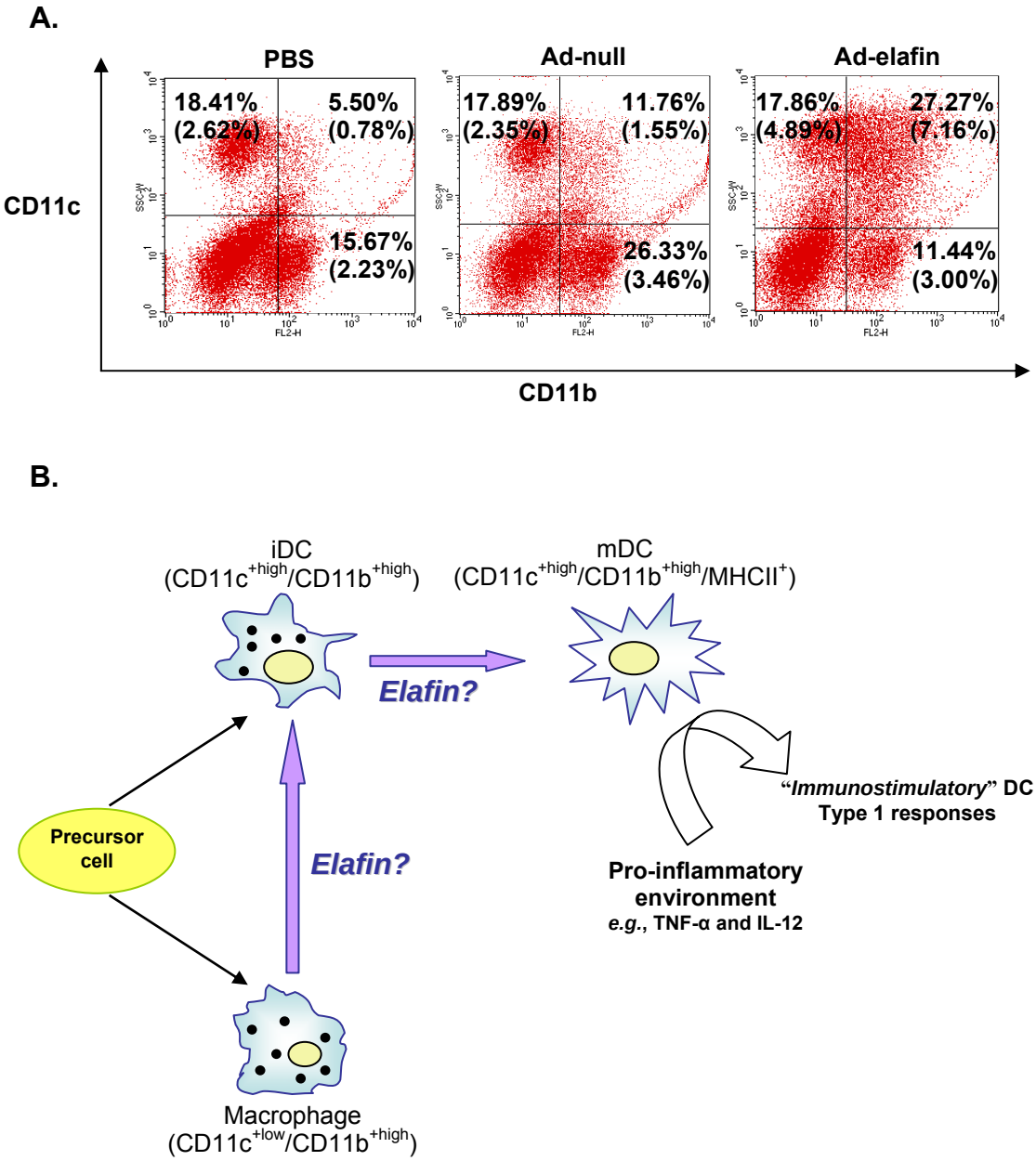


Figure 4.13. Lung CD11b expression analysis 10 days post Ad i.t. instillation

See legends of Figure 4.6 and Figure 4.11. **A**) The digested whole lung cells (pooled from 3 mice/group) were analysed (R1) for CD11c and CD11b expression and subdivided in 4 gates. Corresponding isotype-matched control mAbs were also used to account for unspecific staining (not shown). Numbers in brackets indicates total % of cells (ungated). **B**) Schematic diagram showing how elafin could influence differentiation of monocytes/macrophages ($CD11c^{+low}$ $CD11b^{+high}$) into iDCs ($CD11c^{+high}$ $CD11b^{+high}$) and mDCs ($CD11c^{+high}$ $CD11b^{+high}$ $MHCII^{+high}$) following over-expression in lung tissue.

4.2.1.3.2. The effect of endogenous elafin over-expression (eTg) on lung APCs

To examine whether the elafin eTg mice also behaved in the same way to those Ad-elafin-treated WT mice, eTg mice as well as their corresponding WT controls were treated with Ad-null (2.5×10^8 pfu/mouse) or PBS and their lungs were examined 10 days post treatment, as above.

As expected, since mice do not have an elafin ortholog, elafin was not detected in the BAL fluids of naïve eTg mice receiving PBS or WT control groups receiving either PBS or Ad-null, whereas Ad-null increased elafin secretion in the eTg mouse lungs (4120.09 pg/ml ± 1151.04; *n*=4 mice). As observed above, when BAL differential counts were performed 10 days post treatment, as shown in Table 4.2, Ad-null-treated eTg mice showed increased percentage of lymphocytes compared to the controls. In addition, eTg mice treated with Ad-null had increased BAL fluid neutrophil percentage, whereas there was a reduction in percentage of myeloid cells, although it did not reach significance. Following whole lung cell analysis, compared to the three control groups, Ad-null-treated eTg mice contained higher numbers of $CD11c^{+high}$ $MHCII^{+high}$ and $CD11c^{+high}$ $CD11b^{+high}$ cells (Figures 4.14 and 4.15), although their numbers were not as high as the ones observed in WT mice receiving Ad-elafin (see above). Furthermore, total $CD11c^{+}$ cells (G1) in Ad-null-treated eTg lungs expressed higher levels of CD11b, CD80 and CD86 compared to Ad-null-treated WT and PBS-treated eTg mouse lungs (data not shown). Due to the low recovery, it was not possible to analyse the CSMs in the $CD11c^{+high}$ $MHCII^{+high}$ DC population of eTg mice (G3).

Table 4.2. Bronchoalveolar lavage cell differential (WT/eTg mice)

On day 0, elafin transgenic (eTg) mice and their WT controls were treated i.t. with either PBS or Ad-null (2.5×10^8 pfu). On day 10, mice were sacrificed, BAL was carried out and cell differential analysis was blindly performed (in order to prevent biasing) by Prof. Sarah Howie after centrifugation (2000 × g for 5 minutes). Cytospinning (100 × g for 3 minutes at room temperature) was performed and slides were stained with Diff-quick. *n* represents the number of animals used in the experiment and results are expressed as % of total BAL cells (Values represent mean and standard deviations [in brackets]).

	n	Neutrophils	Myeloid cells	Lymphocytes
WT PBS	3	0.82 (0.98)	99.18 (0.98)	0 (0)
eTg PBS	4	2.14 (2.10)	98.56 (0.24)	0.48 (0.55)
WT Ad-null	2	1.24 (0.04)	96.39 (1.11)	2.38 (1.07)
eTg Ad-null	5	13.09 (21.7)	80.07 (19.66)	6.96 (3.37)

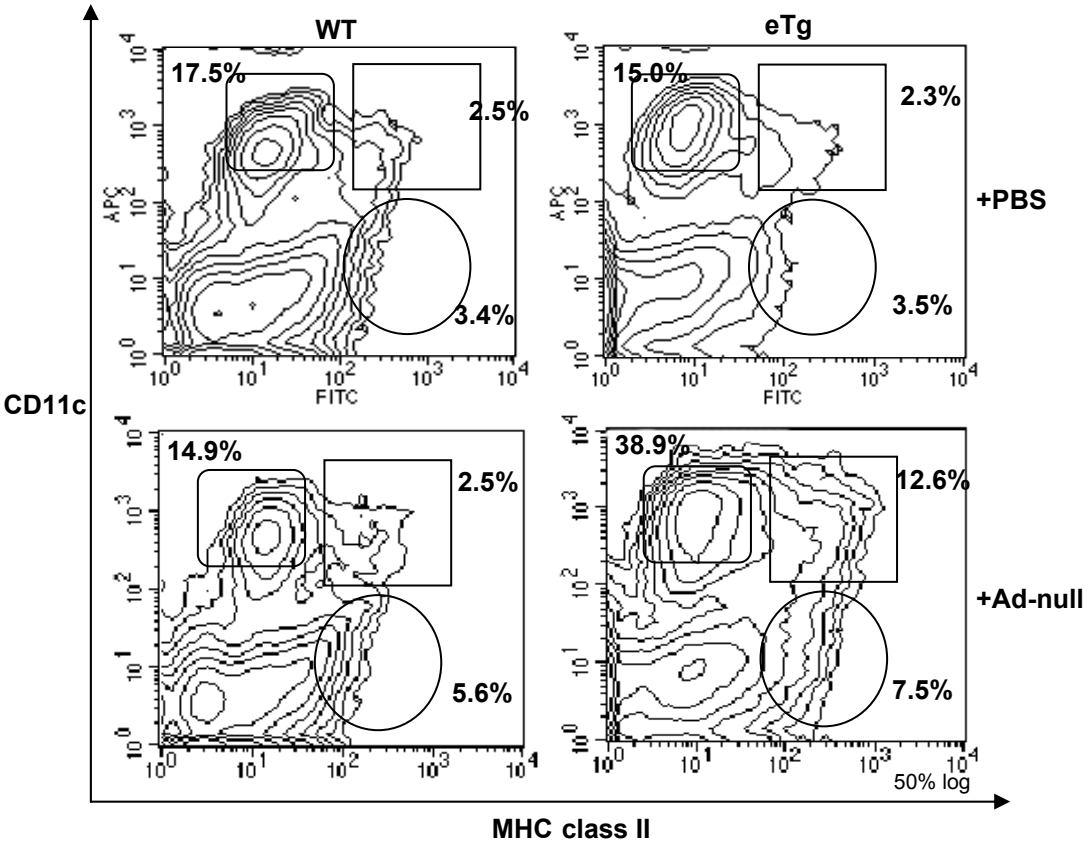


Figure 4.14. eTg mouse lungs myeloid population analysis

See legend of Table 4.2, above. On day 0, eTg mice and their WT controls were treated i.t. with either PBS or Ad-null (2.5×10^8 pfu). The digested lung single cells in R1 (same as above, see Figure 4.11) were analysed for CD11c and MHCII expression. Corresponding isotype-matched control Abs (Armenian hamster [Arm. Ham.] IgG-PE and Rat IgG2aκ-FITC, respectively) to CD11c and MHCII were also used to account for unspecific staining (not shown). Data are representative of 2-3 independent experiments each containing cells pooled from 2-4 mice/group.

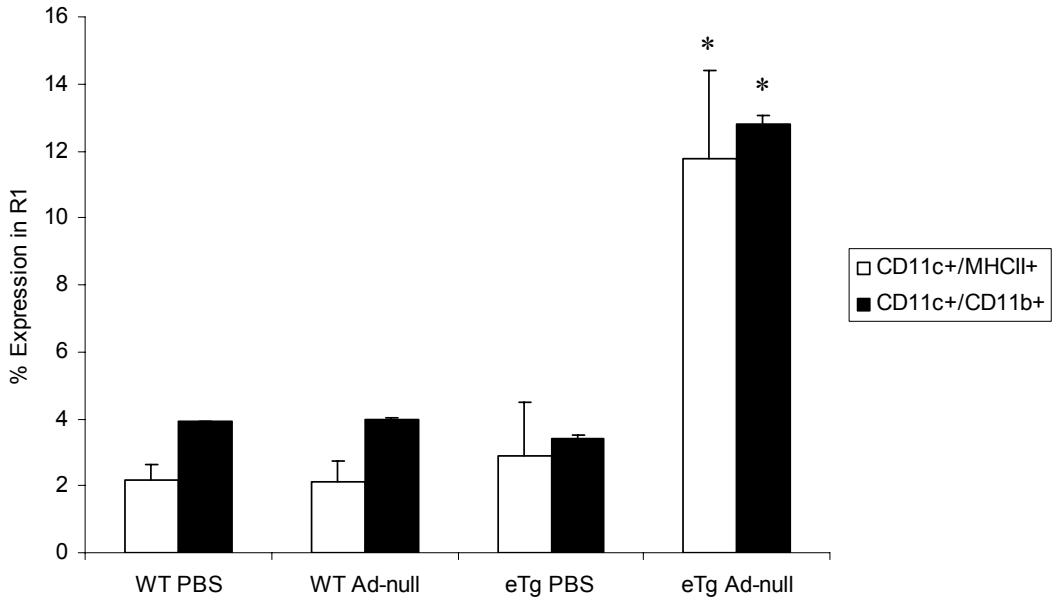


Figure 4.15. Percentage expression of APCs in eTg mouse lungs

See legend of Table 4.2 and Figure 4.14. R1 gate was selected on FCS/SSC plot, analysed for $CD11c^+$ cells and was shown to contain more than 90% of total lung $CD11c^+$ cells. The cells were analysed for CD11c, CD11b and MHCII expression. Data are representative of 2-3 independent experiments each containing cells pooled from 2-4 mice/group. * indicates statistical significance ($p<0.01$), as compared to the Ad-null-WT and PBS-eTg treatments (unpaired *t*-test analysis).

4.2.2. Ad-elafin over-expression for genetic vaccination

Having demonstrated an enhanced *in vivo* primary response (involving an increase in DC numbers and activation) to Ad in WT mice receiving Ad-elafin and in eTg mice receiving Ad-null (see above), our group endeavoured to investigate further the role of elafin on a secondary immune response. This part of the project was realised by Prof. Jean-Michel Sallenave, Mr. Steve D. Williams and Miss. Tara A. Sheldrake, and that my contribution was limited to the preparation and histological analysis of the lung sections to examine lung $CD11c^+$ cell numbers following Ad vaccination. PBS, Ad-null, Ad-elafin and Ad-GMCSF ('positive' control) were administered by i.t. on day 0 and the mice were challenged on day 14 with Ad-LacZ (by the same route), to allow us to assess the efficiency of Ad-elafin vaccination by measuring β-galactosidase expression. Three days later (day 17 of experiment) mice were sacrificed and samples analysed (Figure 4.16). Using the Ad-elafin vaccination and Ad-LacZ challenge protocol as a convenient read-out, our group showed that Ad-elafin treatment was able to induce blocking Abs against Ad-LacZ *in vitro*, and significantly prevented consequent Ad-LacZ infection of murine lungs *in vivo* (see Roghanian *et al*. [2006b] for details).

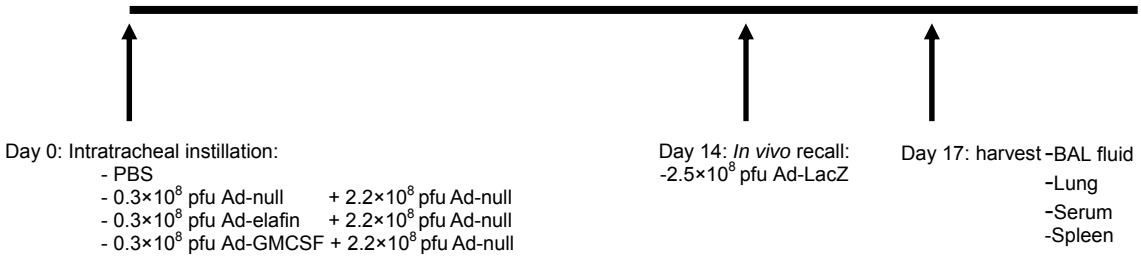


Figure 4.16. Protocol for investigating the role of elafin as a mucosal vaccine adjuvant

To assess the role of elafin on a secondary immune response, WT mice (3-5 mice/group) were treated on day 0 as shown above. Ad-GMCF was included as a positive control. On day 14 of experiment all mice were treated with a second dose of Ad (Ad-LacZ) and were sacrificed on day 17 and various parameters were analysed as shown.

To follow up from previous *in vivo* Ad-elafin gene transfer studies, the presence of lung DCs in this vaccination protocol was studied by immunohistochemistry (Figure 4.17). In line with the FACS data investigating the effect of elafin on lung APCs (see above), many more $CD11c^+$ cells (labelled 'red' with PE; see open arrow) were evident in the airways and in the lung interstitium of Ad-elafin- and Ad-GMCSF-treated mice, as compared to the PBS and Ad-null groups (Ad-elafin vs Ad-null: p=0.0328). Furthermore, these APCs seem to localise around the airways and show the presence of numerous typical dendrites (as indicated with white arrow-heads) suggesting an activated phenotype. PE-conjugated isotype-matched control Ab was also used on histological section and was shown to be negative (not shown).

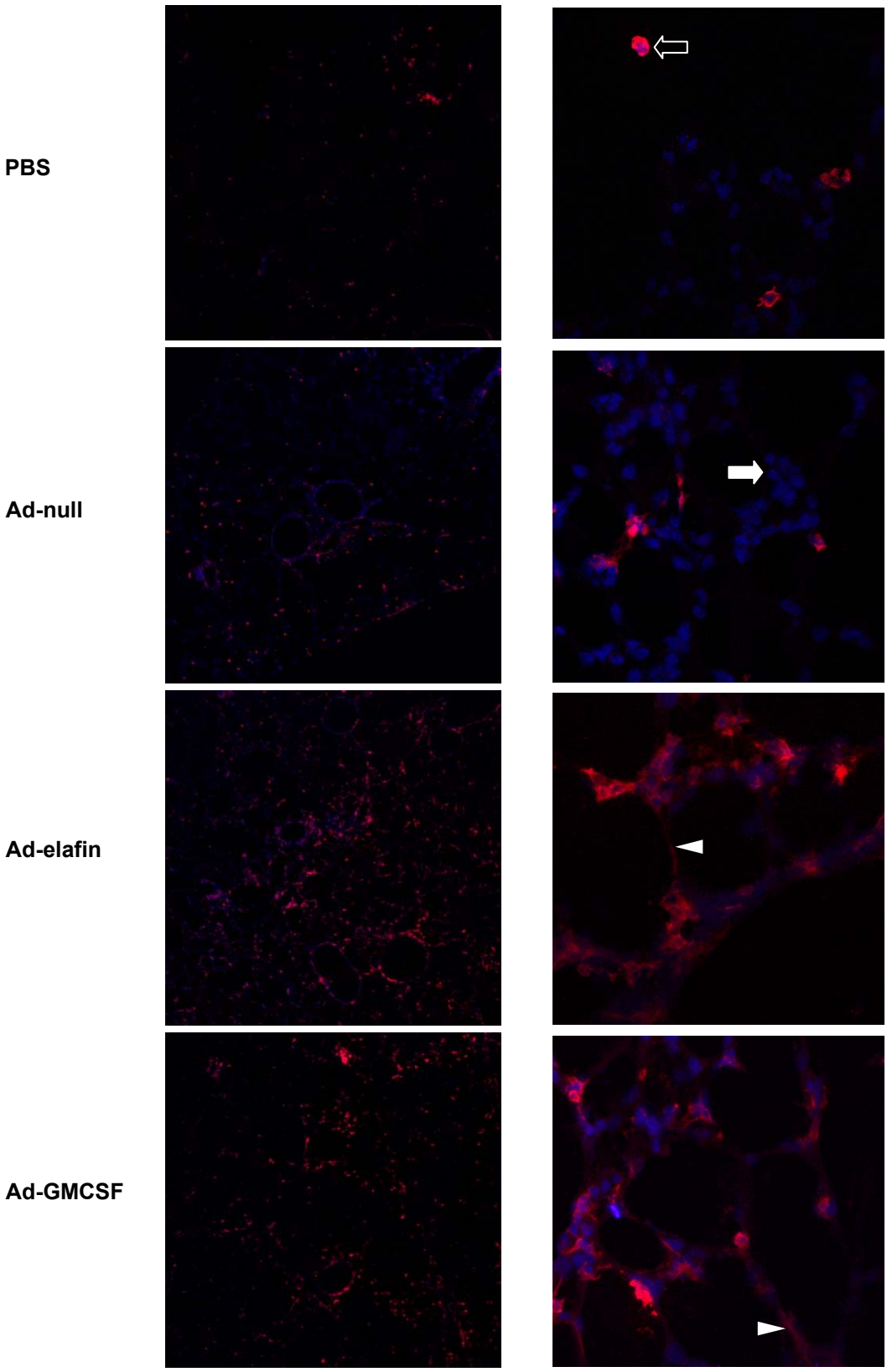


Figure 4.17 (Part A). Histological analysis of CD11c$^+$ cells in lungs given Ad constructs (see next page for figure legend)

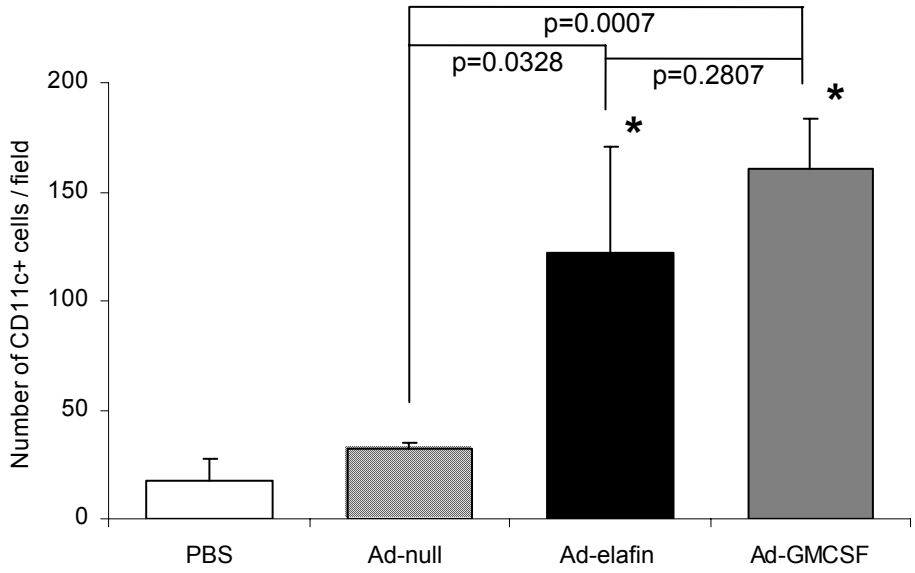


Figure 4.17 (Part B, continued). Histological analysis of CD11c$^+$ cells in lungs given Ad constructs

Mice received i.t. either PBS, Ad-null (0.3×10^8 pfu), Ad-elafin (0.3×10^8 pfu), or Ad-GMCSF (0.3×10^8 pfu) as adjuvant treatments and were simultaneously immunised with Ad-null (2.5×10^8 pfu) via the i.t. route at day 0 (apart from PBS controls). All mice received Ad-lacZ (2.5×10^8 pfu) at day 14 and were culled on day 17. Lungs were obtained and tissue sections (15μm) were cut from OCT-inflamed lungs using a microtome with a cabinet temperature at -30° C, mounted on polylysine-coated slides and fixed with 100% methanol. **A)** Cells were labelled with PE-conjugated hamster anti-mouse CD11c (white arrow) and the nuclei were counterstained with TO-PRO-3. Digital images were acquired with a confocal microscope at various magnifications. White arrow-heads indicate extended dendrites in lungs treated with Ad-elafin and Ad-GMCSF. Representative confocal images of ×5 (left panel) and ×83 (right panel) magnifications are shown. **B)** Quantification of CD11c$^+$ cells was done by counting three fields per experimental group taken using a 10× magnification. Representative figures are shown (*n*=2 mice/group).

4.3. DISCUSSION

The principal novel observation arising from the data in this chapter is that the antimicrobial NE inhibitor 'elafin' is capable of affecting DC phenotype and function, and by doing so could bridge the innate and adaptive branches of the immune system. Discussion of the results presented in this chapter will be divided into two separate sections, as follows.

4.3.1. The *in vivo* effects of elafin on lung APCs and adaptive immunity

Our group has previously shown that the antimicrobial/NE inhibitor elafin, when over-expressed in an Ad vector, is able in an acute model to induce an inflammatory cell influx after lung LPS treatment (Simpson *et al*., 2001a). In that study, a very small dose of Ad-elafin (0.3×10^8 pfu), which was apparently non inflammatory by itself, was able, in conjunction with LPS, to induce the influx of neutrophils and macrophages in the lung 24 hours after LPS treatment (5 days post Ad-treatment). This increase was shown to be due to the LPS-mediated up-regulation of the murine CMV promoter in the Ad-elafin construct (which contains many NF-κB sites), resulting in increased secretion of elafin in the lung. The activity of elafin on innate immune cells was confirmed *in vitro*, using Ad vectors (Simpson *et al*., 2001a), as well as *in vivo*, by using a non-viral approach where newly characterised eTg mice were given LPS i.t. and the lung innate immune parameters were studied 24 hr later (Sallenave *et al*., 2003). Altogether, these results suggested that in addition to its role in an acute model of innate immunity, elafin, a molecule also up-regulated by bacterial LPS and early wave cytokines such as IL-1 and TNF in human epithelial cells (Sallenave *et al*., 1994), may be involved in the initiation of mucosal events leading to adaptive immunity. Therefore, as a model system, our group went on to examine the influence of elafin on immunity against adenovirus (Ad), a well researched pathogen as a potential tool in gene therapy (Volpers *et al*., 2004; Liu *et al*., 2003; Yang *et al*., 1995; Yang *et al*., 1994) and itself a potential infectious agent implicated in chronic lung diseases such as COPD and emphysema (Higashimoto *et al*., 2005; Robbins *et al*., 2004; Hayashi, 2002; Hogg, 2001). Using the dual system

of elafin expression described above (Ad-elafin and eTg mice), it was demonstrated that elafin induces a type 1-biaised inflammatory and immunological response (cellular and humoral) in the lungs and spleens of mice over-expressing elafin (Roghanian *et al*., 2006b). Indeed, in conditions such as psoriasis[1], where a type 1 cytokine pattern predominates (Toichi *et al*., 2006; Lew *et al*., 2004), high concentrations of elafin are found (Pfundt *et al*., 2000; Nonomura *et al*., 1994; Schalkwijk *et al*., 1993); which indicate that elafin might be involved in the skewing of Th1 responses in this situation. Interestingly, in agreement with this, in an earlier study where psoriatic plaque skin-derived DCs were used to support immune responses to resting peripheral blood T cells, these psoriatic plaque skin-derived DCs mediated a Th1 T cell response with high levels of IL-2 and IFN-γ but not IL-4 or IL-10, compared to blood DCs and healthy dermal DCs (Nestle *et al*., 1994).

In order to demonstrate how elafin is able to bridge between the innate and adaptive immunity in the lung, the decision was then taken to examine whether the effects described for elafin were likely to be mediated through the increase in numbers and/or activation status of lung APCs. Firstly, it was necessary to adapt the best protocol to analyse the murine lung APCs, which also supported simultaneous phenotypical analysis of other lung leukocyte populations (see above). By analysing whole lung single cell suspensions, in agreement with von Garnier *et al*. (2005), $CD11c^{+high}$ $MHCII^{+high}$ cells were defined as bona fide lung DCs and $CD11c^{+high}$ $MHCII^{+low}$ cells as lung monocytes/macrophages, the latter population expressing higher levels of the F4/80 marker than $CD11c^{+high}$ $MHCII^{+high}$ cells. Using the Ad-elafin system of delivery (Figures 4.10 and 4.11), it was established that elafin over-expressers exhibited higher numbers of total lung $CD11c^{+high}$ cells and $CD11c^{+high}$ $MHCII^{+high}$ cells (DCs). In addition, Ad-elafin treatment induced higher levels of CD80 and CD86 in the latter cell population, showing that DCs were activated, in accordance with the increased levels of lung TNF-α and IL-12 (a pro-inflammatory cytokine produced abundantly by activated DCs) observed in BAL fluids of treated animals (Figures 4.8 and 4.9 respectively). IL-12 activates a transcription factor,

[1] A common inflammatory skin disease that involves infiltration of leukocytes, activation of skin-resident cells and increased production of numerous cytokines, chemokines and inflammatory molecules. A type 1 cytokine pattern predominates in psoriasis.

STAT4, in Th0 cells that results in IFN-γ production and differentiation into the Th1 phenotype, enhancing cell-mediated immunity against airway infections caused by viruses, mycobacteria, fungi, and some parasites (Michailowsky *et al*., 2001; Hogan *et al*., 1998; Wakeham *et al*., 1998; Hoag *et al*., 1997). Incidentally, mice receiving Ad-elafin also had significantly higher concentrations of IFN-γ in their BAL fluid compared to controls (Figure 4.9), which is in agreement with increased levels of BAL fluid IL-12 and higher percentage of lymphocytes detected in these animals (Table 4.1). Of note, the level of CD40 was low in PBS-treated mice and was not induced by Ad treatments (Figures 4.11 and 4.12). This is in agreement with data published by Dodge *et al*. (2003) showing that murine pulmonary DCs expressed low levels of CD40.

Murine DCs have been traditionally identified by their expression of CD11c and in addition, DC subsets have been described, based on the expression of CD11b, CD8α, and CD45R/B220 (Bjorck, 2002; Nakano *et al*., 2001; Bjorck, 2001). Hence, the presence of plasmacytoid DCs (pDCs) (CD45R/B220) was investigated and it was found that more than 90% of CD45R/B220 cells were present in the $CD11c^{+high}$ $MHCII^{+high}$ cells population (Figures 4.11 and 4.12). However, there was no difference in the % expression of this marker on $CD11c^{+high}$ $MHCII^{+high}$ cells between the experimental treatments, PBS, Ad-null and Ad-elafin (12.95, 12.7, and 11.46 %, respectively), suggesting that elafin does not modulate pDCs.

In the present study the levels of the myeloid marker CD11b on different cell populations (G1-3) was also measured and it was shown that CD11b was elevated on Ad-elafin-treated mouse APCs (Figures 4.11 and 4.12). To analyse the expression of CD11b in more details FACS plots of CD11c/CD11b were generated (Figure 4.13 A). These indicated that compared to mice treated with Ad-null, Ad-elafin-treated mouse lungs showed an increase in $CD11c^{+high}$ $CD11b^{+high}$ cells (11.76% vs 27.27%, respectively); supporting the argument that the precursor cells responsible for the increase in DC numbers following elafin augmentation are myeloid $CD11b^{+}$ monocytes/macrophages (Figure 4.13 B). These observations are further strengthened by data published by our collaborators (Wang *et al*., 2000) showing that

Ad-GMCSF promotes the differentiation and activation of a myeloid DC population primarily by acting on macrophages during pulmonary immune responses. This is also supported by earlier studies suggesting the potential of macrophages to differentiate to DCs (Palucka *et al*., 1998; Zou *et al*., 2000; see Chapter 1, Figure 1.4); as seen in elafin-expressing mice in the current study.

To confirm the effects of elafin seen in WT mice, eTg mouse lungs were also examined following Ad infection to induce elafin expression (see above). As shown in Figures 4.13 and 4.14, in line with previous observations seen in Ad-elafin-treated WT mice, following the induction of the endogenous elafin in eTg mouse lungs the percentage and activation status of APCs was greatly increased.

In summary, the experiments presented in the latter part of this chapter, using a dual approach of gene transfer, showed that the antimicrobial/NE inhibitor molecule elafin is able to augment lung APC numbers and their activation status. The adjuvant property of elafin is particularly important in cases where mucosal tolerance has to be overcome. This is the case in the lung environment where a default Th2 response has been demonstrated in steady state conditions (Dodge *et al*., 2003; Akbari *et al*., 2001; Stumbles *et al*., 1998), with lung DCs exhibiting an immature type 2 phenotype. This phenotype, which may have evolved to keep in check potentially damaging uncontrolled type 1 responses to innocuous inhaled non-replicative antigens, needs to be overcome in the presence of replicative pathogens, such as viruses and intra-cellular pathogens. Additionally, since innate immune products such as NE (Chapter 3; Roghanian *et al*., 2006a) and exogenous stimuli such as CS have been shown both *in vitro* (Vassallo *et al*., 2005; Guinet *et al*., 2004; Nouri-Shirazi and Guinet, 2003) and *in vivo* (Robbins *et al*., 2005; Franco *et al*., personal communication) to cause a reduction in DC numbers and maturation as well as a decrease in their antigen-presenting capacity and/or to skew immunity towards type 2 responses (Vassallo *et al*., 2005), the increase in the number of lung DCs and the type 1 responses elicited by over-expressing elafin (Roghanian *et al*., 2006b), may be beneficial in the context of COPD/CF infections and exacerbations.

4.3.2. How does elafin regulate DC numbers and activation status?

The effects of elafin on lung APCs, in particular DCs, presented here, are very encouraging. However, the exact mechanism is not yet understood and requires further investigation. The possible mechanisms by which elafin may be exerting its effects on APCs are listed below.

The increase in lung $CD11c^+$ cell numbers following Ad-elafin treatment suggests that elafin may be a chemoattractant and/or an activator of lung DCs (see Chapter 5). Alternatively, as indicated above (Figures 4.13 and 4.15), locally secreted elafin might be acting on $CD11b^+$ myeloid monocytes/macrophages and induce their differentiation into DCs (see Chapter 1, section 1.2.6).

On the other hand, locally produced elafin may act via other indirect mechanisms, through for example the inhibition of NE. Indeed, data presented in Chapter 3 indicated that NE, is able to down-regulate DC CSMs, and affects negatively their ability to present antigen to lymphocytes (Roghanian *et al*, 2006a). Hence, over-expression of elafin may rescue DC function by restoring DC activation and or prevent NE-mediated DC inactivation.

It could also be argued that Ad-elafin may have migrated from the lung to the regional lymph nodes (LN) and spleen and have directed transgene expression at these immune sites and skewed a Th1 response in the LNs. This has not been investigated with lung delivery of Ad vectors, but is more likely to occur in eTg mice since elafin RNA has been detected in most tissues including the lymphocyte-rich organs, such as the spleen and thymus (Sallenave *et al*., 2003). It is possible that secretion of elafin in the spleen milieu may increase lymphocyte responses, either directly or through activation of splenic DCs.

Recent evidence indicates that activation of DCs involves cellular communication with neutrophils (Megiovanni *et al*., 2006; van Gisbergen *et al*., 2005a and 2005b; Bennouna *et al*., 2005 and 2003). For instance, murine neutrophils

that were stimulated with tachyzoites of *Toxoplasma gondii (T. gondaii)* have been found to induce DC maturation, as well as IL-12 and TNF-α production by DCs; whereas depletion of neutrophils from *T. gondii*-infected mice inhibits IL-12 and TNF-α production upon re-stimulation of DCs with *T. gondii*. In that context, in addition to activated DCs, *T. gondii*-stimulated neutrophils themselves produced IL-12 and TNF-α (Bliss *et al*., 1999a and 1999b). In a related study, Megiovanni *et al*., (2006) demonstrated that human monocyte-derived DCs acquired *Candida albicans*-derived antigens from both live and apoptotic neutrophils and thus elicited antigen-specific T cell responses *in vitro*. Indeed, over-expression of elafin has been shown to significantly augment LPS-mediated neutrophil migration into the airways *in vivo*, and to significantly enhance neutrophil migration *in vitro* (Simpson *et al*., 2001a). Similarly lung neutrophils were increased in elafin over-expressers in the current study, although this was not significant (Tables 4.1 and 4.3). Therefore, it could be argued that chemoattraction of neutrophils to inflammatory tissues by elafin leads to increased local production of TNF-α, leading to DC activation. Figure 4.18 shows the proposed two-step process by which neutrophils induce DC maturation. The first step involves the formation of a cellular contact zone or synapse between neutrophils and DCs through interactions between the β_2-integrin CD11b (Mac-1) and C-type lectin DC-SIGN (van Gisbergen *et al*., 2005b), followed by the second step which is mediated by TNF-α production by neutrophils (Bennouna *et al*., 2005 and 2003).

Last but not least, increased lung APC numbers following elafin over-expression might be indirectly due to the action of GM-CSF in the lungs of these mice. Indeed, TNF-α has been shown to induce the production of GM-CSF by fibroblasts and airway epithelial cells (Leizer T *et al*., 1990; Hashimoto *et al*., 2000; Daffern *et al*., 1999). Relatedly, TNF-α has been shown to stabilise GM-CSF RNA in stimulated cells (Akashi *et al*., 1991). Although in the current study BAL fluid GM-CSF was not measured, these data suggest that the elafin-induced TNF-α production in mouse lungs could also influence the expression of GM-CSF, which could in turn enhance DC numbers and activity by acting on precursor cells, as previously reported (Wang *et al*., 2000). A way to test this hypothesis in future studies would be to co-administer neutralising Abs to TNF-α along with Ad-elafin

and investigate whether or not, in the presence of anti-TNF-α Abs, local elafin production increases lung APCs as demonstrated here.

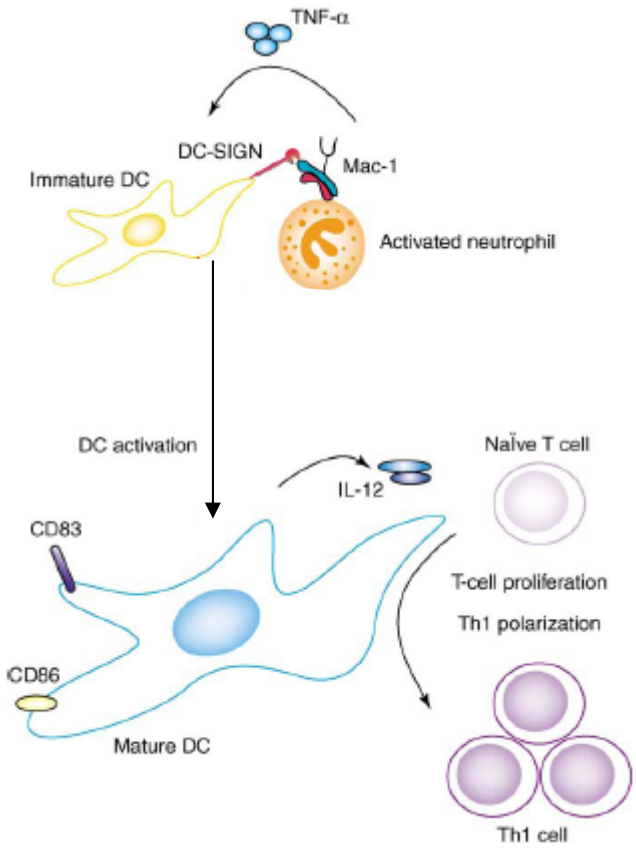


Figure 4.18. Cross-talk between neutrophils and DCs

Activated neutrophils interact with iDCs through binding of receptors such as DC-SIGN by CD11b (Mac-1), to form a synapse that enables transmission of TNF-α from the activated neutrophils to iDCs. TNF-α secretion from activated neutrophils induces maturation of iDCs, which results in up-regulation of CSMs, and the potential of these DCs to strongly stimulate the proliferation of T cells. Activated neutrophils instruct iDCs to mature into IL-12-secreting mDCs that induce Th1 polarisation. Adapted from van Geijtenbeek *et al*., 2005a.

4.4. SUMMARY

The results presented in this chapter demonstrate that Ad-mediated expression of an NE specific inhibitor (elafin) in WT murine lungs, as well as induction of elafin secretion in eTg murine lungs, results in increased number (immunofluorescence) and activation status (flow cytometric measurement) of $CD11c^{+high}/MHCII^{+high}$ lung DCs *in vivo* (as evidenced by higher levels of DC CSMs and higher concentrations of pro-inflammatory cytokines in elafin-producing lungs). These data suggest that elafin is acting either directly or indirectly (*e.g.*, induction of TNF-α) on lung DCs to induce their activation. Alternatively, elafin might be acting as a chemotactic factor for DCs in the lungs (see Chapter 5). In summary these findings suggest that augmentation of the antimicrobial/NE inhibitor elafin is able to bridge the innate and adaptive immunity by activating antigen presenting DCs in the lung and possibly in other mucosal tissues. Figure 4.19 summarises the possible mechanisms by which elafin might be exerting its effects on lung DCs.

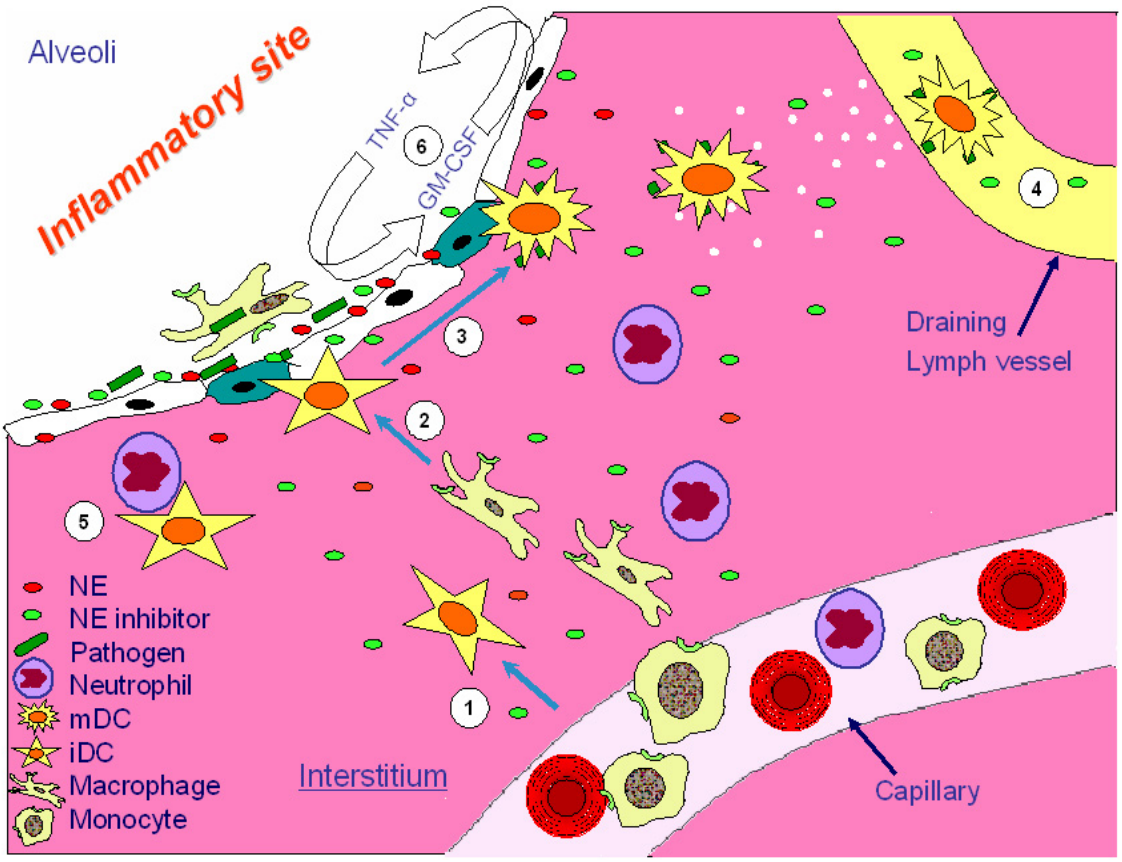


Figure 4.19. Possible effects of elafin augmentation on pulmonary DCs

Schematic diagram demonstrating possible mechanisms by which natural NE inhibitors such as 'elafin' could modulate DCs *in vivo*. **1**) Elafin may be a chemoattractant and/or activator of lung DCs, **2**) elafin might be acting on $CD11b^+$ myeloid monocytes/macrophages and induce their differentiation into $CD11b^+$ $CD11c^+$ DCs, **3**) elafin may rescue DC function by restoring DC activation and or prevent NE-mediated DC inactivation, *e.g.*, cleavage of DC cell surface receptors, **4**) elafin secretion/leakage from the lung to the regional lymph nodes and spleen may increase lymphocyte responses, either directly or through activation of splenic DCs, **5**) Chemoattraction of neutrophils by elafin may result in direct neutrophil:DC interactions and hence increased production of TNF-α by activated neutrophils, leading to DC activation (see legend of Figure 4.18), and **6**) elafin-induced TNF-α production in the lungs could also influence the expression of GM-CSF, which could in turn enhance DC numbers and activity by acting on precursor cells.

CHAPTER 5

IN VITRO EFFECTS OF NATURAL NEUTROPHIL ELASTASE INHIBITORS ON DENDRITIC CELLS

5.1. AIMS AND BACKGROUND

In recent years a number of endogenous antimicrobial molecules (*e.g.*, defensins and LL-37) have been found to profoundly modulate DC function and migration (Yang *et al.*, 1999; Biragyn *et al.*, 2002; Davidson *et al*., 2004). Due to similarities in their expression patterns, structure and antimicrobial properties with natural NE inhibitors (*e.g.*, elafin and SLPI), it was hypothesised that these NE inhibitors could also affect DC responses during infection/inflammation. Moreover, in the preceding results chapter it was demonstrated that the augmentation of an NE specific inhibitor (elafin) could increase lung DC numbers and their activation status (Roghanian *et al*., 2006b). Both SLPI (Nakamura *et al*., 2003; Taggart *et al*., 2005) and α1-protease inhibitor (α1-PI; Spencer *et al*., 2004; Aldonyte *et al*., 2004; Janciauskiene *et al*., 2004) have also been found to have various immunomodulatory properties, rendering them as potential candidates in immunotherapeutic interventions. Thus the primary aim of the work described in this chapter was to investigate the direct effects of respiratory system NE inhibitors on the function and phenotype of DCs *in vitro*. As mentioned in Chapter 4, this was primarily carried out using an adenovirus (Ad)-based strategy. The Ad gene transfer approach uses replication-deficient Ad vectors which are both E1 and E3 deleted, encoding the cDNA for the genes of interest (namely eotaxin, elafin, SLPI and α1-PI used in this part of the project) which are under the control of the powerful murine CMV promoter, to facilitate cellular expression of the transgene (Sallenave *et al*., 1998).

The source of DCs used in this study was the murine (BALB/c) bone marrow (BM), as described in Chapter 3. The experiments described herein were undertaken to investigate the mechanism by which elafin could increase lung DC numbers and activation status; and to examine whether *in vitro* genetic augmentation of NE

inhibitors, namely '*human* elafin', '*murine* SLPI' and '*murine* α1-PI', could modulate DC phenotype and function in a similar fashion to defensins and LL-37 (Yang *et al.*, 1999; Biragyn *et al.*, 2002; Davidson *et al.*, 2004).

5.2. RESULTS

5.2.1. Increasing Ad infection efficiency of DCs *in vitro*

Prior to infection of iDCs with Ad vectors expressing NE inhibitors *in vitro*, since myeloid cells are relatively refractory to Ad infection, the decision was taken to optimise the infection of the cells. To date a few strategies have been described that greatly increase infection efficiency of Ad vectors, some of which are routinely used in our laboratory. These include the use of polycation complexes (Di Nicola *et al*., 2000) such as lipofectamine, calcium phosphate precipitates (Fasbender *et al*., 1998) and the centrifugal methods (Nishimura *et al*., 2001a and 2001b). Employment of such strategies firstly helps to increase the infection efficiency and secondly to reduce the multiplicity of infection (MOI) of Ad needed to infect majority of the cells, hence using fewer immunogenic particles in the experimental system. Since iDCs are highly phagocytic and also are very sensitive to their surrounding microenvironment, it was decided to use the centrifugal method as it is the least manipulative method of the three. In this method, centrifugal force is applied to enhance efficiency of Ad-mediated gene transduction. In this part of the study, Ad-GFP was used to investigate these effects, since GFP expression by infected cells could be viewed by fluorescent microscopy, and also is readily detectable by flow cytometry (on FL1 channel). Figure 5.1 demonstrates that iDCs could be readily infected with Ad vectors (Ad-GFP in this case). Then, to assess the most efficient Ad infection protocol, iDCs were either infected with Ad-GFP at an MOI of 100 on plates or transferred to Eppendorf tubes and infected under centrifugal force, as explained in Chapter 2 (section 2.2.4.2.1). As shown in Figure 5.2, ~70% of the cells cultured on plates were infected with Ad-GFP, compared to ~76% infected by centrifugal force in Eppendorf tubes. Notably, the MFI of the GFP expressing cells following the centrifugal method was 706 (more than double) compared to only 313 in the plate infected DCs. Therefore, it was decided to use the centrifugal method for infecting DCs *in vitro* to achieve higher infection efficiencies in the experiments described throughout this chapter.

It was also important to assess the effect of the centrifugal method on the activation status of DCs and determine whether it had any effects on them. Hence, 24 hours post treatments both uninfected and Ad-GFP infected DCs were stained with mAbs to the CSMs (CD40, CD80 and CD86) and analysed by flow cytometry. As Figure 5.3 demonstrates (for simplicity only CD86 data shown) there was not a notable difference between the plate control uninfected iDCs with the centrifugal control DCs (CD86 MFI 94 vs 111, respectively) indicating that the centrifugal method did not interfere with the downstream experiments, discussed in this chapter. However, control (plate) Ad-GFP-infected DCs expressed lower levels of the CD86 than the centrifugal Ad-GFP-infected cells (MFI 239 vs 312, respectively). Similar results were obtained with CD40 and CD80 (data not shown).

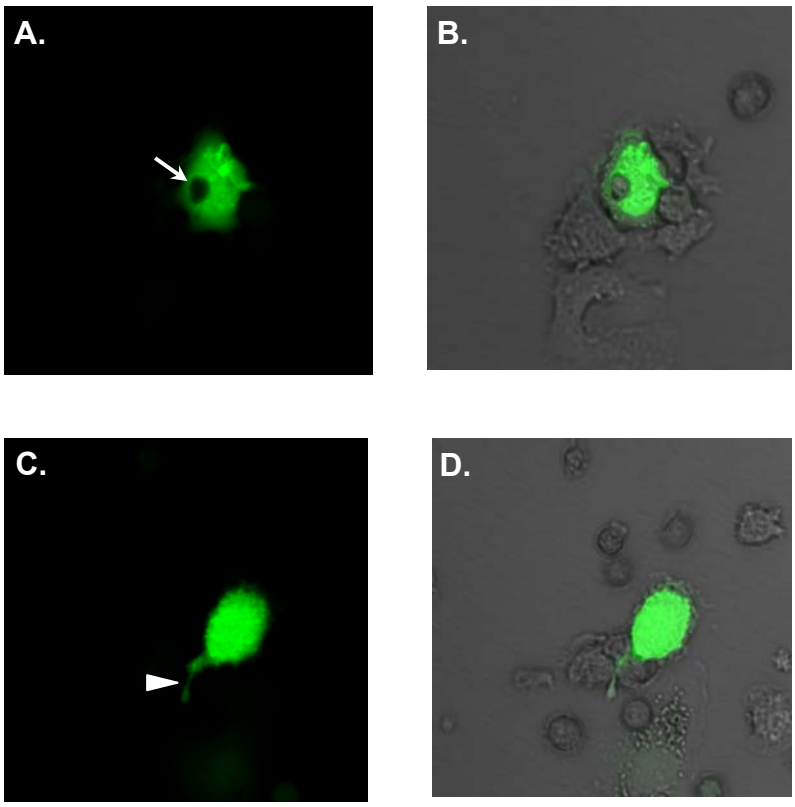


Figure 5.1. Infection of iDCs by Ad

On day 10 of the culture, iDCs were washed with PBS and incubated with Ad-GFP for 1 hour in plates to be infected. They were then washed and supplemented with complete RPMI medium and incubated at 37°C for 24 hours before being examined by fluorescent microscopy. GFP^+ DCs under a fluorescent microscope (×400) are shown above. White arrow indicating nucleus in (**A**) and white arrow head showing DC dendrite in the bottom left picture (**C**). (**B**) and (**D**) show merged light and fluorescent microscope pictures of infected DCs. Representative picture are shown.

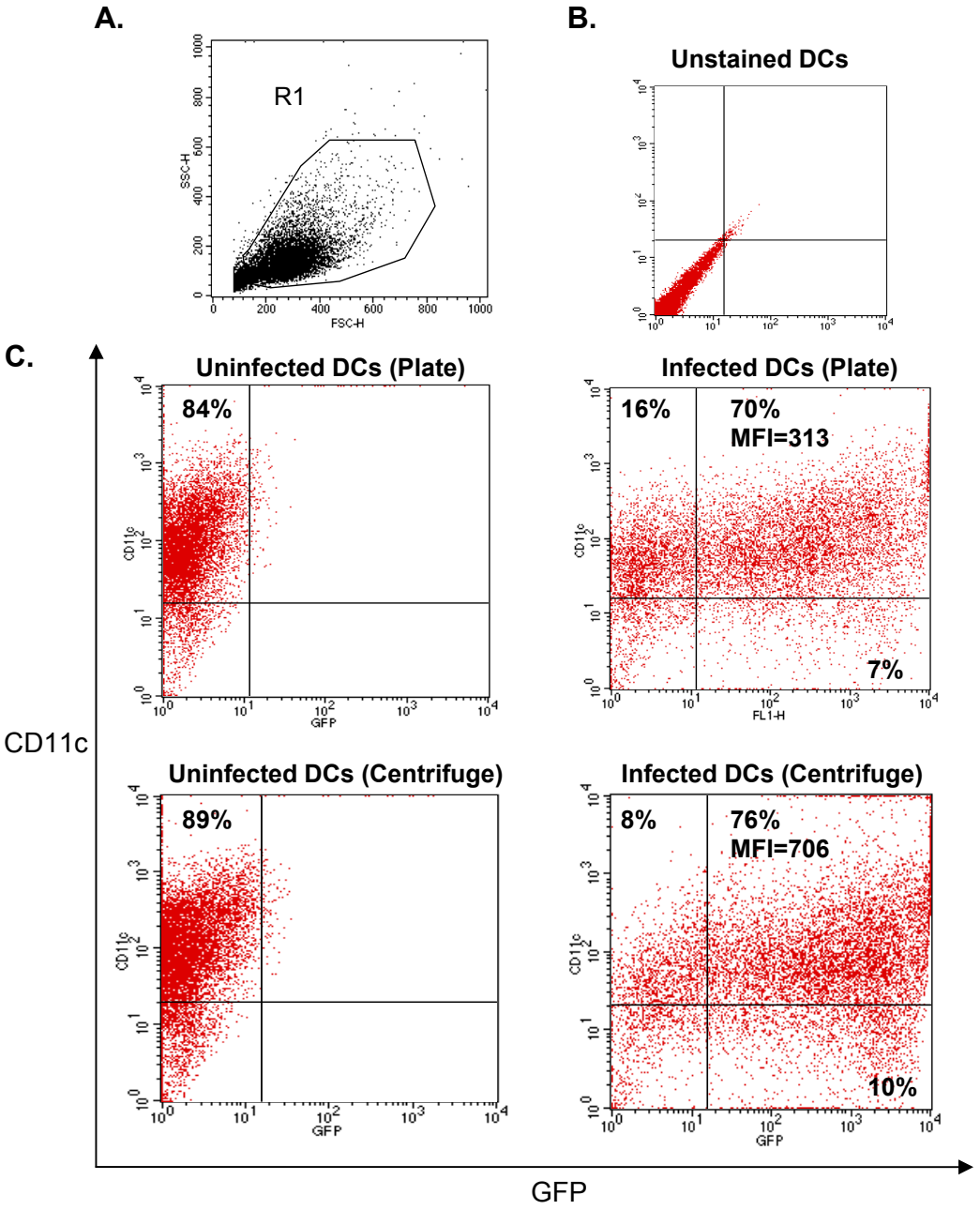


Figure 5.2. Ad-GFP infection of DCs: Centrifugal method to increase infection efficiency

In total 3×10^6 DCs were resuspended in serum-free medium and Ad-GFP used at an MOI of 100 was used. The cells were either cultured in 6-well plates and incubated at 37°C for 1 hour to be infected (top plots) or were transferred to a 1.5ml Eppendorf tube and centrifuged at 2000 × g at room temperature for 1 hour (bottom plots). The infected cells were then washed with PBS to remove free viral particles and resuspended in complete medium containing GM-CSF and incubated at 37°C for 24 hour before being stained with CD11c-APC Ab (isotype-matched Ab staining is not shown) and analysed by FACS. Representative plots of 3 independent experiments are shown; **A**) shows forward and side scatter plot of infected DCs, **B**) shows profile of unstained/uninfected DCs, and **C**) shows profiles of unstained DCs and Ad-GFP-infected DCs using either the conventional plate method (top right plot) or the centrifugal method (bottom right plot).

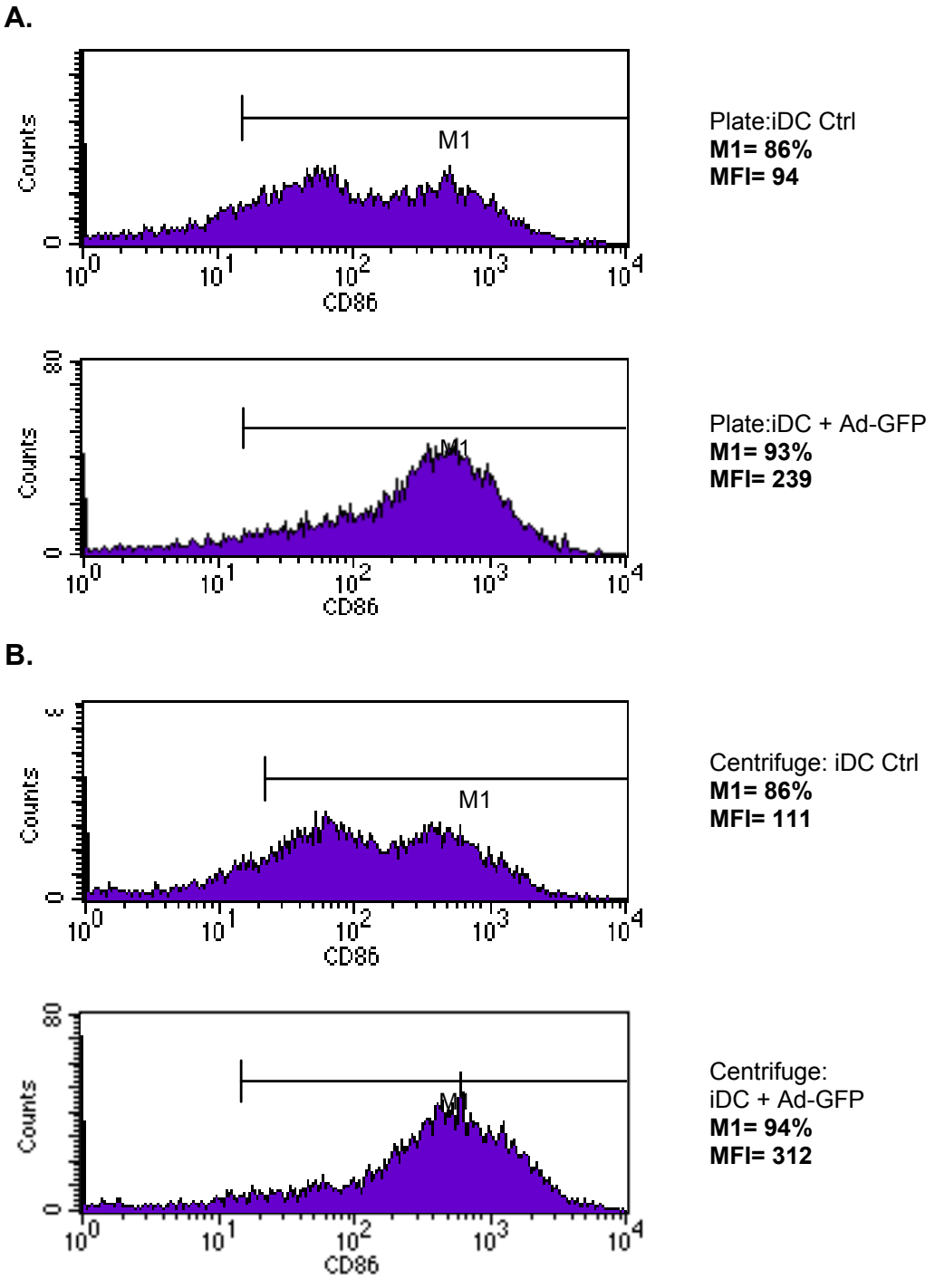


Figure 5.3. Assessment of DC maturation status following infection

DCs were infected with Ad-GFP either in plate or in Eppendorf tube by the centrifugal method, as explained in Figure 5.1 legend, and stained with anti-CD86-PE mAb to assess the activation status of DCs 24 hour following treatment. **A)** Uninfected control (top histogram) and Ad-GFP infected (bottom histogram) DCs cultured in plate, and **B)** Uninfected control (top histogram) and Ad-GFP infected (bottom histogram) DCs using the centrifugal method. Control; ctrl. Representative plots of at least 3 independent experiments are shown.

5.2.2. Optimisation of Ad MOI for DC infection *in vitro*

Once it was established that the centrifugal method greatly enhances the infection efficiency of DCs by Ad-GFP, it was necessary to optimise the MOI of Ad used to infect the cells. As mentioned above, the MOI optimisation would be beneficial for two important reasons; (*i*) using less Ad particles to avoid unnecessary activation of iDCs, and (*ii*) minimising the amount of Ad vector resulting in maximum infection would be more economical. Initial experiments were therefore undertaken to investigate the optimal MOI of Ad for infecting iDCs. Once again, Ad-GFP was used as an indicator of infection and the cells were assessed by fluorescent microscopy and FACS 24 hours following infection. These experiments demonstrated that the optimum MOI of Ad was around 100 where around 70% of the cells were expressing the GFP (Figure 5.4); that using higher MOIs would not be useful.

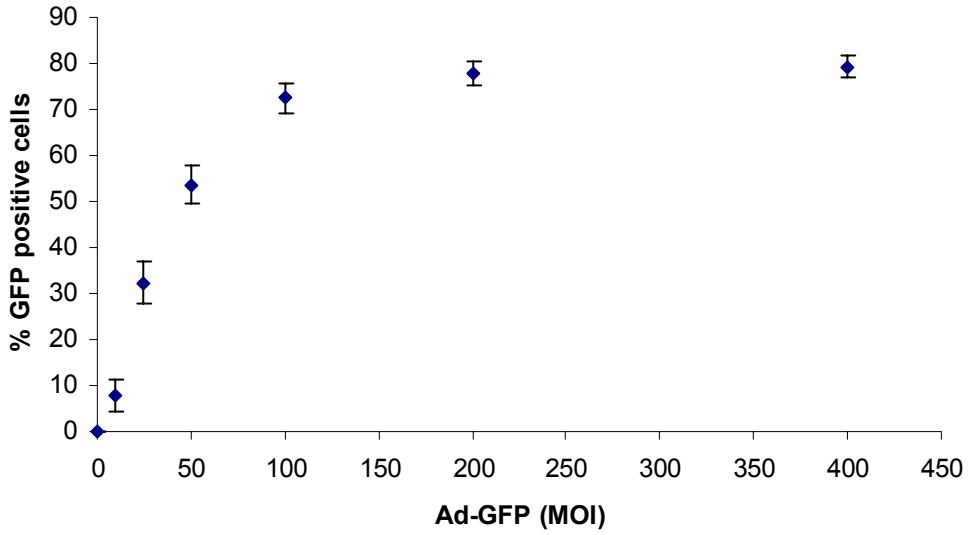


Figure 5.4. Infection of DCs with Ad-GFP

Optimum MOI for DC infection was determined using an Ad-GFP vector. DC enriched populations were infected with increasing MOI of Ad using the centrifugal method for 1 hour as explained in the legend of Figure 5.3. Cells were then washed and incubated at 37°C for a further period of 24 hours. Cells were then harvested and GFP expression determined by flow cytometry. The mean (±SD) of GFP^+ cells from 3 individual titrations was plotted.

5.2.3. NE inhibitors and DCs

To investigate the direct effect of NE inhibitors in isolation on DC activation status, iDCs were infected with Ad vectors expressing one of the three main NE inhibitors (*i.e.*, Ad-elafin, Ad-SLPI/GFP and Ad-α1PI) present in the lung and analysed for expression of the CSMs 24 hours post infection, as described above. In the experiments described here an empty Ad construct (Ad-null) or Ad expressing murine eotaxin (a secreted chemokine of similar molecular mass to elafin and SLPI, with no known effects on DCs) were used as controls. The data from three independent experiments have been summarised in the top graph in Figure 5.5. Compared to uninfected iDCs, the empty Ad-null and Ad-eotaxin (data not shown in this figure, see below) were slightly immunogenic (but not statistically significant), inducing cell activation as assessed by CSM expression levels 24 hours post infection.

It should be noted that, assessment of DCs infected with the Ad vectors showed that Ad-infection did not induce apoptosis/necrosis in DCs as determined by trypan blue dead cell exclusion assay and staining by PI and annexin-V, 24 hours post Ad-infection (not shown).

5.2.3.1. Effect of human elafin on DCs

As shown in Figure 5.5, iDCs infected with Ad-elafin vectors were expressing higher levels of CSMs than the control infected group. Nevertheless, although this observation was consistent, the increased CSMs expression following Ad-elafin infection never reached significance. To assess whether the cells infected with Ad-elafin were able to produce detectable levels of elafin protein, ELISAs were performed on Ad-infected DC supernatants. As shown in Figure 5.6, DCs infected with Ad-elafin secreted detectable levels of elafin in the supernatant. The secretion of elafin was further enhanced by adding LPS/TNF-α to the sample indicating that, as previously observed by our group (Simpson *et al*., 2001a), these mediators could up-regulate the transgene elafin expression presumably by activating the murine CMV

promoter (which is inflammation-inducible; Simpson *et al*., 2001a). Of the two stimuli, TNF-α had the most potent effect and almost doubled the elafin concentration produced by infected DCs. Since murine cells do not code for the elafin gene, as expected, uninfected iDCs and Ad-null-infected cells did not produce any detectable elafin protein in their supernatants. We had previously shown that full-length elafin is able to bind both smooth and rough forms of LPS and modulate consequent macrophage responses to LPS (McMichael *et al*., 2005b). However, it should be noted that Ad-elafin-infected DCs matured (as measured by staining for CD40, CD80, CD86 and MHCII surface markers) in the same way as Ad-null-infected DCs in response to LPS/TNF-α, indicating that the presence of elafin does not (at least at these concentrations) affect DC responses to these stimuli (data not shown).

Since the iDC Ad-elafin experiments were not conclusive, further attempts were made to elucidate whether purified/recombinant elafin was able to directly affect the phenotype and function of DCs *in vitro*. However there was not sufficient material available in-house to perform properly controlled experiments at the time this study was undertaken. To circumvent this hurdle, human elafin was purified from supernatants of Ad-elafin-infected epithelial cells. However, these experiments were hampered by the findings that the purified elafin also contained contaminations such as endotoxins and immunoglobulin (Ig) fragments (washed from the affinity chromatography column) which interfered with the DC activation assay (data not shown) and made it impossible to interpret the data.

Previously, our group had detected the cDNA for elafin in various tissues (including the BM and blood) of the eTg mice generated in house (Sallenave *et al*., 2003). Therefore, in a parallel experiment, BM cells were taken from eTg mice and their WT control littermates, and cultured with GM-CSF to generate DCs (as previously described in Chapter 3). The eTg- and WT-derived iDCs were then stimulated with LPS for a further period of 24 hours and assessed by flow cytometry to investigate whether endogenous elafin production would influence DC differentiation and phenotype. As shown in Figure 5.7, DCs generated from eTg

mice matured in a similar manner to WT-derived DCs in response to LPS stimulation. It should be mentioned that elafin could not be detected by ELISA in supernatants of eTg iDCs/mDCs. Overall these experiments showed that there were no significant differences in CSMs levels expressed on iDCs and mDCs generated from eTg compared to CSM levels of iDCs and mDCs which were derived from WT mice control group.

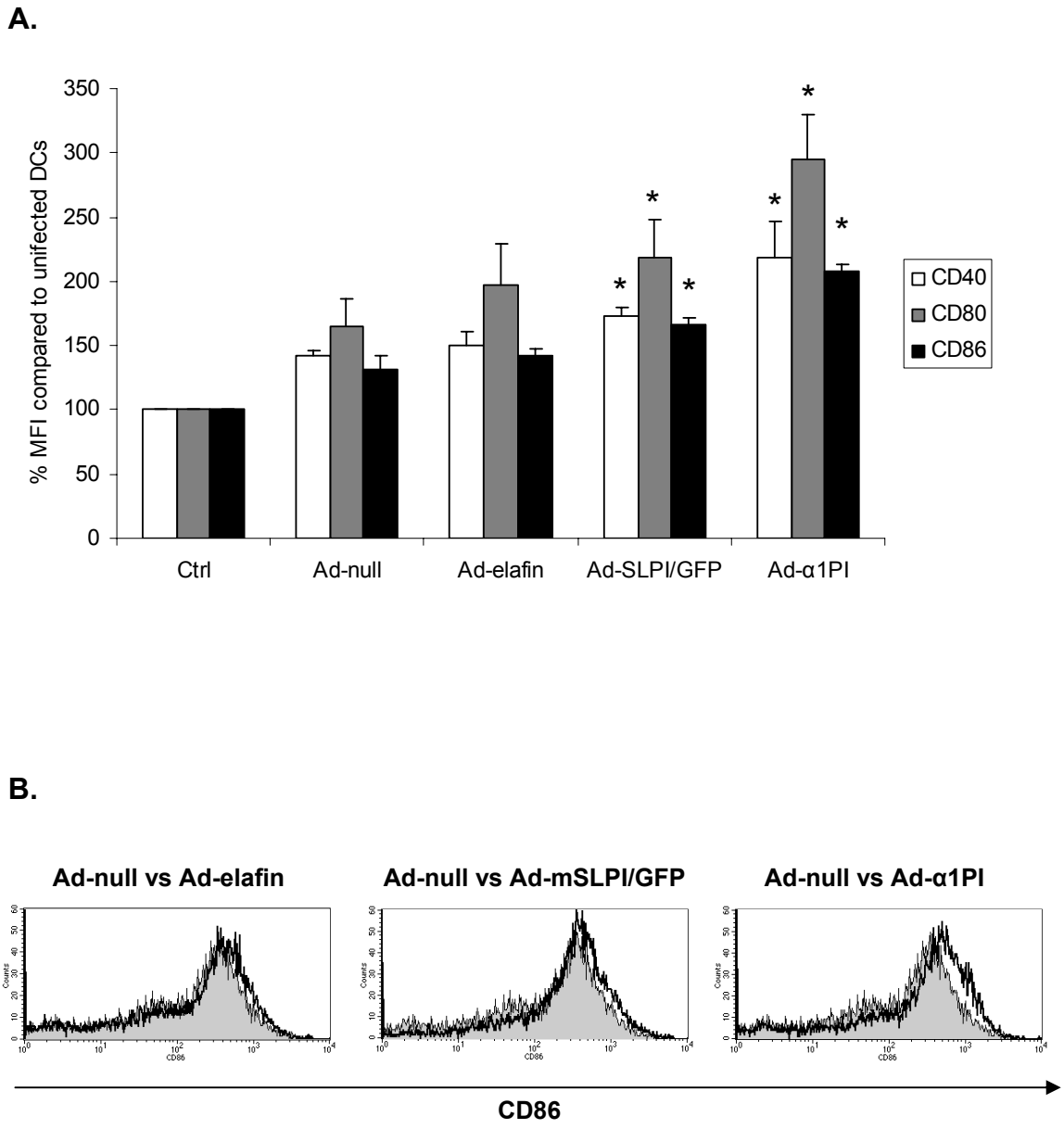


Figure 5.5. Infection of iDCs with Ad expressing NE inhibitors

On day 10 of the culture iDCs were infected with MOI 100 of Ad-elafin, Ad-SLPI/GFP or Ad-α1PI as explained in the legend of Figure 5.3 Ad-null and Ed-eotaxin were used as control for background Ad infection. Twenty four hours post infections cells were washed with PBS and stained with CSM Abs and analysed by flow cytometry. **A)** Graph showing DC CSMs expression following infection by Ad vectors (MFI ± SD of three independent experiments). **B)** Representative FACS histograms for CD86 (left; Ad-elafin, centre; Ad-mSLPI/GFP and right; Ad-α1PI [open black histograms] vs Ad-null [filled grey histogram]). * indicates statistical significance ($p<0.05$) compared to control Ad-null infected DCs; $n=3$ independent experiments.

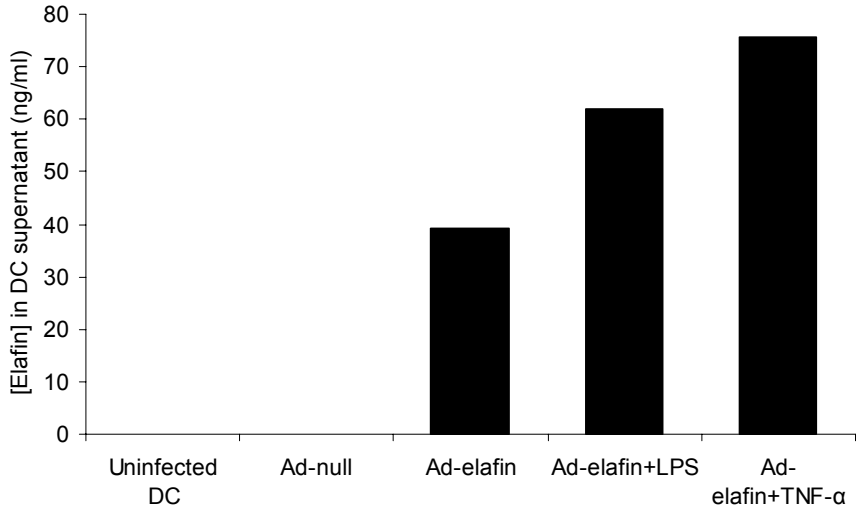


Figure 5.6. Elafin concentrations in infected DC supernatant

10^6 iDCs were infected with MOI 100 of either Ad-null or Ad-elafin on day 10 of the DC culture as previously described. To some Ad-elafin infected cells either LPS or TNF-α (at a final concentration of 100ng/ml and 5ng/ml respectively) were added at the same time of infection. Supernatants were collected 24 hours post treatment and were analysed by elafin ELISA for presence of secreted elafin. Representative data shown (*n*=2).

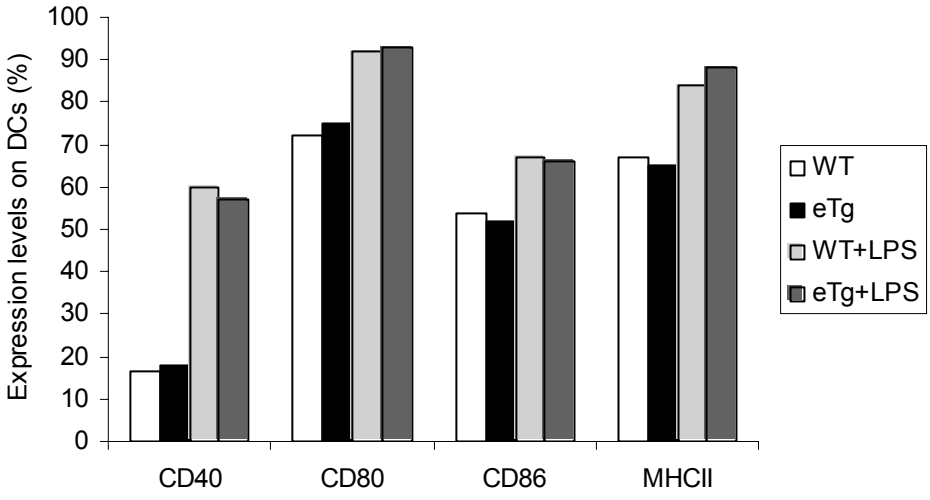


Figure 5.7. Generation of DCs from WT and eTg mice

DCs were generated from BM cells taken from eTg or WT mice (2 mice per group) by culturing cells in complete medium containing GM-CSF, as described in Chapter 2. On day 10 the cells were stimulated with 100ng/ml LPS and 24 hours post treatment the cells were washed and stained with FACS Abs to CSMs. Representative data of 3 independent experiments are shown.

5.2.3.1.1. Chemoattraction of iDCs by elafin

The experiments presented above showed that direct infection of iDCs with Ad-elafin does not induce significant up-regulation of their CSMs, failing to provide any direct link between elafin and DC activation (as measured with CSMs). However, *in vivo* data presented in Chapter 4 clearly demonstrated that elafin over-expression in the lungs of WT and eTg mice resulted in increased number of activated DCs (expressing high levels of CD80 and CD86). Another possible mechanism by which elafin could be involved in the up-regulation of the lung DC numbers is chemoattraction of DCs. To test this hypothesis, the murine Clara (bronchial epithelial) DJS2-2 cell line was employed, as this cell line has been derived from the lung tissue and is a suitable *in vitro* model in this case. Clara cells were infected with Ad-elafin or Ad-null vectors in serum^{-ve} medium containing 0.1% BSA and supernatants were collected 48 hours post infection and placed in the bottom chamber of the polyvinyl uncoated trans-well (5μm pore) plates. Uninfected Clara cell supernatant and the chemokine MIP-3α (a known ligand for CCR6 expressed by iDCs; Dieu *et al*., 1998) were used as the negative and positive controls, respectively. Freshly generated iDCs (1×10^5) were then washed and resuspended in serum^{-ve} medium and placed on the top chamber of the trans-well and incubated for 90 minutes at 37°C. Following incubation, iDCs remaining in the top chambers, iDCs adhered onto the bottom side of the trans-well membranes, and iDCs that had migrated to the bottom chambers were analysed and counted, as described in Chapter 2 (section 2.2.4.2.5.).

Although not controlled for random movement of iDCs (chemokinesis), Figure 5.8 demonstrated that higher number of iDCs were detected in the bottom chambers when elafin-containing Clara media or MIP3α were used, and conversely lower number of iDCs were detected in the top chambers in these groups, compared to Ad-null-Clara medium and Clara medium alone (Figure 5.8 A). On the other hand, when the trans-well membranes were examined, it was found that higher number of cells had migrated through the membranes and had adhered to the lower side of the membranes in elafin-Clara and MIP3α groups (Figure 5.8 B). It should be noted that

this experiment was only performed once and unfortunately there was not enough time and recombinant elafin protein to further optimise this system. Nevertheless, these preliminary data, currently pending further conclusive investigations, are encouraging and hint towards potential effects of elafin on iDCs migration.

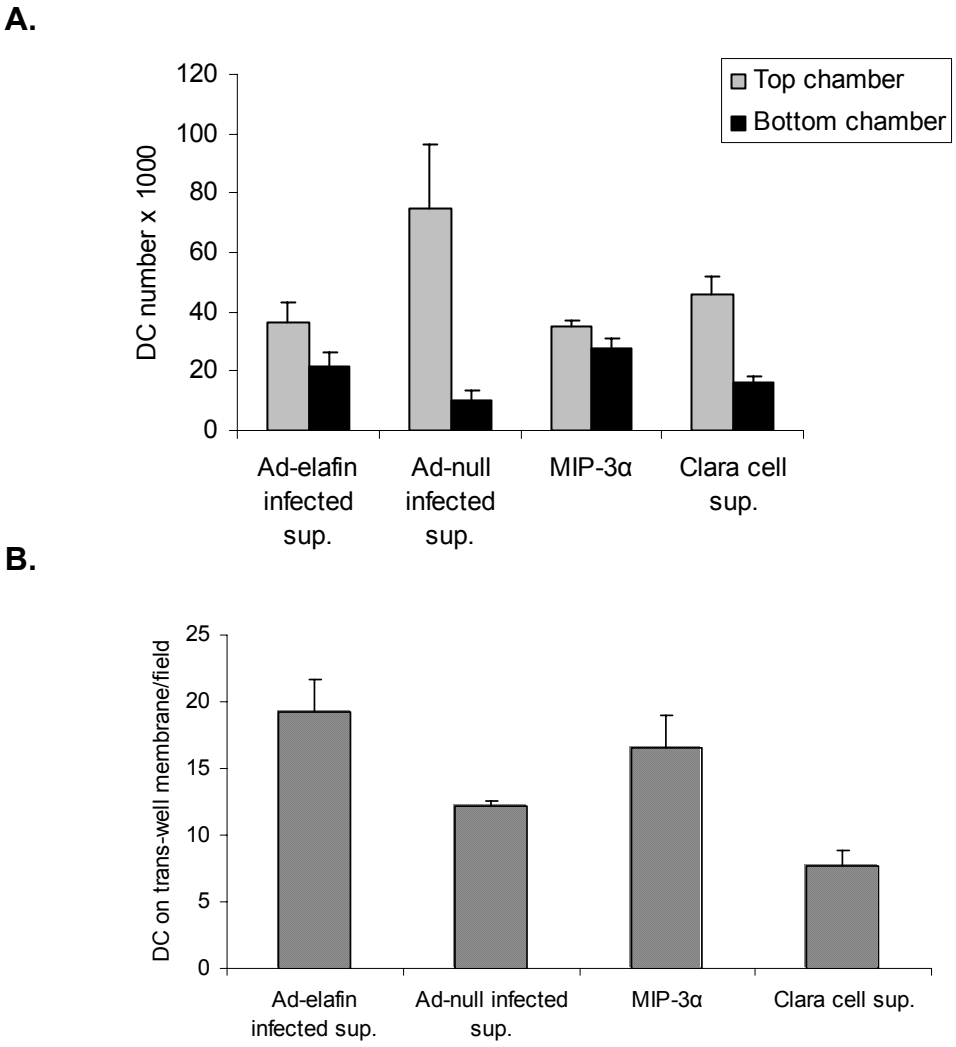


Figure 5.8. Elafin chemoattracts iDCs in transmigration assay

To investigate possible chemotactic properties of elafin on iDCs, Clara cells were infected with MOI 100 of Ad-elafin or Ad-null in serum^{-ve} DMEM medium for 48 hours and 600µl of the collected supernatants were pipetted in the lower chamber of the trans-well Costar plate.10^5 iDCs were resuspended in 100µl DMEM medium containing 0.1% BSA and placed in the upper chamber. The plate was then incubated at 37°C for 90 minutes. Following incubation, **A)** iDCs remaining in the top chambers, iDCs that had migrated to the bottom chambers, and **B)** iDCs stuck onto the bottom side of the trans-well membranes were analysed and counted, as described in Chapter 2 (section 2.2.4.2.5.). Uninfected Clara cell supernatant and the chemokine MIP-3α (100ng/ml in Clara sup.) were used as the negative and positive controls, respectively; *n*=1, duplicate wells.

5.2.3.2. Effect of murine SLPI on DCs

iDCs infected with Ad construct expressing the murine SLPI as well as GFP (Ad-SLPI/GFP), expressed significantly higher levels of CSMs compared to Ad-control infected DCs (Figure 5.5). Since the infected cells also expressed low levels of GFP which was earlier shown to activate iDCs (see Figure 5.3), these observations had to be interpreted with caution and, as for Ad-elafin-infected DCs, were not conclusive. However, recombinant murine SLPI was available in-house and made it possible to investigate the direct effect of this protein on DCs. On day 10 of culture, iDCs were treated with various concentrations of murine SLPI (10-1000 nM) ± LPS (100ng/ml) for 24 hours and analysed by FACS. SLPI treatment of DCs did not affect the DC phenotype at all and LPS was able to fully mature the cells irrespective of the SLPI concentration/presence (data not shown). This is despite the fact that SLPI has previously been shown to suppress monocyte/macrophage responses to bacterial LPS (Taggart *et al*., 2002; Yang *et al*., 2005).

To mimic the experiments performed by Davidson *et al*. (2004) in which investigators showed that LL-37-derived human DCs displayed significantly higher endocytic capacity, modified phagocytic receptor expression and function, up-regulated CSM expression, enhanced secretion of Th1-inducing cytokines, therefore promoting Th1 responses *in vitro*; as opposed to adding recombinant SLPI or infecting cells with Ad-SLPI/GFP on day 7, BM cells were also cultured in the presence of murine SLPI (10-1000 nM) from day 0 of culture. SLPI-derived DCs were counted and analysed on day 10. In addition SLPI-derived DCs were stimulated with LPS and were compared to conventionally generated DCs. However, no differences were observed between all these treatments upon the presence of SLPI in the culture medium (data not shown).

5.2.3.3. Effect of murine α1-PI on DCs

The murine α1-PI expressed by the Ad-α1PI construct was the most potent of the three NE-inhibitors transduced into iDCs, inducing the greatest significant

increase in CSMs and MHCII compared to control Ad-infected iDCs (see Figure 5.5 and below). To confirm that Ad-α1PI was able to infect DCs efficiently and that the transduced cells were able to transcribe and secrete the murine α1-PI protein, supernatants taken from the infected cells were analysed by Western blotting for the presence of α1-PI protein. There is ~60% aa identity between the human and murine α1-PI proteins (Borriello and Krauter, 1991). Therefore, human recombinant α1-PI protein (52kDa) and rabbit anti-human α1-PI Ab (available in the laboratory) were used to detect the murine α1-PI protein in these samples. Preliminary experiments were undertaken to optimise the system (see Chapter 2, section 2.2.4.2.4 for experimental details) and also to determine whether or not the Ab could be used to detect murine α1-PI by Western blot analysis. Indeed the anti-human α1-PI Ab was also able to react with murine α1-PI protein, which is ~51-53 kDa in size (Churg *et al*., 2003; Berger and Baumann *et al*., 1985); when supernatants were analysed from Ad-α1PI-infected cells, murine α1-PI was readily detected (Figure 5.9). The slight difference between the molecular weight of human and murine α1-PI proteins in Figure 5.9 could be due to different glycosylation patterns (post-translational modifications) or the difference in the amino acid compositions of these homologous glycoproteins. Indeed, the mature form of human α1-PI consists of up to 12% carbohydrates by weight, which varies depending the source and preparation of this glycoprotein (Kolarich *et al*., 2006).

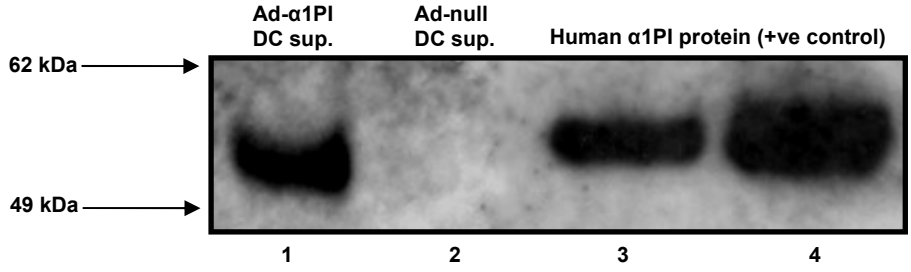


Figure 5.9. Detection of α1-PI by infected DCs

DCs were infected with either MOI 100 of Ad-α1PI (lane 1) or Ad-null (control; lane 2) and were incubated at 37°C for 48 hours allowing them to produce and secrete detectable concentrations of α1-PI. Supernatants were collected at the end of the experiment and were kept at -20°C to be analysed by Western blot. To detect the secreted α1-PI protein, infected DC supernatants were concentrated 10× and ran on a NuPAGE 4-12% Bis-Tris polyacrylamide gel, transferred onto a nitrocellulose membrane and probed with rabbit anti-human α1-PI which was shown to also recognise murine α1-PI in preliminary experiments. Recombinant human α1-PI was used as control in these experiments (lanes 3 and 4, 20ng and 40ng loaded respectively). Representative Western blot of 2 independent experiments is shown.

Secondly, to rule out the potential artefactual nature of DC activation observed in Figure 5.5, it was deemed necessary to rule out the presence of endotoxins in the viral samples used to infect the iDCs in these experiments and in *in vivo* experiments described in Chapter 4. A quick and efficient way to investigate this was to employ the RAW 264.7 murine macrophage cell line which is very sensitive to endotoxins and routinely used in our laboratory (McMichael *et al*., 2005b). Therefore macrophages were stimulated with Ad constructs or LPS, for 4 hours and the supernatants were analysed for TNF-α by ELISA. These experiments showed that macrophages did not release significant concentrations of the pro-inflammatory cytokine TNF-α in response to MOI 100 of Ad vectors compared to PBS treated (Ctrl) cells (Figure 5.10). This suggests that they either did not contain any endotoxins or their levels were minimal in the Ad samples and could not interfere with the parameters analysed in the experiments.

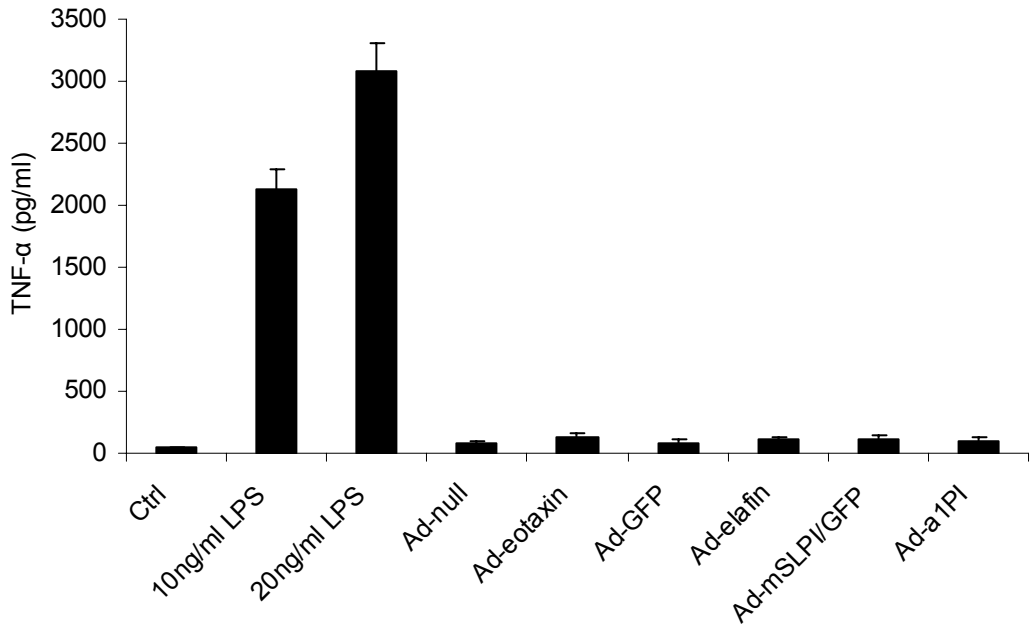


Figure 5.10. Stimulation of RAW 264.7 macrophages with Ad constructs

RAW 264.7 murine macrophages were cultured in 48-well Costar plates overnight. 24 hours later they were washed with PBS and incubated with complete medium containing the Ad constructs (MOI=100) used throughout this thesis, or LPS (10ng/ml and 20ng/ml) as positive control. Four hours post culture, supernatants were collected and analysed by TNF-α ELISA. Results are presented as pooled data from duplicate wells (n=1).

In addition to analysing DC CSMs and MHCII following Ad-α1PI infection, the supernatant of the infected cells were analysed for presence of IL-12p40 by ELISA which, as previously described, is secreted by activated DCs. In line with the previous observations that Ad-α1PI infection activated DCs (Figure 5.5), it was found that the Ad-α1PI infected DCs also produced higher levels of IL-12p40 compared to control uninfected and Ad-null infected DCs (Figure 5.11).

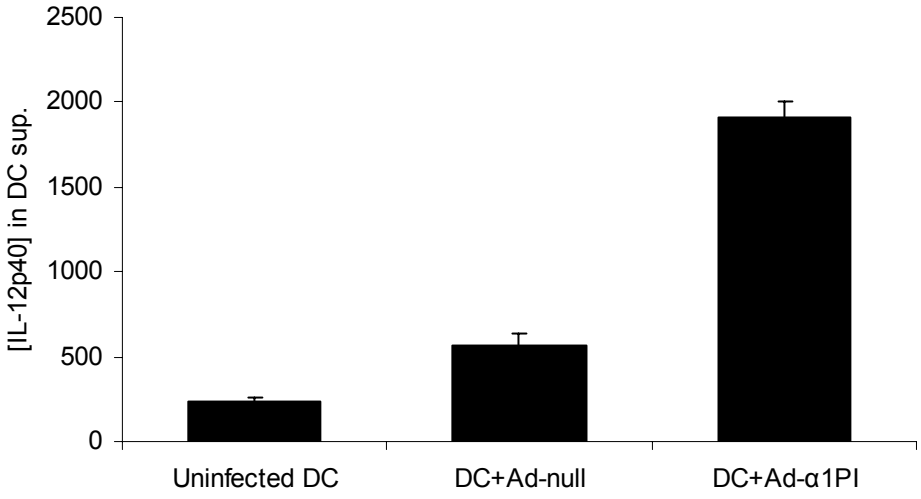


Figure 5.11. IL-12p40 production by Ad-α1-PI infected DCs

DCs were infected as described in the legend of Figure 5.5 and their supernatants were collected 24 hours post infection and analysed by ELISA for the presence of IL-12p40. Concentration units are expressed as 'pg/ml'; duplicate wells were used in ELISA (*n*=2 independent experiments).

Further experiments were carried out to investigate the immunostimulatory effects of murine α1-PI on DCs. The first set of experiments aimed to titrate the MOI of Ad-α1PI used to infect DCs and investigate if there was a dose dependent effect. Here Ad-eotaxin was used as an Ad infection control. As Figure 5.12 demonstrates, although Ad-eotaxin titration also had an affect on the expression levels of DC surface molecules at higher MOIs, Ad-α1PI infection was able to increase the CSMs even when used at an MOI of 10. The expression levels of CSMs and MHCII were maximised when Ad-α1PI was used at MOI of 100 and were comparable to the expression levels induced by LPS (100ng/ml; Figure 5.12, top histograms).

The experiments described hereafter were undertaken to investigate whether the stimulatory effects of α1-PI secretion on Ad-α1PI-DCs were via an extra-cellular mechanism or an intra-cellular one. One way to answer this was to transfer supernatants from Ad-α1PI infected cells onto freshly generated iDCs and assess their CSM and MHCII expression levels following treatment. iDCs were infected with either Ad-eotaxin (control) or Ad-α1PI in serum^{-ve} medium for 24 hours and supernatants were used to culture freshly generated iDCs. As FACS histograms show in Figure 5.13, supernatant transferred from either Ad-eotaxin infected control cells or Ad-α1PI infected cells were unable to induce any increase in CD86 and MHCII levels. Furthermore incubation of iDCs with recombinant human α1-PI (1μg/ml) did not affect the cells (data not shown).

Lastly, attempts were made to inactivate the Ad vectors and examine their effects on iDCs. The purpose of this experiment was to fully elucidate whether the DC activation following Ad-α1PI infection was due to the expression of the α1-PI transgene, or due to other factors that might be influencing DC activation in this system, *e.g.*, viral proteins or endotoxin contaminations in viral samples. To perform this, the fact that U.V. treatment of Ad vectors inactivates their DNA/transgene (while not interfering with Ad uptake by cells, although not tested in the current study) was taken advantage of to investigate whether the stimulatory effects of Ad-α1PI are transgene (α1-PI)-dependent. Preliminary experiments using Ad-GFP indicated that exposing small aliquots (10-20 μl/0.5ml Eppendorf tube) of Ad to direct U.V. light (which inactivates the Ad DNA and the transgene) for 45 minutes resulted in full inactivation of the viral DNA. The inactivation protocol of Ad vectors was tested by infecting iDCs with U.V.-treated Ad-GFP constructs and assessing GFP expression by these cells using a fluorescence microscope 24 hours post treatment (not shown) and by flow cytometry (Figure 5.14 A). Figure 5.14 B and C demonstrate that U.V.-inactivation of Ad-α1PI almost halved the expression levels of CD86 by DCs compared to those infected with fully active Ad-α1PI (MFI 143 vs 263, respectively), indicating that indeed intra-cellular (see Figure 5.13) expression of α1-PI induced DC activation *in vitro*. It should also be noted that Ad-eotaxin inactivation resulted in a small drop in expression levels of CD86 compared to DCs

treated with active Ad-eotaxin (MFI 116 vs 93, respectively), indicating that eotaxin expression could have slight modulatory effects on DCs too. Also, compared to untreated control iDCs, iDCs infected with inactivated Ad vectors were slightly stimulated which could be due interaction of Ad envelope proteins with the DCs (Figure 15.14 B). Based on these data (Figure 5.14.), in the case of CD86, inactivation of these vectors led to a ~46% drop in DC activation following Ad-α1PI infection, compared to only ~19% drop in control Ad-eotaxin-infected cells.

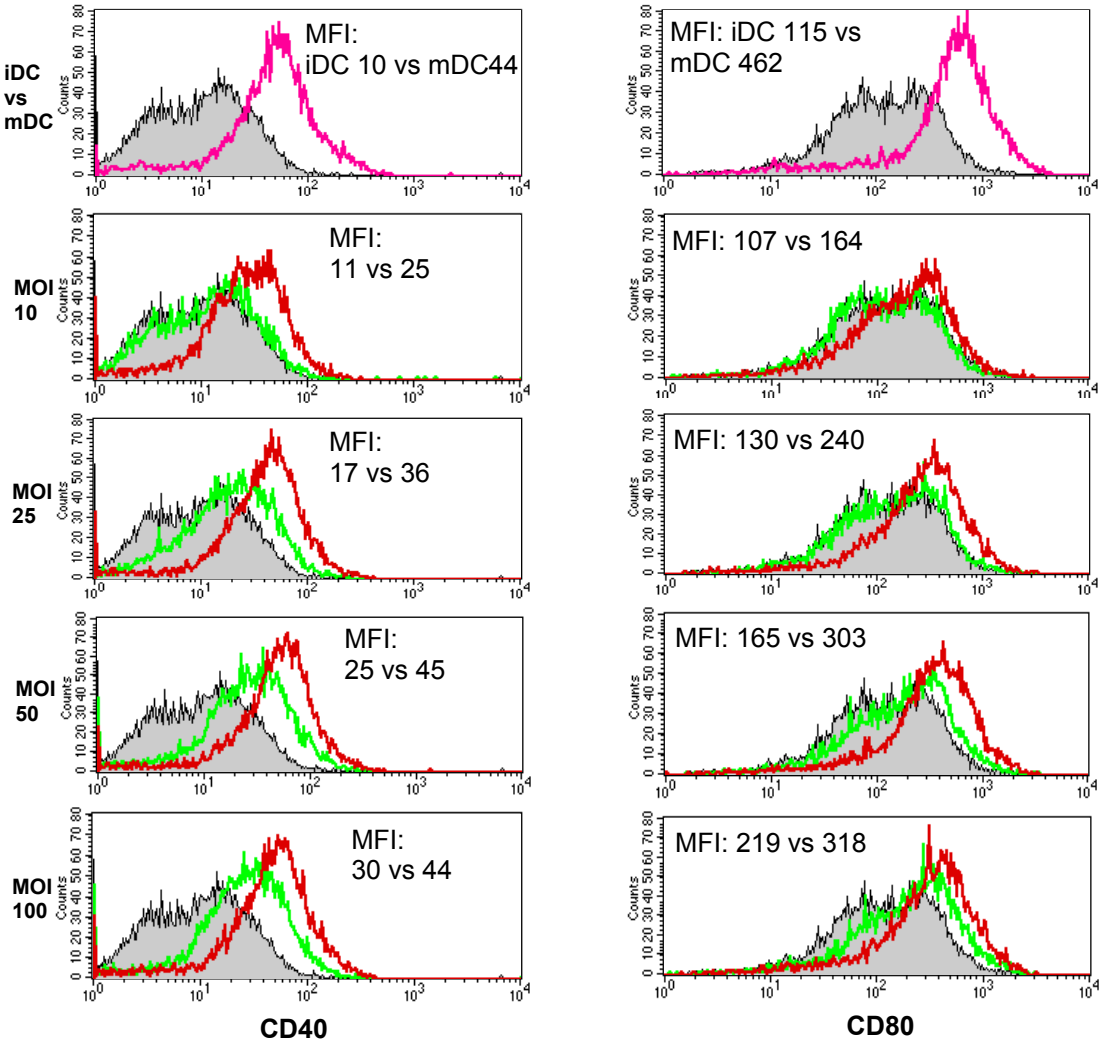


Figure 5.12 (Part A). Titration of Ad-α1PI MOI on DCs

DCs were generated and infected with various MOI (10, 25, 50 and 100) of either Ad-eotaxin or Ad-α1PI and incubated at 37°C for 24 hours, as previously described. Also DCs in one well were left uninfected (negative control) and DCs in another well were stimulated with 100ng/ml LPS as positive control for DC maturation. The uninfected and LPS-stimulated control cells as well as the infected DC groups were washed and stained with FACS Abs to CSMs and MHCII and analysed by flow cytometry for their expression levels. Filled grey histograms = uninfected cells; pink line = LPS-stimulated DCs, green line = Ad-eotaxin infected DCs; red line = Ad-α1PI infected DCs. The MFI values of Ad-eotaxin-infected DCs (left) and Ad-α1PI-infected DCs (right) are indicated on each histogram. Isotype-matched control Ab stainings and gates are not shown for simplicity. Representative plots of 2 independent experiments are shown.

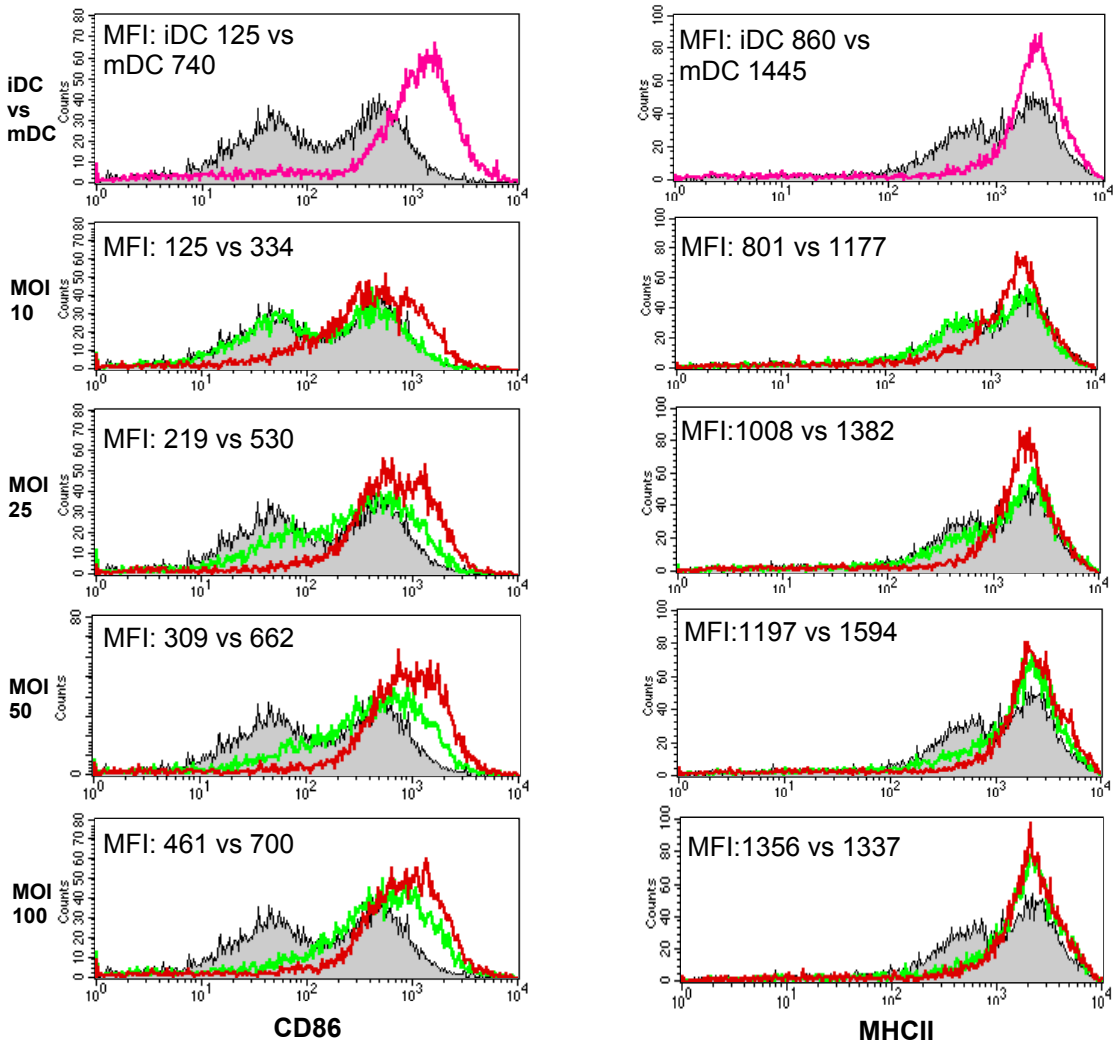


Figure 5.12 (Part B, continued). Titration of Ad-α1PI MOI on DCs

DCs were generated and infected with various MOI (10, 25, 50 and 100) of either Ad-eotaxin or Ad-α1PI and incubated at 37°C for 24 hours, as previously described. Also DCs in one well were left uninfected (negative control) and DCs in another well were stimulated with 100ng/ml LPS as positive control for DC maturation. The uninfected and LPS-stimulated control cells as well as the infected DC groups were washed and stained with FACS Abs to CSMs and MHCII and analysed by flow cytometry for their expression levels. Filled grey histograms = uninfected cells; pink line = LPS-stimulated DCs, green line = Ad-eotaxin infected DCs; red line = Ad-α1PI infected DCs. The MFI values of Ad-eotaxin-infected DCs (left) and Ad-α1PI-infected DCs (right) are indicated on each histogram. Isotype-matched control Ab stainings and gates are not shown for simplicity. Representative plots of 2 independent experiments are shown.

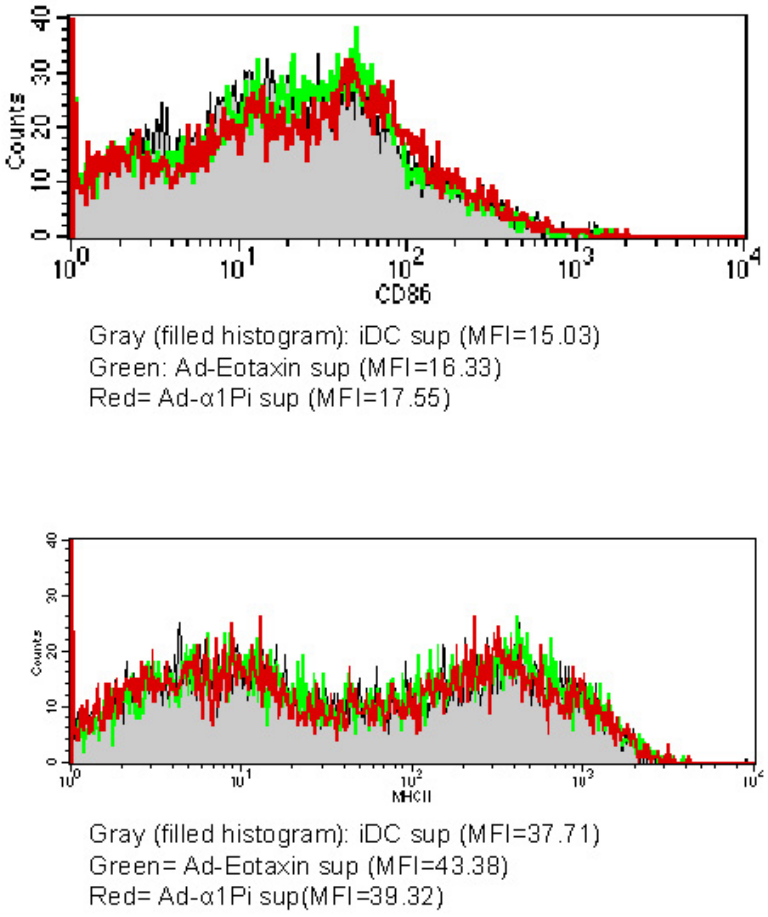


Figure 5.13. Ad-α1PI infected DC supernatant transfer

DCs were infected with either MOI 100 of Ad-eotaxin or Ad-α1PI in serum-free medium and incubated at 37°C for 24 hours. The supernatants from infected cells were collected and transferred onto freshly generated iDCs and incubated for either 4 hours or 24 hours before cells were being washed and analysed by flow cytometry. Representative histograms for CD86 and MHCII are shown (*n*=3).

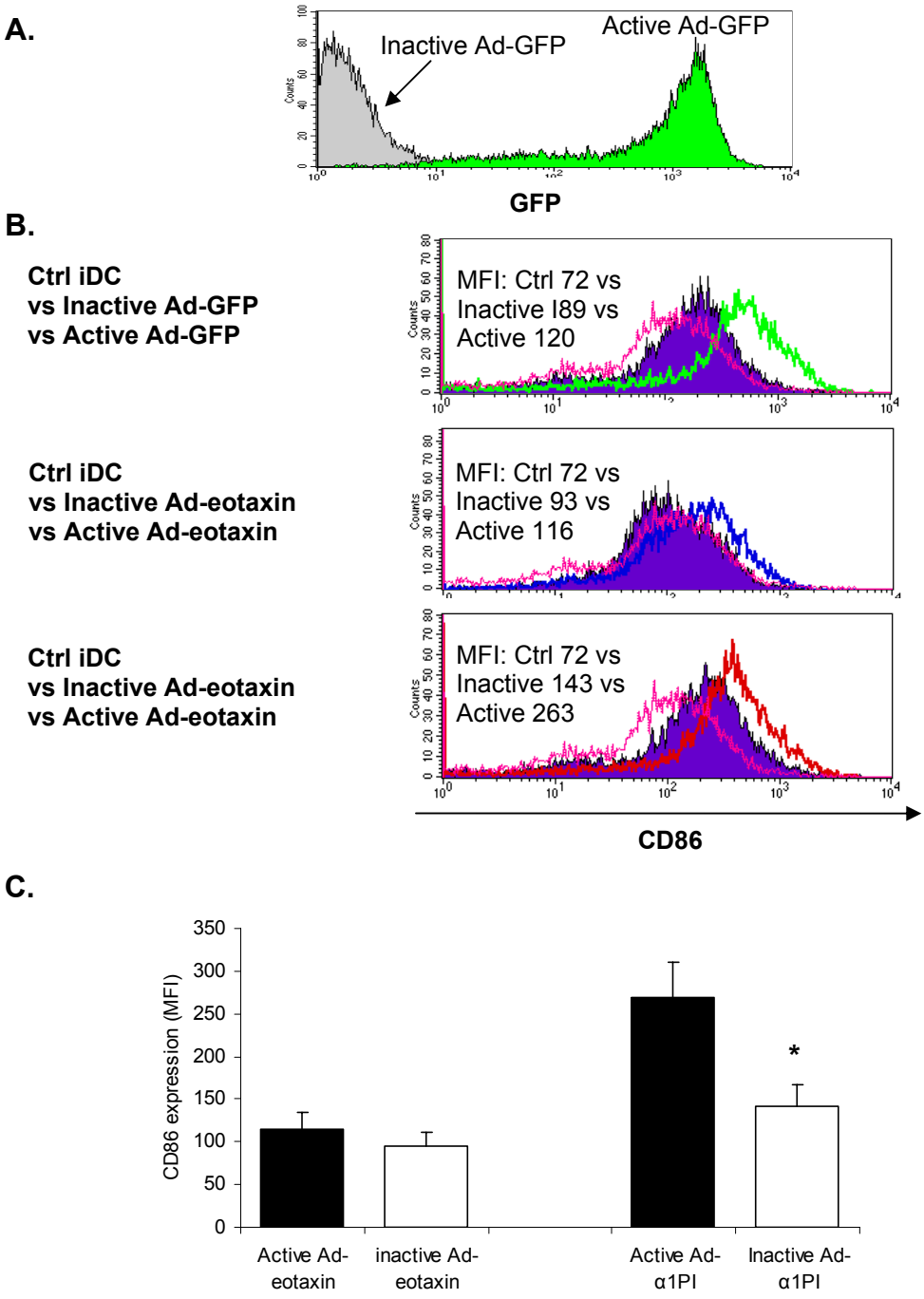


Figure 5.14. U.V. inactivation of Ad-α1PI

Ad vectors were exposed to direct U.V. light for a period of 45 minutes for complete inactivation and then incubated with DCs. **A)** DCs were infected with active and U.V.-inactivated Ad-GFP and GFP expression was assessed by flow cytometry 24 hours post infection. U.V.-inactivated Ad-GFP (filled grey histogram) versus active Ad-GFP (filled green histogram). **B)** Immature DCs (open pink histogram) were infected with either U.V.-inactivated Ad vectors (filled blue histograms) or with active Ad-GFP (open green histogram), Ad-eotaxin (open pink histogram) or Ad-α1PI (open red histogram) at moi of 100. DC activation status was investigated by flow cytometry 24 hours post infection (CD86 expression data shown). Data is representative of two independent experiments. **C**) Pooled data from two experiments shown. * indicates statistical significance ($p<0.05$) compared to control active Ad-α1PI DCs; $n=2$ of duplicate wells.

5.3. DISCUSSION

The principal novel observation arising from the data in this chapter is that some NE inhibitors (*e.g.*, α1-PI) may directly be capable of affecting DC phenotype and function, suggesting their possible roles in bridging the innate and adaptive branches of the immune system. Of the three NE inhibitors investigated, Ad-α1PI infection of iDCs resulted in the highest rate of activation, and elafin and SLPI did not seem to directly be involved in iDC activation. Discussion of the results presented in this chapter will be divided into two separate sections, as follows.

5.3.1. The *in vitro* effects of elafin on DCs

The data presented here demonstrated that elafin does not directly activate iDCs (Figure 5.5). However, as shown in the previous chapter, *in vivo* over-expression of elafin resulted in higher number of mDCs in murine lungs. Therefore it was speculated that elafin might act as a chemoattractant to modulate DC migration in inflammatory tissue. To test this hypothesis, the ability of elafin-rich media to chemoattract iDCs in a transmigration assay was investigated. As shown in Figure 5.8, preliminary experiments showed that iDCs migrated towards elafin-rich media in this *in vitro* assay. Interestingly, the migration of iDCs towards elafin-rich medium was comparable to the migration of iDCs to MIP3α-containg medium (positive control). These early data are encouraging and may in part explain how elafin is controlling DC movement/activation *in vivo*. However, one should be very cautious in interpreting these data, as they are at the preliminary stage and were not properly controlled for. For instance, these experiments did not take into account the random movement of iDCs in culture and did not have control wells where elafin-rich medium would be placed, either at the top/bottom chamber or in both chambers to account for chemokinesis as well as chemotaxis. In addition, the dose of elafin used in these experiments was not titrated and thus could make limitations in interpretion of the data. Therefore, future studies should expand the controls used in this experiment and would ideally have to apply various doses of elafin and different

combinations with respect to the top and the bottom chambers to rule out these limitations and doubts.

Previously, our group had shown that elafin over-expression enhances human neutrophil migration *in vitro*, and that it also augments LPS-mediated neutrophil migration into WT and eTg murine airways *in vivo* (Simpson *et al*., 2001a; Sallenave *et al.*, 2003; also see Chapter 4). As yet, the mechanism by which elafin augmentation enhances neutrophil and monocyte/macrophage/DC chemoattraction in our *in vitro* and *in vivo* models is unclear. As previously mentioned, data generated in Oppenheim's group shows that β-defensins, epithelial antibacterial cationic peptides with six conserved cysteine residues, might have an additional function as potential chemoattractants of iDCs through the chemokine receptor CCR6 (Yang *et al*., 1999; Biragyn *et al*., 2001). In that study Yang *et al*. (1999) showed that human β-defensin was selectively chemotactic for human embryonic kidney 293 (HAK293) cells stably transfected to express human CCR6, a chemokine receptor preferentially expressed by iDCs and memory T cells. Similarly murine β-defensin-2 was shown to act directly on iDCs as an endogenous ligand for TLR4, inducing up-regulation of CSMs and DC maturation (Biragyn *et al*., 2002). The human cathelicidin LL-37 has also been shown to use the formyl peptide receptor-like1 to chemoattract neutrophils, macrophages and T cells (Yang *et al*., 2000). Relatedly, chemerin, a novel protein abundant in inflammatory fluids (which is structurally and evolutionary related to the cathelicidin precursors and cysteine protease inhibitors), has been shown to be a potent chemoattractant of iDCs and macrophages, acting at concentrations similar to that of most chemokines (10pM to 1nM; Wittamer *et al*., 2003). Chemerin is a highly potent and specific agonist of ChemR23. ChemR23 transcripts are abundant in DCs and macrophages, treated or not with LPS, and are related to the subfamilies of chemokine receptors (Samson *et al*., 1998; Wittamer *et al*., 2003).

In a recent study, Yang and colleagues (2005) generated mutant version of SLPI (M73G and M73F) that selectively lost inhibitory function towards chymotrypsin and NE. Using these mutants they were able to conclude that expression of the SLPI protease inhibition mutants suppressed nitric oxide and TNF

production in response to LPS in a similar fashion as WT SLPI in RAW264.7 macrophages. With this in mind, it would be interesting to generate mutated elafin (and oxidised elafin with lost NE inhibitory function) peptides so that they no longer can inhibit proteases, and test their abilities to activate and/or influence DC migration *in vitro* and *in vivo*.

To date no putative cellular receptor(s) for elafin has been characterised. Like chemerin (Samson *et al*., 1998; Wittamer *et al*., 2003), elafin might have its unique receptor(s) on leukocytes. It should be taken into consideration that in addition to human leukocytes, our group has shown that human elafin is also chemotactic for murine leukocytes *in vivo* (Simpson *et al*., 2001a). Therefore, as mice do not have an elafin ortholog, it could be speculated that human elafin signals via other common receptors shared between human and murine leukocytes (*e.g.*, neutrophils and macrophages). Alternatively, it could be speculated that the gene encoding for elafin might have been lost during the evolutionary process in mice, but its receptor has been conserved. Since elafin was shown to chemoattract iDCs *in vivo* (and possibly *in vitro*) in this study, it would be tempting to suggest that, like β-defensins, elafin might also interact with CCR6 or other chemokine receptors expressed on iDCs, *i.e.*, CXCR4, CCR1 and CCR5. One way to test this would be to use the stably transfect HEK293 cells to express these iDC chemokine receptors, as performed by Yang *et al*. (1999), and investigate their migration towards elafin-rich medium. On the other hand, neutralising Abs to iDC chemokine receptors could be used to test whether they could still migrate towards elafin-rich medium or not in these migration assays.

Taken together, these preliminary observations might help explain the data presented in Chapter 4 and could account for at least some of the increase in DC numbers upon elafin augmentation in murine lungs (Roghanian *et al*., 2006b). Although, as mentioned above, one should be extremely careful in making an interpretation of the data presented in Figure 5.8, as this experiment was only performed once and not properly controlled for chemokinesis in addition to chemotaxis, and hence would require further validation experiments.

5.3.2. Activation of DCs by Ad-α1PI infection

The findings presented in the latter part of this chapter demonstrated that Ad-mediated expression of murine α1-PI protein was the most potent of the three NE-inhibitors transduced into iDCs, inducing the greatest significant increase in CSMs and MHCII compared to control Ad-infected iDCs (Figure 5.5). Moreover, Ad-α1PI infected DCs also produced higher levels of IL-12p40 compared to control uninfected and Ad-null infected DCs (Figure 5.11). The effects of α1-PI on DCs seem to be due to the intra-cellular effects of murine α1-PI as medium transfer of murine α1-PI-containg medium (Figure 5.13) or addition of human recombinant α1-PI protein to iDCs did not exert such effects (data not shown).

These data suggest the involvement of α1-PI in activating DC intra-cellular signalling pathways. Although not performed here, measurement of intra-cellular NF-κB levels would help answer this question in future experimental procedures, as NF-κB is implicated in control of CSMs and MHCII transcription in these cells (Ardeshna *et al*., 2000; Sun and Fernandes, 2003; Benveniste *et al*., 2004; Qin *et al*., 2005). The activation of DCs by α1-PI is intriguing because most of the previous published studies in this field have noted inhibition of inflammatory pathways by this NE inhibitor (Stockley *et al*., 2002; Spencer *et al*., 2004; Janciauskiene *et al*., 2004). Janciauskiene and colleagues (2004) showed that native α1-PI (inhibitory) as well as polymerised and oxidised (non-inhibitory) forms of α1-PI inhibited the secretion of IL-1β and TNF-α cytokines by LPS-activated human monocytes, indicating that the anti-inflammatory activity of α1-PI is unrelated to its inhibition of serine proteases. Interestingly, studies performed by Aldonyte *et al*. (2004) showed that both the native and polymerised forms of α1-PI have similar effects as monocyte activators, with pro-inflammatory effects at low doses, and attenuation of pro-inflammatory activities at physiologically normal doses. From the Western blot data (see Figure 5.9) obtained from supernatants of Ad-α1PI-infected DCs, it was estimated that DCs secreted around 0.2μg/ml murine α1-PI over the period of 48 hours. This dose is in the range of the lower dose of α1-PI used by Aldonyte *et al*.'s (2004) and, in agreement with their findings, resulted in pro-inflammatory rather than anti-

inflammatory activities in DCs. To further confirm the observations described in this chapter, future studies should also assess the effects of intra-cellular polymerised and oxidised α1-PI on DCs; investigate whether the activation of DCs by α1-PI is dependant on its NE inhibitor activity/concentration or not. Moreover, future *in vivo* studies such as the ones performed in Chapter 4 (Roghanian *et al*., 2006b) would help to examine the effects of α1-PI on DCs in a physiological setting.

In summary, the stimulatory effects of murine α1-PI on iDCs, as suggested by experiments presented in the latter part of this chapter, could help establish new avenues in this area of research. If these effects are proven in relevant *in vivo* settings and could also be reproduced in a comparable human system, this property of α1-PI (in addition to its antiprotease and anti-apoptotic activities; see Chapter 1, section 1.7.2) could be exploited in vaccination protocols (as an adjuvant to enhance APC function) and in boosting the immune system of immunosuppressed patients, such as those with COPD and CF, and in α1-PI-deficeint individuals (genetic emphysema).

5.4. SUMMARY

The *in vitro* results presented in this chapter demonstrate that NE inhibitors might be capable of inducing DC activation and chemotaxis. Although Ad-elafin infection of iDCs did not induce significant activation (suggesting an indirect mechanism for the *in vivo* elafin-induced activation of lung DCs described in Chapter 4), iDCs were shown to migrate towards elafin-containing medium in preliminary experiment. On the other hand, Ad-α1PI infection of iDCs led to their activation, possibly via intra-cellular signalling of the α1-PI transgene.

Taken together, these preliminary novel observations suggest that natural NE inhibitors might be able to activate the adaptive immune system by chemoattracting or activating iDCs. These properties of NE inhibitors, in addition to their previously described functions (*e.g.*, antimicrobial, antiprotease), could be further exploited to design vaccines and immunotherapeutic protocols.

CHAPTER 6

CONCLUDING REMARKS AND FUTURE DIRECTIONS

It is well accepted that in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), such as emphysema, an imbalance between destructive proteases secreted primarily by activated neutrophils and their natural inhibitors is a determinant factor in the progressive destruction of pulmonary tissue. Indeed, neutrophil mediated inflammation is a major component of parenchymal damage in COPD and CF lungs, and it is widely believed that neutrophil elastase (NE) may be one of the main mediators of such damage. This is because although elafin and other NE inhibitors are highly secreted locally during the course of the disease, their activities might be rendered due to inactivation, *e.g.,* by oxidants (see Chapter 1, section 1.5.1.1). Since the lower airways of most COPD and CF patients are colonised by microorganisms, this might suggest that dendritic cells (DC) in the lungs of these patients are unable to activate the immune system because they fail to efficiently endocytose and/or present antigens. DCs form a network in the upper layers of the epithelium of the airways. In normal lungs inhaled antigens are captured by mucosal DCs across the epithelial tight junction barriers and presented to T cells in local lymph nodes (LN).

In the current study, it was established that exposure of DCs to CSC leads to lower levels of co-stimulatory molecules (CSM), possibly due to CSC-induced DC necrosis, and that exposure of DCs to NE-rich inflammatory sputum secretions collected from COPD and CF patients results in decreased levels of CD80 and CD86 (but not CD40) cell surface CSMs. Similarly, exposure of DCs to purified human NE led to lower levels of CD40, CD80 and CD86, leading to reduced antigen presentation and lower Th1 cytokine output (Chapter 3; Roghanian *et al*., 2006a). Although the *in vivo* effects of these changes remain to be investigated, the reduced Th1 cytokine levels induced by NE treatment of DCs may, in part, explain the reported Th2 imbalance in the lung immune response to bacteria in patients with CF (Brazova *et al*., 2005; Hartl *et al*., 2006) and the reported loss of IFN-γ-secreting

cells in patients with COPD (Tsoumakidou *et al*., 2005). This local Th2-biased phenotype may be instrumental in the inability of these patients to clear semifacultative intra-cellular pathogens in the lung, such as nontypeable *Haemophilus influenzae* (*NTHI*), *Pseudomona*s aeruginosa (*P.a.*) and *Burkholderia cepacia* (*B.c.*), as suggested recently by a variety of animal and human studies (Moser *et al*., 1997; Martin and Mohr, 2000; Abe *et al*., 2002). Although neutrophils are important in early immunity to infection and may regulate adaptive immune responses indirectly through interactions with DCs (van Gisbergen 2005a and 2005b), these data indicate that high numbers of active neutrophils (secreting high levels of proteases) could be deleterious to adaptive immune responses.

Further, in the latter part of this thesis, it was demonstrated that over-expression of an NE specific inhibitor (elafin) *in vivo* results in a higher number of DCs with an activated phenotype in murine lungs, as evidenced by expression of higher levels of CSMs, and higher levels of IL-12 and TNF-α in their BAL fluids (Chapter 4; Roghanian *et al*., 2006b). Elafin is normally produced in a variety of epithelial and mucosal sites, such as the skin and the lung, and is a remarkable effective inhibitor of NE (see Chapter 1, section 1.7.1.1). The production of elafin in the lung has been attributed not only to bronchial epithelial cells and alveolar epithelial cells, but also to alveolar macrophages (Williams *et al*., 2006). Elafin was chosen in the current study because of its immunomodulatory properties, narrow specificity, and its small size (9.9 kDa), which make it a potential candidate for therapeutic use. In line with these data, intra-tracheal (i.t.) treatment of murine lungs with Ad vectors (*e.g*., Ad-GFP, or Ad-LacZ) leads to local expression of the transgene by the epithelial cells as well as alveolar macrophages, and is an ideal *in vivo* tool for such studies. In addition, the RNA message for human elafin has been detected in lung tissues of elafin transgenic (eTg) mice and the protein is also detected following LPS treatment of the eTg murine lungs (Sallenave *et al*., 2003).

In the final part of the study, *in vitro* infection of DCs with Ad constructs expressing one of the three major respiratory system NE inhibitors (either elafin, SLPI or α1-PI) showed that only α1-PI-expressing DCs were highly activated

(Chapter 5). However, preliminary experiments indicated that elafin might affect iDC migration, as iDCs were shown to migrate towards elafin-rich supernatants *in vitro*. This suggests that elafin could directly and/or indirectly affect the number and the activation status of DCs and might help to interpret the *in vivo* data presented in Chapter 4. Taken together the novel data presented in this thesis help advance our understanding of the interactions of NE and NE inhibitors with DCs, and the ability of these molecules to affect the outcome of an immune response.

To complete the data presented here, further investigations into whether direct administration or over-expression of endogenous NE inhibitors (*e.g.*, elafin) may induce autoimmune responses against such molecules are necessary. It is expected that in humans (and the eTg mice) antibodies (Ab) against elafin should not be mounted as autoreactive clones would have been deleted in the thymus during early development of the immune system. Although low levels of peripheral autoreactive T cells could exist, these will not be activated in the absence of danger signals. Therefore, in view of the 'danger model', it could be speculated that Ad vectors may activate DCs which consequently may endocytose locally secreted elafin in an activated fashion and then present it to the autoreactive peripheral T cell clones and induce autoimmune disease. Alternatively, like other antimicrobial proteins, elafin which is up-regulated by inflammatory cytokines/infectious agents (Sallenave *et al*., 1994, Williams *et al*., 2006) may have the properties of an immune 'alarmin'[1] and act as a natural adjuvant during an episode of mucosal infection/inflammation (Oppenheim and Yang, 2005, Matzinger, 2002; Gallucci *et al*., 1999). For instance, Biragyn and colleagues (2003) exploited the alarmin property of defensins in immunising mice with fusion proteins consisting of murine active β-defensins linked to a non immunogenic lymphoma antigen, suggesting that fusion constructs with inflammatory mediators targeting immature DCs could elicit protective anti-tumour immunity. Furthermore, future experiments should measure the presence of Abs to the over-expressed molecule as well as any specific $CD4^+$ and $CD8^+$ T cell responses,

[1] 'Alarmin' is a new terminology recently proposed by Oppenhiem and Yang (2005) to describe structurally diverse endogenous mediators of innate immunity which are rapidly released in response to infection or tissue injury, have both chemotactic and activating effects on APCs, and exhibit particularly potent *in vivo* immunoenhancing activity. This subset of mediators alerts host defences by augmenting innate and adaptive immune responses to tissue injury and/or infection.

such as the clones specific to the elafin transgene. Indeed, the medical literature is full of claims and counter claims concerning the risk of autoimmune disease as a consequence of vaccination (Wraith *et al*., 2003). Incidentally, the antimicrobial LL-37, which possesses similar properties to the NE inhibitors, has been shown to inhibit leukocyte apoptosis (Barlow *et al*., 2006). On the other hand, recent data demonstrate that inhibition of DC apoptosis can independently result in autoimmunity in mice (Chen *et al*., 2006). Therefore, studying the effects of NE inhibitors on leukocyte apoptosis is desirable, as apoptosis in the immune system is critical for maintaining self-tolerance and preventing autoimmunity. Recently, the first phase in the clinical trial of elafin has been successfully completed by Proteo Biotech AG[2]. In this trial, elafin was tested on 32 healthy male volunteers in Kiel, Germany. All intravenously applied doses of recombinant human elafin were well tolerated and no severe adverse events occurred. The successful conclusion of this trial demonstrates that the use of human elafin at various doses, without the use of a carrier, for therapeutic purposes (inflammatory diseases, particularly in lung and circulatory disorders) could be tolerated.

The danger model currently favours the notion that the APC acts as the key regulator of the immune system by passively collecting and integrating signals from the tissue milieu and then deciding whether to initiate immunity or tolerance. Matzinger has recently suggested that the tissues themselves, not the APC, should be considered as the ultimate controllers of immunity (Matzinger, 2007). The new concept that tissue cells could actively secrete biochemical requests for immunity is a radical idea in contrast with the accepted image of immunity as a stand-alone system for protecting tissues. As described before, in the steady state, lung DCs do not synthesise IL-12 and preferentially stimulate Th2 rather than Th1 immunity (Stumbles *et al*., 1998; Constant *et al*., 2002) via secretion of anti-inflammatory cytokines (*e.g.,* IL-6 and IL-10) or by production of indoleamine 2,3-dioxygenase (IDO) which suppresses T cell responses (see Chapter 1, section 1.2.10). Therefore, the local microenvironment is of absolute importance in shaping DC phenotype, and the secretion of NE inhibitors such as elafin by the epithelial cells could be directly

[2] Proteo owns the rights for the production and exploitation of recombinant elafin. Source: www.proteo.de/pages/presse.php?DATUM=2005-12-13&BACK=/pages/presse_01_02.php&lang=en

involved in such processes. The observation that the Ad delivery of elafin promotes a Th1 response in the mouse lungs leads one to speculate that this response might be dependent upon the presence of the Ad vector itself as a type 1 'danger signal' (see above; even though such an effect has also been described in eTg mice, independently of the Ad mode of delivery) and does not necessarily indicate that elafin will always act as a type 1 adjuvant, regardless of the initiating signal. However, the very strong type 1 response elicited in both WT mice receiving Ad-elafin and eTg mice (Roghanian *et al*., 2006b) indicate that this strategy could be used in a variety of other applications, for viral or intra-cellular pathogens (*e.g.,* HIV, *Mycobacterium tuberculosis*, *Chlamydia*) which normal route of entry are mucosal surfaces. Although cytokines could be used in these approaches as adjuvants, the use of elafin, a unique pleiotropic molecule with antimicrobial and anti-NE properties would be particularly relevant in anti-infective protocols (see below).

Viruses have acquired numerous biological properties over millions of years of evolution which allow them to effectively recognise and enter cells, traffic within the cytosol to the nucleus, translocate into the nucleus and express their genes in the host cell. If virus constructs are to be used in gene therapeutic protocols to augment NE inhibitors in the respiratory tract, further experiments and many more large-scale clinical trials are required to examine the direct effect of virus vector-derived antigens on host's immune system and its safety *in vivo*. Despite the clinical hazards associated with viral vectors they, along with liposomes, currently remain amongst the vehicles of choice for gene transfer in gene therapy trials in the UK and worldwide. In the UK alone, eighty eight clinical trials were registered with the Gene Therapy Advisory Committee in 2004[3] (Relph *et al*., 2004). Currently, Ad vectors remain one of the popular viral vectors in clinical trials because of their ability to transduce a broad range of dividing and non-dividing cells. In addition, Ad vectors can be produced in high titres, and they replicate episomally and do not insert their genome into that of the host cell, which ensures less disruption of vital cellular genes. However, the efficacy of Ad delivery might be severely hampered because most people have been exposed to natural Ad infections. Therefore, a successful viral gene

[3] GTAC webpage: www.advisorybodies.doh.gov.uk/genetics/gtac

therapy vector ideally needs to be protected from recipient's circulating neutralising Abs and exhibit a preferential tropism for target cells in the organ where gene therapy is aimed at. In a novel approach, Fisher *et al*. (2001) coated surfaces of Ad particles with a multivalent hydrophilic polymer which proved to be an effective way of changing its tropism and interaction with the immune system. Nevertheless, as mentioned above, Ad vectors remain the most immunogenic of all the viral vectors currently in use (Relph *et al*., 2004) and their use *in vivo* may cause further unpredicted complications. *In vivo*, Ad vectors at high titers could induce the expression of various cytokines and inflammation-associated genes in innate immune cells such as macrophages and non-innate targets such as epithelial and endothelial cells (Liu and Muruve, 2003). Indeed, in one of the early clinical trials (The 'Jesse Gelsinger Case') where a very high dose of Ad vector (3.8×10^{13} viral particles) was used the patient died only four days post treatment (Lehrman *et al*., 1999; Ferber, 2001; Relph *et al*., 2004). It appeared that, in line with the danger model, his immune system had launched a raging attack on the Ad vector, setting off an overwhelming cascade of organ failures, starting with jaundice, and progressing to a blood-clotting disorder, kidney failure, lung failure, and ultimately brain death. Also Ad might indirectly promote leukaemia by perturbing the immune surveillance of a spontaneously occurring pre-malignant B cell clone, simply by being present in those cells. Certain HLA types that are associated with an increased risk for common acute lymphoblastic leukaemias (cALL) potentially cooperate with Ad in this process (Dorak, 1996). In addition to developing safer replication-deficient vectors, alternative methods of gene delivery with a less invasive nature should be explored to over-express NE inhibitors in future studies. For instance, adeno-associated virus (AAV), which is far less immunogenic than Ad vectors and is currently one of the most popular gene delivery systems in animal experiments (Wu *et al*., 2006) could be employed to over-express NE inhibitors in lungs of people suffering from COPD and CF. This is mainly because of the long-term and efficient AAV's transgene expression in various cell types such as lung cells (Flotte, 2005; Halbert *et al*., 2007).

Future studies *in vivo* should not be limited to examining the dynamics of APCs and should also take into account the presence of NK cells. NK cells play a

crucial role as a first line of defence against viral and bacterial infections. The former are a heterogeneous population, expressing a mosaic of inhibitory and activating cell-surface NK receptors. The balance of inhibitory and activating signals transmitted following engagement of NK receptors with their ligands (on target cells) determines the response of a particular NK cell (Lanier, 2005). Recent studies indicate that NK cells constitute a key component of the response to replication-deficient Ad *in vivo* (Ruzek *et al*., 2002; Xu *et al*., 2004). In addition, replication-deficient Ad vectors have been shown to stimulate NK cell recognition (Tomasec *et al*., 2007). Specifically, Tomasec *et al*. (2007) showed that primary human fibroblasts transduced with replication-deficient Ad vectors were more susceptible to NK cell-mediated lysis- this was due to the expression of ligands of the potent activating NK receptor, NKG2D. NKG2D ligands might act as a CSM for $CD8^+$ CTLs and γδ T cells and might also have a profound effect on stimulating both innate and adaptive immune responses through the release of cytokines (Tomasec *et al*., 2007). Consequently, an investigation into the kinetics of NK cells following i.t. Ad-elafin treatment would be useful in order to dissect the mechanisms by which elafin modulates the immune system. To date no such study has been performed, and as yet the effects of NE inhibitors on the phenotype and/or function of NK cells remain unexplored. Over-expression of elafin might also affect the cross-talk between NK cells and DCs. The cross-talk between NK cells and myeloid DCs leads to NK cell activation and DC maturation. During lung inflammation, NK cells are rapidly recruited from the blood and engage DCs in a reciprocally regulating interaction. Mature DCs (via secretion of IL-12) activate NK cytotoxic function and production of TNF-α and IFN-γ, whilst activated NK cells enhance DC maturation and IL-12 secretion (Moretta *et al*., 2005). The net result is selection of DCs that polarise naïve αβ T cells to a Th1 phenotype, as seen in our study (Roghanian *et al*., 2006b). Therapeutic approaches, like elafin augmentation (Roghanian *et al*., 2006b), that stimulate DCs to produce NK-cell-activating cytokines might directly activate NK cells (and consequently augment adaptive immune responses), thus minimising the toxic side effects of systemic cytokine administration.

In summary, the findings presented in this thesis suggest that, the presence of high concentrations of active NE is harmful to DCs *in vitro*, whereas high concentrations of NE inhibitors at the sites of inflammation/infection are essential in maintaining DC phenotype and function and therefore may influence the outcome of disease pathology and the consequent immune responses. In addition, this study established that augmentation of the antimicrobial/NE inhibitor elafin is able to bridge between the innate and adaptive arms of the immune system by activating DCs in lung, and possibly in other mucosal tissues (not investigated in the current study). Recent studies have demonstrated that respiratory pathogens such as *Chlamydia pneumoniae*, *NTHI*, *P.a.* and *B.c.* could reside intra-cellularly (Sethi, 2000), especially in macrophages, and in the sub-epithelial zone in human respiratory tissues. These bacteria are protected from antibiotics and bactericidal Abs, and may act as reservoirs of infection in COPD and CF lungs. Undoubtedly, vaccinating these groups of patients (*i.e.*, people prone to developing COPD, and children diagnosed with mutations in the CFTR gene), where an imbalance between the NE and its inhibitors exists and high incidents of frequent/chronic infections are evident, with a Th1-inducing molecule such as elafin against these common respiratory intra-cellular pathogens may significantly reduce morbidity and mortality. Indeed, while classical adjuvants such as mycobacterium extracts enhance inflammation to provide non-specific 'danger signals' that spark a strong adaptive response, transgene adjuvants representing endogenous mediators, such as elafin, are likely to have highly specific effects on individual components of the immune response as shown here (see above).

In conclusions, the findings described in this thesis (see Figure 6.1) may further contribute to the understanding of the modulation of pulmonary DCs by the elastase:anti-elastase molecules of the lung milieu during different episodes of inflammation/infection.

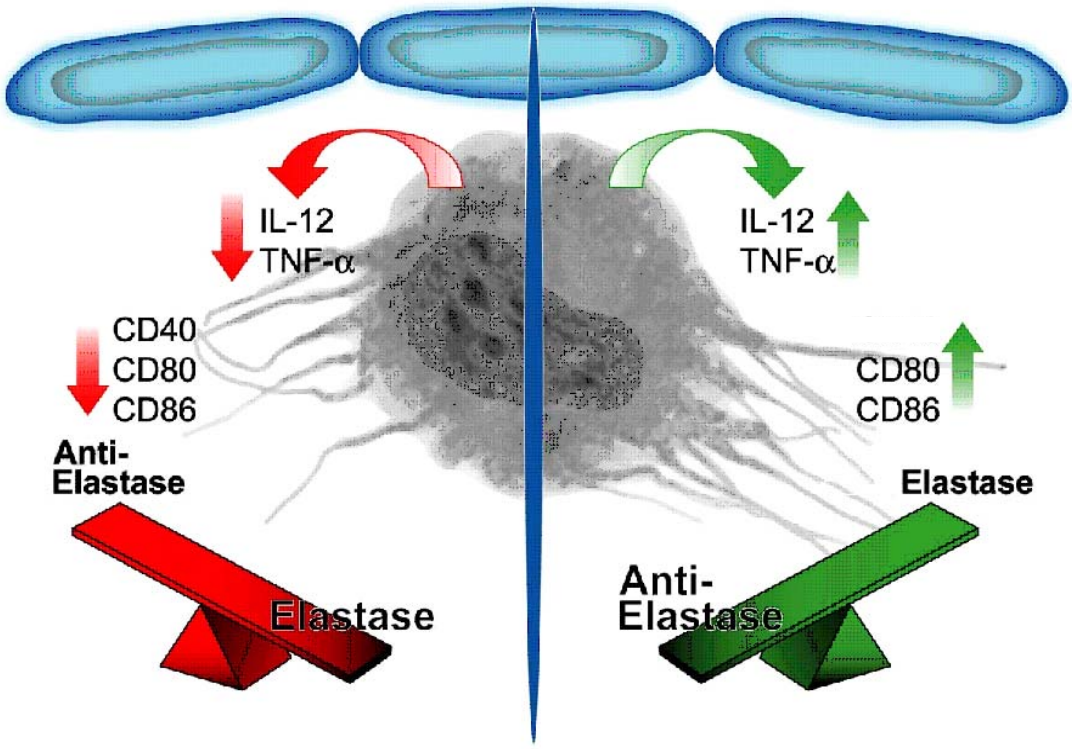


Figure 6.1. Effects of NE:NE inhibitor imbalance scenarios on DCs

Schematic diagram demonstrating the net effects of imbalance of NE and its inhibitors on the phenotype/activation status of DCs. High concentrations of uninhibited NE in inflammatory tissues result in down-regulation of DC co-stimulatory molecules (CSM; namely CD40, CD80 and CD86), and subsequently lower secretion of pro-inflammatory cytokines (IL-12 and TNF-α) (left panel; Roghanian *et al*., 2006a). On the other hand, NE inhibitor-rich tissues contain higher numbers of activated DCs with increased levels of CSMs, secreting higher concentrations of the pro-inflammatory cytokines (right panel; Roghanian *et al*., 2006b).

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