# The Macrophage in Cystic Fibrosis

Thesis submitted for the degree of

Doctor of Philosophy

By

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August 2007

To all past, present and future individuals

who have been or will be affected by cystic fibrosis

# Statement of Originality

The accompanying thesis submitted for the degree of Doctor of Philosophy is entitled 'The Macrophage in Cystic Fibrosis.' This thesis is based on work conducted by the author in the Department of Infection, Immunity and Inflammation at the University of Leicester during the period between January 2004 and August 2007. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

This work has not been submitted for another degree in this or any other University.

Signed ..... Date 4th April 2008.....

# Acknowledgments

Firstly I wish to thank my family for supporting a large part of my education and moral encouragement throughout. My thanks also go to Emma Johnson and Stuart Terington whose support and faith gave me the initial encouragement and confidence to undertake a PhD. My thanks go to the children's research fund whose funding enabled me to begin this journey.

During this time I have met so many friends who have spurred me on when times were tough and laughed with me, usually during a beer, when things were good. I have singled out just a few for special mention. Dr Satish Rao for doing much of the dull stuff by getting the project up and running, his clinical expertise and introducing me to the cystic fibrosis teams. Dr Simon Range, who made me feel part of the clinical team for two years and permitted access to adult patients. Also to specialist nurse Bernie Donaghy for helping me obtain samples and who put up with me hanging around the clinic and ward all the time. Also to Nicky, Linda, Julia, Lorraine, Diane and everyone at the respiratory unit who made me feel welcome at the hospital and gave me plenty of advice and encouragement. Melanie Todner for teaching me the art of phlebotomy. Thanks to Prof. Chris O'Callaghan for access to paediatric cystic fibrosis patients. Also thanks to everybody in Gauting, Germany for their advice, friendship and molecular assistance and making me feel welcome in their lab.

My heartfelt thanks go to all the patients and healthy volunteers who have given up their time, blood and sputum (often on several occasions) to participate in this project. Particularly the Gregory and Westmoreland brothers, the Williams sisters and also Deepa, Ben, Jo, Stephen, Terrie and Tom. I couldn't have done it without you! I extend equally important thanks to Dr Jonathan Grigg and in particular Prof. Löms Ziegler-Heitbrock who have given great supervision, advice, and wisdom over the years. Many thanks also to the rest of the lab, Annette, Assad, Ayman, Bernie, Brent, Ghada, Helen, Karl and Rasheedah. In particular to Karl and Bernie for their friendship, support, lots of experimental advice and for looking over this thesis.

My thanks to the rest of the post-graduate community, members of the 'Q club', the thesis writer's association team, and 'Micro' football team. Thanks to my housemate Paul for choosing the correct words of encouragement and introducing me to so many things. Last but not least thanks to Angela Robinson for all of her love and banter through the final stages.

# Abstract

**Name: Adam Wright**

**Thesis Title: The Macrophage in Cystic Fibrosis**

**Background:** Cystic fibrosis (CF) is caused by absent/defective CF transmembrane conductance regulator protein (CFTR). CF is characterized by thick airway mucus, chronic infection and neutrophil inflammation leading to respiratory failure. I analysed airway macrophages (MΦs) and their expression of pattern recognition receptors (PRRs) in CF, since these cells are crucial to airway immune defence and they can orchestrate inflammation. I also performed transcript analysis of CF monocyte-derived MΦs (MDMs).

**Methods:** Sputum was induced in CF paediatric and adult cohorts. Phenotype and function of CF MΦ were determined by flow cytometry and compared to controls. Monocytes (>92% purity) were grown *in vitro* to generate MDMs ($n$=15). Transcripts encoding the entire human genome were analysed (n=5) and expression of individual genes were confirmed by RT-PCR.

**Results:** In classical CF ($n$=10) there was an increase in the proportion of monocyte-like small MΦs (of total MΦ) compared to controls ($n$=10) (73 ± 18% and 16 ± 8%, respectively, $p<$ 0.0001). In non-classical CF ($n$=4), with milder lung disease, small MΦs increased to 31 ± 20% ($p$>0.05). PRRs were absent on small MΦs from CF and control. In contrast, clear expression could be detected on large MΦs from control but not CF. In line with this, CF small MΦs showed a strongly reduced uptake of particles compared to controls. Microarray analysis of MDMs revealed α- and β-tryptase as being significantly higher under constitutive and stimulated conditions in CF compared to control. However, using RT-PCR, expression of α- and β-tryptase was similar between groups.

**Conclusions:** The phenotype of small MΦs in CF suggests that these cells are newly recruited monocytes from blood. Low expression of PRRs on these cells in CF and their reduced uptake indicates a reduced capacity to clear inhaled particles, which may contribute to further damage in CF. Further to this I was unable to confirm any transcript differences between CF and control MDMs due to mutant CFTR.

# Abbreviations

α - Alpha

AA – Arachidonic Acid

ABC – Adenosine Tri-Phosphate Binding Channel

AF - Autofluorescence

ANOVA – Analysis of Variance

APC - Allophycoerythrin Cyanin

ARDS - Acute Respiratory Distress Syndrome

ASL – Airway Surface Liquid

ATP – Adenosine Tri-Phosphate

β - Beta

BAL – Bronchoalveolar Lavage

BD – Becton Dickinson

Bp – Base Pair

CD - Cluster of Differentiation

cDNA – Complementary Deoxyribonucleic acid

CF - Cystic Fibrosis

CFTR – Cystic Fibrosis Transmembrane Conductance Regulator

$Cl^-$ - Chloride

CLR – C-Type Lectin Receptor

$CO_2$ – Carbon dioxide

COPD – Chronic Obstructive Pulmonary Disease

CR – Complement Receptor

cRNA – Complementary Ribonucleic Acid

CSF – Colony Stimulating Factors

CTLD – C-Type Lectin-like Domain

DC – Dendritic Cell

DC-SIGN – Dendritic Cell Specific Intercellular Adhesion Molecule Grabbing Non-Integrin

DEPC - Diethylpyrocarbonate

ΔF508 – Deletion of Phenylalanine (F) at position 508

$dH_2O$ – Deionised Water

DNA – Deoxyribonucleic Acid

Abbreviations

dNTPs – Deoxynucleotide Phosphates

D-PBS – Degassed Phosphate Buffered Saline

DTT - Dithiothreitol

EAA – Extrinsic Allergic Alveolitis

EC – Epithelial Cells

*E. coli - Escherichia coli*

ECM – Extracellular Matrix

EDTA - Ethylenediaminetetracetic Acid

ENaC – Epithelial Sodium Channel

ε - Epsilon

$F(ab')_2$ - Fragment (Antibody Binding')$_2$

Fc – Conserved Fragment

FCS – Fetal Calf Serum

$FEV_1$ - Forced Expiratory Volume in One Second

FITC - Fluoroscein Isothiocyanate

FL - Fluorescence

FMO – Fluorescence Minus One Strategy

FSC – Forward Scatter

γ - Gamma

GM – Geometric Mean

GM - CSF – Granulocyte/Monocyte – Colony Stimulating Factor

$HCO_3^-$ - Hydrogen Bicarbonate

*H. influenzae – Haemophilus influenzae*

HLA - Human Leukocyte Antigen

HMC-1 – Human Mast Cell

$H_2O$ - Water

$H_2O_2$ – Hydrogen Peroxide

HOCl – Hypochlorite

hrs - Hours

IFN - Interferon

Ig – Immunoglobulin

IKK – Inhibitor Kappa Kinase

IL - Interleukin

IL-1Ra – Interleukin-1 Receptor Antagonist

IPF – Idiopathic Pulmonary Fibrosis

IRAK – IL-1R Associated Kinase

$K^+$ - Potassium

KCL – Potassium Chloride

$KHCO_3$ – Potassium Hydrogen Carbonate

$KH_2PO_4$ – Potassium Dihydrogen Phosphate

KO – Knockout

LD – Large Depletion

LDL – Low Density Lipoprotein

LPB – Lipopolysaccharide Binding Protein

LPS – Lipopolysaccharide

LRI – Leicester Royal Infirmary

LRR – Leucine Rich Repeats

$LTB_4$ – Leukotriene $B_4$

mAb – Monoclonal Antibody

MACS –Magnetic Activated Cell Sorting

MAPK – Mitogen Activated Protein Kinases

MARCO – Macrophage Receptor with Collagenous Structure

MBL – Mannan Binding Lectin

M-CSF - Monocyte – Colony Stimulating Factor

MDM – Monocyte-Derived Macrophages

MHC – Major Histocompatibility Complex

min - Minute

MIP – Macrophage Inflammatory Protein

ml – $10^{-3}$ Litres

MM6 - MonoMac 6

MMP – Matrix Metalloproteinase

MP – Molecular Pattern

MΦ – Macrophage

MPO - Myeloperoxidase

Abbreviations

mRNA – Messenger Ribonucleic Acid

MS – Mini Separation

MyD88 – Myeloid Differentiation Factor 88

*n* - Number

N/A – Not Applicable

$Na^{2+}$ - Sodium

NaCl – Sodium Chloride

$Na_2HPO_4$ – Disodium Hydrogen Phosphate

NBD – Nuclear Binding Domain

NF-κB – Nuclear Factor – Kappa Binding

ng – $10^{-9}$ Grammes

$NH_4Cl$ – Ammonium Chloride

nm – $10^{-9}$ Metres

NSG – No Significant Growth

*P. aeruginosa – Pseudomonas aeruginosa*

PBMC – Peripheral Blood Mononuclear Cells

PBS - Phosphate Buffered Saline

PC5 - Phycoerythrin-Cyanin 5

PCR – Polymerase Chain Reaction

PE - Phycoerythrin

PerCP - Peridinin Chlorophyll Protein

pg – $10^{-12}$ Grammes

PK – Protein Kinase

PMT - Photomultiplier Tube

PRR – Pattern Recognition Receptors

R – Regulatory Domain

RNA – Ribonucleic Acid

ROI – Reactive Oxygen Intermediates

rpm – Revolutions Per Minute

RT – Reverse Transcription

s - Seconds

*S. aureus - Staphylococcus aureus*

Abbreviations

SD – Standard Deviation

SLPI – Secretory Leukocyte Protease Inhibitor

SLR – Signal Log Ratio

SMG – Submucosal Gland

sp. - Species

SP – Surfactant protein

SR – Scavenger Receptor

SRCR – Scavenger Receptor Cysteine Rich Domain

SSC – Side Scatter

TAE – Tris-Acetate-Ethylenediaminetetracetic Acid

TGF – Transforming Growth Factor

TIMP – Tissue Inhibitors of Matrix Metalloproteinase

$TiO_2$ – Titanium Oxide

TIR – Toll- Interleukin-1 Receptor

TIRAP – TIR Associated Protein

TLR – Toll-Like Receptor

TNF - Tumour Necrosis Factor

TRAF – TNF Associated Factor

TRIF – TIR Death Domain Containing Adaptor Protein Inducing IFN beta

$\mu g – 10^{-6}$ Grammes

$\mu l – 10^{-6}$ Litres

ULA – Ultra Low Attachment

$\mu m – 10^{-6}$ Metres

V – Volts

WT-Wild Type

$\zeta$ - Zeta

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# 1 Introduction

## 1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is a common (1 in 2600 caucasians are affected at birth in the United Kingdom) monogeneic disease that is inherited in an autosomal recessive manner and exhibits multi-organ pathology with fatal consequences (WHO 2002; Gibson, Burns *et al.* 2003). An extremely high infant mortality rate was a feature of CF in the first half of the 20th century. Today however, CF patients, of which there are approximately 60,000 worldwide, survive well into adulthood (Gibson, Burns *et al.* 2003). Over the last few decades, improved diagnostics and earlier, more aggressive therapeutic care have continued to increase the median age of survival to 35 years (Gibson, Burns *et al.* 2003). However CF places a huge burden on health care providers and is associated with significant costs. The estimated total cost for all CF patients in Germany (3582 patients) to receive one year of treatment has been calculated to be €105 million (Baumann, Stocklossa *et al.* 2003). Much of this treatment is aimed at ameliorating symptoms or secondary consequences of the disease rather than treating the root cause. Therefore this figure shows no sign of decreasing, since there is currently no cure.

### 1.1.1 Clinical Features

The genetic abnormality that gives rise to CF reduces or abolishes the expression of a cell surface and intracellular transmembrane chloride ($Cl^-$) channel with diverse roles (Engelhardt, Yankaskas *et al.* 1992; Di, Brown *et al.* 2006). Exocrine gland epithelia, particularly, are mostly affected whereby altered anion transport into the lumen of the gland initiates the formation of thickened luminal secretions that accounts for many of the clinical manifestations of CF (Zuelzer and

Newton 1949; Salinas, Haggie *et al.* 2005). For example, pancreatic insufficiency arises as a consequence of thickened fluid production in the exocrine ducts of the pancreatic acini, which blocks the passage of pancreatic enzymes required for food digestion leading to maldigestion and malabsorption (Davis, Drumm *et al.* 1996). Indeed the term CF refers to the development of epithelial-lined, concretion filled cysts surrounded by fibrous tissue that occur in the pancreas soon after birth (Anderson 1938; Zuelzer and Newton 1949).

Further to this gastrointestinal manifestations of CF may arise due to reduced $Cl^-$ secretion by the gastric parietal cell that impairs the formation of gastric acid in the stomach demonstrated in a murine model of CF (Sidani, Kirchhoff *et al.* 2007). Thus after birth, a failure to thrive, steatorrhea (fatty stools), and/or meconium ileus (neonatal intestinal obstruction) associated with pancreatic insufficiency are classic features suggesting a CF diagnosis (Anderson 1938; Zuelzer and Newton 1949; Davis, Drumm *et al.* 1996). Further to this the male vas deferens and seminiferous tubules become blocked and are then resorbed leading to male infertility and consequently an inability to palpate the vas deferens at birth (Davis, Drumm *et al.* 1996). The fertility of CF females is reduced but rarely absent (Super 1992).

When CF was first described the principle cause of death immediately following birth in CF is by meconium ileus and highlights the rapidity within which pancreatic changes occur (Anderson 1938; Zuelzer and Newton 1949). When surviving this initial period, affected children died of respiratory failure due to severe large airway plugging (Figure 1-1), caused, in part, by thickened exocrine gland secretions (Gibson, Burns et al. 2003). Other contributors to respiratory failure include repeated endobronchial infection and persistent leukocyte recruitment (inflammatory response) to the lungs (Zuelzer and Newton 1949; Sobonya and Taussig 1986). Chronic lung

infection and the inflammatory response both contribute to dilatation of the small and large airways (bronchiolectasis and bronchiectasis respectively) in almost all patients, associated with fibrotic changes, which over time may also destroy alveolar tissue (emphysema), though the alveolar tissue is often spared (Sobonya and Taussig 1986; Durie, Kent et al. 2004). The progression, severity and extent of lung disease varies substantially (Sobonya and Taussig 1986) but currently is the primary cause of death ending in respiratory failure in ~80% of CF affected individuals (Gibson, Burns et al. 2003). The progression and extent of respiratory disease is usually measured by lung function testing (spirometry) and/or chest X-ray and are good predictors of morbidity in CF patients of all ages (Kosorok, Zeng et al. 2001; Emerson, Rosenfeld et al. 2002; Ranganathan, Stocks et al. 2004). Spirometric assessment of lung function often involves measurement of a maximum inhalation of air followed by maximum forced expiration over one second (s) ($FEV_1$). The $FEV_1$ (in litres), compared to a large cohort of sex, age, race and height matched healthy controls, is then expressed as a percentage. A low $FEV_1$ percentage is an indicator of poor lung function compared to a healthy control population. Accordingly, those with the lowest $FEV_1$ exhibit the greatest disease severity and are thus associated with the greatest healthcare cost (Baumann, Stocklossa et al. 2003). It is one of the most important and widely used outcome measures to assess lung disease progression (Demko, Byard et al. 1995; Emerson, Rosenfeld et al. 2002) and in assessing clinical interventions (Suri, Marshall et al. 2003; Brennan and Geddes 2004). It is also used to assess the occurrence of an acute worsening or exacerbation of lung disease is associated with a wide range of clinical systemic symptoms over and above what is considered to be 'normal' for the affected individual. This may include chronic (daily) cough, production of increased volumes of bronchial mucus with accompanying leukocytes (sputum), a change in sputum colour from an initial purulent yellow/green to light brown and/or a decrease in $FEV_1$ (Gibson, Burns et al. 2003; Goss and Burns 2007).

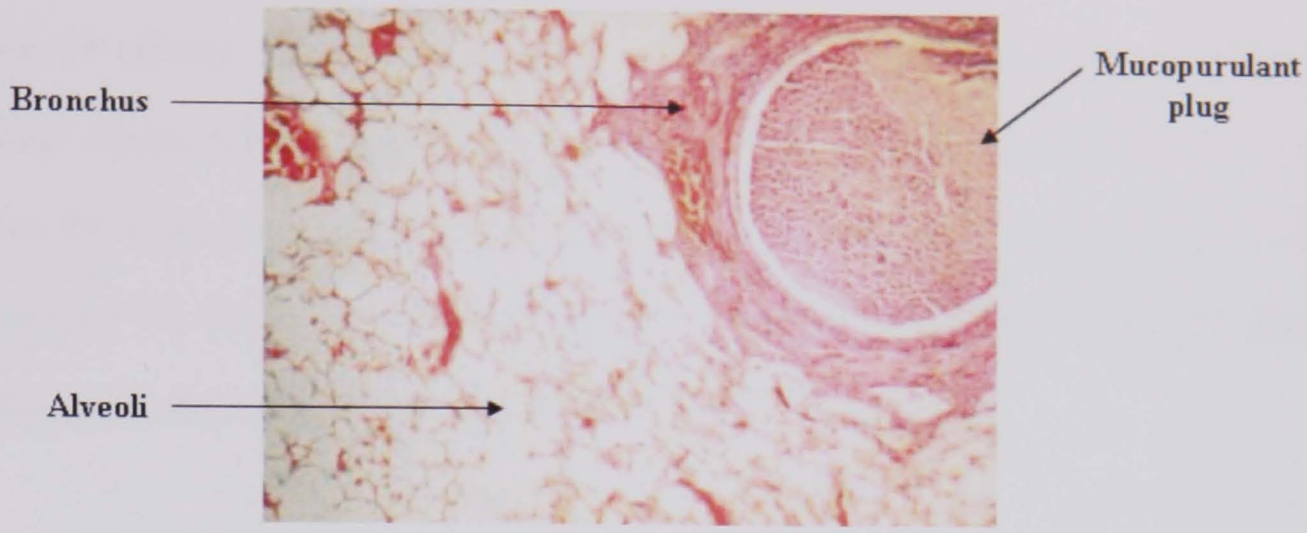


**Figure 1-1 Bronchial plugging is a prominent feature of CF (Gibson, Burns *et al.* 2003)**

A high infant mortality at the beginning of the 20th century and a broad range of symptoms complicated the clinical identification, diagnosis and characterisation of the basic defect (Anderson 1938; Zuelzer and Newton 1949) until identification of defective $Cl^-$ transport (Di Sant'Agnese, Darling *et al.* 1953). Di Sant'Agnese *et al.* (1953) described an excess of salt in the sweat of children affected with heat prostration i.e. dehydration and weakness in CF affected individuals. Elevated sweat sodium ($Na^+$) and $Cl^-$ represents a failure by cells lining the sweat gland to reabsorb $Cl^-$ (and thus $Na^+$) due to an absent $Cl^-$ transmembrane protein (Rowe, Miller *et al.* 2005). By contrast in the airway lining, the concentration of NaCl (and pH) has been reported as being similar to that of normal and disease control airway surface liquid (ASL) and submucosal gland (SMG) fluid (Knowles, Robinson *et al.* 1997; Matsui, Grubb *et al.* 1998; Jayaraman, Joo *et al.* 2001; Thiagarajah, Song *et al.* 2004) although this is disputed by others (Smith, Travis *et al.* 1996). Interestingly the sweat gland also does not produce thickened secretions and therefore a different $Cl^-$ transport mechanism to that in other exocrine glands such as those in the lung and pancreas are likely to be in operation (Rowe, Miller *et al.* 2005). Based

on Di Sant'Agnese's earlier work, elevated sweat $Cl^-$ concentrations ≥ ~60 mmol/litre indicates a possible CF affected individual (De Boeck, Wilschanski *et al.* 2005), when also supported by additional clinical features (such as those described above) since other conditions may cause an elevated $Cl^-$ level (Stern 1997). The sweat test is still one of the gold standard diagnostic indicators for CF, despite the advance in genetic technologies and the subsequent elucidation of the basic molecular defect in 1989 (De Boeck, Wilschanski *et al.* 2005).

### 1.1.2 Identification of the Cystic Fibrosis Gene

Landmark publications in the 1980's (Kerem, Rommens *et al.* 1989; Riordan, Rommens *et al.* 1989; Rommens, Iannuzzi *et al.* 1989) identified the gene responsible for CF. Their aim was to create a clone of genomic deoxynucleic acid (DNA) and through DNA hybridisation identify conserved regions between animal species that would be suggestive of house keeping and tissue specific genes (Rommens, Iannuzzi *et al.* 1989). A DNA sequence was obtained based on overlapping regions that contained an open reading frame capable of encoding 1480 amino acids (Riordan, Rommens *et al.* 1989).

A transcript of the putative CF gene was found in the nasal polyps and pancreas and to a lesser extent in the lung, colon, sweat gland, placenta, liver and parotid gland (Riordan, Rommens *et al.* 1989), a finding that concurred with the multi-organ pathology of the majority of CF affected individuals. Comparisons were then made between CF and non-CF genomic DNA sequences (Riordan, Rommens *et al.* 1989). Riordan *et al.* (1989) noted that a three base pair deletion leading to loss of a phenylalanine residue at amino acid position 508 (ΔF508) of the polypeptide was the most significant and consistent difference. Oligonucleotides carrying ΔF508 were shown to hybridise to 68% of CF chromosomes but not those of 198 unaffected individuals (Riordan,

Rommens *et al.* 1989). Since then, in a United States cohort of 293 CF patients, a ΔF508 mutation on the CF gene has been confirmed as the most prevalent (71% of patients) mutation (Kerem, Corey *et al.* 1990). From the same study, homozygosity for ΔF508 (ΔF508 +/+) (52% of subjects) was the most common mutation and correlates with pancreatic insufficiency in almost all individuals, reduced weight and a greater severity of lung disease i.e. a greater decline in $FEV_1$ (Kerem, Corey *et al.* 1990).

To date more than 1500 mutations have been identified in the CF gene (CF mutation database held at www.genet.sickkids.on.ca/cftr/StatisticsPage.html accessed 20th August 2007). The frequency of a given CF mutation is subject to substantial variation according to geographical area and ethnicity (Lucotte, Hazout *et al.* 1995; Hamosh, FitzSimmons *et al.* 1998; WHO 2002; Bossi, Casazza *et al.* 2004). Caucasians exhibit the highest incidence of CF, particularly Ireland (1 in 1800 births) (WHO 2002), as a result of an estimated carrier frequency of 1 in 25 (Gibson, Burns *et al.* 2003). The reason for this Caucasian bias is unclear but there are suggestions of a heterozygous advantage during cholera infection (by limiting secretory diarrhoea) and having a protective effect from cardiac arrythmias and ischaemia (Davis, Drumm *et al.* 1996; Guilbault, Saeed *et al.* 2006). Understandably CF research is most prominent in Western Europe and countries of white Caucasian origin e.g. Australia, Canada and the United States.

The identification of the genetic defect marked a watershed in CF research and diagnosis. Screening for CF, based on raised immunoreactive trypsin levels, was already established but genetic detection offers earlier and a greater specificity and sensitivity of detection. Genetic analysis has also enabled identification of a previously overlooked atypical population of CF adults who due to a borderline sweat test and lack of clinical symptoms have escaped the CF

diagnosis (Nick and Rodman 2005; Rodman, Polis *et al.* 2005). Thus, CF is no longer confined to paediatrics and represents a broad continuum of mild clinical manifestations (De Boeck, Wilschanski *et al.* 2005; Rodman, Polis *et al.* 2005). Most atypical individuals carry two mild CF causing mutations, lower sweat $Cl^-$, and are pancreatic sufficient, have significantly milder lung disease (i.e. increased $FEV_1$), colonisation/infection with a different spectrum of bacteria and fewer exacerbations requiring hospitalisation, consequently living to old age (Nick and Rodman 2005). Thus the rate of progression and severity of lung disease and pancreatic involvement varies with the type of CF mutation. However other genes (so called 'modifier genes') encoding immunoregulatory proteins and hereditary/life-style factors also contribute to the disease phenotype (Garred, Pressler *et al.* 1999; Drumm, Konstan *et al.* 2005; Plant, Gallagher *et al.* 2005; Brazova, Sismova *et al.* 2006).

### 1.1.3 The Structure and Function of the Cystic Fibrosis Gene Product

Riordan *et al.* (1989) predicted that the CF protein contains two membrane spanning motifs and therefore a transmembrane protein. They also found sequences that resemble nucleotide binding folds termed nucleotide binding domains (NBDs) 1 and 2. Comparisons with other membrane spanning molecules such as multidrug resistance P-glycoprotein led to the suggestion that the CF gene product was involved in ion transport across the cell membrane (Riordan, Rommens *et al.* 1989). The CF gene product has since been known as the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and is part of the adenosine tri-phosphate (ATP) binding channel (ABC) group of cell membrane transporters (Figure 1-2).

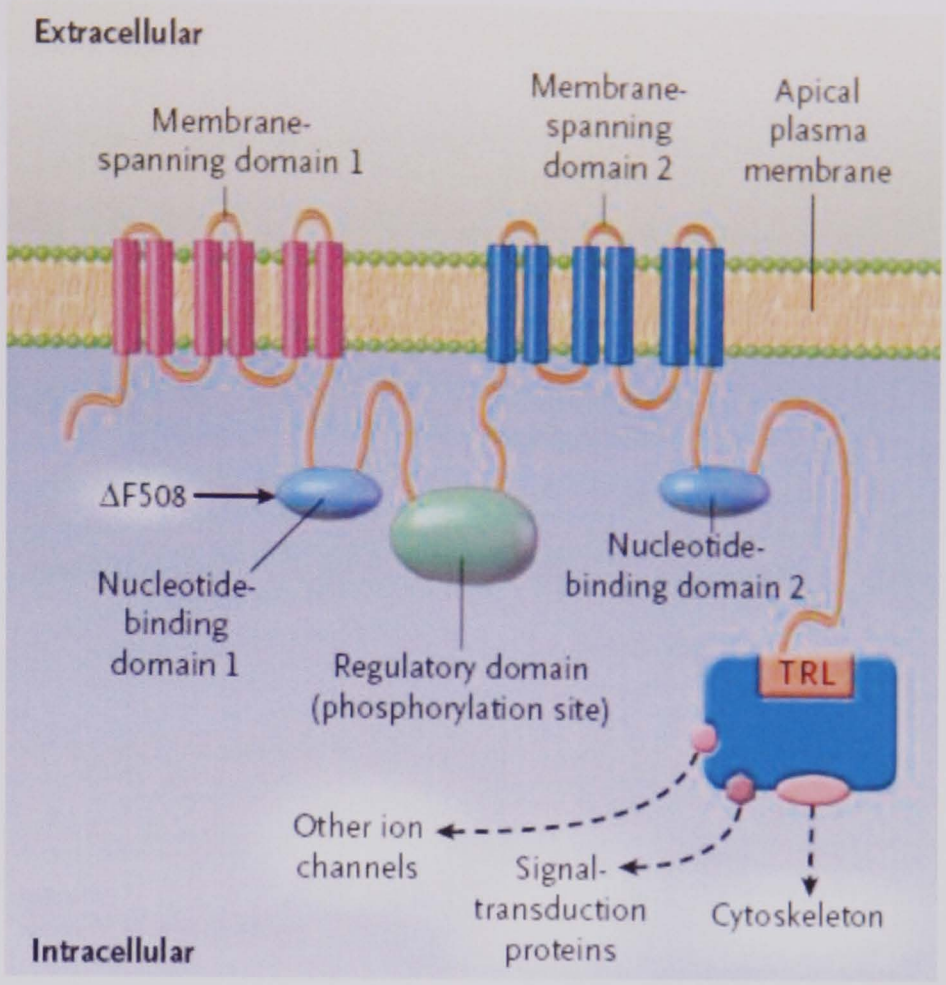


**Figure 1-2 CFTR protein inserted into the cell membrane (Rowe, Miller *et al.* 2005)**

CFTR exists as a monomer (Chen, Chang *et al.* 2002) and contains a unique regulatory (R) domain, which lies between NBD 1 and 2 (Figure 1-2). The R domain is a highly conserved region (Ostedgaard, Baldursson *et al.* 2001) that plays a role in regulating channel activity (Rich, Gregory *et al.* 1991) though it is not clear how it performs this function. Cyclic adenosine monophosphate dependant protein kinase A (PKA) and C (PKC) have an important role in regulating channel opening (Jia, Mathews *et al.* 1997) possibly via phosphorylation of the CFTR R domain, a process that is regulated by endogenous phosphatases (Ostedgaard, Baldursson *et al.* 2001; Gadsby, Vergani *et al.* 2006). However it is not clear which phosphorylated amino acid residues in the R domain cause inhibition or stimulation of channel activity (Ostedgaard, Baldursson *et al.* 2001). One view is that phosphorylation of the R domain by PKA increases the

affinity of the NBDs for ATP (Ostedgaard, Baldursson *et al.* 2001) another posits that phosphorylation of the R domain is required after ATP binding to allow opening of the channel pore (Riordan 2005). The function of the R domain is currently obscure, though channel opening and closing has recently been demonstrated to be dependent on ATP binding and hydrolysis at NBDs (Vergani, Lockless *et al.* 2005).

In non-CF cells, following gene transcription, CFTR is transported through various organelle compartments where it can be co-translationally folded (Kleizen, van Vlijmen *et al.* 2005) and subject to post-translational modifications (e.g. glycosylation) before insertion into the apical cell membrane in an efficient and stable manner (Varga, Jurkuvenaite *et al.* 2004). In CF, ΔF508 is present in NBD 1 (Figure 1-2) thus causing misfolding of the CFTR protein, which marks it for degradation by the endoplasmic reticulum quality control machinery in an ubiquitin dependent manner, forming part of the endoplasmic reticulum-associated degradation pathway (Gelman, Kannegaard *et al.* 2002; Younger, Chen *et al.* 2006). Mutant CFTR is detected by a large molecular complex involving heat shock proteins, ubiquitin ligases and endoplasmic reticulum membrane proteins such as RMA1 (Younger, Chen *et al.* 2006), which gives rise to an activation of this degradation pathway (Gelman, Kannegaard *et al.* 2002; Vij, Fang *et al.* 2006).

ΔF508 homozygosity is a class II mutation that prohibits the insertion of CFTR into the apical cell membrane (Engelhardt, Yankaskas *et al.* 1992) and is thus proteolytically degraded as described (Figure 1-3). Other classes of mutation (e.g. class III G551D and class IV R347P) escape this quality control checkpoint (Gibson, Burns *et al.* 2003) and insert themselves into the membrane but may lack proper functionality (Figure 1-3) (Engelhardt, Yankaskas *et al.* 1992). Still, low levels of membrane CFTR may be sufficient to ameliorate some of the disease

symptoms (Davis, Drumm *et al.* 1996) since individuals with other classes of mutation have less severe disease compared to homozygous (ΔF508) affected patients (Kerem, Corey *et al.* 1990; Rodman, Polis *et al.* 2005).

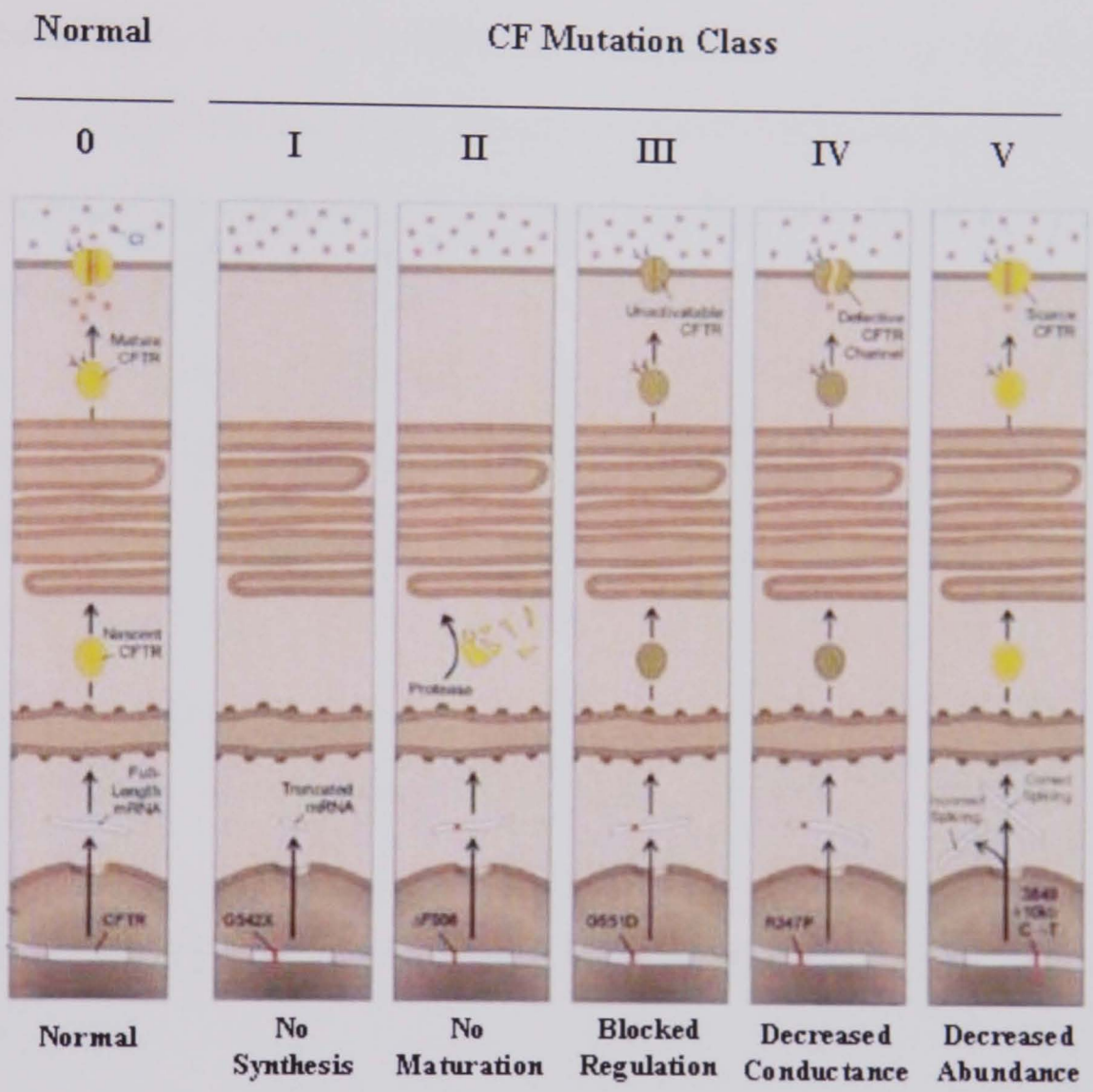


**Figure 1-3 Different CFTR mutations are processed differently (Gibson, Burns *et al.* 2003)**

Rich *et al.* (1990) demonstrated that CFTR has a major role in the efflux of $Cl^-$ ions, further substantiated by others (Anderson, Gregory *et al.* 1991), but also that the CF defect of abolished $Cl^-$ transport could also be reversed by wild-type (WT) CFTR. Unfortunately, to date, correction of the genetic defect, through the administration of WT CFTR, has not yet ameliorated the clinical manifestations of CF. CFTR may be rescued from the degradation pathway by a variety

of other mechanisms, including downregulation of heat shock proteins (Denning, Anderson *et al.* 1992; Wang, Venable *et al.* 2006), which also remain to be exploited therapeutically.

Further functions of CFTR, in addition to $Cl^-$ transportation, have been demonstrated *in vitro* and include the transport of bicarbonate ($HCO_3^-$) (Poulsen, Fischer *et al.* 1994; Illek, Yankaskas *et al.* 1997) and the extracellular antioxidant glutathione (Linsdell and Hanrahan 1998; Gao, Kim *et al.* 1999), which have both been linked to CF pathophysiology (Machen 2006). CFTR has been shown to play a role in cell membrane recycling (Barasch, Kiss *et al.* 1991; Bradbury, Jilling *et al.* 1992), $Cl^-$ dependent endosome fusion (Biwersi, Emans *et al.* 1996) and in other organelle compartments, acidification of endosomes (Barasch, Kiss *et al.* 1991; Di, Brown *et al.* 2006; Painter, Valentine *et al.* 2006) though this has been refuted (Lukacs, Chang *et al.* 1992; Biwersi, Emans *et al.* 1996; Seksek, Biwersi *et al.* 1996). CFTR also interacts with a wide array of cytosolic and transmembrane proteins that are involved in fluid and electrolyte homeostasis, cell growth and development, ion transport and the immune response (Xu, Liu *et al.* 2006). Therefore the role of CFTR beyond that of a $Cl^-$ channel is becoming increasingly apparent (Li and Naren 2005; Mehta 2005; Xu, Liu *et al.* 2006). However it is still not fully resolved how disruption in the transport of these molecules (including $Cl^-$), caused by loss of CFTR, is associated with the various manifestations of the final CF disease phenotype (Machen 2006). Further work in these areas is important to our understanding of how mutant CFTR leads to a systemic exocrinopathy causing infertility aswell as gastrointestinal and pulmonary complications.

### 1.1.4 The Lung is the Primary Site of Pathology

Mutant CFTR does not appear to have an effect on the development and maturity of foetal lungs, which have been described as being normal at birth (Hubeau, Puchelle *et al.* 2001). However the

majority of respiratory symptoms begin within three months of birth underscoring the vital role CFTR plays to maintain respiratory health (Zuelzer and Newton 1949). The human lung is divided into the following sterile areas, which divide continuously in a dichotomous fashion: the oropharynx, trachea, bronchi (surrounded by cartilage) and bronchioles (cartilage free) that terminate in the alveoli where the gas exchange interface mediated by type I epithelial cells (ECs) are located. The exocrine SMG are located within the cartilaginous airways of the lung i.e. the bronchi (Figure 1-4) (Rogers 2003; Ballard and Inglis 2004). The most prominent respiratory changes in CF include plugging of the bronchi and bronchioles with inspissated secretions from the bronchial and tracheal SMGs (Zuelzer and Newton 1949). Other associated changes include increased desquamation and slower maturation of ECs (Zuelzer and Newton 1949; Hajj, Lesimple *et al.* 2007), bronchiectasis with occasional SMG dilatation (Sobonya and Taussig 1986), thinning of the subepithelial basal membrane, collagen and elastin degradation (Zuelzer and Newton 1949; Durieu, Peyrol *et al.* 1998), increased airway smooth muscle mass (Hays, Ferrando *et al.* 2005) and finally airway fibrosis (Sobonya and Taussig 1986). Emphysema is not a prominent feature (Sobonya and Taussig 1986).

In line with these pathological descriptions is the identification and localisation of CFTR throughout the respiratory tree. At birth, CFTR mRNA and protein expression is patchy, of low abundance along the airway epithelium and localised to the bronchioles (Engelhardt, Yankaskas *et al.* 1992; Engelhardt, Zepeda *et al.* 1994), SMGs (Engelhardt, Yankaskas *et al.* 1992; Tizzano, O'Brodovich *et al.* 1994) and possibly alveolar type II cells (McCray, Wohlford-Lenane *et al.* 1992). The expression of CFTR protein on the airway surface epithelium was shown to be strong in very few cells (Engelhardt, Yankaskas *et al.* 1992). Closer inspection of CFTR protein

localisation in the SMG (Figure 1-4) has identified the origin for much of the inspissated secretions observed in the CF airways.

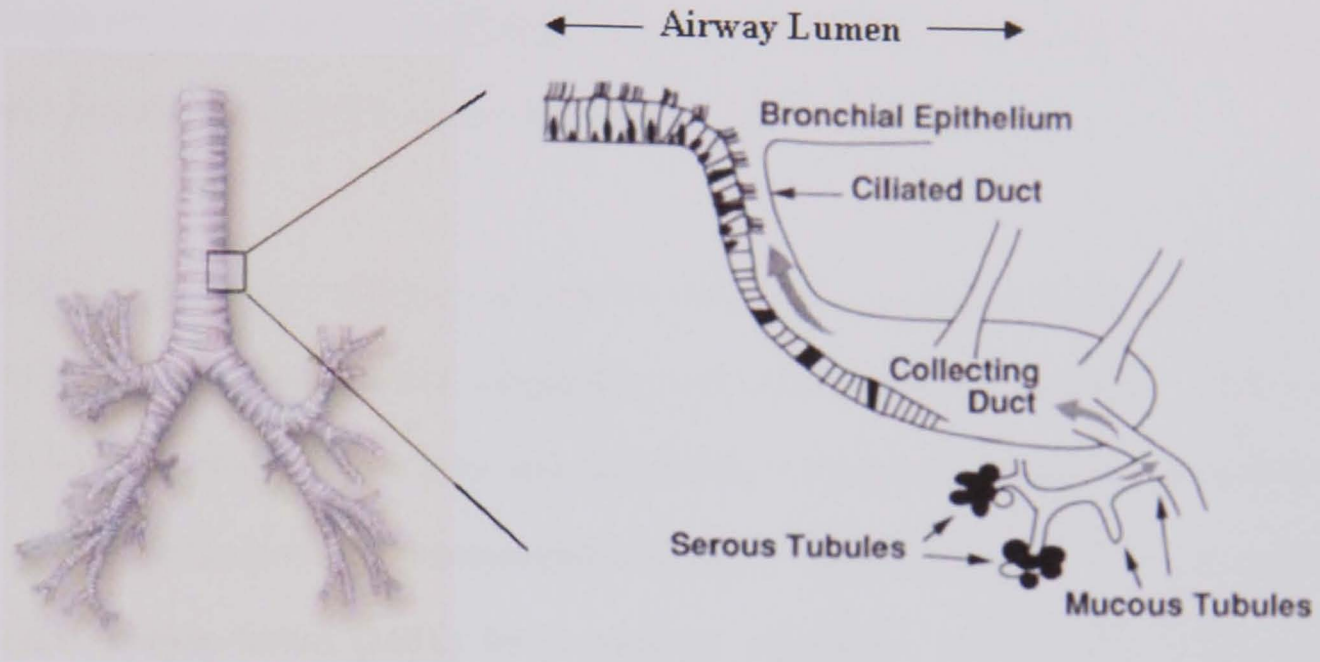


**Figure 1-4 A diagrammatic representation of the airways (left) and the SMG (right) showing high CFTR expressing regions (in black) (Engelhardt, Yankaskas *et al.* 1992; Rowe, Miller *et al.* 2005)**

The SMG is divided into a proximal ciliated region, a middle collecting duct and distal region characterised by the presence of either serous or mucous tubules (Engelhardt, Yankaskas *et al.* 1992). In the distal region of the SMG, serous tubule cells all had very high levels of CFTR protein whereas CFTR could not be detected in the mucous tubule cells (Engelhardt, Yankaskas *et al.* 1992). Serous cells are responsible for secreting the watery fluid that hydrates mucins produced by mucous tubule cells and the airway surface bronchial goblet cells (Rogers 2003). Serous cells help maintain the composition and volume of the ASL (Boucher 2004; Salinas, Haggie *et al.* 2005), which lies over the bronchial epithelium and are an important source of proteins that also help to protect the airway from infection and collateral damage from the immune responses (Joo, Lee *et al.* 2004). The ASL bathes respiratory epithelia, facilitates cilia

beat, gas exchange at the respiratory bronchioles and alveoli, lubricates the lung mechanics (via surfactant) during breathing and protects the respiratory epithelium against infection and consequences of an immune response. It is thus in the bronchial region, where absent CFTR leads to an alteration of ASL and compromised antibacterial defences.

A plethora of diverse, cell-derived proteins make up a 'chemical shield' within the ASL to protect the lung from bacterial colonisation and inflammation by-products. These may have direct anti-bacterial effects e.g. α- and β-defensins, cathelicidins, lysozyme, lactoferrin or may coat (opsonise) bacteria e.g. immunoglobulins (Igs), surfactant proteins (SP) A-D, complement, mannan binding lectins (MBL) for recognition, engulfment and digestion (phagocytosis) by resident and recruited leukocytes of the innate immune system. The ASL also protects the epithelium from leukocyte derived degradative enzymes and oxidants via anti-proteases ($\alpha_1$-anti-trypsin, $\alpha_1$-anti-chymotrypsin, secretory leucocyte protease inhibitor (SLPI), tissue inhibitors of metalloproteinase (TIMPs)) and via anti-oxidants (e.g. CFTR transported glutathione). The mucociliary escalator and cough clearance are also very important mechanical mechanisms (which complement the chemical shield and leukocyte defences) for clearing inhaled particles and bacteria. These mechanisms rely on appropriate ASL viscosity and volume (Knowles and Boucher 2002; Randell and Boucher 2006). ASL volume sensing and composition is maintained by active ion/water membrane transport channels (CFTR and Epithelial $Na^+$ Channel (ENaC), amongst others), adenosine (and their receptors), serine proteases and protease activated receptors as well as other, yet to be defined mechanisms (Boucher 2004; Myerburg, Butterworth *et al.* 2006; Randell and Boucher 2006; Factor, Mutlu *et al.* 2007).

In homozygous ΔF508 individuals, apical CFTR protein is absent from those areas shown in Figure 1-4 (Engelhardt, Yankaskas *et al.* 1992). SMG dysfunction likely precedes the onset of infection and an immune response (Zuelzer and Newton 1949; Mall, Grubb *et al.* 2004; Salinas, Haggie *et al.* 2005). Absent CFTR leads to a deficiency of serous cells to hydrate the mucus that collects in the SMGs ready for transport to the airway surface and instead a thicker fluid is produced (Jayaraman, Joo *et al.* 2001; Thiagarajah, Song *et al.* 2004; Salinas, Haggie *et al.* 2005; Joo, Irokawa *et al.* 2006). Thiagarajah *et al.* (2004) were also able to determine that reduced water content had a more prominent role than elevated protein secretion in generating thicker secretions using a CFTR inhibitor ($CFTR_{inh}^{-172}$) on *ex vivo* pig and human SMG tissue. However, absent CFTR may have a different effect on SMG fluid hydration compared to its effects on airway surface hydration (Joo, Irokawa *et al.* 2006). The most prominent theory to link loss of CFTR to inspissated bronchial secretions and abolished anti-bacterial defence mechanisms is known as the ASL volume depletion theory (Figure 1-5) (Knowles and Boucher 2002).

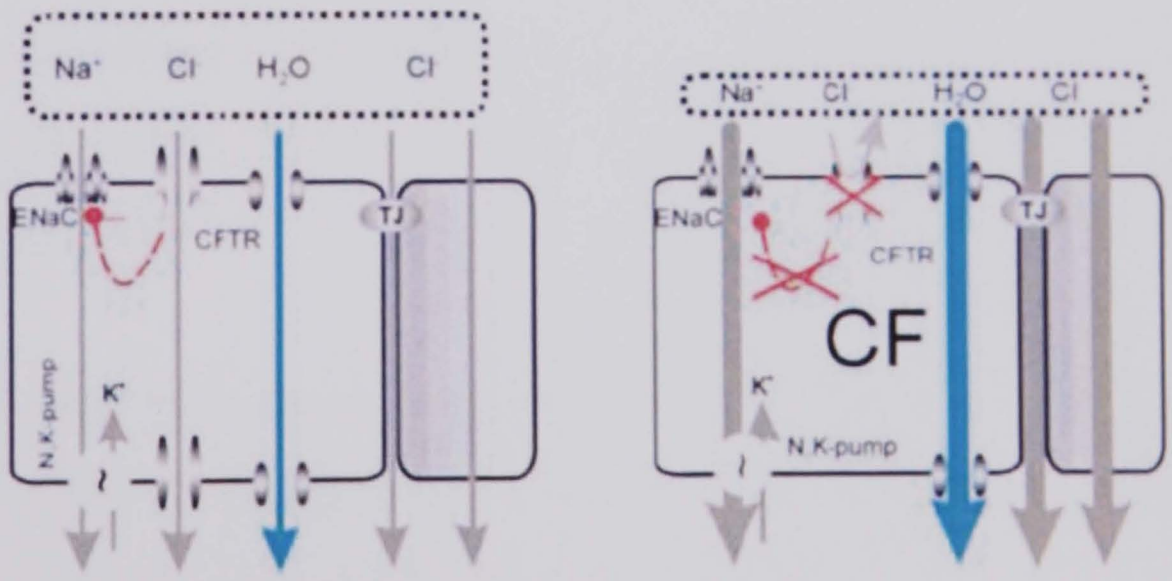


**Figure 1-5 Role of CFTR in maintaining ASL volume in normal respiratory epithelia (left) and the consequences of absent CFTR in CF (right) (Gibson, Burns *et al.* 2003)**

When the ASL volume is high the ENaC present in the apical membrane is highly active (Boucher 2004). The absorption of $Na^+$ through ENaC allows $Cl^-$ to flow concomitantly through

a paracellular route and via CFTR thus driving water ($H_20$) absorption through the epithelial lining and reducing ASL volume (Figure 1-5, left) (Boucher 2004). When the volume is low $Cl^-$ is driven out of the cell by CFTR allowing $Na^+$ ions to pass paracellularly to restore volume by the concomitant movement of $H_2O$ out of the cell. The restoration of ASL volume by CFTR does not occur in CF affected individuals. CFTR has been proposed to inhibit ENaC activity through currently undefined mechanisms but NBD1 has been shown to be important (Schreiber, Hopf *et al.* 1999; Boucher 2004) and therefore the absence of CFTR removes the inhibitory effect and elevated ENaC activity drives $H_20$ hyperabsorption on airway surfaces (Figure 1-5, right) (Boucher 2004; Mall, Grubb *et al.* 2004; Myerburg, Butterworth *et al.* 2006). The volume depletion theory and its dependence on $Na^+$ transport is also supported by the generation of a CF mouse model that overexpress ENaC and exhibits features of human CF disease such as volume depletion, SMG enlargement, mucus plugging and retention of bacteria (when added experimentally) in the airways (Mall, Grubb *et al.* 2004). However when inhibiting $Na^+$ channel activity in a randomized, double blind, placebo-controlled clinical study the investigators did not find any improvement in pulmonary function, bacterial densities or sputum volume (Graham, Hasani *et al.* 1993).

Taken together viscous mucus, mucus stasis and weakening of anti-bacterial mechanical and chemical defences facilitate acute infection by a select group of organisms and the generation of hypoxic mucopurulant plugs (Smith, Travis *et al.* 1996; Matsui, Grubb *et al.* 1998; Knowles and Boucher 2002; Worlitzsch, Tarran *et al.* 2002; Gibson, Burns *et al.* 2003; Moraes, Plumb *et al.* 2006). This scenario is initiated from birth and the cycle of chronic infection and a chronic leukocyte infiltration are major contributors to respiratory failure in CF.

### 1.1.5 Lung Infection is a Major Complication

*Staphylococcus aureus* (*S. aureus*) and non-typeable *Haemophilus influenzae* (*H. influenzae*) are the most common bacterial isolates in CF lung disease within the first years of life (Abman, Ogle *et al.* 1991; Armstrong, Grimwood *et al.* 1995; Gibson, Burns *et al.* 2003). This is followed by an increased prevalence of *Pseudomonas aeruginosa* (*P. aeruginosa*) colonisation and infection (Abman, Ogle *et al.* 1991) between 11-17 years, which persists throughout adulthood in ~80% of CF patients (Bauernfeind, Bertele *et al.* 1987; Gibson, Burns *et al.* 2003). However an antibody response towards *P. aeruginosa* can be detected as early as the first 15 months for 90% of CF infants (Burns, Gibson *et al.* 2001). *P. aeruginosa* is an opportunistic pathogen associated with infection only when the immune system may already be compromised. Other CF associated pathogens include *Aspergillus* species, *Mycobacteria* species, *Burkholderia cepacia* and as CF patients age, other organisms are increasingly isolated (e.g. *Stenotrophomonas maltophilia*) and are associated with lung disease and antibiotic resistance (Lyczak, Cannon *et al.* 2002; Lambiase, Raia *et al.* 2006). In contrast to bacterial infections, viral infections (e.g. Influenza, Respiratory Syncytial Virus) have not been described in CF with any higher frequency than found in a normal population (Wat and Doull 2003; van Ewijk, van der Zalm *et al.* 2005). However, viral infections may increase the severity of lower respiratory disease, decrease the $FEV_1$ and facilitate colonisation by CF-associated bacterial lung pathogens (Wat and Doull 2003; van Ewijk, van der Zalm *et al.* 2005).

The organisms described above are almost pathognomic CF pathogens whose predeliction for the CF lung cannot be explained by viscous mucus alone and thus the role of CFTR in infection remains to be clarified (Chmiel, Berger *et al.* 2002; Gibson, Burns *et al.* 2003). Cell surface CFTR has been shown to bind *P. aeruginosa* (via LPS) and thus in CF, ECs may have lost an

important mechanism for eradicating this organism (Pier, Grout *et al.* 1996; Pier, Grout *et al.* 1997). Conversely CF cells display an increased density of modified glycolipids (asialoGM1), which are proposed attachment sites for *P. aeruginosa* (Saiman and Prince 1993). However, a close association between the airway epithelium and *P. aeruginosa* could not be found (Worlitzsch, Tarran et al. 2002). Therefore, why the CF airways exhibit a high predilection for *P. aeruginosa* is unknown (Pier 2002). In addition an array of *P. aeruginosa* derived proteins (elastase and cathepsins) allows it to establish an acute infection by degrading opsonins (Mariencheck, Alcorn et al. 2003; Alcorn and Wright 2004; Malloy, Veldhuizen *et al.* 2005), their receptors (Berger, Sorensen *et al.* 1989) and anti-microbial and anti-biofilm proteins such as lactoferrin (Britigan, Hayek *et al.* 1993; Singh, Parsek *et al.* 2002; Rogan, Taggart *et al.* 2004). Further to this, *P. aeruginosa* collagenase is an important contributor to epithelial cell detachment *in vitro* (Venaille, Ryan *et al.* 1998).

Once *P. aeruginosa* colonisation begins, most likely from the environment, single variants may persist for as long as 8 years (Romling, Fiedler *et al.* 1994; Lyczak, Cannon *et al.* 2002) where they adapt to the CF lung environment (Worlitzsch, Tarran *et al.* 2002; Matsui, Wagner *et al.* 2006; Smith, Buckley *et al.* 2006). In an *in vitro* model mimicking the CF lung (containing 8% mucin solids), *P. aeruginosa* adapts to the thickened environment by down-regulating virulence factors (e.g. Type III secretion system, flagella, production of proteases, lipopolysaccharide (LPS) modification) (Smith, Buckley *et al.* 2006) and upregulate alginate production, necessary for persistent colonisation and infection (Worlitzsch, Tarran *et al.* 2002; Matsui, Wagner *et al.* 2006). Overproduction of alginate, a bacterial exopolysaccharide, leads to the development of sessile biofilms (Cobb, Mychaleckyj *et al.* 2004) and the conversion of non-mucoid strains to a mucoid phenotype. Conversion to a mucoid, non-motile, phagocytosis resistant (Krieg, Helmke

*et al.* 1988; Mahenthiralingam, Campbell *et al.* 1994) phenotype enables *P. aeruginosa* to establish chronic infection. The mucoid phenotype of *P. aeruginosa* is associated with significant lung disease progression in comparison to non-mucoid phenotypes (Demko, Byard *et al.* 1995; Lyczak, Cannon *et al.* 2002).

*P. aeruginosa* has been observed as growing in 'macrocolonies' (> 100 μm in diameter) within CF mucus *ex vivo* and *in vitro* (Worlitzsch, Tarran *et al.* 2002; Matsui, Wagner *et al.* 2006). It was noted *in vivo* that these macrocolonies are not associated with the surface epithelium and instead are concentrated within the airway lumen (Worlitzsch, Tarran *et al.* 2002). Over time, mucus stasis, further mucus secretion and biofilm formation occlude the lumen of the bronchi and bronchioles. Oxygen consumption by ECs and bacteria generate 'hypoxic' (average $pO_2$ of 2.5 mmHg) areas where *P. aeruginosa* produce further alginate and form macrocolonies (Worlitzsch, Tarran *et al.* 2002). Taken together, biofilm and macrocolony formation within a viscous ASL protect the bacterium from the cellular (Leid, Willson *et al.* 2005) and humoral (Meluleni, Grout *et al.* 1995) immune system.

Following acquisition of *P. aeruginosa*, colonised CF patients are more likely to have a chronic cough and hospital admissions for a respiratory complication than *S. aureus* or *H. influenzae* colonised patients (Abman, Ogle *et al.* 1991). *P. aeruginosa* colonisation is associated with respiratory exacerbations, as measured by spirometry ($FEV_1$), chest X-ray, volume and colour of sputum, which contributes to further lung function decline (Abman, Ogle *et al.* 1991; Pier 2000; Kosorok, Zeng *et al.* 2001; Emerson, Rosenfeld *et al.* 2002; Lyczak, Cannon *et al.* 2002; Nixon, Armstrong *et al.* 2002; Lambiase, Raia *et al.* 2006). Furthermore, *P. aeruginosa* colonised patients have a higher risk of death than non-colonised CF patients (Emerson, Rosenfeld *et al.*

2002). Progressive lung function decline has been attributed to the presence of bacterial infection and the concomitant immune response within the lungs of CF patients (Dakin, Numa *et al.* 2002; Mayer-Hamblett, Aitken *et al.* 2007). Both persistent infection and the cellular immune response are major contributors to CF morbidity and ultimately mortality.

### 1.1.6 Innate Immunity Contributes to Lung Disease

Elie Metchnikoff has been credited with the birth of a cellular (as opposed to humoral) theory of host defence based upon his observations of leukocytes engulfing a thorn or yeasts in *Daphnia* in the 1880s, which were later termed phagocytes and are now known as the macrophage (MΦ) and the neutrophil (Metschnikoff 1884; Silverstein 1989; Ambrose 2007). However, similar observations of host defence in a rodent model (Slavjansky 1869) and in humans (Osler 1875; Ambrose 2006) existed before Metchnikoff's reports.

The innate immune system of the lung is phylogenetically conserved and comprised of cellular (e.g. MΦs, neutrophils, eosinophils, basophils and mast cells) and ASL soluble factors i.e. the chemical shield, described previously (Section 1.1.4). A broncho-alveolar lavage (BAL) with sterile saline is the most common method for sampling components of the innate and acquired immune system in the airways. The lung MΦ is the most recoverable and numerous leukocyte (Rutgers, Timens *et al.* 2000) being evenly distributed between large and small airways throughout the respiratory tree (Battaglia, Mauad *et al.* 2007). Together with ECs and the chemical shield, resident lung MΦs act as sentinels through evolutionarily conserved pattern recognition receptors (PRRs) and initiate acute immune responses (inflammation) to injury/infection through the production of cell derived proteins (cytokines) and other mediators (Delves and Roitt 2000).

Inflammation is a general term used to describe the following cardinal signs: redness ('*rubor*'), heat loss ('calor'), pain ('dolor'), tissue swelling ('tumor') and sometimes though not always, loss of function ('functio laesa') and varies in severity and duration. Resident MΦs assist in eliciting inflammation in response to detection of a variety of molecular patterns (MPs), which may be pathogen-associated or endogenous MPs some elicited by tissue injury (known as alarmins) (Medzhitov and Janeway 2000; Stuart and Ezekowitz 2005; Bianchi 2007). An inflammatory response is initially dominated by an influx of neutrophils (Maus, Huwe *et al.* 2002; Maus, von Grote *et al.* 2002), which engage in phagocytosis. Resident MΦs assist in phagocytosis but also promote clearance of accumulated dying neutrophils (Savill, Wyllie *et al.* 1989; Hanayama and Nagata 2005) from the airway lumen/tissue (resolution) once neutrophil phagocytosis has been completed. Resident and recruited MΦs restore the homeostasis of the lung and return to being the predominant cell type present in the airways of healthy individuals regardless of sampling method (Rutgers, Timens *et al.* 2000). If the infectious organism or particulate cannot be removed then acute inflammation gives rise to chronic inflammation that can take a variety of forms but is associated with morbidity and mortality in a variety of diseases, for example chronic obstructive pulmonary disease (COPD), asthma as well as CF (Nathan 2002).

In CF, thickened ASL and compromised mechanical and chemical antibacterial defences contribute to establishment and persistence of infection within the CF lung. A persistent neutrophil dominated (> 95% of total leukocytes), chronic immune response is a hallmark of CF and COPD (O'Donnell, Breen *et al.* 2006) and contributes to morbidity and mortality.

### 1.1.6.1 Role of the Neutrophil

It is acknowledged (Cantin 1995) that lung inflammation in CF starts early in life (within 1 year), likely to be in response to infection, with an unrelenting recruitment of neutrophils (Birrer, McElvaney *et al.* 1994; Balough, McCubbin *et al.* 1995; Khan, Wagener *et al.* 1995; Noah, Black *et al.* 1997; Sagel, Kapsner *et al.* 2001; Dakin, Numa *et al.* 2002; Conese, Copreni *et al.* 2003; Muhlebach, Reed *et al.* 2004). The quantity of airway neutrophils and protein for a neutrophil recruiting cytokine - CXCL-8 correlates with bacterial burden (Balough, McCubbin *et al.* 1995; Muhlebach, Stewart *et al.* 1999; Dakin, Numa *et al.* 2002; Muhlebach, Reed *et al.* 2004). CXCL-8 is a cytokine that, once bound to appropriate receptors (CXCR-1 and CXCR-2) on target cells (e.g. neutrophils), directs their migration up a concentration gradient (chemotaxis) and is therefore termed a chemokine. CXCL-8 mediates between 63 and 77% of the neutrophil migration across endothelial layer *in vitro* in response to LPS and proinflammatory cytokines (Baggiolini, Walz *et al.* 1989; Huber, Kunkel *et al.* 1991). CF sputum demonstrates neutrophil chemotactic activity mediated by CXCL-8, which increases with the severity of disease and decreases in patients who have been discharged from hospital (Richman-Eisenstat, Jorens *et al.* 1993). Other neutrophil chemoattractants that have been identified and are elevated in CF sputum include Leukotriene $B_4$ ($LTB_4$) and the complement component C5a (Fick, Robbins *et al.* 1986; Konstan, Walenga *et al.* 1993; Lawrence and Sorrell 1993). CXCL-8 (Mayer-Hamblett, Aitken *et al.* 2007) and $LTB_4$ (Greally, Hussein *et al.* 1993) have been negatively correlated with $FEV_1$. Therefore, other neutrophil chemotactic proteins such as $LTB_4$, $C5_a$, and collagen fragments via CXCR-2 (Weathington, van Houwelingen *et al.* 2006) may also have an important role in recruiting neutrophils to the airspace. Neutrophil recruitment in CF is thus more likely driven by neutrophil specific chemoattractants, since CF neutrophils *per se* have normal chemotactic activity (Church, Keens *et al.* 1979; Pizurki, Morris *et al.* 2000; Moraes, Plumb *et al.*

2006). However, in the presence of CF-like mucus *in vitro* Matsui *et al.* (2005) have demonstrated reduced chemotactic capability.

Chronic inflammation in CF is dominated by an overwhelming influx of neutrophils, which fail to digest bacteria in phagolysosomes (Painter, Valentine *et al.* 2006), and consequently undergo programmed cell death (apoptosis) (Gibson, Burns *et al.* 2003). Apoptotic neutrophils are a major component of CF sputa compared with non-CF bronchiectetic controls (Vandivier, Fadok *et al.* 2002). Apoptotic cells, unless removed by pulmonary MΦs (Barker, Erwig *et al.* 2002; Sexton, Al-Rabia *et al.* 2004) or via other mechanisms, become necrotic potentially leaking their degradative enzymes (e.g. elastase, myeloperoxidase (MPO), Hypochlorite (HOCl), Cathepsin G) and other potentially harmful metabolites (reactive oxygen intermediates (ROIs), hydrogen peroxide ($H_2O_2$)) and high molecular weight DNA into the airway milieu (Goldstein and Doring 1986; Vandivier, Fadok *et al.* 2002; Henke, Renner *et al.* 2004).

Neutrophil elastase, in particular, has been shown to overwhelm the normal quantities of protease inhibitors found in paediatric and adult CF ASL and create a protease/protease inhibitor imbalance (Goldstein and Doring 1986; Birrer, McElvaney *et al.* 1994; Khan, Wagener *et al.* 1995). A protease/protease inhibitor imbalance has an important role in explaining many features of CF pathophysiology and is central to the latest mechanism that attempts to describe chronic infection. *P. aeruginosa* phagocytosis is compromised by the action of neutrophil elastase that cleave complement receptor (CR) 1 (a receptor for the complement opsonin C3b) and the opsonic-ligand for CR3, iC3b creating an 'opsonin-receptor mismatch' (Berger, Sorensen *et al.* 1989; Tosi, Zakem *et al.* 1990). Further to this neutrophil elastase cleaves CXCR1 on neutrophils, preventing phagocytic killing and exacerbating CXCL-8 production by TLR 2

expressing cells (Hartl, Latzin *et al.* 2007) Aerosolising the main inhibitor to neutrophil elastase (α1-anti-trypsin) to CF patients has been shown to restore the protease/protease inhibitor imbalance and restore neutrophil killing of *P. aeruginosa* within CF ASL compared with control (McElvaney, Hubbard *et al.* 1991; Hartl, Latzin *et al.* 2007).

Neutrophil elastase has been shown to contribute to further neutrophil recruitment and airway plugging with mucopurulant material: *in vitro* elastase induces further CXCL-8 secretion (and thus neutrophil recruitment) by a human bronchial epithelial cell line (Nakamura, Yoshimura *et al.* 1992). In addition respiratory mucins increase during CF exacerbations (Henke, John *et al.* 2007) and neutrophil elastase may play a role in this by the demonstration that it potentiates further mucin secretion (Kohri, Ueki *et al.* 2002; Shao and Nadel 2005), in a time and dose dependent manner *in vitro* (Park, He *et al.* 2005). However in contrast to disease (chronic bronchitis) control, CF sputum contained much less quantities of specific mucins and greater quantities of DNA, which have been attributed to neutrophil decomposition causing further increases in CF ASL viscosity (Gibson, Burns *et al.* 2003), *P. aeruginosa* biofilm formation (Walker, Tomlin *et al.* 2005) and decline in $FEV_1$ (Henke, Renner *et al.* 2004).

Other neutrophil products such as Cathepsins and MPO play a role in airway epithelial cell injury (Venaille, Ryan *et al.* 1998) and are associated with decreased $FEV_1$ and thus increased severity of lung disease (Sloane, Lindner *et al.* 2005). Matrix metalloproteinases (MMPs) contribute to just under half of the elastase burden in the CF airways (Bruce, Poncz *et al.* 1985). MMPs are metzincin proteases that together with their inhibitors (TIMPs) play an important role in extracellular matrix turnover (Page-McCaw, Ewald *et al.* 2007). MMP-8, 9, 11,12 and TIMP-1 activity was found following an MMP screen in CF sputum (Gaggar, Li *et al.* 2007).

Interestingly, MMP-9 has been shown to significantly correlate with increased neutrophils, elevated CXCL-8 and a decline in $FEV_1$ (Sagel, Kapsner *et al.* 2005), although this latter observation could not be replicated by others (Gaggar, Li *et al.* 2007). Neutrophil elastase can contribute to elevated MMP-9 activity by cleaving pro-MMP-9 or by degrading TIMP-1 (Gaggar, Li *et al.* 2007). Similarly MMP-9 activity may further play a role in CF by inactivating airway protease inhibitors (Page-McCaw, Ewald *et al.* 2007).

Over the course of many years infection and inflammation lead to destruction of bronchial cartilage (Ogrinc, Kampalath *et al.* 1998), collagen and elastin fibres present in airway support tissue (Bruce, Poncz *et al.* 1985; Stone, Konstan *et al.* 1995; Durieu, Peyrol *et al.* 1998) and this leads to bronchiectasis (Sobonya and Taussig 1986; Gibson, Burns *et al.* 2003). Neutrophil elastase contributes to more than half of this breakdown (Bruce, Poncz *et al.* 1985; Power, O'Connor *et al.* 1994; Stone, Konstan *et al.* 1995) and has been shown to inversely correlate with severity of lung disease measured by $FEV_1$ (Power, O'Connor *et al.* 1994; Hartl, Latzin *et al.* 2007; Mayer-Hamblett, Aitken *et al.* 2007) and chest x-ray (Bruce, Poncz *et al.* 1985) in CF patients. Bronchiectasis is not confined to CF and is a feature of other diseases (chronic bronchitis) that have persistent infection and neutrophilic inflammation causing substantial morbidity, culminating in respiratory failure (King, Holdsworth *et al.* 2006). Like CF, neutrophilic inflammation in COPD increases with the severity of disease (indicated by a fall in $FEV_1$) (O'Donnell, Peebles *et al.* 2004) and is also associated with disease exacerbations (Qiu, Zhu *et al.* 2003). This disease however becomes apparent later in life with smoking being a major cause and unlike CF, emphysema is a prominent feature of disease (O'Donnell, Breen *et al.* 2006). Elucidating the mechanisms driving airway neutrophil recruitment is a research goal for both of these important chronic airway diseases.

### 1.1.6.2 Neutrophil Recruitment in the Absence of Infection

In CF, the persistent neutrophilic inflammation is so excessive that efforts have been made to identify whether mutant CFTR causes an intrinsic defect in the immune response, which could contribute to prolonged neutrophilic recruitment to the lungs. A comprehensive review by Machen (2006) suggests that mutant CFTR is not directly involved in altering the immune response but absent CFTR leads to an altered lung physiological environment that promotes activation of pro-inflammatory signaling cascades, through airway ECs, without the need for an infection.

In support of these views, CF infants with negative bacterial and viral cultures still maintain a low level inflammatory response indicated by elevated neutrophil recruitment, coupled with higher levels of CXCL-8 in BAL fluid (Konstan, Hilliard *et al.* 1994; Balough, McCubbin *et al.* 1995; Khan, Wagener *et al.* 1995; Noah, Black *et al.* 1997; Muhlebach, Stewart *et al.* 1999). There was also a trend towards higher CXCL-8 levels in CF infants infected with *H. influenzae* compared with control infants infected with the same pathogen, however this was not significant (Noah, Black *et al.* 1997) possibly due to the small number of individuals analysed ($n$=3). When infected control and uninfected CF infants were compared, the median level of CXCL-8 was seven-fold higher in CF (Noah, Black *et al.* 1997) or similar (Muhlebach, Stewart *et al.* 1999) compared to controls. Despite this conflicting data, neutrophils and CXCL-8 are significantly increased in CF BAL fluids compared to control even at the same level of LPS activity (Muhlebach, Stewart *et al.* 1999; Muhlebach and Noah 2002). In contrast, Armstrong *et al.* (1997), looking at the same immunological parameters (CXCL-8, neutrophil numbers and elastase) as Khan, Wagener *et al.* (1995), provide compelling evidence that in CF infants, with a mean age of 2.4 months, that there is no sign of increased inflammation over healthy controls.

When tracking lung development in aborted CF foetuses there was also no evidence of an intrinsic inflammatory response (Hubeau, Puchelle *et al.* 2001). These studies indicate that early inflammation does not originate *de novo* but may originate in response to infection however this does not exclude absent/defective CFTR from having a role in a defective immune response in CF. Until more sensitive techniques for detection of infectious agents are available, such findings of elevated inflammation are always open to claims of undetected bacterial/viral infection (Machen 2006). To address this issue, investigators have turned their attention to mouse models and *in vitro* systems where the environment is better characterised and infection can be ruled out as a confounding factor.

The elevated levels of CXCL-8 reported by Noah *et al.* (1997) are similar to those observed by Tirouvanziam, de Bentzmann *et al.* (2000) who looked at human CF airway grafts implanted onto immunodeficient mice kept under pathogen free conditions for evidence of inflammation compared to non-CF grafts. Here CF airway grafts produced eight-fold more CXCL-8 compared to non-CF grafts (Tirouvanziam, de Bentzmann *et al.* 2000), exhibited luminal bronchiolar neutrophilic inflammation (Tirouvanziam, Khazaal *et al.* 2002) and when challenged with *P. aeruginosa,* epithelial exfoliation was more intense than in a non-CF cohort (Tirouvanziam, de Bentzmann *et al.* 2000). In addition, in a CF murine model with upregulated ENaC activity, neutrophils accumulate in the sterile airway lumen post natally (but not in newborns) in association with significantly elevated levels of MΦ Inflammatory Protein-2 (MIP-2), the murine homologue of human CXCL-8 (Mall, Grubb *et al.* 2004). Interestingly epithelial cell fluid did not contain elevated MIP-2 thereby implicating sources other than airway ECs for inititating, at least early, neutrophil chemotaxis to the airways such as resident MΦs. Furthermore in a recent study by Perez, Issler *et al.* (2007) using a CFTR inhibitor ($CFTR_{inh}^{-172}$) on a normal human

tracheal cell line *in vitro*, basal but not apical CXCL-8 was significantly increased over controls and again further in response to *P. aeruginosa.*

Further evidence for an elevated immune response, this time in response to infection, has come from mouse models of CF exhibiting either a null (S489X) mutation (Heeckeren, Walenga *et al.* 1997) or a class III mutation (G551D) (Thomas, Costelloe *et al.* 2000; McMorran, Palmer *et al.* 2001; Vij, Fang *et al.* 2006). All of these models exhibited excessive intraluminal accumulation of neutrophils in the airways. Bacterial load was identical between the murine S489X model and controls but the former exhibited significantly greater levels of the pro-inflammatory cytokine tumour necrosis factor (TNF) and MIP-2 (Heeckeren, Walenga *et al.* 1997). This aggressive immune response without clearance of pulmonary infection could have contributed to the high death rate in this model. In support of a dysregulated immune response, in a class III murine model of infection, TNF and MIP-2 were both elevated at day 1 following infection compared to controls. However both of these cytokines were lower than controls on day 2, which could possibly contribute to a hyporesponse to infection thus contributing to increased persistence of infection.

A number of theories have been put forward to explain a pulmonary hyper-inflammatory response in mutant CFTR expressing lung airway EC in the absence of infection (Machen 2006). All of these theories link either non-functional CFTR or CFTR deficiency to an altered metabolic response that culminates in the activation and increased translocation of the Nuclear Factor – Kappa B (NF-κB) transcription factor to the nucleus. NF-κB then activates the transcription of many pro-inflammatory genes (including both CXCL-8 and TNF) leading to persistent recruitment of neutrophils. In support of this has been the demonstration of elevated constitutive

NF-κB translocation present in mutant CFTR affected cell lines (Knorre, Wagner *et al.* 2002; Vij, Fang *et al.* 2006). These theories (Machen 2006) include; 1) elevated $Na^-$ influx through ENaC, followed by $Na^+$ efflux via the basolateral $Na^+$-$K^+$ ATPase causing increased oxygen consumption by mitochondria and the generation of ROIs with subsequent NF-κB activation; 2) Reduced glutathione and $HCO_3^-$ efflux by mutant CFTR increases the oxidation and acidity of the ASL, which than activates NF-κB; 3) ER stress by misfolded CFTR elevates cytosolic calcium thereby activating NF-κB translocation.

## 1.2 The Lung Macrophage in Cystic Fibrosis

To date, resident lung MΦs have received little attention in the aetiology of CF despite their contribution to the control of infection and inflammation. Lung MΦs (Figure 1-6) defend the epithelial surface against environmental (microbial and non-microbial particulates) insult (Kulkarni, Pierse *et al.* 2006), clear tissue debris/leukocytes and their products which could cause tissue damage (Sexton, Al-Rabia *et al.* 2004), actively participate in the initiation and regulation of innate and adaptive immune responses (Lohmann-Matthes, Steinmuller *et al.* 1994) and produce enzymes involved in tissue remodelling and repair processes (Nathan 1987). Given these important roles and location, resident and/or recruited lung MΦs could thus be important in controlling CF bacterial colonisation/persistence and in perpetuating the self destructive chronic inflammation, and may provide a target to ameliorate disease.

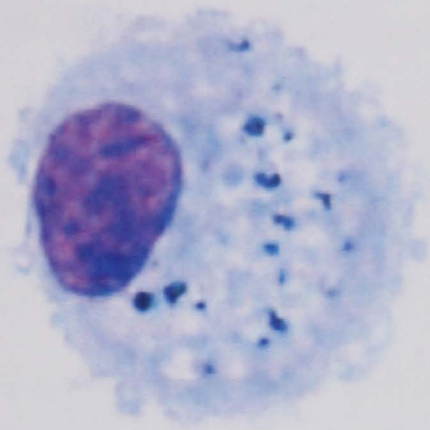

**Figure 1-6 Light microscopy of an airway MΦ with ingested particulate matter (black) (Kulkarni, Pierse *et al.* 2006)**

The MΦ exists throughout many of the tissue compartments of the major organs, where different environmental niches confer heterogeneity in MΦ phenotype but bound by common functional attributes and origins (van Furth, Cohn *et al.* 1972). As well as the lung MΦ other tissue MΦs include liver Kuppfer cells, bone associated osteoclasts, nervous system microglia and the peritoneal MΦ.

The MΦ by definition is tissue resident and typically large but can vary from 10-25 μm in diameter (van Furth, Cohn *et al.* 1972) to anything upto 25-50 μm (Ross and Auger 2002). They have an eccentrically placed nucleus that is round or kidney shaped, with a ruffled membrane and microvilli (Ross and Auger 2002) as shown in Figure 1-6. The presence of large cytoplasmic vacuoles and large granules reflect their active phagocytic and metabolic activity (Ross and Auger 2002).

### 1.2.1 Origins and Kinetics

Metchnikoff was not the first to describe phagocytosis, where this phenomonon was also observed by others, notably by Slavjansky in 1869 and William Osler, the latter looking at the

pathology of miner's lung (Osler 1875). In 1875, Osler noted the ingestion of carbon particles by "usually round, sometimes oval, occasionally irregular corpuscles" and "these are variable in size...approaching the size of colourless blood corpuscles...or attain five or six times their size" (Osler 1875). These descriptions and those of Slavjansky are perhaps the earliest observations of lung phagocyte (most likely MΦ) activity in animals such as the Guineapig (Slavjansky 1869) and in humans (Osler 1875; Ambrose 2006). However what set Metchnikoffs studies apart was his firm belief (and determination) that phagocytosis formed part of an important defensive capability (allied to humoral immunity championed by Paul Erhlich) to render harmless what may have been harmful.

In the lung different types of MΦ have been recognised based on their location throughout the respiratory system (Lohmann-Matthes, Steinmuller *et al.* 1994). MΦs reside in the ASL in close association with ECs throughout the large airways (bronchial MΦ) but particularly in the alveoli (alveolar MΦ) and connective tissue (interstitial MΦ) spaces of the lung (van oud Alblas and van Furth 1979; Lohmann-Matthes, Steinmuller *et al.* 1994). Resident alveolar MΦs are the best described due to their traditional sampling by BAL in humans and rodents and their large numbers, where they can constitute over 90% of total cells in children (Riedler, Grigg *et al.* 1995) and adults (Spiteri, Clarke *et al.* 1992). Examination of human CF alveolar MΦs by electron microscopy does not reveal any significant differences from control MΦs, both containing "...a spectrum of organelles and inclusions...moderate number of phagosomes containing myelin...some primary lysosomes...." (Thomassen, Demko *et al.* 1980). Alveolar and bronchial MΦs are considered to represent the paradigm of mature lung MΦs due to their large size and status as terminally differentiated cells (Lohmann-Matthes, Steinmuller *et al.* 1994). In contrast, interstitial MΦs are smaller, may constitute upto 40% of total MΦs and it has been suggested that

they may be an intermediate in the development of alveolar MΦs (Laskin, Weinberger *et al.* 2001). This has now been demonstrated in a mouse model whereby BAL and parenchyma MΦ reconstitution with donor monocytes was tracked following bone marrow grafts (Landsman and Jung 2007)

The life span of lung MΦs has been estimated at approximately three months in humans (Thomas, Ramberg *et al.* 1976; Ross and Auger 2002) or ~ 27 days in mice (van oud Alblas and van Furth 1979), where they are continually being replaced throughout adult life by the constitutive recruitment of MΦ precursors from the bone marrow via the blood stream (Thomas, Ramberg *et al.* 1976; van oud Alblas and van Furth 1979; Ross and Auger 2002; Maus, Janzen *et al.* 2006). The bone marrow origin of the lung MΦ was demonstrated in humans following a sex mis-match bone marrow transplant allowing identification of host and donor MΦs (Thomas, Ramberg *et al.* 1976). However rodent models of lung MΦ origins demonstrate that there may be other cell sources during prenatal life, before development of the bone marrow, including the murine yolk sac (Moore and Metcalf 1970; Ross and Auger 2002), 'angular cells' in rat prenatal lungs (Sorokin, McNelly *et al.* 1992) or local precursors in the lung (Summer, Kotton *et al.* 2004).

In the bone marrow, multi progenitor cells under the influence of haematopoietic growth factors known as colony stimulating factors (CSFs) become monoblasts yielding two pro-monocytes which undergo mitosis to become circulating monocytes - a process termed 'monocytopoiesis' (Figure 1-7). Monocytopoiesis is important because although human alveolar MΦs retain some proliferative capacity (Golde, Byers *et al.* 1974; Pforte, Gerth *et al.* 1993; Nakata, Gotoh *et al.* 1999; Gordon and Taylor 2005) rodent studies show that transmigration of monocytes from the

circulation into the lung is the dominant pathway for the replenishment of ~95% of tissue (including lung) MΦs under constitutive and inflammatory conditions (Nakata, Gotoh *et al.* 1999; Ross and Auger 2002; Xu, Manivannan *et al.* 2005).

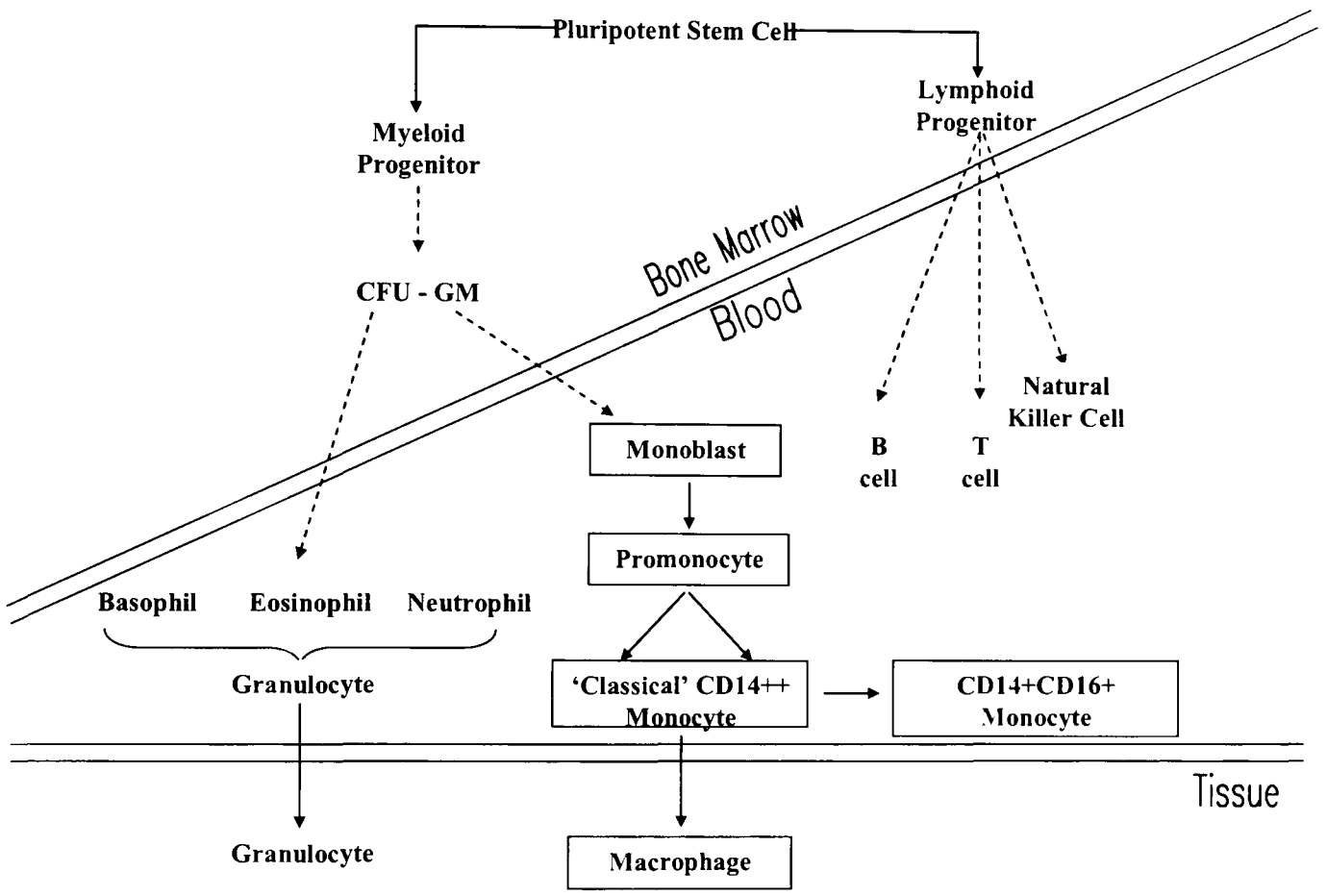


**Figure 1-7 Tissue MΦs are repopulated by monocytopoiesis.** Progenitors committed to the MΦ lineage are shown in boxes

## 1.2.2 The Monocyte: A Macrophage Precursor

Two of the most important CSFs that are required for the generation of monocytes from multi-potent progenitor cells include MΦ-CSF (M-CSF) and Granulocyte/MΦ–CSF (GM-CSF). Both of these growth factors may be derived from resident MΦs, or MΦ-stimulated endothelial cells or fibroblasts present within the bone marrow (Ross and Auger 2002). M-CSF is important for constitutive production of monocytes whereas GM-CSF and IL-3, both produced by MΦ stimulated T cells, are considered to be important in upregulating production in response to infection (Ross and Auger 2002). M-CSF binding to its receptor (c-fms) present on the cell

surface of progenitor cells stimulates the differentiation of monoblasts and promonocytes to mature monocytes (Motoyoshi 1998; Ross and Auger 2002) and prolongs monocyte survival (Becker, Warren *et al.* 1987).

Human monocytes circulate in the blood-stream including the marginal pool, constituting 1-6% of leukocytes (300-700 cells per μl of peripheral blood) (Steppich, Dayyani *et al.* 2000; Ross and Auger 2002) and ~10% of all mono-nucleated cells (PBMCs). Therefore monocytes are particularly amenable to isolation and *in vitro* culture, which under the appropriate conditions generates monocyte-derived MΦs (MDM) to assess cell function. Monocytes are typically much smaller than MΦs being just 10-14 μm in diameter, they have a less undulating membrane and exhibit an oval or indented nucleus that takes up a much larger proportion of the cytoplasm than the MΦ (van Furth, Cohn *et al.* 1972). *In vivo,* monocytes are committed to the generation of MΦs and their pulmonary phenotypic variants (Van Furth, Diesselhoff-den Dulk *et al.* 1973; Gordon and Taylor 2005). It has been proposed that in man, monocytes may circulate for up to 70 hours before proportional (based on organ size) migration, across the endothelium into tissues (Ross and Auger 2002). In mice monocytes have been demonstrated to engraft in the lung where maturation towards a terminally differentiated MΦ occurs further in response to growth factors and cytokines in the local environment (Kennedy and Abkowitz 1998).

At first thought to be one homogenous population, human monocytes are now considered to constitute two major populations (Ziegler-Heitbrock 2000), discriminated via expression of Cluster of Differentiation (CD) antigen 14, part of the LPS receptor complex (Simmons, Tan *et al.* 1989; Wright, Ramos *et al.* 1990), and CD16 (an IgG receptor) present on the monocyte cell surface (Passlick, Flieger *et al.* 1989). Approximately 90% (266 ± 127 cells/μl) of total

monocytes can be detected with strong expression of CD14 (therefore considered to be a human monocyte marker) but little or no CD16. These cells were subsequently named 'classical' monocytes. The remaining ~10% (45 ± 35 cells/μl) express medium levels of CD16 but medium to low amounts of CD14 (Passlick, Flieger *et al.* 1989). The latter CD16 cells are slightly smaller than their classical counterparts (average of 13.8 μm vs 18.4 μm) and are thought to derive from classical monocytes (Passlick, Flieger *et al.* 1989; Gordon and Taylor 2005). Classical monocytes are believed to be play an important role in recruitment to inflamed tissue sites (Gordon and Taylor 2005) whereas CD16 expressing monocytes may become resident MΦs in health (Landsman, Varol *et al.* 2007). Recruitment of monocytes during inflammation may be facilitated by high TNF producing (CD16 positive) monocytes, which have recently been shown to 'patrol' blood vessels (Auffray, Fogg *et al.* 2007).

### 1.2.3 Monocyte Migration into the Lung

Under constitutive conditions *ex vivo* alveolar ECs (Rosseau, Selhorst *et al.* 2000; Eghtesad, Jackson *et al.* 2001) and bronchial ECs (Koyama, Rennard *et al.* 1989; Pisabarro, Leung *et al.* 2006) release chemotactic proteins e.g. CCL-2 causing monocyte but not neutrophil (Muller and Weigl 1992) transmigration. CCL-2 (Eghtesad, Jackson *et al.* 2001; Maus, von Grote *et al.* 2002) binding to monocyte CCR-2 or dendritic cell and monocyte chemokine-like protein binding to as yet unidentified G-protein coupled receptors (Pisabarro, Leung *et al.* 2006) are important for constitutive monocyte chemotaxis to the airways. Depletion of alveolar MΦs in an *in vivo* murine model of constitutive trafficking indicates that ECs are more important than MΦs in controlling this migration via CCL-2 (Maus, Koay *et al.* 2002).

Under steady state conditions in mice, 15% of total monocytes may be recruited to the alveoli and interstitial tissue (compared with 56% for the liver and 8% for the peritoneal cavity) (van oud Alblas and van Furth 1979). Each year ~40% of resident MΦs are replaced by newly recruited monocytes (Maus, Janzen *et al.* 2006). In contrast under conditions that elicit inflammation monocyte recruitment is greater in speed and magnitude (Van Furth, Diesselhoff-den Dulk *et al.* 1973; Maus, von Grote *et al.* 2002) leading to replacement of ~85% of resident lung MΦs within 2 months (Maus, Janzen *et al.* 2006). In a murine model of infection monocytes emigrate from the bone marrow under the influence of the monocyte chemokine CCL-2 binding to its receptor CCR-2, and enter the circulation (Serbina and Pamer 2006). CXCL-8, demonstrated to be upregulated in CF airways, could be important in initiating monocyte (as well as neutrophil) transmigration during inflammation (Gerszten, Garcia-Zepeda *et al.* 1999).

Adherence to the vascular endothelium occurs constitutively and transiently ('tethering'), mediated by monocyte and endothelial expressed selectins, causing monocyte 'rolling' along the endothelium (von Andrian and Mackay 2000; Imhof and Aurrand-Lions 2004). Firm attachment, a pre-requisite for transmigration, is initiated by endothelial integrins in response to upregulation by chemokines (e.g. CCL-2) and cytokines like TNF or bacterial products e.g. LPS (Hogg and Doerschuk 1995). $\beta_1$ (VLA-4) and $\beta_2$ integrins (e.g. CD11a/CD18 or CD11b/CD18) on monocytes and neutrophils play an important role in lung migration under constitutive and inflammatory conditions (Li, Miyasaka *et al.* 1998; Rosseau, Selhorst *et al.* 2000; Maus, Huwe *et al.* 2002; Imhof and Aurrand-Lions 2004). Similar mechanisms and receptors are involved in monocyte and neutrophil migration to the bronchial region, though when migrating to the alveoli they may differ in their dependency upon $\beta_2$ integrins (Doerschuk 2000).

In a rodent model of acute inflammation monocyte and neutrophil migration occur in a co-ordinated biphasic pattern in the airways (Doherty, Downey *et al.* 1988; Maus, Huwe *et al.* 2001; Maus, von Grote *et al.* 2002; Henderson, Hobbs *et al.* 2003). In reponse to intratracheal instillation of LPS and CCL-2, monocytes are elevated rapidly at 48 hours (hrs) then rapidly decrease by ~90% at 72 hrs (Maus, von Grote *et al.* 2002). In contrast neutrophil recruitment was elevated earlier and quicker at 12 hrs then decreased by ~90%, also at 72 hrs, suggesting an important temporal recruitment of monocytes and neutrophils to the lungs that causes transient pulmonary leakage (Maus, von Grote *et al.* 2002). To illustrate the importance of co-migrating monocytes as a regulator of neutrophil recruitment, in an identical model of acute lung inflammation, neutrophil recruitment in response to LPS is 10-fold lower without CCL-2 compared to when under the same conditions with CCL-2 (Maus, Koay *et al.* 2002), despite the fact that monocytes but not neutrophils express CCR-2 (Traves, Smith *et al.* 2004). In addition, depletion of resident murine lung MΦs reduced neutrophil recruitment in to and out of the airways during *P. aeruginosa* infection (Kooguchi, Hashimoto *et al.* 1998). This response was also associated with significantly higher levels of neutrophil recruiting chemokines and bacterial survival 48 hrs post infection (Kooguchi, Hashimoto *et al.* 1998) although these findings have been refuted in similar models of infection (Cheung, Halsey *et al.* 2000). Taken together these rodent studies suggest that the monocyte/MΦ lineage could play a role in the excessive neutrophilic recruitment into the airways in CF.

As monocytes transmigrate over the endothelial surface into the pulmonary tissue space and mature as tissue MΦs monocytes experience profound changes at both gene (Srivastava, Jung *et al.* 2005) and protein levels (Jin, Opalek *et al.* 2004). The development of the monoblast to the MΦ is coincident with a decreased replicative capacity and an increased ability to perform

degradative phagocytosis (Ross and Auger 2002). There is also a downregulation of monocyte antigens (e.g. CD14) and upregulation of those important to MΦ phagocytic function e.g. CD16 and other PRR such as CD68 (Passlick, Flieger *et al.* 1989; Viksman, Liu *et al.* 1994; van der Kooij, von der Mark *et al.* 1997; Staples, Smallie *et al.* 2007). Once the MΦ is present in the lung interstitium local growth factors, particularly GM-CSF, ensure terminal differentiation and maturation towards an alveolar MΦ phenotype (Shibata, Berclaz *et al.* 2001), an important pre-requisite to carry out its sentinel, anti-microbial and immunoregulatory functions.

### 1.2.4 Lung Macrophage Function in Health and Cystic Fibrosis

MΦs are mature effector cells of the innate immune system involved in maintaining homeostasis by opsonic (Section 1.2.4.1) and non-opsonic phagocytosis (Section 1.2.4.2), acting as sentinels for the presence of pathogens (Section 1.2.4.3), shaping adaptive immune responses via antigen presentation (Section 1.2.4.4), shaping innate responses through cytokine and other mediator production (Section 1.2.4.5), and tissue remodelling (Section 1.2.4.6). To carry out these protective/restorative functions the alveolar and interstitial MΦ relies on the action of para-/autocrine secreted cytokines, chemokines, growth factors and Igs amongst others for which the MΦ also has a plethora of receptors (Lohmann-Matthes, Steinmuller *et al.* 1994; Ross and Auger 2002). It has recently come to light that an array of receptor ligation events by exogenous and/or endogenous stimuli, encode several different gene transcription and functional responses by MΦs leading to different forms of MΦ activation (Gordon 2003).

The early and repeated susceptibility of CF patients to lung infection and the on-going neutrophilic inflammation, suggests that innate immune function is compromised either inherently by CFTR and/or due to the altered ASL environment. Mimicking CF mucus viscosity

in an *in vitro* model it has been demonstrated that the movement and function of neutrophils (Matsui, Verghese *et al.* 2005) and the diffusion of lactoferrin (Matsui, Wagner *et al.* 2006) are compromised by thickened mucus compared to non-CF like mucus. Furthermore a viscous and hypoxic ASL, described previously, may also compromise the diffusion of important determinants on MΦ metabolism and terminal differentiation such as oxygen tension (Cohen and Cline 1971) and GM-CSF, respectively (Shibata, Berclaz *et al.* 2001). Reduced diffusion of mediators may have an impact on many of the functions described above and on other cross-talk mechanisms that exist between airway ECs and resident MΦs (Reynolds, Reynolds *et al.* 2007), compromising MΦ function.

### 1.2.4.1 Opsonic Phagocytosis

The alveolar MΦ in contrast to the interstitial MΦ is considered to be the most important phagocytic MΦ within the lung since they have a greater avidity for phagocytosis and enhanced potency for bacterial killing (via ROI) (Lohmann-Matthes, Steinmuller *et al.* 1994).

Acute infection of murine models have been utilised to discern the role of healthy MΦs in the clearance of infection. In the context of CF, airway MΦs have been demonstrated to play an important active phagocytic role during *S. aureus* infection (Rehm, Gross *et al.* 1980; Broug-Holub, Toews *et al.* 1997; Cheung, Halsey *et al.* 2000) but not *P. aeruginosa* infection (Rehm, Gross *et al.* 1980; Cheung, Halsey *et al.* 2000). Interestingly, Kooguchi *et al.* (1998) demonstrated that depleting airway MΦs increased the *P. aeruginosa* burden significantly early on during infection (8 hrs) but had a more pronounced effect on the outcome of the immune response to infection 48 hrs later. Similarly human alveolar MΦs *ex vivo* have been shown to phagocytose *S. aureus*, *H. influenzae, Streptococcus pneumoniae* and *P. aeruginosa*, the latter at

low levels (Thomassen, Demko *et al.* 1980; Jonsson, Musher *et al.* 1986; Berger, Norvell *et al.* 1994), whereas monocytes grown in culture to generate MDM were unable to kill *P. aeruginosa* even at a 1:1 ratio (Mathy-Hartert, Deby-Dupont *et al.* 1996). Taken together airway MΦs appear to play an important role in preventing infection by CF-associated pathogens except *P. aeruginosa* and in the latter case may be more important in modulating the cytokine environment.

Within the lung opsonic phagocytosis relies on antibodies, complement components (e.g. C1q and C3 fragments; MBL) and SPs that are able to coat bacteria allowing their uptake by specific lung MΦ receptors (Berger, Norvell *et al.* 1994; Lohmann-Matthes, Steinmuller *et al.* 1994; Wright 2005). It has been noted by several investigators (Biggar, Holmes *et al.* 1971; Thomassen, Demko *et al.* 1980; Fick, Naegel *et al.* 1981; Thomassen, Demko *et al.* 1982; Hornick and Fick 1990; Berger, Norvell *et al.* 1994) that CF serum significantly reduces *P. aeruginosa* phagocytosis by inhibiting Ig dependent mechanisms on normal pulmonary MΦs from animals or humans in *in vitro* phagocytic assays. Notably the phagocytic ability of monocytes was unaffected (Thomassen, Demko *et al.* 1982), whereas neutrophils were either unaffected (Fick, Naegel *et al.* 1981; Thomassen, Demko *et al.* 1982) or showed moderate inhibition (Hornick and Fick 1990).

Human airway MΦs from CF and healthy individuals demonstrated a modest increase in phagocytosis of *P. aeruginosa* with increasing concentrations of anti-pseudomonal IgG (and thus conserved fragment (Fc) receptor ligands) but not complement proteins (Berger, Norvell *et al.* 1994). This dependency on the presence of Ig, but not necessarily complement, reflects MΦ Fc cell surface receptor expression. On alveolar MΦs from healthy donors, Fcγ receptor II (CD32 – low affinity IgG receptor) is expressed to the greatest extent (~75% of MΦs), followed by III

(CD16 – low affinity) then I (CD64 – high affinity), ~53% and ~38%, respectively (Berger, Norvell *et al.* 1994). By contrast, the percentage of CF alveolar MΦs expressing all three Fc receptors (and complement receptors), were shown to be reduced by approximately half (Berger, Norvell *et al.* 1994). Igs constitute 10-15% of the total protein in the ASL, are composed of different classes (e.g. IgA, G, M, D and E) and subclasses (e.g. $IgG_{1-4}$) (Naegel, Young *et al.* 1984) and *in vivo* induce anti-/pro-inflammatory immune responses based on the presence/absence of sialylation, respectively, of the Fc region (Kaneko, Nimmerjahn *et al.* 2006). CF patients have been shown to mount a high IgA and IgG response towards infection suggesting that a local Ig deficiency is not responsible for the lack of phagocytosis (Shryock, Molle *et al.* 1986; Pedersen, Espersen *et al.* 1990; Kronborg, Fomsgaard *et al.* 1992). The inhibitory effect of CF serum was attributed to the presence of large quantities of circulating $IgG_2$ and $IgG_4$ against pseudomonas LPS (Shryock, Molle *et al.* 1986; Hornick and Fick 1990), which despite $IgG_2$ and $IgG_4$ demonstrating normal avidity for LPS (Fick, Olchowski *et al.* 1986), also demonstrates the lowest affinity for alveolar MΦ Fcγ receptors amongst the entire subclass of Igs (Naegel, Young *et al.* 1984). Thus airway MΦ Fc mediated phagocytosis of *P. aeruginosa* is blocked by non-opsonic antibodies that may have an important role during the early course of infection.

SPs were not investigated in these studies but are also likely to be an important opsonin within the lung and are produced constitutively by alveolar type II cells, SMG cells and Clara cells (Wright 2005). SPs are evolutionarily conserved PRRs in the collectin family of opsonins (e.g. C1q of complement and MBL), that bind clustered oligosaccharides through carbohydrate binding C-type lectin-like domains (CTLD) (Wright 2005). SP-A and -D both bind *S. aureus*, *P.aeruginosa*, apoptotic cells (e.g. neutrophils) and DNA, all important players in CF pathophysiology (Wright 2005). Surfactant proteins can augment phagocytosis through a variety

of mechanisms such as binding to specific receptors (e.g. CD91/Calreticulin, C1q receptor) on MΦs, inducing cellular activation and upregulating the presence of cell surface non-opsonic phagocytic receptors on alveolar MΦs (Beharka, Gaynor *et al.* 2002; Wright 2005).

Phagocytosis of apoptotic neutrophils can also occur through the phosphatidylserine receptor (Vandivier, Fadok *et al.* 2002), which like the Fc receptor and CR3 is reduced on CF MΦs possibly through neutrophil and pseudomonas derived elastases as described previously (Tosi, Zakem *et al.* 1990; Mariencheck, Alcorn *et al.* 2003). In summary, Fc receptor and SP mediated phagocytosis are important mechanisms for lung MΦ mediated clearance of bacteria and apoptotic neutrophils both of which have been shown previously to play a role in CF pathophysiology.

The downstream intracellular killing mechanisms mediated by MΦ bactericidal enzymes are conserved between different tissue MΦs, with the exception of the alveolar MΦ due to the plentiful supply of oxygen (Ross and Auger 2002). Alveolar MΦs rely on both glycolysis and oxidative metabolism for optimum phagocytosis (Cohen and Cline 1971). Specifically, oxygen tensions at or below 25 mmHg such as that observed at the epithelial cell membrane in the CF lung (Worlitzsch, Tarran *et al.* 2002), will severely impair intracellular killing (Cohen and Cline 1971), described below. Interestingly opsonised but not non-opsonised phagocytic material ingested by airway MΦs *in vitro* leads to the generation of oxygen metabolites required for intracellular killing, indicating different signalling events as well as receptors (Kobzik, Godleski *et al.* 1990).

Following phagocyte receptor-ligand interaction, the MΦ cell membrane invaginates forming a cleft, whose projections surround the engulfed particle allowing the formation of an intracellular

vesicle (phagosome). Primary lysosomes are intracellular vacuoles, which contain acid hydrolases and have a pH of ~4-5 (Steinman, Mellman *et al.* 1983) required for optimum activation of proteases (elastases and cathepsins) (Stuart and Ezekowitz 2005). Fusion of the phagosome and primary lysosome generates a secondary lysosome (phagolysosome). In the phagolysosome the phagocytosed contents are in contact with proteases, HOCl and ROIs generated via a 'respiratory burst', in an acidic (pH ~ 4.7) environment (Ross and Auger 2002; Stuart and Ezekowitz 2005; Di, Brown *et al.* 2006). In human and murine alveolar MΦs acidification is generated by the phagolysosomal transmembrane V-ATPase proton pump and CFTR (Di, Brown *et al.* 2006). Interestingly absence of CFTR in CF airway MΦs leads to the alkalinazation of this membrane compartment and thus an inability to kill and digest bacteria (Di, Brown *et al.* 2006).

### 1.2.4.2 Non-Opsonic Phagocytosis

Non-opsonic phagocytosis can occur through two large sub-groups of PRR known as C-type lectin receptors (CLR) and scavenger receptors (SR) (McGreal, Martinez-Pomares *et al.* 2004; Mukhopadhyay and Gordon 2004). Although non-opsonic phagocytosis has been known for some time (Stahl, Rodman *et al.* 1978), only recently has the diversity of these PRRs and their contribution to innate immunity been appreciated (Medzhitov and Janeway 2000).

CLRs are soluble (e.g. MBL) or membrane bound (e.g. mannose receptor) and involved in recognition of exposed carbohydrate (mannose or galactose) through specific recognition domains (CTLD) (McGreal, Martinez-Pomares *et al.* 2004). CLRs are involved in cell-cell adhesion and endocytosis of MPs present on plasma glycoproteins and in bacterial recognition (Lee, Evers *et al.* 2002; McGreal, Martinez-Pomares *et al.* 2004).

SRs are a large family of cell surface, intracellular or soluble receptors subdivided into 6 groups (A-F) based on their tertiary structure (Sarrias, Gronlund *et al.* 2004). However they all share a cystiene-rich binding domain (SRCR domain) and ability to bind and endocytose modified low density lipoproteins (LDL) (Sarrias, Gronlund *et al.* 2004). Of particular interest are those with roles in clearance of infection such as CD14 (involved in LPS recognition), SR A I and II and the MΦ receptor with collagenous structure (MARCO) and those involved in apoptotic cell removal (e.g. SR A, CD36, CD14). In contrast increased expression of intracellular CD68 (a group D SR for oxidised LDLs) (van der Kooij, von der Mark *et al.* 1997) has been used to indicate increasing MΦ maturation and detection of tissue MΦs (Kelly, Bliss *et al.* 1988; Pulford, Rigney *et al.* 1989; Staples, Smallie *et al.* 2007). Though opsonic phagocytic receptors have been studied in CF and their function with CF-associated pathogens, little however is known about the expression or role of non-opsonic PRRs in the CF airways. Despite this, MΦ non-opsonic phagocytosis of CF associated pathogens has also been studied. Non-opsonic phagocytosis by human and rodent MΦs but not monocytes play a role in the clearance of non-mucoid *P. aeruginosa* (Lee, Hoidal *et al.* 1984; Cabral, Loh *et al.* 1987; Speert, Wright *et al.* 1988; Mahenthiralingam, Campbell *et al.* 1994) but not mucoid strains (Cabral, Loh *et al.* 1987; Krieg, Helmke *et al.* 1988). Furthermore *S. aureus* and *E. coli* can also be cleared by non-opsonic mechanisms (Lee, Hoidal *et al.* 1984; Devalon, Elliott *et al.* 1987; Arredouani, Yang *et al.* 2004; Arredouani, Palecanda *et al.* 2005).

In addition to their phagocytic role an increasing body of evidence have identified roles for both CLR and SR in modulating cytokine production in response to infection (Cotena, Gordon *et al.* 2004; Jozefowski, Arredouani *et al.* 2005; Hollifield, Bou Ghanem *et al.* 2007), possibly through interaction with other signalling PRR e.g. Toll-like receptors (TLRs) (McGreal, Martinez-

Pomares *et al.* 2004; Tachado, Zhang *et al.* 2007). Thus non-opsonic receptor expression and phagocytic mechanisms may also play an important role in CF.

### 1.2.4.3 Toll-like Receptors

TLRs, of which to date 11 have been identified in mammals, are transmembrane non-phagocytic, signalling receptors on MΦs and on other leukocytes and ECs that bind to a wide range of self/non-self MPs via an extracellular leucine rich repeat (LRR) domain (Akira and Takeda 2004; Tsan and Gao 2004). TLR expression has not been studied to date on CF MΦs, though normal and CF epithelial cell lines have been reported as having similar TLR expression and response to a variety of TLR agonists including LPS (Muir, Soong *et al.* 2004; Greene, Carroll *et al.* 2005).

In the context of CF, Gram-positive (e.g. *S. aureus*) bacterial products such as lipoteichoic acid, peptidoglycan (both TLR2) and Gram negative (e.g. *P. aeruginosa*) components e.g. LPS (TLR4) and flagellin (TLR5) are recognised by this system (Akira and Takeda 2004; Mukhopadhyay, Herre *et al.* 2004; Zhang, Louboutin *et al.* 2005). TLR-ligand interaction subsequently induces the maturation of monocytes into MΦs and dendritic cells (DC) (Krutzik, Tan *et al.* 2005). TLRs also co-operate with and upregulate vitamin $D_3$, Fc receptors and SRs (e.g. CD14, SR-A, MARCO) present on the cell surface of MΦs (Mukhopadhyay, Herre *et al.* 2004). The sensing of LPS by TLR4 via the CD14/LPS binding protein (LBP) complex and subsequent pro-inflammatory signalling cascade (Figure 1-8), is one of the best characterised interactions between a SR (CD14 and LBP) and TLR (Akira and Takeda 2004). Sensing of LPS by TLR4, induces a signalling cascade via Toll-IL-1R (TIR) domains and myeloid differentiation primary response protein 88 (MyD88) dependent and independent pathways ending in pro-inflammatory

cytokine and chemokine production (e.g. TNF and CXCL-8) via NF-κB and mitogen activated protein kinase (MAPK) activity, respectively (Figure 1-8).

TLR4 signalling, particularly by MΦs and to a lesser extent ECs, in response to LPS plays an important role in neutrophil recruitment to the airway demonstrated in TLR4 KO mice (Hollingsworth, Chen *et al.* 2005). Further to this TLR 4 and TLR 2 expressing cells have been implicated in mediating CXCL-8 production in response to CXCR1 cleavage (Hartl, Latzin et al. 2007). Thus TLRs have important roles in mediating the inflammatory response of MΦs to infection. This is further supported by polymorphisms in human TLR components (e.g. IRAK-4) that have been attributed to disease (Cook, Pisetsky *et al.* 2004) and murine MyD88 KO models that demonstrate an altered neutrophil recruitment and cytokine production (Power, Peng *et al.* 2004). Therefore TLR expression by MΦs may have an important contribution to the initiation of pro-inflammatory immune responses.

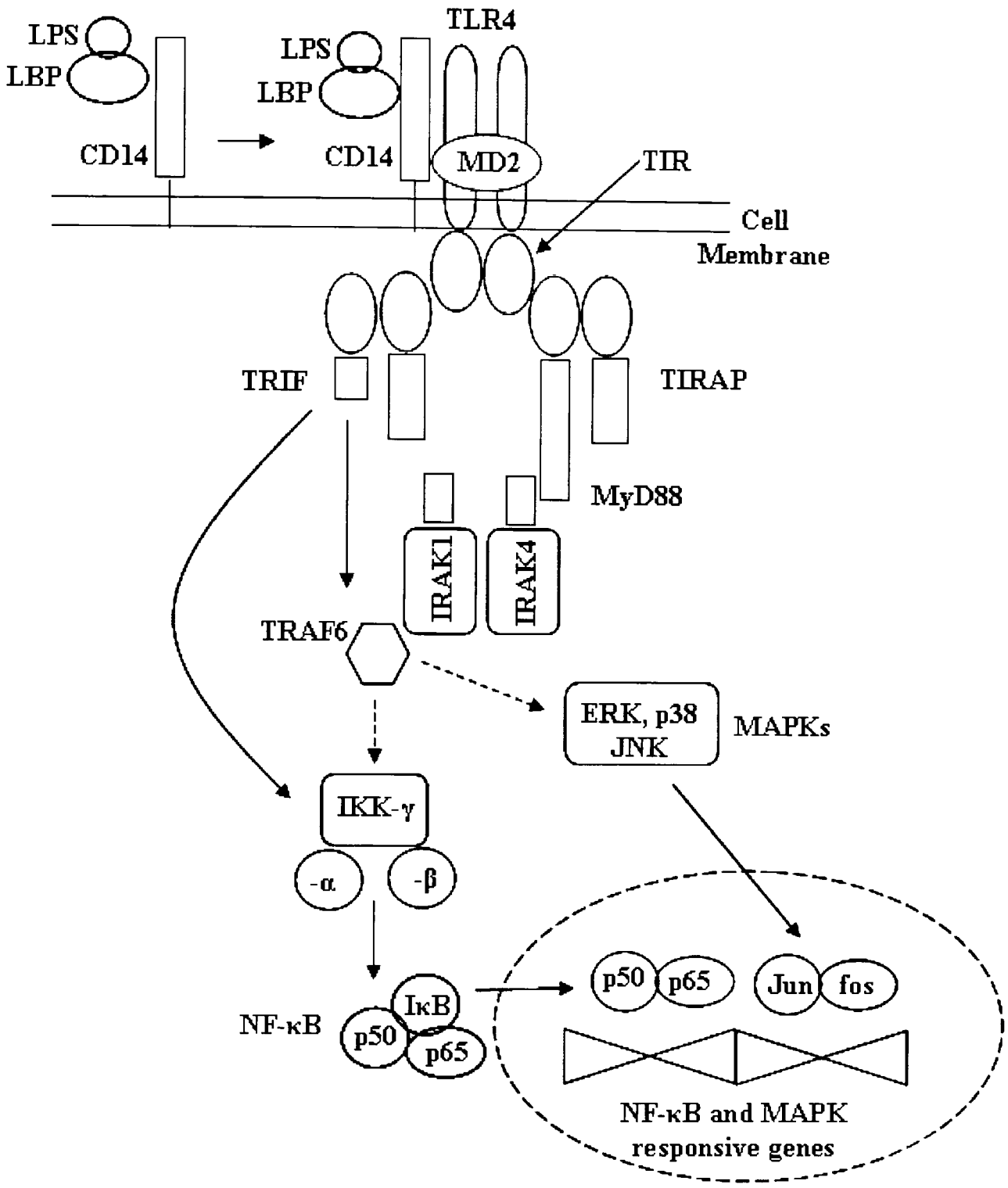


**Figure 1-8 Co-operativity between CD14 and TLR4 induces a signalling cascade leading to gene transcription via NF-κB and MAPKs in response to LPS (Aderem and Ulevitch 2000; Akira and Takeda 2004)**

### 1.2.4.4 Antigen Presentation

Breakdown and recycling of ingested components forms an important pre-requisite for cell surface peptide presentation (antigen presentation) to pulmonary lymphocytes and activation of adaptive immunity. Innate immune recognition by resident antigen presenting cells such as the MΦ via PRRs have an important role in directing the adaptive immune response towards appropriate targets (Fearon and Locksley 1996). Given the high quantity of circulating Ig in CF the CF antibody response to infection is intact. Whilst alveolar MΦs are more potent at phagocytosis compared to interstitial MΦs, the latter cells are better at presenting antigen (Lohmann-Matthes, Steinmuller *et al.* 1994), which probably takes place in the bronchus associated lymphoid tissue (Agostini, Chilosi *et al.* 1993; Moyron-Quiroz, Rangel-Moreno *et al.* 2004). Despite this DCs are the most potent presenters of antigen (Hance 1993)

Presentation of peptide is mediated by a group of proteins known as the major histocompatibility complex (MHC). The MHC are split into two classes and are further subdivided into different types and variants – class I (A, B and C) and class II (DR, DP and DQ) - presenting endogeous (e.g viral/self peptides) or exogenous (bacterial peptides) peptides, respectively. In humans they are known as human leukocyte antigen (HLA) class I and II. Pertabations of HLA function are associated with chronic inflammatory and infectious diseases (Friese, Jones *et al.* 2005; Jones, Fugger *et al.* 2006). Interestingly, in CF, antigen presentation is almost abolished during severe end-stage disease (Knight, Kollnberger *et al.* 1997) and HLA class II (but not I) polymorphisms have been associated with increased or decreased colonisation by *P. aeruginosa* (Aron, Polla *et al.* 1999), further hinting that MΦ function may be important in CF airway disease.

### 1.2.4.5 Mediator Secretion and the Control of Inflammation

MΦs secrete a plethora of enzymes, opsonins including complement components, ROIs, derivatives of arachidonic acid (AA) and other lipid mediators, cytokines and their inhibitors and a variety of other products that have pro- and anti-inflammatory effects (Nathan 1987; Ross and Auger 2002). Of these MΦ products, cytokines and chemokines have received the most attention in respiratory disease (including CF) since they can be easily quantitated, and have diverse pro- and anti-inflammatory roles during inflammation thus contributing to the pathophysiology of CF. Therefore MΦs need to be held under control to prevent over/under-production that could contribute to a dysregulated immune response.

Cell-cell contact with airway ECs and lymphocytes is an important mechanism that allows MΦs to suppress their own pro-inflammatory activity or those of lymphocytes, respectively (Fireman, Ben-Efraim *et al.* 1993; Takabayshi, Corr *et al.* 2006). Alveolar MΦs produce Transforming growth factor (TGF)-β, which requires cleavage of its inhibitor (latent activating peptide) by epithelial cell integrins ($\alpha v\beta_6$) to allow binding of TGF-β to receptors on MΦs and other cell types (Takabayshi, Corr *et al.* 2006). Under quiescent conditions TGF-β has been demonstrated to act in an autocrine and paracrine manner to suppress MΦ activity and thus immune responses in a murine model *in vivo* (Takabayshi, Corr *et al.* 2006). Interestingly in a CF cohort of nearly 500, polymorphisms in the promoter region of the TGF-β gene (leading to increased expression) correlate with a significantly decreased $FEV_1$ in these patients (Drumm, Konstan *et al.* 2005). In contrast Brazova *et al.* (2006) could not confirm these findings but noted a non-significant association between extremes of TGF-β production and lower lung function. The mechanisms by which the anti-inflammatory effects of TGF-β are mediated have yet to be elucidated in CF. In a murine model *in vivo* the presence of LPS or flagellin bound to MΦ (but not epithelial) TLR4 and

5 respectively, initiated MΦ actin re-organisation, removed epithelial $\alpha v\beta_6$ mediated cleavage of TGF-β latent activating peptide and thus removed the anti-inflammatory effect of TGF-β on MΦ activity (Takabayshi, Corr *et al.* 2006). Increased TGF-β activity could therefore raise the threshold required for activation of alveolar MΦs that could lead to an early hyporesponse towards infection.

Free of TGF-β suppression, alveolar MΦs respond with an 'acute phase response' via eicosanoid mediators (prostaglandins, leukotrienes and thromboxanes) that are responsible for many of the cardinal signs of acute inflammation (Funk 2001). Subsequently alveolar MΦs release a plethora of cytokines and chemokines in a time and dose dependent manner that is quantitatively different from airway ECs (Thorley, Ford *et al.* 2007). In a murine model of airway *P. aeruginosa* infection both airway MΦs and ECs had qualitatively and temporally divergent roles in the release of cytokines and chemokines such as CXCL-8 thereby suggesting distinct roles (Hajjar, Harowicz et al. 2005).

CXCL-8 aside however, there appears to be little consensus on the quantities and role of cytokines and other chemokines in the CF airway. Hubeau *et al.* (2001), examining CF foetuses, and Armstrong *et al.* (1997) looking at CF infants under 6 months of age noted the absence of any intrinsic inflammatory abnormalities when looking at an array of different cytokines and chemokines including CXCL-8. Despite these two observations CXCL-8 protein has consistently been shown to be considerably higher in CF airways in infants and adults, in some cases without demonstrable infection (Balough, McCubbin *et al.* 1995; Bonfield, Panuska *et al.* 1995; Khan, Wagener *et al.* 1995; Osika, Cavaillon *et al.* 1999; Sagel, Kapsner *et al.* 2001; Dakin, Numa *et al.* 2002; Nixon, Armstrong *et al.* 2002; Muhlebach, Reed *et al.* 2004; Xiao, Hsu *et al.* 2005; Alexis,

Muhlebach *et al.* 2006; Hartl, Griese *et al.* 2006). Mean quantities of CXCL-8 were considerably (three-fold) higher in CF patients compared to other airway diseases such as non-CF bronchiectasis (Osika, Cavaillon *et al.* 1999), COPD and asthma (Xiao, Hsu *et al.* 2005). In addition, a murine homologue of CXCL-8, MIP-2, was also significantly elevated together with neutrophilic inflammation in the airway fluid of an infection naïve (Mall, Grubb *et al.* 2004) or infection challenged (Heeckeren, Walenga *et al.* 1997; McMorran, Palmer *et al.* 2001) CF mouse model. To date, it has not been established as to which cell(s) are producing this chemoattractant *in vivo* yet there is evidence to suggest that MΦs are in part responsible.

*In vitro* normal human alveolar MΦs demonstrated earlier and greater basal (but not stimulated) production of CXCL-8, compared with airway ECs (Thorley, Ford *et al.* 2007). Moreover MΦ derived CXCL-8 has been shown to exhibit a two-fold greater affinity for neutrophils compared with epithelial derived CXCL-8 (Hebert, Luscinskas *et al.* 1990). Bonfield *et al.* (1995) identified strong CXCL-8 protein expression in 20% of CF alveolar MΦs by intracellular staining but not those of control, unless stimulated with LPS suggesting that CF airway MΦs may be 'classically activated' (Gordon 2003). In addition CF alveolar MΦs produce elevated transcript levels of CXCL-8, in comparison to control, that positively correlate with CXCL-8 protein present in CF lavage fluid (Khan, Wagener *et al.* 1995). In addition, other chronic lung diseases characterised by neutrophil recruitment such as idiopathic pulmonary fibrosis (IPF) have also correlated MΦ CXCL-8 transcript content with protein found in lavage fluid (Carre, Mortenson *et al.* 1991).

Further evidence, this time directly implicating CFTR, suggest that the monocyte/MΦ lineage is an important area for investigation in CF. Zaman *et al.* (2004) noted a hundred-fold higher

sensitivity to LPS by CF monocytes compared to controls, attributed to increased MAPK (ERK and p38) signalling that led to elevated basal and stimulated CXCL-8 production. This study however has not been corroborated by analysis of pulmonary MΦs in CF under the same experimental conditions. Intriguingly, in the ENaC murine model MΦs were implicated (though not directly assessed) as the source of MIP-2 production since elevated MIP-2 could not be found on epithelial cell cultures (Mall, Grubb *et al.* 2004).

Other potent neutrophil chemotaxins produced by alveolar MΦs include complement fragments (e.g. C5a) (Fick, Robbins *et al.* 1986), derivatives of AA ($LTB_4$) (Rankin, Sylvester *et al.* 1990; Peters-Golden 2004) and CXCL-1 (growth related oncogene-α) (Thorley, Ford *et al.* 2007). In response to LPS, alveolar MΦs secrete $LTB_4$ earlier (between 1 and 3 hours) than CXCL-8 (following 3 hours) suggesting that $LTB_4$ may be responsible for the early recruitment of neutrophils and CXCL-8 for a sustained, later recruitment (Rankin, Sylvester *et al.* 1990). CXCL-8 and $LTB_4$ also induce exocytosis of neutrophil granules, increase $\beta_2$ integrin receptor expression, cytokine generation and respiratory burst of neutrophils (Baggiolini, Walz *et al.* 1989; Peters-Golden 2004).

In addition to CXCL-8 CF alveolar MΦs also stained intracellularly for TNF-α, Interleukins (IL) 1β, 6, 10 and IL-receptor antagonist (IL-Ra), whereas control MΦs did not, unless stimulated with LPS (Bonfield, Panuska *et al.* 1995). Neutrophil chemotaxis into the airways may also be increased via MΦ derived pro-inflammatory cytokines such as TNF and IL-1β that act on ECs and MΦs in an autocrine or paracrine fashion to release more chemoattractants including CXCL-8 (Baggiolini, Walz *et al.* 1989; Huber, Kunkel *et al.* 1991; DeForge, Kenney *et al.* 1992; Thorley, Ford *et al.* 2007). TNF and IL-1β are also elevated in the CF airways (Balough,

McCubbin *et al.* 1995; Bonfield, Panuska *et al.* 1995; Osika, Cavaillon *et al.* 1999; Xiao, Hsu *et al.* 2005) though this is not always the case (Alexis, Muhlebach *et al.* 2006). In CF, TNF is increased seven-fold over non-infected COPD and asthma subjects (Xiao, Hsu *et al.* 2005) but not those with bronchiectasis (Osika, Cavaillon *et al.* 1999). In addition TNF negatively correlates with $FEV_1$ and positively correlates with leukotrienes (e.g. $LTB_4$) in a young (11 years) CF cohort (Greally, Hussein *et al.* 1993). TNF and IL-1β gene transcription is mediated by the same transcription factor as CXCL-8, NF-κB. Therefore elevated constitutive NF-κB translocation (Knorre, Wagner *et al.* 2002) or increased signalling via MAPKs (Zaman, Gelrud *et al.* 2004) in CF MΦs could play an important role in maintaining elevated levels of these pro-inflammatory cytokines. In line with this, human CF MDMs exhibited a hundred-fold (Pfeffer, Hueckstead *et al.* 1993) and murine bone marrow CF MΦs a ten-fold (Thomas, Costelloe *et al.* 2000) higher sensitivity to LPS than controls. The CF airways responds to infection with a more vigorous production of cytokines (e.g. CXCL-8) compared to disease control patients (Muhlebach, Stewart *et al.* 1999) and a greater sensitivity to LPS could play a role in this response. This sensitivity to LPS is important because although alveolar MΦs secrete substantially less neutrophil recruiting chemokines (including CXCL-8) in the long term (at 24 hrs), MΦs release TNF and IL-1β basally, earlier and in significantly greater quantities in response to LPS (measured over 24 hrs) compared to airway ECs (Hajjar, Harowicz *et al.* 2005; Thorley, Ford *et al.* 2007). Thus the MΦ is one of the main sources of TNF within the airways. Early TNF and IL-1β may thus be important to the pathogenesis of CF since they have a pleiotropic effect on a wide range of cells and in LPS stimulated whole blood assays were shown to have a synergistic effect that gave rise to a second wave of CXCL-8 production as part of a biphasic response (DeForge, Kenney *et al.* 1992). TNF also stimulates MMP-9 secretion (Vaday, Hershkoviz *et al.* 2000) and primes neutrophils for HOCl production (She, Wewers *et al.* 1989).

Taken together, resident MΦs are one of the most potent producers of pro-inflammatory cytokines such as TNF and IL-1β and are an important source of neutrophil chemoattractants such as CXCL-8, CXCL-1 and $LTB_4$ upon exposure to LPS and thus warrant further investigation in CF.

In addition, TNF, IL-1β and IL-12, also produced by MΦs in response to LPS, elicit the release of Interferon (IFN)-γ from natural killer cells and T cells thereby augmenting MΦ pro-inflammatory mechanisms such as enhancing intracellular killing and antigen presentation via MHC class II (Fearon and Locksley 1996). IFNγ and TNF can also synergise to enhance positive feedback mechanisms for further MΦ release of TNF and ROIs (Nathan 1987). Despite CF alveolar MΦs demonstrating reduced phagocytic and bactericidal capacity, IFNγ protein (a potent enhancer of MΦ respiratory burst) is either increased in CF airway fluid (Hartl, Griese *et al.* 2006) or equal to controls (Xiao, Hsu *et al.* 2005; Alexis, Muhlebach *et al.* 2006). MΦs require IFNγ for the killing of some organisms (e.g. *Mycobacteria* and *Listeria* species; non-alginate producing *P. aeruginosa*) but not others (e.g. *S. aureus*) (Lohmann-Matthes, Steinmuller *et al.* 1994; Leid, Willson *et al.* 2005). Interestingly lower production of IFNγ by CF mononuclear cells correlates with a greater reduction and rate of decline in $FEV_1$ and more frequent colonisation of *P. aeruginosa* compared to high responders (Moser, Kjaergaard *et al.* 2000; Brazova, Sediva *et al.* 2005).

In a murine model of *P. aeruginosa* infection the release of IL-1β and signalling via the IL-1 receptor culminating in NF-κB activation was demonstrated to be critical in allowing eradication of *P. aeruginosa* from the airways (Reiniger, Lee *et al.* 2007). Interestingly NF-κB translocation was blocked when bronchial epthelial cells were co-cultured with IL-1Ra and it was previously

demonstrated that CF patients' exhibit increased IL-1Ra in serum compared to bronchiectetic controls (Osika, Cavaillon *et al.* 1999). IL-1Ra, demonstrated in MΦs (Bonfield, Panuska *et al.* 1995), could be an important source of this cytokine and thus play an important role in determining the course of the immune response to *P. aeruginosa*.

In response to *P. aeruginosa* there appears to be a T helper 2 immune response in CF subjects characterised by elevated IL-4, IL-13, significant elevation of T helper 2 cells, T helper 2 chemokines (e.g. CCL-17) as well as neutrophil influx and this is associated with a poorer lung function (Brazova, Sediva *et al.* 2005; Hartl, Griese *et al.* 2006). IL-12, a cytokine that promotes the development of a T helper 1 response, has not been assessed with respect to CF. The production of IL-12 and other IL-12 family members (particularly IL-23) by MΦs may be important given the identification of elevated pro-inflammatory IL-17 levels from IL-17 expressing T cells ($T_h$-17 Cells) in the CF airways (McAllister, Henry *et al.* 2005). In such an environment as this, TNF could also promote a T helper 2 immune response by skewing MΦs towards an antigen presenting DC phenotype that supports antibody production (Chomarat, Dantin *et al.* 2003). *P. aeruginosa* may thus skew the immune response away from cellular immunity and towards antibody production that ultimately prevents eradication by mechanisms already discussed.

Deleterious and prolonged inflammation in the lung can impair epithelial integrity and consequently gas exchange. Thus resident airway MΦs are held under immunosuppressive mechanisms and release anti-inflammatory cytokines and derivatives of AA to reduce inflammation. Nitric oxide also has a broad range of anti-inflammatory and anti-microbial functions (Bogdan 2001) that may derive from airway MΦs via constitutive and inducible nitric

oxide synthase activity (Wooldridge, Deutsch *et al.* 2004). Interestingly nitric oxide has been shown to be reduced in the CF airway (Moeller, Horak *et al.* 2006) but not others (Wooldridge. Deutsch *et al.* 2004). It is also becoming increasingly recognised that pro-inflammatory cytokines/mediators also induce the production of anti-inflammtory cytokines/mediators to suppress the immune response (Wanidworanun and Strober 1993; Serhan and Savill 2005).

LPS and to a much lesser extent TNF (but not IL-1) (Wanidworanun and Strober 1993; Mosmann 1994) can stimulate concomitant production of the anti-inflammatory cytokine IL-10 by MΦs. IL-10 inhibits further pro-inflammatory mediator release by MΦs, inhibits production of ROIs, reduces the capacity for antigen presentation (Allavena, Piemonti *et al.* 1998), enhances phagocytosis by increasing expression of Fc and SRs (Allavena, Piemonti *et al.* 1998; Ross and Auger 2002; Jung, Sabat *et al.* 2004), increases the inhibitor of NF-κB (Chmiel, Berger *et al.* 2002), increases the sensitivity of lymphocytes to TGF-β mediated suppression (Mocellin, Marincola *et al.* 2004) and promotes the maturation of monocytes to MΦs (Allavena, Piemonti *et al.* 1998). The concentration of IL-10 in CF airway lavage fluid has been demonstrated to be much lower than controls in humans (Bonfield, Konstan *et al.* 1995; Bonfield, Panuska *et al.* 1995; Osika, Cavaillon *et al.* 1999; Hartl, Griese *et al.* 2006) and CFTR KO mice (Soltys, Bonfield *et al.* 2002) but this has not been replicated by other groups who observed either no difference (Noah, Black *et al.* 1997; Muhlebach, Reed *et al.* 2004; Brazova, Sediva *et al.* 2005) or an increase of IL-10 in CF airways (Xiao, Hsu *et al.* 2005; Alexis, Muhlebach *et al.* 2006) and serum (Casaulta, Schoni *et al.* 2003) compared to controls. MΦ derived IL-10 may thus be important but it is unknown what contribution it has in CF in attempting to prevent inflammation.

Monocytes and MΦs are also involved in 'class switching' early phase pro-inflammatory eicosanoids (e.g. prostaglandins and leukotrienes) to anti-inflammatory lipoxins (Serhan and Savill 2005; Ariel and Serhan 2007). Like LPS and TNF, pro-inflammatory prostaglandins also inititiate the transcription of anti-inflammatory lipid mediators, thus providing signals for the resolution of acute inflammation (Serhan and Savill 2005). It was noted that, in comparison to controls with similar CXCL-8 and neutrophil levels, lipoxin $A_4$ concentrations were suppressed substantially (Karp, Flick *et al.* 2004). In a murine model of chronic lung infection with *P. aeruginosa,* administration of a lipoxin analogue reduced neutrophil recruitment to the lung parenchyma and airway (Karp, Flick *et al.* 2004). Other lipid mediators include eicosapentanoic and docosahexanoic acid, which are converted to resolvins of the E and D series, respectively (Serhan and Savill 2005). Deficiencies in docosahexanoic acid have been noted in CF tissue biopsies (Freedman, Blanco *et al.* 2004). Such deficiencies may be related to dietary malabsorption, which could lead to further impairment in the production of lipids by MΦs that resolve acute inflammation.

### 1.2.4.6 Tissue Remodelling

A variety of diseases are associated with chronic inflammation and dysregulated tissue remodelling e.g. asthma, rheumatoid arthritis and COPD (Nathan 2002). Successful tissue remodelling is a fine balance between tissue destruction, deposition of extracellular matrix (ECM) and other structural components and re-epithelialization to restore function (Meneghin and Hogaboam 2007; Wynn 2007). Through the production of pro-angiogenic (e.g. vascular endotheial growth factor, some CXC chemokines) and other proliferative growth factors, MΦs have an important role in repair, wound closure and in restoring functional tissue (i.e re-epithelialization) (Strieter, Polverini *et al.* 1995; Meneghin and Hogaboam 2007).

In order to carry out airway remodelling MΦs produce degradative enzymes such as lysosomal acid hydrolases (lipases, proteases, DNases) and in particular over 20 MMPs that have been identified in humans and mice (Shapiro and Senior 1999; Ross and Auger 2002). Resident alveolar MΦs are not potent producers of proteases unlike recruited 'inflammatory MΦs' (Ross and Auger 2002; Srivastava, Jung *et al.* 2005). In CF such cells are exposed to bacterial products, cytokines, and Fc receptor ligation at inflammatory sites which can induce their release (Ross and Auger 2002). In the case of MMPs, which are kept in check by TIMPs, a variety of roles have been defined based on knock out animal models (Page-McCaw, Ewald *et al.* 2007). MMPs are important for path clearing through ECM during cellular migration, initiating cell (e.g. neutrophil) recruitment in response to ECM cleavage, airway remodelling by degrading intercellular epithelial junctions and in the activation (e.g. TGF-β) or deactivation of cytokines and chemokines (Takabayshi, Corr *et al.* 2006; Weathington, van Houwelingen *et al.* 2006; Page-McCaw, Ewald *et al.* 2007). Some MΦ derived MMPs have also been shown to be major contributors to smoking induced pulmonary emphysema in murine models, if they are over produced or overwhelm their tissue inhibitors (Hautamaki, Kobayashi *et al.* 1997; Ofulue and Ko 1999; Churg, Wang *et al.* 2004).

As a consequence of chronic bronchial inflammation in CF tissue remodelling is dysregulated since there is significant destruction and alteration of connective tissue, ECM and epithelia within the airways (Bruce, Poncz *et al.* 1985; Stone, Konstan *et al.* 1995; Ogrinc, Kampalath *et al.* 1998; Hays, Ferrando *et al.* 2005). There is also a significant increased mass of CF airway smooth muscle that may contribute to bronchial hyperreactivity in some patients (Hays, Ferrando *et al.* 2005). Degradative products of elastin (desmosine and isodesmosine) and collagen (hydroxyl-/lysylpyridinoline) were seen in the urine of CF patients (Bruce, Poncz *et al.* 1985; Stone,

Konstan *et al.* 1995). Moreover in proximal regions of the bronchi, cartilage destruction was evident in CF autopsy specimens and interestingly MΦs were located in these areas (Ogrinc, Kampalath *et al.* 1998). Primarily, such destructon impairs lung function but ECM fragments can also enhance further neutrophil recruitment via CXCR-2 into the airways (Weathington, van Houwelingen *et al.* 2006).

However where functional tissue cannot be replaced due to inhibition of reparative mechanisms (i.e. through persistent damage by chronic inflammation through either self/non-self mechanisms) it is replaced by non-functional ECM deposition (fibrosis) (Rennard 1999). Fibrosis is a common feature in both the pancreas and lung in CF (Anderson 1938; Zuelzer and Newton 1949; Durieu, Peyrol *et al.* 1998) and in CFTR KO murine models (Durie, Kent *et al.* 2004) and MΦs may play a role in this process. Fibrosis is accompanied by increased presence and activation of interstitial fibroblasts who are the main producers of ECM, following activation by MΦ products (Meneghin and Hogaboam 2007). This may be augmented further by MΦ cytokines such as IL-1 stimulated fibroblast proliferation, whilst platelet derived growth factor and TGF-β augment secretion of ECM components from fibroblasts. In addition a T helper 2 immune response, as seen in CF, is associated with increased fibrosis (Jakubzick, Choi *et al.* 2003; Wynn 2004). IL-4 and IL-13, elevated in CF, are particularly pro-fibrogenic cytokines (Jakubzick, Choi *et al.* 2003).

Chronic inflammation in CF is recognised to be an important therapeutic target to try and halt the rapid decline towards respiratory failure (Koehler, Downey *et al.* 2004). Through many of the functions outlined above, MΦs could have a hitherto under appreciated role in CF lung disease.

### 1.2.5 Sampling Human Lung Macrophages

To determine the role of MΦs in CF a few different approaches are available for obtaining MΦs from the lung, used both in health and disease. Currently the most common method for investigating airway cellular (including MΦs) and fluid phase mediators from humans and rodents is the BAL. However it is invasive, technically demanding and unpleasant for volunteers. Similarly, lung tissue obtained by biopsy, suffers from the same draw backs. Therefore non-invasive sampling methods are increasingly being used to measure various indices of inflammation including MΦs, most notably induced sputum and exhaled breath condensate. Induced sputum, at the forefront of non-invasive techniques to sample the lung, is a well characterised method of sampling the large airways (Alexis, Hu et al. 2001; Keatings and Nightingale 2001; Kelly, Efthimiadis et al. 2001). Moreover cellular and fluid phase mediators from sputum have now been correlated with disease severity in CF, demonstrating the utility of performing sputum induction for research and clinical investigations (Mayer-Hamblett, Aitken *et al.* 2007).

Sputum induction involves the inhalation of hypertonic saline to draw water via ENaC dependent osmosis (Donaldson, Bennett *et al.* 2006) from the ECs lining the airway surfaces. Hydration of the airway surfaces facilitates expectoration of sputum, which can be collected and assessed in downstream applications. Sputum induction normally involves three separate inhalations after which the volunteer is asked to cough vigorously into a pot. Sputum induction is a safe, valid and well tolerated technique for sampling MΦs and fluid phase mediators in health and in mild to severe lung disease (De Boeck, Alifier et al. 2000; Spanevello, Confalonieri et al. 2000; Henig, Tonelli et al. 2001; Kelly, Efthimiadis et al. 2001; Pizzichini, Pizzichini et al. 2002; Suri, Marshall et al. 2003). In addition to this sputum induction has also been demonstrated

therapeutically to help improve lung function in CF when performed regularly over a 1 year period (Donaldson, Bennett *et al.* 2006; Elkins, Robinson *et al.* 2006).

Alexis *et al.* (2001) have demonstrated in healthy volunteers using radiolabelled particles that induced sputum derives mainly from the central or bronchial airways, rather than the peripheral regions (alveoli) of the lung. Sputum induction is thus a relevant technique for measuring inflammatory indices in respiratory disease where the majority of the (immuno) pathology occurs in the bronchial region such as in CF and COPD (Rutgers, Timens *et al.* 2000; Gibson, Burns *et al.* 2003). Sputum consists of mucus, inflammatory cells and ECs and is non-sterile by virtue of the fact that it is transported through the trachea and into the mouth for expectoration. An analysis of induced sputum content in over a hundred healthy adult volunteers has shown the cellular content to consist of mainly MΦs (~69%) and neutrophils (~27%) with the remainder comprised of lymphocytes (1%), ECs (1.5%) and eosinophils (0.6%) (Spanevello, Confalonieri *et al.* 2000) similar to other published investigations (D'Ippolito, Foresi *et al.* 1999; Belda, Leigh *et al.* 2000). However some authors, following sequential analysis of induced sputum, have observed a percentage of MΦs similar to BAL of over 80% (Holz, Jorres *et al.* 1998; Alexis, Hu *et al.* 2001; Reinhardt, Chen *et al.* 2003). In contrast to healthy individuals, sputum induction derived from paediatric CF patients demonstrated elevated percentages of neutrophils (67%), lymphocytes (6%) and eosinophils (16%) compared with controls (Reinhardt, Chen *et al.* 2003). On the other hand MΦs decrease as a percentage of total cells (26%) (Reinhardt, Chen *et al.* 2003). Interestingly, in a study of induced sputum from paediatric stable CF patients (Reinhardt, Chen *et al.* 2003) and in COPD (Rutgers, Timens *et al.* 2000) the absolute number of MΦs recovered from sputum remained similar to healthy controls.

MΦs retrieved by induced sputum may have a different functional and cell surface phenotype than those recovered by BAL or biopsy, such as an increased phagocytic capacity and oxidative burst (Lohmann-Matthes, Steinmuller *et al.* 1994; Lensmar, Elmberger *et al.* 1998; Alexis, Soukup *et al.* 2000). MΦ cell surface antigens are utilised in order to identify sub-populations that may exist and/or compare functions relevant to pathophysiology.

### 1.2.6 Identification of Different Macrophage Populations

Prior to the advent of monoclonal antibodies (mAbs) in the 1970s, MΦ identification relied on morphology and histochemistry for non-specific esterase and acid phosphatase using the light microscope. Since this time, increasing characterisation of MΦ cell surface antigens and functions, using flourophore labelled mAbs and fluorescence detection based methods, have enabled detection of MΦ sub-populations or activation states (Lensmar, Elmberger *et al.* 1998; Alexis, Soukup *et al.* 2000; Gordon 2003; Gordon and Taylor 2005).

Lung MΦs have shared, consistently expressed epitopes that have aided their identification in health and disease. HLA-DR is an MHC class II protein that has been consistently detected on MΦs recovered from different compartments within the lung, in line with their antigen presentation capacity. Whilst it is not specific for the MΦ lineage, ~90% of MΦs recovered by BAL, bronchial lavage, induced sputum and tissue biopsy (interstitial MΦs) from healthy and diseased subjects express HLA-DR (Gant and Hamblin 1985; Noble, Du Bois *et al.* 1989; Kiemle-Kallee, Kreipe *et al.* 1991; Spiteri and Poulter 1991; Rankin, Marcy *et al.* 1992; Spiteri, Clarke *et al.* 1992; Lensmar, Elmberger *et al.* 1998; Frankenberger, Menzel *et al.* 2004).

In contrast, CD68 is a ~120 kD, predominantly intracytoplasmic, lysosomal membrane SR but also a cell surface antigen (Kelly, Bliss *et al.* 1988; van der Kooij, von der Mark *et al.* 1997; Umino, Skold *et al.* 1999) that is specific for cells of the monocyte/MΦ lineage including MΦs that exist in different tissue compartments (Kelly, Bliss *et al.* 1988; Pulford, Rigney *et al.* 1989; Laskin, Weinberger *et al.* 2001; Stout and Suttles 2004). Monocytes also express CD68 (Kelly, Bliss *et al.* 1988; Pulford, Rigney *et al.* 1989) and expression of this antigen can increase significantly during *in vitro* culture (Staples, Smallie *et al.* 2007) but monocytes express CD68 to a much lower level than tissue MΦs, thus CD68 has come to reflect MΦ maturation. On ~98% of BAL and airway lavage MΦs CD68 can be detected (Spiteri and Poulter 1991; Rankin, Marcy *et al.* 1992; Spiteri, Clarke *et al.* 1992; Umino, Skold *et al.* 1999) and tissue biopsies have demonstated CD68 positive MΦs as being the dominant cell type in the bronchial wall of healthy subjects (Power, Burke *et al.* 1994). In comparison Lensmar *et al.* (1998), perhaps due to using a different antibody to those studies described above, detected a lower percentage of CD68 positive cells from BAL (34%) and induced sputum (61%) samples. Other mAbs used to identify MΦs have included 25-F9 and 27-E10 (Striz, Wang *et al.* 1993; Rosseau, Hammerl *et al.* 2000; Laskin, Weinberger *et al.* 2001), RFD1 and RFD7 (Spiteri, Clarke *et al.* 1988; Spiteri and Poulter 1991) and those of the Ki-M series (Kiemle-Kallee, Kreipe *et al.* 1991).

Alterations in MΦ sub-populations in chronic inflammatory lung diseases e.g. sarcoidosis, acute respiratory distress syndrome (ARDS), IPF and COPD compared to the healthy airways have been noted (Spiteri, Clarke *et al.* 1988; Noble, Du Bois *et al.* 1989; Kiemle-Kallee, Kreipe *et al.* 1991; Striz, Wang *et al.* 1993; Rosseau, Hammerl *et al.* 2000; Frankenberger, Menzel *et al.* 2004). These studies have identified elevated monocytic influx using cell surface antigens that are more highly expressed on monocytes (or newly recruited MΦs), such as CD14 and 27-E10

(calprotectin), compared to resident mature MΦs or alterations in the staining pattern of RFD1 (HLA class II) and RFD7 mAbs. The identification of MΦ sub-populations are important because alterations in phenotype are co-incident with disease and have been associated with decreases in lung function (Krombach, Gerlach *et al.* 1996; Rosseau, Hammerl *et al.* 2000; Taylor, Noble *et al.* 2000) thus possibly contributing to pathophysiology. Taken together, detection of cell surface or intracellular antigens on MΦs using fluorophore labelled mAbs permits the identification of MΦs and potential subsets that may have an important contribution to understanding disease pathophysiology in CF. Today, this application can be performed routinely by flow cytometry.

### 1.2.7 Flow Cytometry

Flow cytometry has a long history and use in the identification and characterisation of cells of the immune system including those of the monocyte/MΦ lineage (Shapiro 2003). It relies on the differential (large and small angle) light scattering properties (i.e. the refractive index) of cells as they pass through a light source whilst travelling in a cell suspension in single file. Small (scattered in the forward direction) and large (scattered at right angles to the incident beam) angle scattered light are detected by photo-diode or photo-multiplier tubes (PMT), respectively. Light signals detected in this way are a reflection of relative cell size (forward scatter - 'FSC') and internal granularity or roughness (side angle scatter – 'SSC') and permit the identification of the major leukocyte subpopulations by these parameters (Alexis, Soukup *et al.* 2000; Shapiro 2003; Frankenberger, Menzel *et al.* 2004). Additional properties can be determined with the use of fluorophore conjugated mAbs directed against cell surface or intracellular molecules. Light scatter and light emission spectra from fluorophore conjugated mAbs permits the discrimination of cell types from heterogenous samples.

Flow cytometry has several advantages over other cell identification methods, like microscopy. Flow cytometry can analyse a large number of cells in a short space of time and on each cell assess several parameters simultaneously. Furthermore a uniform flow rate and brief excitation to light ensure that photobleaching does not occur (Shapiro 2003) compared with fluorescence microscopy methods. In the present study I have used flow cytometry to identify and characterise airway sputum MΦs from patients with CF.

## 1.3 Thesis Aims

- To identify control and CF MΦs from paediatric induced sputum samples using flow cytometry
- To demonstrate that control and CF sputum MΦs exist as two populations - small and large.
- Show that small MΦs predominate in the CF airways.
- Characterise the cell surface phenotype and function of small sputum MΦs and compare this to control MΦs.
- Compare control and CF MDMs for differences in function due to mutant CFTR that could contribute to CF lung disease pathophysiology

# 2 Materials and Methods

## 2.1 Ethics

Ethics approval was granted by the Leicestershire Local Research Ethics Committee (Reference 7183) to obtain blood and sputum samples from paediatric and adult CF affected patients registered with the University Hospitals Leicester Royal Infirmary (LRI) and Glenfield Hospital NHS Trust, Leicestershire, UK, respectively. Approval was also granted to approach healthy children from the local area to act as a paediatric control group. Adult control donors represented volunteer work colleagues that work in the same department or building.

Initial contact, via an invitation letter, was made through the parent or guardian of the child concerned, complete with relevant information regarding details of the study. Adult CF patients were also approached by invitation letter and / or during their outpatient clinic visit. Throughout, parents and paediatric/adult subjects were reminded that they could withdraw from the study at any time without any consequences regarding their clinical management. Written informed consent was taken from each individual taking part in the study including the parent/guardian of the child. Whilst participating in this study all subjects were given a unique code so that all data collected could be kept confidential.

## 2.2 Subjects

Subjects were classified as children if they were 16 years of age or below at the point of sampling. A clinical history and biometric data were recorded from each participant. Healthy

controls were all non-smokers, had an $FEV_1$ greater than 70% predicted and were asymptomatic at the time of sampling.

Paediatric and adult CF patients were selected for consideration based on their attendance to a dedicated CF outpatient clinic or hospital ward, either at the LRI (paediatrics) or Glenfield Hospital (adults). All CF patients had undergone genetic screening (City Hospital, Nottingham) at or following diagnosis for the 29 most common CF alleles affecting 85-90% of the Caucasian population. A genetic mutation described as being 'unknown' suggest that a mutation is present but outside of the 29 routinely tested mutations.

A diagnosis of CF was made for the paediatric and the majority of the adult cohort based on the following criteria: a positive sweat test (>60mmol/l), detection of two established CF causing alleles by polymerase chain reaction (PCR), frequent lung infections requiring hospitalisation and other organ involvement (i.e. pancreatic insufficiency). Such patients are described in this thesis as having a 'classical' CF clinical presentation (Table 3-2), therefore this term has been used throughout this thesis. In contrast, 'non-classical' patients (Table 3-2) present later in life being detected with other manifestations of CF for example, in males, congenital absence of the vas deferens. Such patients have a borderline sweat test and as in this case may be homozygous for two mild disease causing CFTR mutations. Non-classical CF patients presented in this thesis were pancreatic sufficient, not experiencing any nutritional symptoms, have mild lung disease and less frequent hospitalisations compared to classical CF. This clinical distinction was performed by the CF consultant respiratory physician (Dr Simon Range) based on clinical and genetic criteria outlined above and has been described previously (Rodman, Polis *et al.* 2005). CF patients were compared against a control population that was recruited from the University of

Leicester and LRI or Glenfield Hospital personnel. All control donors were age matched where possible and were non-smokers (unless otherwise stated) and had no symptoms of disease.

## 2.3 Blood Collection

Peripheral blood from control and (classical) CF donors was collected into a standard laboratory syringe by ante-cubital venepuncture, performed by trained personnel. For monocyte purification (Section 2.5) peripheral blood was prevented from clotting using 10 units/ml of Heparin-Sodium (Ratiopharm, Ulm, Germany). All other experiments using peripheral blood monocytes (Section 2.6.1 and 2.6.3) were collected into Sarstedt (Nümbrecht, Germany) monovette blood collection bottles, pre-loaded with an ethylenediaminetetraacetic acid (EDTA) anti-coagulant (#05.1167.001).

## 2.4 Sputum Collection

### 2.4.1 Infection Control Guidelines

Sputum induction was performed in a well-ventilated laboratory at the Children's Asthma Centre, Windsor building, University Hospitals LRI or in a dedicated out patient clinic side room at Glenfield Hospital by trained personnel. Special care was taken to monitor bronchoconstriction and to prevent cross infection. Medical cover was provided by trained personnel to ensure the safety of the participants.

The following guidelines were strictly adhered to:

- Strict separation of *Pseudomonas* from non-*Pseudomonas* infected individuals
- The nebuliser handset was wiped down with alcowipes; impact plate, nebuliser head, mouthpiece and valves were washed and autoclaved after each use by the sterilisation unit at the LRI.

- The work-surfaces and spirometer were wiped with 70% ethanol after each induction, whilst sterile disposable spirometer mouthpieces (and filters) were used for each subject
- Disinfection of shared spacers was ensured by immersion in disinfectant overnight.
- The Glenfield Hospital respiratory unit provided disposable, single use, spirometer mouthpieces and filters, aprons, surgical masks and sterile reusable volumatic spacers ensuring that the procedure was carried out according to good infection control standards.

### 2.4.2 Collection Protocol

Spirometry of paediatric control and CF cohorts was perfomed using a Vitalograph (Buckinghamshire, England) 2120 spirometer, supported by Spirotrac IV software programme (Vitalograph). Spirometry of adult control and CF cohorts were performed using a handheld 'Micro plus' spirometer (Micro Medical, Kent, UK). Participants were asked to take a deep breath in and to blow out as hard and as quickly as possible into the spirometer thereby recording the $FEV_1$. The $FEV_1$ (% predicted) was used to assess the overall 'respiratory fitness' of the participant(s).

Two inhalations of a short acting $\beta_2$ agonist, Salbutamol (200mcg total dosage, Baker Norton, London, UK), were administered using a volumatic spacer and participants were asked to wait for 15 min. This was administered to counter acute constriction that could occur during the induction procedure (Dr Satish Rao, Personal Communication). After 15 min, lung spirometry was repeated and the $FEV_1$ was taken as a 'baseline value'. Therefore any adverse reactions would be indicated by a fall in the $FEV_1$. Prior to commencing sputum induction participants were asked to clear out any naso/oro-pharyngeal secretions by blowing their nose and washing their mouth.

Three, 5 min inhalations of 5.4% hypertonic saline (#G005027, Nova Laboratories, Leicestershire, UK) were then performed using an ultrasonic nebuliser (Clement Clarke International #3605204, Essex, UK or Schill, #MN 80180, Probstzella, Germany), set to maximum output (0.5ml/min). Since differences in induced sputum could not be found when using high (3ml $min^{-1}$) or low (0.4ml $min^{-1}$) output nebulisers in control and COPD cohorts (Kelly, Brown *et al.* 2002), between males and females (Belda, Leigh *et al.* 2000) and when performed twice within a week I did not believe that any of these parameters would bias the data I collected (Pizzichini, Pizzichini *et al.* 1996). After each inhalation, participants were asked to clear their naso/oro-pharyngeal secretions to reduce contamination and repeat spirometry measurements. Following spirometry, participants were encouraged to cough any secretions into a large (140mm) sterile Petri dish (Bibby Sterilin, Staffordshire, UK) or a standard medical specimen pot. Productive coughs produced at other time points during the procedure were also collected into the same pot.

In some instances there was a fall in $FEV_1$ between 10 and 20% that necessitated a further two inhalations of Salbutamol (200mcg), administered as described earlier. Sputum induction was continued if the $FEV_1$ improved to within 10% of the baseline or terminated if it remained greater than 10%. Where microbiological analysis was required a representational portion of induced sputum was sent to the LRI pathology services.

### 2.4.3 Sputum Processing

Expectorated sputum was examined by eye. The colour and consistency was noted and classified as mucoid (colourless/white in appearance), purulent (yellow/green) or muco-purulent (in between). The sputum selection method (Kelly, Efthimiadis et al. 2001) was used as a guide for

picking appropriate material. Excessive EC contamination of sputum can be a problem with healthy donors therefore white, well-circumscribed material (referred to as 'plaques') was selected using sterile forceps into a pre-weighed centrifuge tube. Selected portions of sputum are richer in non-squamous viable cells and have less contamination from ECs compared to the residual portion (Pizzichini, Pizzichini *et al.* 1996). Plaques are areas of sputum that are rich in leukocytes that are bound together by mucins. Identification of plaques was aided using an inverted microscope. Saliva or 'spit' was avoided in order to further reduce contamination by squamous ECs. CF sputum is often very homogenous due to the excessive amount of purulant material, thus the whole sputum sample was taken for analysis. Bias was reduced using an inverted microscope by ensuring that leukocytes were not lost in unselected control fractions.

The weight (*g*) of the selected/whole portion of sputum was determined and four times the weight (in ml) of 10% Dithiothreitol (DTT) was added, followed by a vigorous vortex. Concentrated DTT (Sputolysin®, #560000, Calbiochem, California, USA) was diluted 1 in 10 with sterile distilled water to yield 6.5 mM DTT in 100 mM phosphate buffer, pH 7.0. For large samples, DTT was freshly prepared with distilled $H_20$ ($dH_20$) and stored at 4°C, or for small samples stored in single use (1.5ml) aliquots at -20°C. DTT facilitates homogenisation and breakdown of sputum by reducing disulphide bonds, through the addition of hydrogen, between cysteine rich domains on sputum mucins (Cleland 1964; Hirsch, Zastrow *et al.* 1969; Voynow 2002).

The sputum/DTT mix was homogenised on ice for 15 min on a ThermoFisher Scientific bench rocker (Basingstoke, UK). Later, an equal volume (to DTT) of sterile standard laboratory grade phosphate buffered saline ('PBS' composed of NaCl, KCl, $Na_2HPO_4$, $KH_2PO_4$, adjusted to pH 7.4) was added to the sputum/DTT mixture and subsequently subjected to a vigorous vortex. The

whole sample was passed over a pre-wetted, non-sterile cotton medical gauze (#18508. Lohmann Rauscher, Germany) in a powder funnel (#FPH-455-010Y, Fisher Scientific, Leicestershire, UK) followed by a 48-μm nylon (#03-48/31, Serofar, Lancashire, UK) gauze (twice) in order to remove any large mucin/EC aggregates that can be problematic when analysing cells by flow cytometry. Each filter was washed through with 3 ml of PBS after filtering the sputum cells, to minimise cell loss. Cells that passed through the nylon were then subjected to centrifugation at 800g for 10 minutes at 4°C. The supernatant was pipetted off and either kept at –80°C or discarded. Pelleted cells were resuspended in 1 ml of PBS and 10 μl mixed with an equal volume of 0.4% Trypan Blue (#T-8154, Sigma-Aldrich, Dorset, UK) for a viable cell count and to determine the proportion of ECs (Table 3-1 and 3-2). Though this method is well recognised it is less sensitive and specific than current flow cytometry protocols that can differentiate viable from non-viable leukocytes. A high proportion of non-viable cells, unless gated out of the analysis by flow cytometry, can increase the degree of non-specific binding and lead to false interpretation of data. Cells were subsequently centrifuged and resuspended in PBS and used for downstream experiments as described for the separation of sputum MΦs (Section 2.4.4), cytospin preparation (Section 2.4.5) or flow cytometry analysis of sputum MΦs (Section 2.6).

### 2.4.4 Separation of Sputum Macrophages

Sputum cells were prepared, as above, and resuspended in 1ml of PBS (+2% Fetal Calf Serum (FCS) purchased from Autogen Bioclear, UK) on ice. Peripheral blood (2 ml) from a healthy donor was collected as described (Section 2.3) into a heparinized syringe and subsequently diluted 1:1 with tissue culture grade (filtered) PBS. Filtered PBS was generated by filtering over columns (UltraSteriset™ columns, Gambro, Meyzieu, France) to remove traces of bacterial products. Blood/PBS was layered gently over an equal volume of density gradient medium

(Ficoll-Paque™ preparation, GE Healthcare Uppsala, Sweden) and centrifuged at 400 *g* for 30 min at room temperature without applying a brake.

PBMCs appear like a white cloud above the density gradient medium at the interphase. The interphase and supernatant were discarded and an aliquot of pelleted mononuclear cell depleted erythrocytes were recovered. Thirty microlitres of erythrocytes were added to the sputum cells along with 50 µl of a monocyte enrichment mAb cocktail ('RosetteSep', #15028, Stem Cell Technologies (Vancouver, Canada) and incubated at room temperature for 20 min.

Sputum MΦs are notoriously difficult to isolate by conventional magnetic bead based protocols (as given for whole blood in Section 2.5.1) due to the sticky nature of sputum and the large aggregates present that can block fluid flow through magnetised columns as determined by our collaborating laboratory in Munich, Germany. Furthermore fluorescence activated cell sorting is technically demanding and requires access to specialist trained personnel and equipment. Therefore this 'RosetteSep' cocktail was chosen to circumvent these problems.

This 'RosetteSep' cocktail contains mAbs directed towards the non-monocyte/MΦ antigens (CD2, 3, 8, 19, 56, 66b) and an anti-glycophorin-A mAb cross-linked through their Fc regions. During the 20 min incubation time unwanted leukocytes are cross-linked via the glycophorin-A antigen on erythrocytes forming 'large immunorosettes' that have a large density. Subsequently 1 ml of PBS (2% FCS) was added to dilute the sputum cell suspension 1:1 before layering over a Ficoll-Paque solution of equal volume (2 ml). Sputum cells were centrifuged again at 400g for 30 min at room temperature. Immunorosetted leukocytes and erythrocytes have a higher density and so are pelleted by centrifugation through the gradient leaving just mononuclear cells

containing sputum MΦs and ECs only. This was determined by preparing cytospins and the extent of EC contamination was recorded in the results section under particle uptake (Section 4.3). Cells were recovered from the interphase, washed in one volume of PBS and 10 µl was taken and mixed with an equal volume of 0.4% Trypan Blue for cell viability determination (Section 4.3). Isolated cells were subsequently used for morphology analysis by cytospin (prepared as described below) and for particle uptake assays (Section 2.6.4).

### 2.4.5 Cytospin Preparation

A cell suspension in PBS (75 µl) was loaded into a cyto-centrifuge cup and centrifuged using a cytocentrifuge (ThermoFisher Scientific) on to a microscope slide at 450 rpm for 6 min. The slide was allowed to air dry before being stained with 'Diff Quick' (#130832, Medion Diagnostics, Düdingen, Germany). Each slide was immersed ten times in a fixative solution (Fast green in methanol) then blotted on tissue to remove excess fluid. This was repeated for Eosin G in PBS pH 6.6 (stain solution 1) and then finally for the Thiazine counterstain also in PBS pH 6.6 (stain solution 2). Slides were then washed with standard $dH_20$, blotted, and allowed to air dry, before microscopy examination. Thiazine is a cationic dye that binds negatively charged constituents such as nuclear material, whereas anionic dyes such as eosin bind to cytoplasmic proteins (Kiernan 1999).

## 2.5 Cell Culture

### 2.5.1 Purification of Monocytes from Whole Blood

Heparinized whole blood was taken from each subject as described (Section 2.3) and diluted 1:1 with filtered PBS under sterile conditions. Blood/PBS was mixed by pipette then gently layered over an equal volume of Ficoll-Paque density gradient medium. Once layered, samples were

centrifuged as described (Section 2.4.4). During this time filtered PBS (25 ml) containing 1% FCS and a final concentration of 2 mM EDTA (Sigma-Aldrich) was prepared for degassing (D-PBS). D-PBS was recommended for use with magnetic activated cell sorting (MACS) purification columns by the manufacturer (Miltenyi Biotec, Bergisch-Gladbach, Germany). D-PBS was obtained by placing it under vacuum for 30 min in order to extricate any gas bubbles.

Following centrifugation a thick PBMC layer can be discerned at the interphase and is rich in monocytes but also lymphocytes and platelets. This layer was gently aspirated and pooled into a centrifuge tube. Cells were pelleted by centrifugation at 400 *g* for 5 min at room temperature unless otherwise stated. The cell pellet was resuspended in filtered PBS and counted as follows: 10 µl of cell suspension was placed underneath a Neubauer haemocytometer and PBMCs were counted to give the number of cells ($10^7$)/ml. PBMCs were re-pelleted and resuspended in 80 µl of D-PBS and 20 µl of CD16 microbeads (#130-045-701, Miltenyi Biotec) per $10^7$ cells and incubated on ice for at least 15 min. I wanted to reduce contamination by CD16 expressing monocytes therefore I chose to deplete CD16 positive cells prior to CD14 enrichment in order to obtain a better defined population of monocytes (classical CD14++ monocytes) for subsequent analysis. CD16 expressing monocytes, as discussed, are phenotypically (Passlick, Flieger *et al.* 1989) and functionally (Auffray, Fogg *et al.* 2007) different, thus differences found between controls and CF could be caused by differing proportions of these cells.

Subsequently, the total volume was made up to 10 ml with D-PBS and pelleted as previously. Cells were subsequently resuspended in D-PBS (2 ml) prior to application to the magnetized columns. Sterile, single use Large Depletion (LD #130-042-901, Miltenyi Biotec) columns were housed in the VarioMACS cell separation unit (#130-090-282, Miltenyi Biotec), surrounded by a

magnet. The LD column was primed twice with 2 ml of D-PBS, which was allowed to run through into a discard pot. Resuspended cells (2 ml) were applied to the magnetized column and allowed to elute through into a sterile centrifuge tube. Cells pass through the column slowly so that even weakly expressing CD16 positive cells will be adhered to the column. Columns were then washed with fresh D-PBS three times to ensure that there were no CD16 negative cells trapped within the column. LD columns were subsequently discarded. CD16 depleted cells collected from this preparation were pelleted and resuspended in filtered PBS and counted as described.

CD16 depleted cells were pelleted as previously and re-suspended in a further 80 µl of D-PBS and 20 µl of CD14 microbeads (#130-050-201, Miltenyi Biotec) per $10^7$ cells and incubated for at least 15 min on ice. Following this, cells were re-pelleted and resuspended in 1 ml of D-PBS and kept on ice, whilst sterilisation of the MiniMACS magnetic unit was being undertaken using 70% ethanol. Single use, sterile Mini Separation (MS, #130-042-201, Miltenyi Biotec) columns were housed in the MiniMACS magnetic unit (#130-042-102, Miltenyi Biotec) and 2 x 500 µl of D-PBS was used to prime the MS column and allowed to run through into a discard pot. CD16 depleted PBMCs (1 ml) containing CD14 microbeads were passed over the magnetized columns. Here, cells pass through the column quickly and so only strongly CD14 positive cells (i.e. monocytes) will be trapped on the column. Following washing (3 x 500 µl) with D-PBS, monocytes remain attached to the column whilst lymphocytes and platelets pass through the column and are discarded. Positive selection was chosen for monocyte isolation because it is quicker and less expensive than negative selection. Positive selection may cause activation of monocytes, however because both control and CF monocytes were treated in an identical fashion I did not believe this would compromise my ability to identify true differences between groups

upon subsequent examination. Further to this, it was evident that positive selection did not cause a sustained activation since the quantity of TNF transcripts was negligible (Section 5.4).

To retrieve the monocytes from the column, it was necessary to remove the MS column from the MiniMACS magnetic unit and thus remove the magnetic field imparted by the magnet on the MS column. The CD14 expressing monocyte/microbead complex can then be eluted and collected for analysis. The MS column was placed over a 15 ml centrifuge tube and through the addition of 1 ml of PBS to the column and forcefully applying a plunger (four times), CD14 expressing monocytes were collected into a fresh sterile tube and stored on ice.

MACS separation yielded a monocyte cell suspension to a high degree of purity (>90%), which was demonstrated by taking a small aliquot for flow cytometry (Section 2.6.5). These cells were then used for generation of MDMs, described below.

### 2.5.2 Generation of Monocyte-Derived Macrophages

MACS purified monocytes were counted using a Neubauer haemocytometer, as previously, and resuspended in cell culture medium. The cell culture medium was composed of the following reagents: very low endotoxin RPMI 1640 medium (#F1415, Autogen Bioclear, UK), 1% non-essential amino-acids (#K0293, Autogen Bioclear); 1% Glutamine (#25030-024, Invitrogen, UK), 2% Penicillin and Streptomycin (#15140-122, Invitrogen); 1% OPI (Oxaloacetate, Pyruvate, Insulin) media supplement (#O5003, Sigma-Aldrich) reconstituted with tissue culture water (#W3500, Sigma-Aldrich). Before use, cell culture medium was filtered using UltraSteriset™ columns (Gambro) and supplemented with FCS (10%). Monocytes isolated simultaneously from control and CF donors were resuspended in cell culture medium to the same

cell density (~1x $10^6$/ml) and at 2 ml per well, seeded in a 24-well ultra low attachment (ULA) costar cell culture plates (Corning, New York, USA). Monocyte suspensions (2 ml) were supplemented with M-CSF (100 ng/ml final) (Ex3-001, kindly donated by The Genetics Institute, Massachusetts, USA).

To generate MDMs, control and CF monocytes were incubated at 37°C, 5% $CO_2$ for 5 days. After 3 days, cell culture medium (1 ml) was replaced with fresh medium (1 ml) containing serum and fresh M-CSF (100 ng/ml final). M-CSF and adherence are important determinants of monocyte survival, priming and augment monocyte differentiation (by increasing size, granularity, antigen presentation and phagocytic capacity) towards MΦs in the presence but not in the absence of serum (Becker, Warren *et al.* 1987; Andreesen, Brugger *et al.* 1990; Sporn, Eierman *et al.* 1990; Brugger, Kreutz *et al.* 1991). Following 5 days in culture, monocytic cells loosely adhere to the well surface and take upon features of MΦs such as a larger cell size and formation of processes. Previous analysis of maturation markers (CD68) and morphology of MDMs over a period of 3-5 days incubation concluded that this length was appropriate for the generation of MΦs (Staples, Smallie *et al.* 2007).

On day 5 adult MDMs were harvested by vigorous pipetting for flow cytometry analysis of cell surface CD14 and CD16 expression (Section 2.6.6), and LPS stimulation (Section 2.5.3) before re-suspending in 200 µl of TRI-reagent (#T-9424, Sigma-Aldrich). By contrast paediatric monocytes were seeded and cultured in the same way, however due to logistical constraints I did not isolate monocytes as paired control and CF samples at the same point in time. In contrast to MDMs from adult donors paediatric MDMs were harvested and without stimulation, stored in TRI-reagent (200 µl). All TRI-reagent samples were kept for later ribonucleic acid (RNA)

extraction (Section 2.7), reverse transcription (RT) and subsequent transcript analysis by PCR (Section 2.8 and 2.10, respectively).

### 2.5.3 Lipopolysaccharide Stimulation of Monocyte-Derived Macrophages

MDMs were harvested from 24-well ULA culture plates by vigorous pipetting after 5 days in culture. MDMs were, pelleted, resuspended and counted as described. MDMs were further pelleted and resuspended in cell culture medium to give a density as close to 1 x $10^6$/ml as possible. Into 4 wells in a fresh 24-well ULA plate, control (2 x 1 ml) and CF (2 x 1 ml) MDMs were aliquoted to an equal density and M-CSF (100 ng/ml final) was added. LPS (from *Salmonella minnesota* (#L-6261, Sigma-Aldrich) was added to two wells (either 1 or 10 ng/ml final), one from each experimental group; remaining wells were left as untreated controls. MDMs were incubated for 3 hrs at 37°C, 5% $CO_2$. MDMs were harvested as previously described into separate eppendorf tubes, pelleted in a cooled (4°C) microcentrifuge for 5 min. The supernatant from each pellet was taken off and stored at –80°C. The remaining pellet was resuspended in TRI-Reagent (200 µl) and kept for later RNA extraction (Section 2.7), RT and subsequent PCR analysis (Section 2.8 and 2.10, respectively).

### 2.5.4 Culture of Human Mast Cell and Macrophage Cell Lines

A human monocyte/MΦ cell line, known as Mono Mac 6 (MM6) (Ziegler-Heitbrock, Thiel *et al.* 1988), was grown in cell culture medium (10% FCS) until semi confluent at 37°C; 5%$CO_2$. MM6 cells were continually passaged and an aliquot (1 x $10^6$/ml) taken for downstream experiments. A human mast cell line (HMC-1) was supplied in collaboration with Prof. Peter Bradding (University of Leicester Hospitals, UK) and an aliquot of 1 x $10^6$/ml cells were provided by Aline Dupont (University of Leicester, UK). MM6 and HMC-1 cell lines were used

as positive controls for alpha (α) and beta (β) tryptase RT-PCR experiments (Section 2.10), respectively. For this an aliquot from each cell line was pelleted and lysed in TRI-reagent (200 µl) and kept at -80°C. RNA extraction and RT into cDNA was performed as for control and CF donor MDMs (Section 2.7 and 2.8, respectively).

## 2.6 Flow Cytometry

Flow cytometry of leukocytes, particularly sputum MΦs, presents several methodological problems for the investigator. These include cell clumping, presence of non-viable cells, non-specific staining and autofluorescence (AF) of MΦs. I attempted to minimise, as much as possible, the effects of these factors on the identification of sputum MΦs. The efforts that were taken to do this are highlighted, where necessary, below and in Section 3.0 and discussed in Section 6.0.

Specific mouse anti-human (unless otherwise stated) mAbs used in this thesis are given in Table 2-1. Appropriate isotype controls were used to determine non-specific staining and were purchased from the same company as the specific antibody unless otherwise stated. All reagents were stored at 4°C and in the dark. Cell suspensions were prepared as previously described and stained (using dilutions recommended by the manufacturer unless otherwise stated), as described below, for the identification of sputum MΦs (2.6.1), PRR expression on MΦs (2.6.2) and monocytes (2.6.3), MΦ particle uptake (2.6.4), determination of monocyte purity (2.6.5), and CD14 and CD16 expression on MDMs (2.6.6). Stained cell suspensions were aliquoted into appropriate round bottom flow cytometry tubes prior to examining on a FACSCalibur™ Flow Cytometer both of which were purchased from Becton Dickinson (BD), Wycombe, UK. This is equipped with a 4-colour PMT, dual laser (488 nm ('blue') argon laser and 633 nm ('red') diode

laser) connected to an Apple Macintosh computer (Operating System 9). This 4-colour instrument restricted my ability exclude both non-viable cells and MΦ autofluorescence as confounding variables on the identification of MΦs. The optics layout including flourescence (FL) channels and filter settings for this instrument are given in Figure 2-1.

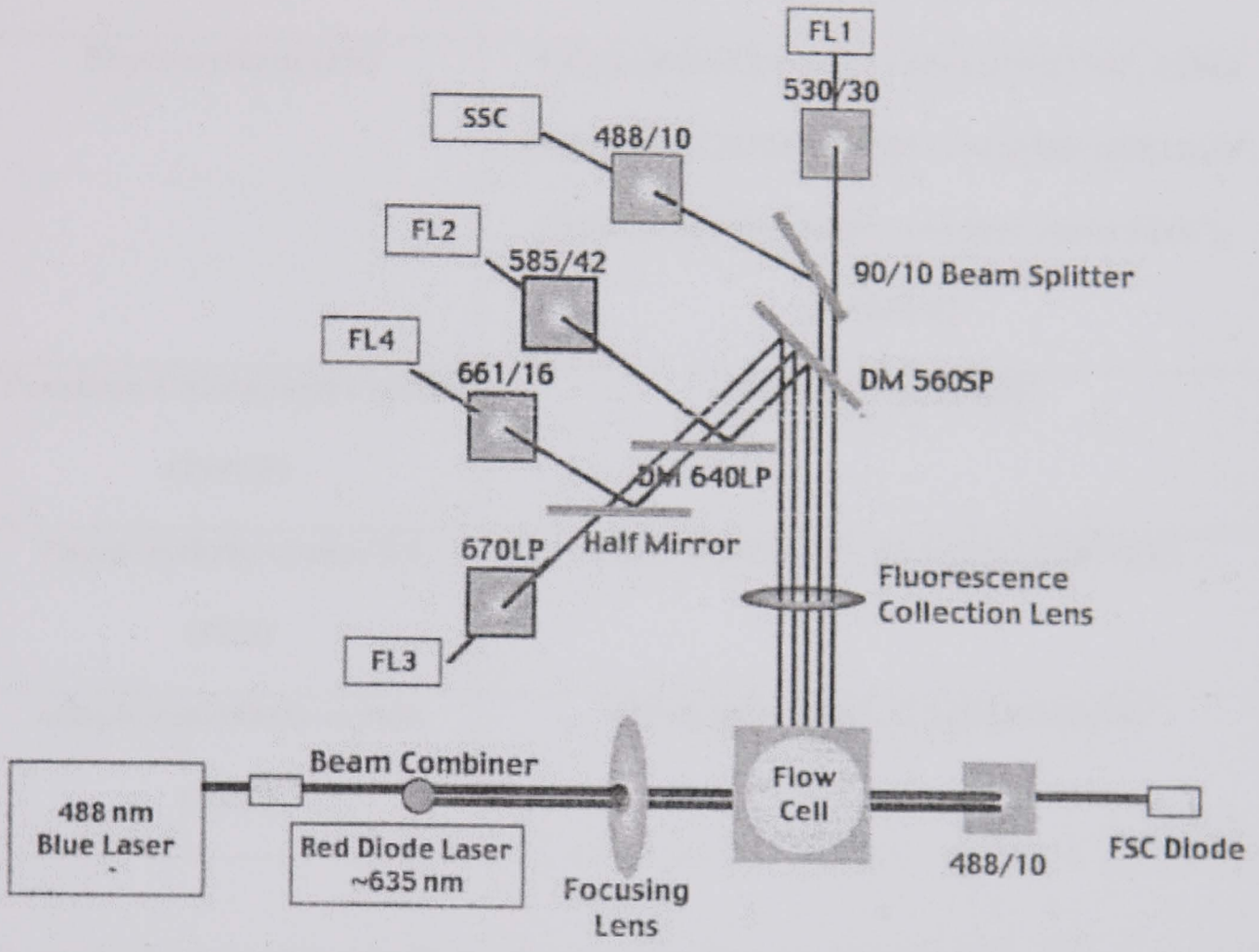


**Figure 2-1 Optics layout for the FACSCalibur (provided by Becton Dickinson Ltd, UK)**

Data was acquired and analysed (using CellQuest™ Pro software, also supplied by BD) and expressed as channels. FSC was measured on a linear scale therefore the arithmetic mean was taken for comparison, where appropriate. All fluorescence (and SSC) data was acquired on a 10-decade logarithmic scale therefore the geometric mean (GM) was obtained as a measure of staining intensity or degree of light refraction, respectively. The GM is preferred when using logarithmic data in order to reduce the effects of outliers on the mean (Reed, Holmes *et al.* 1998).

**Table 2-1 Specific flourophore conjugated mAbs used in this thesis**

| Fluorophore Conjugate | Antibody |
|---|---|
| Fluoroscein isothiocyanate (FITC) | CD14 (#6635011)[1], CD16b (#MCA1725F)[2], CD66b (#MCA216F)[2], CD66b (#555724)[3], |
| Phycoerythrin (PE) | CD14 (#MHCD1404)[4], CD16 (#332779)[3], CD68 (#556078)[3], CD206 (#555954)[3], CD209 (#551265)[3], Goat anti-mouse heavy and light chain F(ab')$_2$ (#IM0855)[1] |
| Peridinin Chlorophyll Protein (PerCP) | HLA-DR (#347402)[3] |
| Phycoerythrin-cyanin 5.5 (PC5) | CD14 (#IM2640)[1], HLA-DR (#A07793)[1] |
| Allophycoerythrin-cyanin (APC) | CD14 (#IM2580)[1], CD45 (#IM2473)[1] |

**Legend:** [1]Beckman Coulter (Marseilles, France), [2]Serotec (Oxford, UK), [3]BD, [4]Caltag (UK)

## 2.6.1 Identification of Sputum Macrophages

Sputum cells in PBS (100 µl) were stained with FITC-conjugated CD66b, CD16b, PC5-conjugated HLA-DR ($IgG_1$) and APC-conjugated CD14 ($IgG_{2a}$) or an appropriate isotype control (in brackets). The 'fluorescence minus one' (FMO) strategy (Perfetto, Chattopadhyay *et al.* 2004) was used whereby, using this approach (Table 2-2), background fluorescence (i.e. non-specific staining by mAbs due to Fc-Fc receptor interaction or bleaching into other PMTs caused

by the presence of other fluorophores) can be determined more accurately as a true reflection of the total fluorescence background signal in a PMT channel. MΦs contain many Fc receptors (as discussed in Section 1.2.4.1) and therefore by using this strategy non-specific binding by specific antibody could be taken into account. Cells were incubated with mAbs on ice for 15 min followed by a wash in 1 ml PBS (+2% FCS) and pelleted. The supernatant was removed and resuspended in 250 µl of Cytofix/Cytoperm™ (#51-2090k, BD) and incubated on ice for a further 20 min. This step fixes the cell surface bound antibodies prior to permeabilisation with Perm/Wash™ Buffer kit (#51-2091k2, BD), containing saponin to permit intracellular staining.

Sputum cells were pelleted and resuspended in 1 ml of Perm/Wash buffer (diluted 1 in 10 with PBS from concentrate), pelleted again and resuspended in 100 µl of diluted Perm/Wash to prevent reversibility of cell membrane permeabilisation. Cells were then stained with PE-conjugated intracellular CD68 (or an isotype control, $IgG_{2b}$, kappa), for 30 min, in the dark, on ice. PBS (400 µl) was added to increase the volume for flow cytometry acquisition. No less than 300 MΦ events were collected into the final light scatter dot plot for comparison, though in most samples this figure was over 1000. All analysis regions were set and then re-drawn if required by the principle investigator (Prof. Ziegler-Heitbrock) on the same day for both groups, using the same criteria to minimise bias.

As a point of comparison PBMCs were generated from whole blood (as described) from adult CF sputum donors. PBMCs were subsequently stained as described above for sputum cells and analysed using the same instrument settings as for sputum. Flow cytometer acquisition instrument settings were initially finalised for paediatric sputum then adult sputum MΦs and monocytes, which are given below in Figure 2-2. Paediatric instrument settings were identical

except that the FL4 voltage setting was 550 rather than 650V. We did not examine CD14 expression between paediatric and adult sputum MΦs but despite this the FL4 voltage was increased when analysing adult sputum cells for better display of CD14 positive events. Cellular events were acquired, analysed and are presented in chapters 3 and 4.

**Table 2-2** Experimental set up for sputum MΦs

| | | Tube Number | | | |
|---|---|---|---|---|---|
| **Fluorescence Channel** | **Antibody** | **1** | **2** | **3** | **4** |
| **FL1** | **CD66b/16b** | + | + | + | + |
| **FL2** | **CD68** | + | **$IgG_{2b}$** | + | + |
| **FL3** | **HLA-DR** | + | + | **$IgG_1$** | + |
| **FL4** | **CD14** | + | + | + | **$IgG_{2a}$** |

```
Cytometer Type:   FACSCalibur

Detectors/Amps:
Param   Detector  Voltage   AmpGain      Mode
P1        FSC       E-1       5.08       Lin
P2        SSC       250       1.00       Log
P3        FL1       600       1.00       Log
P4        FL2       510       1.00       Log
P5        FL3       630       1.00       Log
P6        FL2-A               1.00       Lin
P7        FL4       650                  Log

Threshold:
Primary Parameter:   FSC
Value:  52

Secondary Parameter:   None

Compensation:
FL1 - 10.8 % FL2
FL2 - 41.7 % FL1
FL2 - 30.1 % FL3
FL3 - 30.0 % FL2
FL3 - 3.7 % FL4
FL4 - 30.8 % FL3
```

**Figure 2-2 Instrument settings for the acquisition of adult sputum MΦs**

### 2.6.2 Pattern Recognition Receptor Expression on Sputum Macrophages

From control and CF donors paired sputum and (EDTA) blood samples were taken for analysis of PRRs. Sputum cells were prepared as previously described (Section 2.4.3) for flow cytometry analysis. Cell surface expression of three MΦ PRRs (CD206, CD209, and MARCO) was determined on adult sputum MΦs.

Cells (100 µl) were stained for CD206 expression with the following mouse anti-human mAbs: FITC-conjugated CD16b and CD66b, PE-conjugated CD206, PerCP or PC5-conjugated HLA-DR and APC-conjugated CD14. PE-conjugated $IgG_1$, kappa was substituted for PE-CD206 to act as an isotype control using the same 'FMO' strategy as described previously. Cells and antibodies were incubated on ice for 15 mins before adding PBS (400 µl) for flow cytometry acquisition. CD209 expression was determined as above except PE-conjugated CD209 was used instead of CD206. In this case PE-conjugated $IgG_{2b}$, Kappa was used as an isotype control.

To detect MARCO expression on sputum MΦs a different approach was taken because the mouse anti-human mAb MARCO (an $IgG_3$ antibody, clone PLK-1) was not conjugated to a fluorophore. Clone PLK-1 was donated by Lester Kobzik at the Harvard School of Public Health (Boston, USA) and has been previously used to detect cell surface MARCO expression (Bunn, Kobzik *et al.* 2004; Arredouani, Palecanda *et al.* 2005). MARCO was stored as two 2.5 mg/ml vials at -80°C in PBS. Before use MARCO was thawed, serially diluted with filtered PBS to determine the optimum final concentration (10 µg/ml) required for staining (data not shown) and stored in the dark at 4°C. Sputum cells (100 µl) were incubated with the primary MARCO (10 µg/ml) mAb for 30 minutes on ice. Cells were washed by pelleting at 4°C and resuspending twice in PBS (+2% FCS) to a final volume of 100 µl. To control for non-specific binding by the primary

antibody a non-specific un-conjugated $IgG_3$ kappa (FLOPC-21) was used from Sigma-Aldrich (product discontinued) at the same concentration for use in PRR staining experiments. To detect binding of the primary mAb, a PE-conjugated secondary goat anti-mouse mAb (Table 2-1) was used at the same concentration (10 μg/ml) and incubated as described for the primary antibody. Cells were washed as previously to remove excess secondary antibodies. To block the remaining binding sites for murine IgG on the secondary antibody, an excess of $IgG_3$ was added and incubated in the dark on ice. Subsequently FITC-conjugated CD16b and CD66b, PerCP or PC5-conjugated HLA-DR and APC-conjugated CD14 were added as before to aid identification of sputum MΦs. As a point of comparison, PRR expression on sputum MΦs was compared with blood monocytes (described below) taken at the same time of sampling. No less than 1000 gated light scatter events were used to determine PRR expression.

### 2.6.3 Pattern Recognition Receptor Expression on Blood Monocytes

Control and CF peripheral blood was collected into EDTA blood collection tubes (Section 2.3). Whole blood (100 μl) was incubated with FITC-conjugated CD14, PerCP or PC5-conjugated HLA-DR and PE-conjugated CD206 or CD209 or an appropriate isotype control. Cells were incubated on ice for 15 min, following which 3ml of an ammonium chloride based erythrocyte lysis buffer solution (4.15g $NH_4Cl$, 0.5g $KHCo_3$, 26μl 0.5M EDTA in 500ml $dH_2O$) was added and left to stand at room temperature in the dark for 20 min before cell acquisition.

To examine monocytes for MARCO expression, whole blood (100 μl) was treated in the same way as sputum, except that following blocking of free binding sites on the secondary antibody with unconjugated $IgG_3$ kappa, cells were co-stained with FITC-conjugated CD14 and PC5 or PerCP-conjugated HLA-DR. For all three PRRs, monocytes were identified in the same way

using differential light scatter and positive cell surface expression of HLA-DR and CD14 (Chapter 4). In one instance less than ~600 monocyte events were gated to determine PRR expression. In all other instances no less than 1000 monocyte events were analysed.

### 2.6.4 Particle Uptake by Sputum Macrophages

Following separation of MΦ from sputum (Section 2.4.4), cells were pelleted once more and resuspended in cell culture medium (without FCS) and kept on ice. To assess the role of MARCO in particle uptake, sputum MΦs were aliquoted (50 µl) and anti-human MARCO (clone PLK-1 acting as an inhibitor) or $IgG_3$ (AbCam #9405-1 UK) were added and incubated as described previously for PRR expression except that the final concentration of PLK-1 was 20µg/ml. Following incubation, Fluoresbrite Carboxylate YG 1.0 µm (#15702, Polysciences Inc, USA) spheres in cell culture medium were vortexed vigorously and added at a ratio of 5 fluorospheres per MΦ. Cells with and without beads were agitated and incubated under similar conditions (37°C; 5%$CO_2$ for 1 hr) as described by Arredouani *et al.* (2005), who also examined particle uptake by airway MΦs. Cells were further agitated gently following 30 min incubation. Following 1 hr incubation, cells were removed from the incubator and placed on ice. To each of the samples an aliquot of APC-conjugated CD45 was added and incubated on ice for 15 minutes as per the manufacturer's recommendations. CD45 was added to aid identification of CD45 expressing sputum MΦs from CD45 negative ECs and debris by gating on CD45 high events using flow cytometry. Following incubation PBS was added (350 µl) and trypan blue (0.08 µg/µl final concentration) to quench exogenous fluorescence (Cramer, Yamanishi *et al.* 2003) and incubated on ice for 15 min. Subsequently sputum MΦs were acquired and analysed by flow cytometry. No less than 1000 events were collected in the final light scatter small MΦ window. Flourospheres only were also analysed but could not be detected by the flow cytometer.

### 2.6.5 Determination of Monocyte Purity

Control and CF purified monocytes (50 µl) were incubated with either PC5 or PE-conjugated CD14 or a non-specific isotype control ($IgG_{2a}$). Cells were incubated on ice for 15 min. after which PBS (300ul) was added prior to flow cytometry. Approximately 10,000 light scatter events were gated in a 'monocyte gate' (Figure 2-3, below, upper panel 'R1') and examined in a histogram plot for CD14 staining intensity on a logarithmic scale (lower panel). The proportion of CD14 positive events (lower panel red line) was determined against the isotype control (green line) and expressed as percentage purity. Only monocyte purity levels that reached greater than 90% were used for the generation of MDMs (Section 2.5.2).

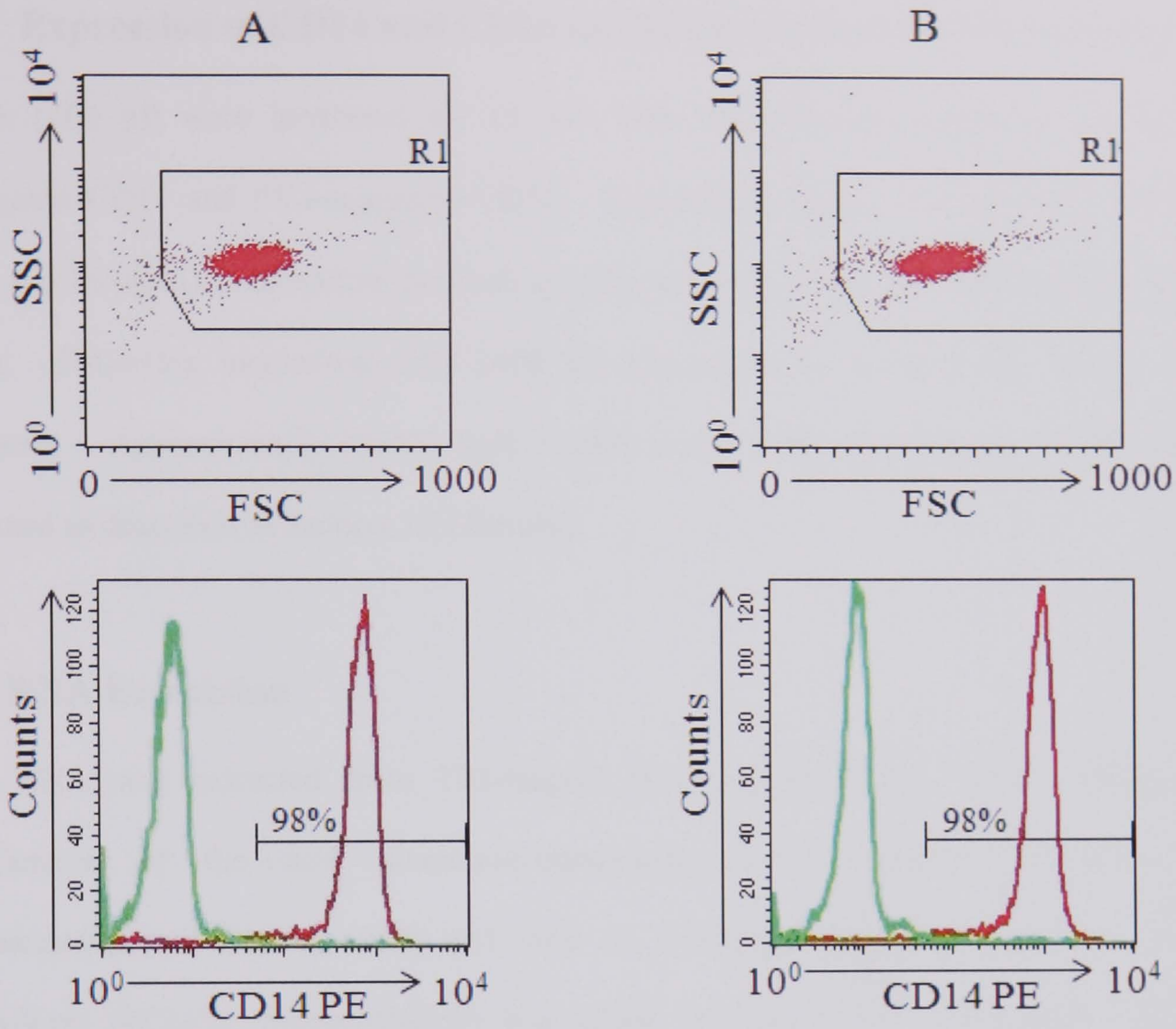


**Figure 2-3 Monocyte purity following MACS purification from a control (column A) and a CF (column B) donor**

*In column A and B, upper panel, total light scatter events are displayed. A region ('R1') was placed around total cells and gated onto a fluorescence histogram to examine CD14 expression (lower panel). CD14 positive events (red line) were determined in comparison to an isotype control (green line). Events that fell to the right of the isotype control are CD14 positive (horizontal bar) and given as percentage of total gated events. In this example, 98% of control events (column A) were CD14 positive (i.e. monocytes). Similarly 98% of CF events (column B) were also CD14 positive. For each example an approximately equal number of events are shown.*

### 2.6.6 Expression of CD14 and CD16 on Monocyte-Derived Macrophages

MDMs (100 µl) were incubated for 15 min with the following antibodies on ice: FITC-conjugated CD14 and PE-conjugated CD16. Appropriate isotype controls were used (FITC-$IgG_{2b}$ and PE-$IgG_1$) to substitute for each specific antibody, using the FMO strategy, discussed earlier. Following incubation, PBS (400 µl) was added to increase the volume for flow cytometry. Approximately 10,000 light scatter events were gated in an 'MDM gate' and examined as described in Section 5.2 (Results).

## 2.7 RNA Extraction

Total RNA was extracted from TRI-reagent (Chomczynski 1993) lysed MDMs using a modification of the acid guanidium-thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987) and used as previously (Staples, Smallie *et al.* 2007). Chloroform (#C2432, Sigma-Aldrich) was added (40 µl) to each TRI-reagent aliquot and vortexed vigorously to form a white emulsion, which was allowed to stand on ice for 5 min to separate out. Following centrifugation at 12,000 *g* for 15 min at 4°C, three layers become evident. The top layer is aqueous and contains RNA, the bottom layer is organic and constitutes mainly protein, whilst in between is the interphase, which contains DNA (Chomczynski 1993). Equal proportions of the aqueous RNA containing layer (100 µl) from each sample was mixed with standard laboratory grade isopropanol and centrifuged as above to precipitate and pellet the RNA. The supernatant was discarded and the pellet resuspended in 500 µl of 75% ethanol (standard lab grade, in 0.1% diethylpyrocarbonate (DEPC, #D-5758, Sigma-Aldrich) treated $dH_20$). RNA was pelleted at 12,000 *g*, for 10 min at 4°C and the supernatant was discarded. To remove any remaining ethanol the RNA pellet was placed under vacuum for 10 min after which

the RNA was resuspended in 20 µl of 0.1% DEPC treated $dH_20$ and kept at -80°C for reverse transcription into cDNA (Section 2.8, below).

## 2.8 Reverse Transcription of RNA into cDNA

Extracted RNA (4 µl) and an equal aliquot of 0.1% DEPC treated $dH_2O$ (negative control) were placed into separate eppendorf tubes and incubated in a Progene Thermocycler (Techne, Cambridge, UK) for 5 min at 70°C to linearise the RNA. Linearised RNA was reverse transcribed for 1 hr at 42°C in a reaction volume of 20 µl containing 1 mM deoxynucleotide phosphates (dNTPs), 0.4 U/µl AMV Reverse Transcriptase (#M510A), 1 x AMV buffer (#M515A), 1.6 U/µl RNAsin (#N251B) and 10 ng/µl hexanucleotides (Amersham Pharmacia #27-2166-01) and was terminated at 90°C for 5 min. dNTPs were purchased as individual bases, Adenine (#U120A), Guanine (#U121A), Cytosine (#U122A) and Thymidine (#U123A). Each dNTP was diluted 1 in 10 with 0.1% DEPC treated $dH_2O$ to form a 10mM working solution. All products, unless specified, were purchased from Promega (Wisconsin, USA). All stocks and working solutions were kept at -20°C prior to use. Complementary DNA (cDNA) was kept at 4°C prior to PCR analysis (Section 2.10).

## 2.9 Microarray Analysis of Monocyte-Derived Macrophages

RNA extraction and preparation of cDNA for microarray analysis (Affymetrix version Human genome U133 plus 2.0, Max-von-Pettenkofer Institute, Munich, Germany) was performed by Dr Thomas Hofer at our collaborating group (Clinical Cooperation Group, "Inflammatory Lung Diseases", Gauting, Germany). From the cDNA preparation streptavidin-PE labelled complementary RNA (cRNA) was generated and fragmented into 25-200 base pairs (bp) and hybridised to a series of 25 bp oligonucleotides for 16 hrs at 45°C (Hardiman 2004). Detection

was performed using a primary goat anti-streptavidin and biotinylated goat-IgG secondary. Addition of fresh streptavidin-PE enabled detection hybridised probes by a confocal laser and Agilent GeneArray scanner (Hardiman 2004). Microarray preparation and analysis was performed and recorded by Dr Reinhard Hoffman at the Max-von-Pettenkofer Institute (Ludwigs-Maximilians University, Munich, Germany). Transcript expression was analysed by the author with the assistance of Prof. Löms Ziegler-Heitbrock (University of Leicester, UK) in Excel, using data generated from microarray suite version 5.0 (MAS 5.0) software. Robust differences were selected on the basis of transcript detection (present), qualitative change and the relative change in transcript abundance between comparisons as given by the signal log ratio (SLR) where an SLR of 1.0/-1.0 indicates a 2-fold increase/decrease respectively.

## 2.10 Real-Time PCR

PCR for genes of interest was performed using the Roche (Mannheim, Germany) LightCycler™ capillary technology on cDNA generated from control and CF MDM specimens. Total cDNA were measured for the house keeping gene using primers for α-enolase by Dr Thomas Hofer (Gauting, Germany) using the same Roche LightCycler technology as used in this thesis. This enabled all genes that were studied to be compared against a common baseline. The following sense (5') and anti-sense (3'), α- and β-tryptase primers were designed and used previously by Schwartz *et al.* (2003). TNF primers were designed and provided by Dr Thomas Hofer. All PCR primers for α- and β-tryptase, TNF and enolase were purchased from MWG Biotech (Ebersberg, Germany). All primers were kept as a working solution of 15 μM in 0.1% DEPC $dH_2O$ at -20°C before use. Given below are the following primers used in this thesis:

α-Tryptase primers:

5' primer: 5'-TGC AGC AAG CGG GTA TCG T-3';

3' primer: 5'-AGT CTG GAT GAT GTA GAA CTG T-3'

β-Tryptase primers:

5' primer: 5'-TGC AGC GAG TGG GCA TCG T-3';

3' primer: 5'-GAT CTG GGC GGT GTA GAA CTG T-3'

TNF primers:

5' primer: 5'-CAG AGG GAA GAG TTC CCC AG-3';

3' primer: 5'-CCT TGG TCT GGT AGG AGA CG-3'

α-enolase (house keeping gene):

5' primer: 5'-GTT AGC AAG AAA CTG AAC GTC ACA-3';

3' primer: 5'-TGA AGG ACT TGT ACA GGT CAG-3'

An aliquot of the cDNA (3 μl) to be tested was amplified in a reaction mixture containing 1 x SyBr Green Taq ready mix capillary formulation (#S-1816, Sigma-Aldrich), 1.5 mM $MgCl_2$ and 0.375 μM of each primer in a final reaction volume of 20 μl (Staples, Smallie *et al.* 2007). Each sample was placed into Roche LightCycler capillaries (#11909339001) and pelleted at 400 *g* for 10 s at 4°C. This ensures the PCR reaction is being carried out at the bottom of the capillary. LightCycler capillaries were transferred to a Roche PCR LightCycler for real time analysis and analysed on LightCycler software (version 3.5). Each reaction was run under the following PCR conditions: cDNA was denatured at 95°C for 10 min; amplification of primer targets was

performed as follows, 96°C for 10 s; 60°C for 10 s; 72°C for 25 s. Tryptase primers were examined following 45 cycles of PCR, whereas TNF primers were examined following 55 cycles. For each PCR reaction a melting curve was generated by heating to 96°C for 2 s, cooling to 65°C and subsequently increasing by 1°C per s to 96°C to determine the melting point of the PCR products. The melting temperature for each primer product was consistent for each of the primers examined in that a single consistent peak at a given temperature indicated a single specific PCR product. All enolase and TNF PCR runs produced a single peak at 89.30°C and 88.63°C, respectively. Transcripts from MM6 and HMC-1 were run simultaneously as CF and control MDM preparations as positive controls for α- (MM6) and β- (HMC-1) tryptase. Only melting points that matched the positive controls (90.25 ± 0.24 ($n$=5) and 90.42 ± 0.34 ($n$=2), respectively) were used for comparison. All PCR data performed for genes of interest and house keeping genes were examined in the same way (i.e. noise band threshold of 0.1; analysis threshold of 0.3). Cycle numbers for each sample were generated for specific reactions only, representing the degree of fluorescence and thus gene expression.

### 2.10.1 Electrophoresis

A 2% (high gel strength) agarose gel (Melford Laboratories, # MB1200, Ipswich, UK) in Tris-Acetate-EDTA (TAE) electrophoresis buffer (composed of Tris base, glacial acetic acid, EDTA and 0.1% DEPC treated $dH_2O$) containing ethidium bromide (0.25 μg/ml) was melted on a low power setting in a standard microwave for 5 min, thoroughly mixing throughout. A BioRad (Hertfordshire, UK) gel container was set up for 8 wells and the gel, once poured in, was allowed to cool. Once set, the gel was placed into the electrophoresis chamber connected to a PowerPac™ basic electrical supply unit (both BioRad) and submerged with TAE buffer containing ethidium bromide.

α- and β-tryptase PCR products were examined for specificity of tryptase primers. A 2% agarose gel was chosen as being suitable for the length of the expected PCR product (278 bp) determined by Schwartz *et al.* (2003). PCR products (20 µl) were centrifuged at 400 *g* for 10 s into small eppendorfs and mixed with 4 µl loading buffer (1 in 5 dilution) (Bioline, London, UK). Wells 1-5 were loaded with MM6 (1 and 3), HMC-1 (2 and 4) or MDM (5) PCR amplification products and well 6 was loaded with 5 µl of a base pair ladder (HyperLadder IV, New England Biolabs, USA) (Figure 5-2). Ensuring that samples were 'running' from black (negative) to red (positive) electrodes, an electrical current was applied to the PCR products under the following conditions: 100 V (69 mA) for 1 h. A photograph was taken of the gel using a standard photographic digital camera connected to a Personal Computer.

## 2.11 Statistics

Graphic (except flow cytometry plots) and statistical analysis were carried out using GraphPad Prism software (version 3.00, GraphPad Inc., California, USA). Significance ($p \leq 0.05$) was calculated using an appropriate statistical test as given in the text. Microarray SLR $p$ values were calculated using Wilcoxon signed rank test.

# 3 Results: Identification of Small Macrophages in Sputum

## 3.1 Introduction

CF airway infection and neutrophil dominated inflammation occur during the first few years of life and continues into adulthood (Khan, Wagener *et al.* 1995; Konstan and Berger 1997). By the time adulthood is reached most CF patients experience chronic endobronchial infection and inflammation that is associated with severe respiratory disease and ends in respiratory failure (Zuelzer and Newton 1949; Sobonya and Taussig 1986). In the bronchial airway of healthy individuals, MΦs predominate and are the largest (25-50μm) leukocytes that can be recovered (Belda, Leigh *et al.* 2000; Ross and Auger 2002). In the CF and COPD bronchial airway, there is a proportional (but not absolute) decrease in the number of MΦs as they are overwhelmed by the influx of neutrophils (Rutgers, Timens *et al.* 2000; Reinhardt, Chen *et al.* 2003). Moreover, COPD large sputum MΦs are outnumbered by smaller (10.1 ± 4.3 μm) sputum MΦs (Frankenberger, Menzel *et al.* 2004).

Small sputum MΦs have a ~100-fold and ~50-fold higher CD14 and HLA-DR cell surface expression, respectively, than large MΦs from the same donor (Frankenberger, Menzel *et al.* 2004). In COPD and control donors, small sputum MΦs are the greatest contributors to TNF production following LPS stimulation compared to large MΦs (Frankenberger, Menzel *et al.* 2004). Thus a greater proportion of these cells may contribute to maintaining persistent inflammation. Given the similarities in airway inflammation between COPD and CF, I tested the hypothesis that small sputum MΦs would also be the predominant MΦ in CF airways even during mild airway disease. Small MΦs could provide a hitherto unrecognised driver of chronic inflammation in the CF airways. I sought to investigate this in induced sputum from CF patients

with mild (paediatric cohort) and more severe (adult cohort) pulmonary disease using flow cytometry. Presented in this chapter is a strategy to identify sputum MΦs in paediatric and adult healthy control and CF cohorts so that this hypothesis could be tested.

## 3.2 Identification of Macrophages in Control Sputum

Following sputum processing (Section 2.4.3) a homogenous cell suspension was obtained that was amenable to staining (Section 2.6.1) and flow cytometry analysis (described below). To separate MΦs from other sputum cells (mainly neutrophils) by flow cytometry a previous study employed fluorophore conjugated antibodies to common monocyte/MΦ cell surface antigens (HLA DR-PC5, CD14-APC) and granulocyte antigens (CD66b-FITC, CD16b-FITC) (Frankenberger, Menzel *et al.* 2004). To increase the specificity of this approach I used a cell permeabilisation protocol to identify the predominantly intracellular pan tissue MΦ antigen (using a PE-labelled mAb) CD68 (Kelly, Bliss *et al.* 1988; Umino, Skold *et al.* 1999; Staples, Smallie *et al.* 2007) for a more accurate identification of sputum MΦs.

MΦ AF is a useful property that can be exploited by flow cytometry to separate MΦs from other sputum cells and has been utilised by other investigators (Viksman, Liu *et al.* 1994; Umino, Skold *et al.* 1999; Garn 2006). When excited by a 488 nm laser MΦs emit an AF peak at 541 nm, tailoring down to 580 nm (Viksman, Liu *et al.* 1994). Using a filter set on the BD FACSCalibur as described (Table 2-2) such emission will be detected mostly by FL1 (530/30 filter) but also to a lesser extent FL2 (585/42). I reasoned that the AF exhibited by the monocyte-like nature of small MΦs would be low (Frankenberger, Menzel *et al.* 2004) and thus may not assist in the identification of these cells. AF can be detrimental for analysis unless the

contribution made by AF can be controlled for by comparing unstained with stained cells or quenched.

In a four-colour flow cytometry approach I was able to identify sputum MΦs in induced sputum from paediatric (Figure 3-1, below, page 101) and adult control volunteers (Figure 3-3, upper panel, page 103). There were no differences in gating strategy between paediatric and adult control donors. Total sputum cells were visualised according to their light scatter characteristics (size and granularity) from which a region of interest was drawn to select cells for further analysis (Figure 3-1 A). Shown in Figure 3-1 (B) are gated paediatric sputum light scatter events demonstrating specific staining for HLA-DR (*y*-axis) and CD14 (*x*-axis) cell surface antigens. A region of interest was drawn (Figure 3-1, B, 'R1') based on positive expression of HLA-DR (present on all antigen presenting cells) and CD14 - two established monocyte/MΦ antigens.

Specific expression by a mAb is determined, here, by the FMO strategy (Perfetto, Chattopadhyay *et al.* 2004), as described previously (Section 2.6.1). For example, Figure 3-1, E and F demonstrate non-specific binding of labelled isotype controls, $IgG_1$ and $IgG_{2a}$ for HLA-DR (*y*-axis) and CD14 (*x*-axis), respectively. For confirmation that HLA-DR and CD14 positive events are MΦs I gated R1 events onto a further dot plot (Figure 3-1, C) comparing expression of two granulocytic antigens – CD66b and CD16b (*y*-axis) against CD68 expression (*x*-axis). MΦ events were identified from other leukocytes/debris by using a mAb against CD68. Specificity of staining for CD68 (Figure 3-1, B, 'R2') was demonstrated using a PE-labelled isotype control (Figure 3-1, F), $IgG_{2b}$. In paediatric (Figure 3-1) and adult control donors (Figure 3-3, upper panel, page 103) I was able to confirm that the majority of HLA-DR and CD14 positive events in 'R1' are also CD68 positive ('R2') and thus represent total sputum MΦs. Despite exhibiting high

fluorescence in FL-1 such cells are unlikely to be CD66b or CD16b positive and may represent, in part, AF though this was not directly tested.

When these sputum MΦs were plotted back onto a light scatter plot (Figure 3-1, D) the majority exhibited a high FSC (*x*-axis) and SSC (*y*-axis) signal for paediatric (Figure 3-1, D, 'R3') and adult controls (Figure 3-3, C, 'R3'). FSC and SSC give a rough but not absolute indication of size and granularity respectively (Shapiro 2003). Hence in paediatric and adult control donor samples, sputum MΦs are quite large and granular compared to other sputum cells. I also noted MΦ gated events that exhibited lower FSC and SSC (Figure 3-1 D, and Figure 3-3, C, 'R4'). These constituted 7% (paediatric) and 11% (adult) of the total MΦ population in the examples shown.

## 3.3 Identification of Macrophages in Cystic Fibrosis Sputum

Having successfully identified sputum MΦs from healthy controls I, turned to paediatric (Figure 3-2, below, page 102) and adult (Figure 3-3, lower panel, page 103) CF sputum MΦs using the same four-colour approach. Total sputum cells were visualised according to their light scatter characteristics as previously (Figure 3-2, A) and gated onto a dot-plot to examine HLA-DR and CD14 expression. HLA-DR and CD14 positive events were identified (Figure 3-2, B, 'R1') and specific staining was confirmed using identical isotype controls (Figure 3-2, E and F respectively) as described above. HLA-DR and CD14 positive events were then examined for specific CD68 expression (Figure 3-2, C) against an isotype control ($IgG_{2b}$) (Figure 3-2, G). The intensity of CD68 staining of CF gated HLA-DR and CD14 positive events appeared lower than that observed for controls. Paediatric and adult CF HLA-DR, CD14 and CD68 positive events were subsequently labelled as 'sputum MΦs' and gated onto a light scatter plot (Figure 3-2, D

and Figure 3-3, F) as described above. Similar to the paediatric control, CF paediatric sputum MΦs with a large FSC and SSC signal were identified (Figure 3-2, D, 'R3'). In contrast to the paediatric control the majority of CF MΦs had a lower FSC and SSC signal (Figure 3-2, D, 'R4'). In this example they represented the majority (67%) of total MΦs ('R3 and R4') compared to 7% shown in paediatric control sputum. In comparison sputum MΦs with lower FSC and SSC in an adult case with CF (Figure 3-3, F, 'R4') represented 85% of total MΦs (again 'R3 and R4') compared to 11% in an adult control sputum sample. I therefore concluded that the majority of CF MΦs in this sample were of lower size and granularity compared to control. I named these lower FSC and SSC MΦ events 'small sputum MΦs' in contrast to 'large MΦs'. In the two CF examples shown small MΦs constituted the major MΦ population and are identified in CF for the first time.

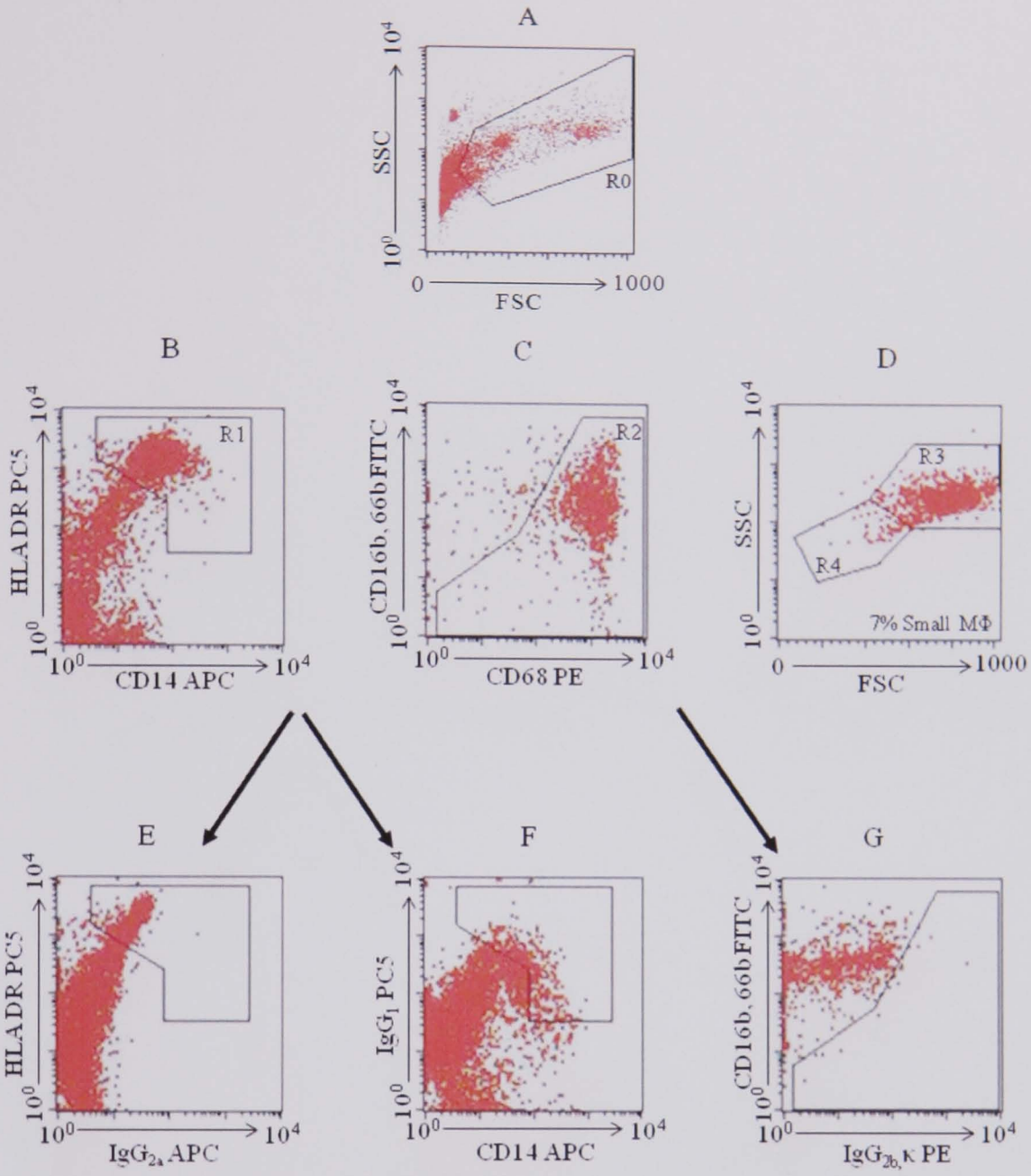


**Figure 3-1 Identification of paediatric control sputum MΦs by flow cytometry**

*Paediatric light scatter cell events (A) were gated for HLA-DR (y-axis) and CD14 (x-axis) expression (B). Double positive cells (B, 'R1') were gated with reference to an isotype control (E and F respectively). The cells in 'R1' were then analysed for CD68 expression (C, x–axis). CD16b and CD66b were included (C, y-axis) to remove contaminating granulocytes. CD68 positive cells (C, 'R2') were gated with reference to an isotype control (G). HLA-DR, CD14 and CD68 positive events were gated onto a light scatter plot (D) demonstrating relative cell size (x-axis; FSC) and granularity (y-axis; SSC). In this light scatter plot two populations were identified - 'large' ('R3') and 'small' ('R4'). The percentage of small MΦs (7%) in 'R4' was determined relative to total MΦs ('R3' and 'R4'). Similar numbers of events are shown in each plot*

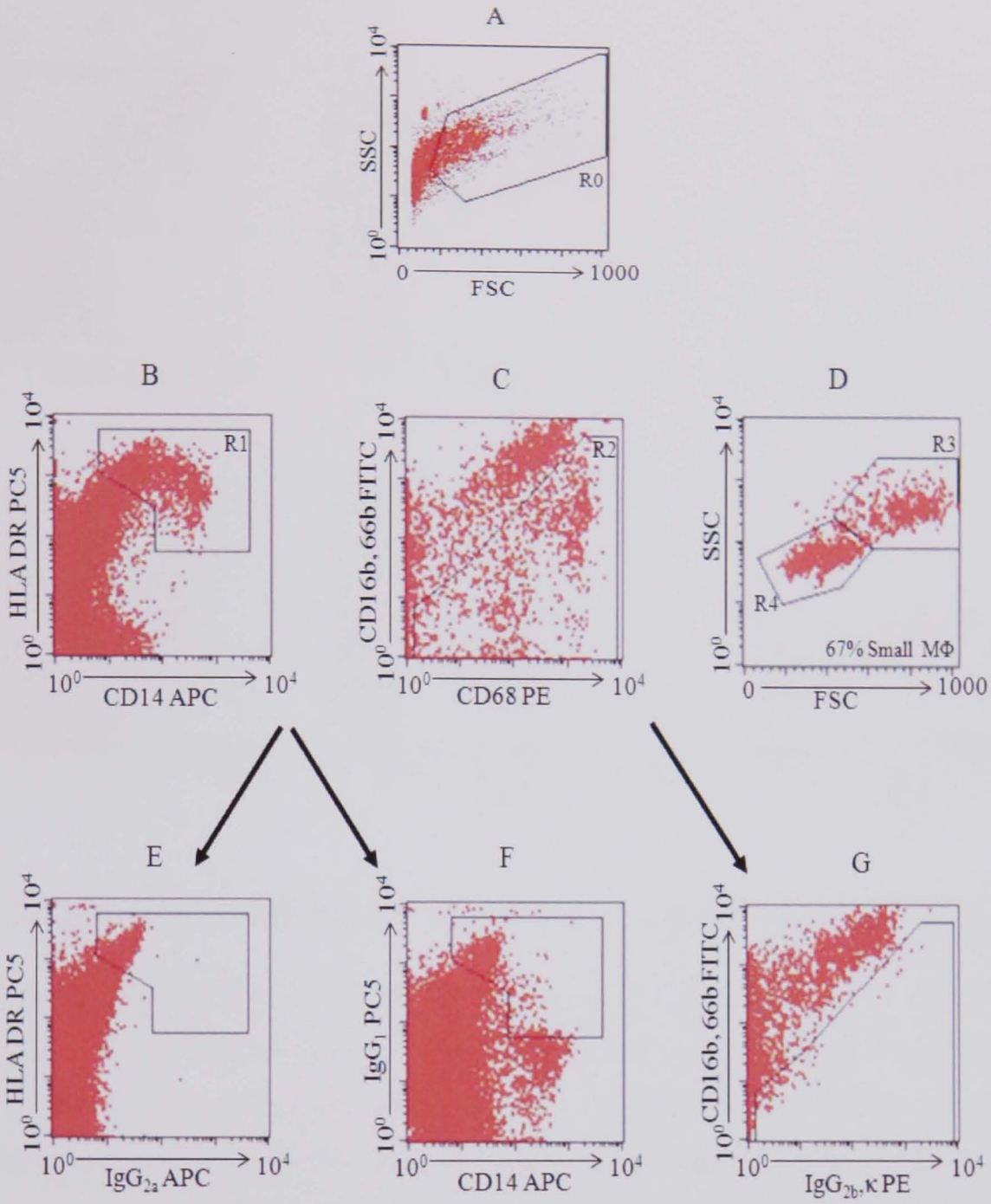


**Figure 3-2 Identification of paediatric CF sputum MΦs by flow cytometry**

*Paediatric sputum cells were analysed as described for Figure 3-1. In contrast, however, CD68 positive events (C, 'R2') when gated with reference to an isotype control (G), demonstrated one population with higher and one with lower CD68 expression (y-axis). HLA-DR, CD14 and CD68 positive events were gated onto a light scatter plot (D) demonstrating relative cell size (x-axis; FSC) and granularity (y-axis; SSC). In the light scatter plot in D two populations were identified as for Figure 3-1 - 'large' ('R3') and small ('R4'). The percentage of small MΦs in 'R4' was determined (67%) relative to total MΦs ('R3 and 'R4'). Similar numbers of events are shown in each plot.*

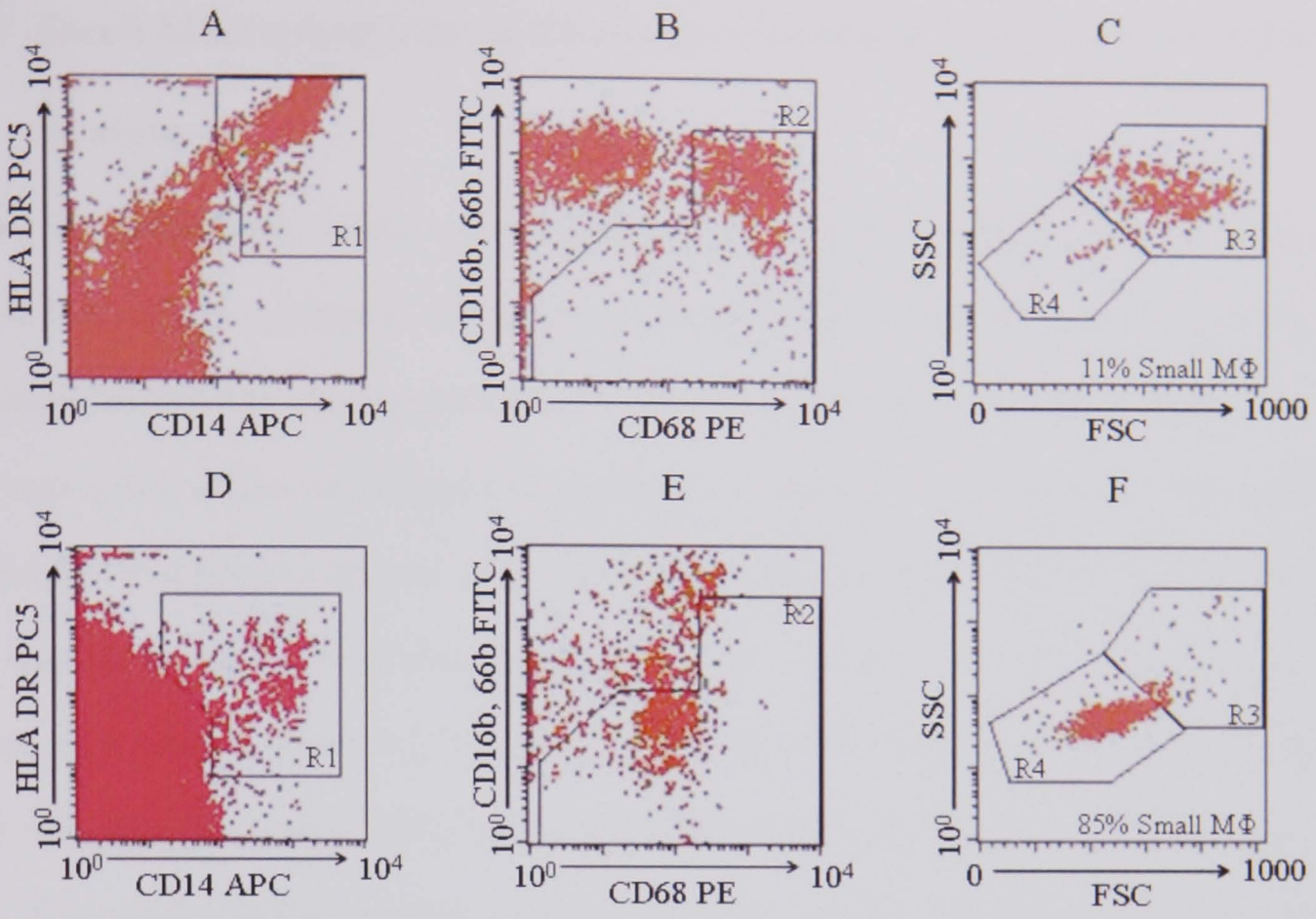


**Figure 3-3 Adult sputum MΦs from a control (upper panel) and CF (lower panel) donor**

*Events were gated as described in Figure 3-1 and Figure 3-2 for both groups. Discrimination of small and large MΦs for each sample was performed by the principle investigator (Prof. Ziegler-Heitbrock) on the same day for both cohorts, using the same criteria to minimise bias. Control sputum MΦs (upper panel, plot C) contained a proportion of small MΦs (11%) that was much lower than that observed in CF (lower panel, plot F) sputum (85%). An approximate equal number of events are shown in each plot.*

## 3.4 Small Macrophages are a Consistent Feature of Adult but not Paediatric Sputum

Using the method outlined above I determined whether the observation of a large percentage of small MΦs were a consistent feature of CF lung disease. Furthermore I chose to sample a paediatric cohort with mild lung disease to examine whether these small MΦs are present during the early phase of disease progression. I sampled a cohort of 13 paediatric control donors and performed 18 inductions in total. In the CF cohort I sampled 13 paediatric donors and performed 20 inductions overall. From these inductions a final control cohort of 8 individuals and a CF cohort of 5 patients (Table 3-1, below) yielded an adequate number of cells for comparison (no less than 300 MΦ events). Cell viability for the paediatric samples studied is also given in Table 3-1. Cell viability in these paediatric (and adult, Table 3-2) cohorts are similar to that reported by Belda *et al.* (2000) of a mean sputum cell viability of 69% in 96 healthy subjects and in CF (71%) by Alexis *et al.* (2007). The final paediatric CF cohort had an average age of 12.2 ± 2.5 years that was almost identical to the control cohort (12.8 ± 3 years). $FEV_1$, though lower in the CF cohort (80 ± 20%), was not significantly different from the control cohort (96 ± 10%) ($p \geq 0.05$ using a Mann-Whitney U-test). However when looking at the percentage of small sputum MΦs (Figure 3-4, below) there was an overall significantly higher mean percentage of small MΦs in the paediatric CF group (50 ± 35% SD) compared with controls (13 ± 13% SD; $p=0.0186$ using an Mann-Whitney U-test).

**Table 3-1 Paediatric control and CF cohort data**

| | Control | CF |
|---|---|---|
| **Donors (*n*)** | 8 | 5 |
| **Age (mean years ± SD)** | 12.8 ± 3 | 12.2 ± 3 |
| **Sex (Female:Male)** | 4:4 | 3:2 |
| **Ethnicity (Caucasian:Asian)** | 6:2 | 4:1 |
| **Genotype (*n*)** | N/A | ΔF508 +/+ (3); ΔF508 +/R347 (1)<br>ΔF508 +/G551D (1) |
| **Last Known Culture (*n*)** | N/A | NSG (2); *Mycobacterium sp.* (1);<br>*P. aeruginosa* (1);<br>*Aspergillus sp.* (1) |
| **Inhaled Steroids (Yes:No)** | N/A | 2:3 |
| **% Viability (mean ± SD)** | 67 ± 9[†] | 63 ± 15[†] |
| **% $FEV_1$ (mean ± SD)** | 96 ± 10 | 80 ± 20 |
| **% Small MΦ (mean ± SD)** | 13 ± 13 | 50 ± 35* |

**Legend:** *$FEV_1$ – Forced expiratory volume in one second, n – number, N/A – Not Applicable, NSG - No Significant Growth; sp. – species; SD – Standard deviation; [†] one sample could not be determined and in some samples less than 100 cells were counted, * p=0.0186 vs Control (Mann-Whitney U-test)*

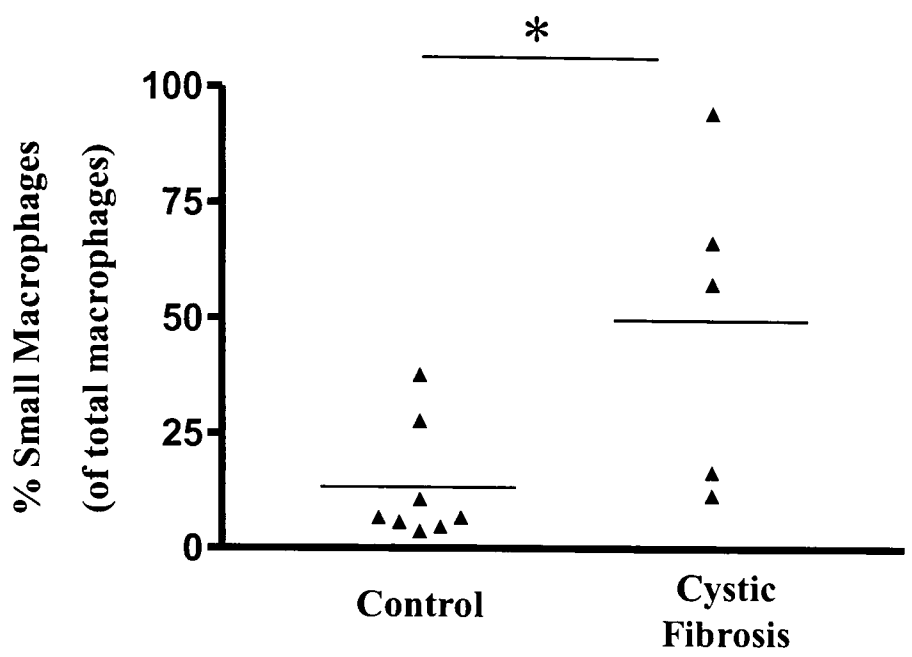


**Figure 3-4 Percentage of small MΦs (*y*-axis) from paediatric control and CF cohorts (*x*-axis)** (Horizontal bars represent mean values) * $p$=0.0186

To confirm these findings I also examined a cohort of adult CF patients ($n$=14) and controls ($n$=10). None of the donors are or were smokers. Here the larger lung volumes compared to paediatric donors yielded a greater volume of sputum for analysis such that more parameters could be assessed with greater ease. Moreover, CF adult patients are at a more advanced stage of lung disease compared with paediatric patients and would allow us to establish 'proof of principle' that small MΦs could be involved in the pathophysiology of CF lung disease and are not a transient feature of our paediatric cohort.

I examined two adult CF cohorts (Table 3-2, below) that differ in severity of disease with one group ($n$=10) having a higher severity ('classical') and the other ($n$=4) milder pulmonary disease ('non-classical'). This distinction was made by the consultant respiratory CF physiologist as described previously (Section 2.2). CF cohorts were compared to a cohort of non-smoking adult controls. The age and $FEV_1$ of the classical (but not non-classical) CF cohort was significantly

different than the control ($p$=0.0023 for age and $FEV_1$ using an Mann-Whitney U-test). Cell viability of sputum cells appeared greater but non-significant (using a Mann-Whitney U test ) in the CF cohort compared to control. However for the majority of control samples less than 100 cells were counted compared to over 100 cells being counted in the majority of CF samples.

**Table 3-2 Adult control and CF cohort data**

| | **Control** | **Classical CF** | **Non-Classical CF** |
|---|---|---|---|
| **Donors (*n*)** | 10 | 10 | 4 |
| **Age (mean years ± SD)** | 35.2 ± 12 | 21.5 ± 4* | 42.5 ± 10 |
| **Sex (Female:Male)** | 3:7 | 7:3 | 1:3 |
| **Ethnicity (Caucasian:Asian)** | 6:4 | 8:2 | 4:0 |
| **Genotype (*n*)** | N/A | ΔF508 +/+ (6)<br>ΔF508 +/N5098R (1)<br>ΔF508 +/unknown (3) | ΔF508 +/R117H (2)<br>A455E/A4326delTC (1)<br>G542x/PolyT5/9 (1) |
| **Last Known Culture (*n*)** | N/A | *P. aeruginosa* (9);<br>NSG (1) | NSG (3)<br>*Candida* sp. (1) |
| **Inhaled Steroids (Yes:No)** | N/A | 5:5 | 0:4 |
| **Antibiotics (*n*)** | N/A | Colomycin (4);<br>Ciprofloxacin (2);<br>Azithromycin (2);<br>Vancomycin (1); | None |
| **% Epithelial Cells** | 10 ± 7† | 12 ± 18 | 11 ± 6 |

| | | | |
|---|---|---|---|
| **% Viability (mean ± SD)** | 66 ± 13[†] | 78 ± 10 | 64 ± 16 |
| **% $FEV_1$ (mean ± SD)** | 89 ± 15 | 62 ± 19* | 93 ± 16 |
| **% Small MΦ (mean ± SD)** | 16 ± 8 | 73 ± 18** | 31 ± 20 |

**Legend:** *$FEV_1$ – Forced expiratory volume in one second, n – number, N/A – Not Applicable, NSG - No Significant Growth; sp. – species; SD – Standard deviation, Unknown – refers to a CF mutation that is outside of those commonly tested, [†] less than 100 cells were counted in most cases, * $p=0.0007$ vs control; ** $p<0.0001$ vs control (Mann-Whitney U-test).*

A comparison of small sputum MΦs (Figure 3-5, below) reveals a significantly higher mean percentage of small MΦs in the classical CF group (73 ± 18%) compared to control (16 ± 8%; $p$ <0.0001 using a Mann-Whitney U-test). The proportion of small sputum MΦs, despite being higher in the non-classical CF group (31.2 ± 19.7%) were not significantly different to control ($p$=>0.05, using a Mann-Whitney U-test).

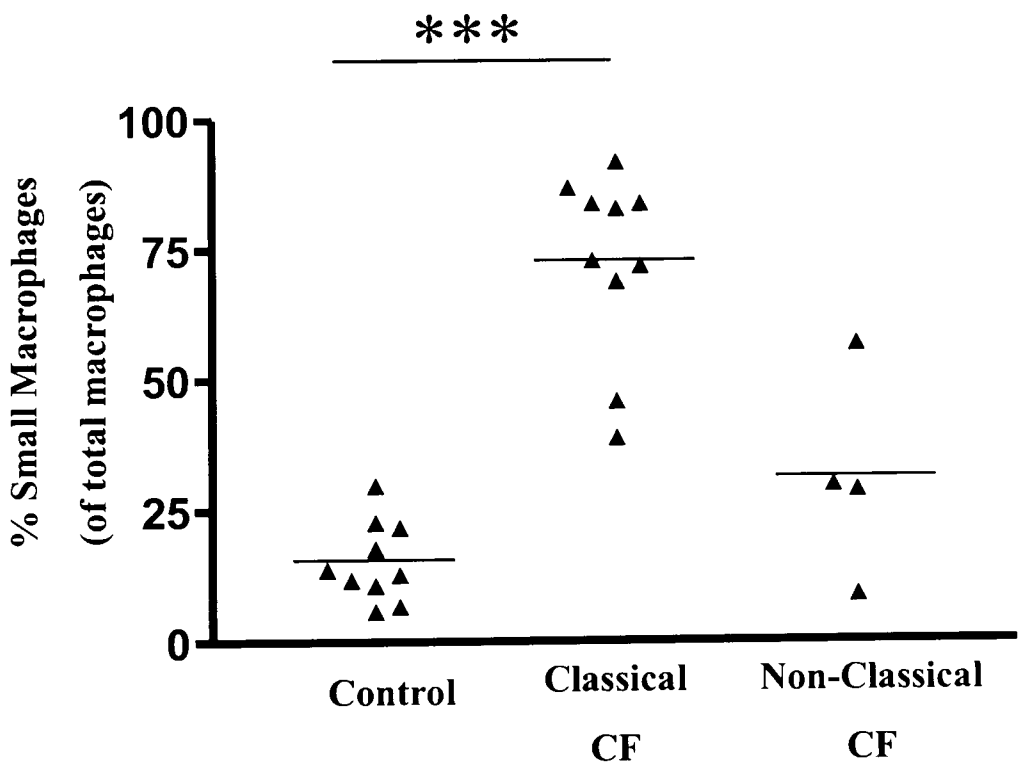


**Figure 3-5 Percentage of small MΦs ($y$-axis) from adult control, classical and non-classical CF cohorts ($x$-axis)** (Horizontal bars represent mean values) ***$p<0.0001$

## 3.5 Small Macrophages Resemble Monocytes

Next, I wished to determine whether CF small MΦs, because of their similar light scatter characteristics to neutrophils, would resemble blood monocytes rather than tissue MΦs. I thus examined whether PBMCs isolated from the same adult CF donors as described previously ($n$=5) have similar light scatter characteristics to CF small MΦs (Table 3-3).

**Table 3-3 Average (mean±SD) parameter channel values determined by flow cytometry from five cystic fibrosis donors**

| | FSC | SSC | CD14 | HLA-DR | CD68 |
|---|---|---|---|---|---|
| **Monocytes** | 444 ± 36 | 98 ± 23 | 229 ± 217 | 63 ± 21 | 78 ± 56 |
| **Small MΦ** | 442 ± 24 | 93 ± 35 | 163 ±191 | 469 ± 545 | 146 ± 85 |
| **Large MΦ** | 685 ± 19 | 419 ± 88 | 130 ± 152 | 1897 ± 1052 | 737 ± 314 |

The light scatter characteristics of CF small MΦs, though variable, suggest a monocyte-like rather than a MΦ-like phenotype. Large MΦs scatter laser light (FSC and SSC) to a significantly greater degree ($p$=0.0079 Mann-Whitney U-test) than both small MΦs and monocytes, which both scattered light to a similar extent (non-significant). There were no significant differences between monocytes and MΦs with respect to CD14 expression but a trend towards higher CD14 expression in monocytes and small MΦs compared to Large MΦs. Also, Large MΦs had a significantly higher expression of HLA-DR compared to small MΦs ($p$=0.03) and monocytes ($p$=0.0079), using a Mann-Whitney U-test. Small MΦs and monocytes also differed from each other ($p$=0.0079) with respect to HLA-DR expression. Furthermore, CD68 fluorescence intensity was much lower in small MΦs and blood monocytes (146 ± 85 and 78 ± 56 fluorescence channels, respectively) compared to large MΦs (737 ± 314 flourescence channels, $p$=0.0079

using a Mann-Whitney U test). Small MΦs and monocytes were not significantly different from each other with respect to CD68 expression. Monocytes express lower levels of CD68 (Staples, Smallie *et al.* 2007) and this data, in addition to differential light scatter, support the monocyte-like appearance of CF small MΦs. To further support the monocyte-like nature of small sputum MΦs I took photographs of control and CF sputum MΦs and compared them to blood monocytes (Figure 3-6).

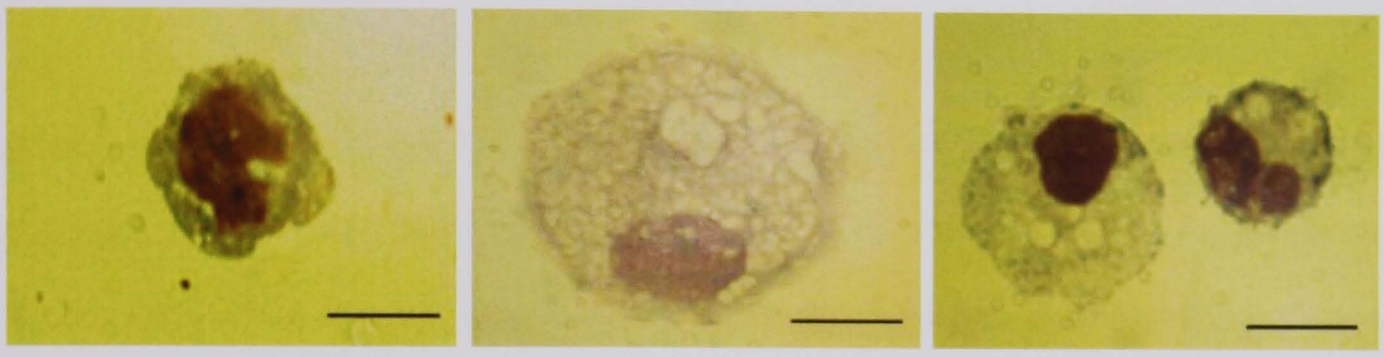

**Figure 3-6 Light microscopy photograph of a blood monocyte (left) and sputum MΦs from control (centre) and CF (right) donors.** Bars represent 10 μm.

## 3.6 Correlation between the Percentage of Small Macrophages and Clinical Data

Upon identifying CF small MΦs I originally set out to examine clinical parameters (such as infection status and/or therapy) against the percentage of small MΦs to further understand the relationship between small MΦs and respiratory disease in CF. However due to the small sample size this was not possible and instead I examined the clinical data taken at the time of sampling for those that had a high or low percentage of small MΦs, summarised in Figure 3-7.

In light of CF clinical data taken at the time of sputum donation (Table 3-1), paediatric patients with culture positive sputa (donors 1, 4 and 5) were reported as being symptomatic (Dr S Rao,

personal communication) at the time of induction. Microbiological analysis of sputum from donors 1 and 4 demonstrated the presence of *Mycobacterium* sp. and *P. aeruginosa*, respectively. Donors 1 and 4 exhibited a high percentage of small MΦs (58 and 67%, respectively) and lower $FEV_1$ values, taken at the time of induction, of 53 and 66%, respectively. In contrast, sputum donor 5 was positive for *Aspergillus* sp., had a higher $FEV_1$ score (102%) and a low percentage of small MΦs (12%).

Culture negative donors (2 and 3) from the paediatric cohort both reported feeling 'well' and had a high $FEV_1$ score of 94 and 84%, respectively. In line with this donor 2 had a similar percentage of small MΦs (17%) compared with controls (13%). In contrast donor 3 (95%) had a very high percentage of small MΦs despite having a culture negative sputum.

In conclusion, increases in small MΦs are an inconsistent feature of paediatric CF sputa but in some cases are associated with culture positive sputa or an exacerbation. From these data, it is unclear what relationship they have with paediatric CF lung disease.

Taking into account the clinical data for the adult classical CF cohort I noted that two donors could be described as having an exacerbation, which could account for the higher percentage of small MΦs observed from these donors. Donor 2 was admitted to the ward following clinic attendance for intravenous antibiotics despite feeling 'well'. The second (donor 10) was given a further dose of ciprofloxacin for increased production of 'greenish' sputum. The remaining classical (and non-classical) patients were described as being stable at the time of analysis i.e. requiring no additional medication, a decision made by the consultant respiratory physician (Dr

Simon Range). Despite this, donors 1 and 6 had light macroscopic evidence of haemoptysis (and were thus included in the exacerbation group in Figure 3-7).

In comparison to our paediatric cohort, all except one classical adult CF subject (Table 3-2, above) had culture positive sputa for *P. aeruginosa* either before or on the day of the clinic visit. A high prevelance of *P. aeruginosa* is characteristic amongst CF patients of increasing age (Gibson, Burns *et al.* 2003). In our study this was associated with a consistently and significantly high percentage of small MΦs. One donor however, despite having a culture negative sputum also had a significantly high percentage of small MΦs (84%).

In summary the paediatric and adult (classical) patients were sub-divided into physician diagnosed 'exacerbation' and 'stable' (but infected) groups and the percentage of small MΦs in each group were examined (Figure 3-7).

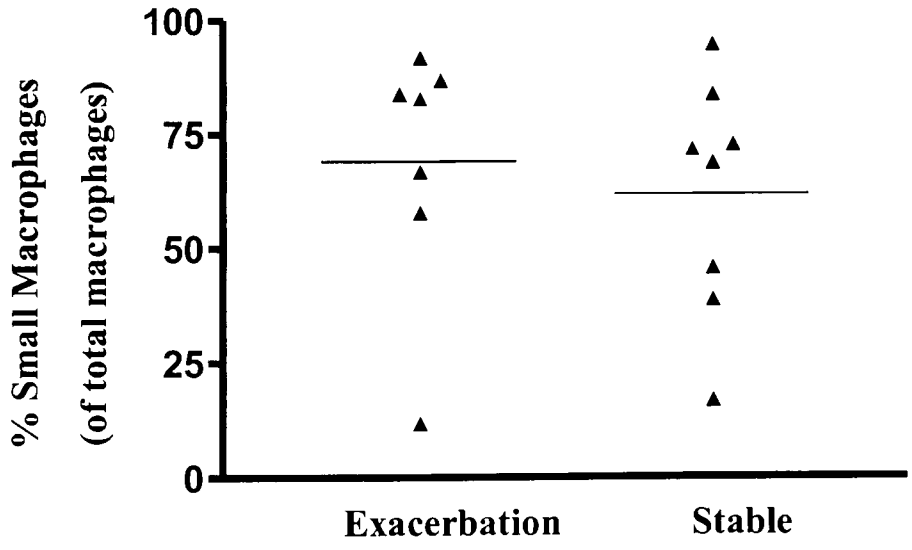


**Figure 3-7 The percentage of CF small MΦs according to clinical status**

In contrast to the classical group the non-classical CF group were not receiving treatment for respiratory symptoms, were culture negative for CF bacterial pathogens and were feeling well. I noted that there were a slightly higher mean percentage of small MΦs in this group compared to controls, though this was non-significant. When looking at the clinical data I noted that two donors with a comparatively high percentage of small MΦs (57% and 29%) also have a history of smoking. One of these is a current smoker (57%) and the other an ex-smoker (29%). An increase in the proportion of small MΦs has been identified in COPD, a disease induced by smoking (Frankenberger, Menzel *et al.* 2004).

Taken together, in stable (but mostly infected) paediatric and adult (classical) CF patients, the latter with more advanced disease, there was a significant and consistent elevation in the proportion of small MΦs compared to controls. In those with physician diagnosed respiratory exacerbation there was a trend towards a further elevation in the percentage of small MΦs.

Within the classical paediatric and adult CF cohorts there was a broad range of FEV1 values (36-102%) that did not correlate with the percentage of small MΦs. However when taking both classical (paediatric and adult) and non-classical CF cohorts together (n=19) I noted a significant negative correlation (Pearson correlation r= -0.59, p=0.0082) between the percentage of small MΦs and FEV1 (Figure 3-8, below).

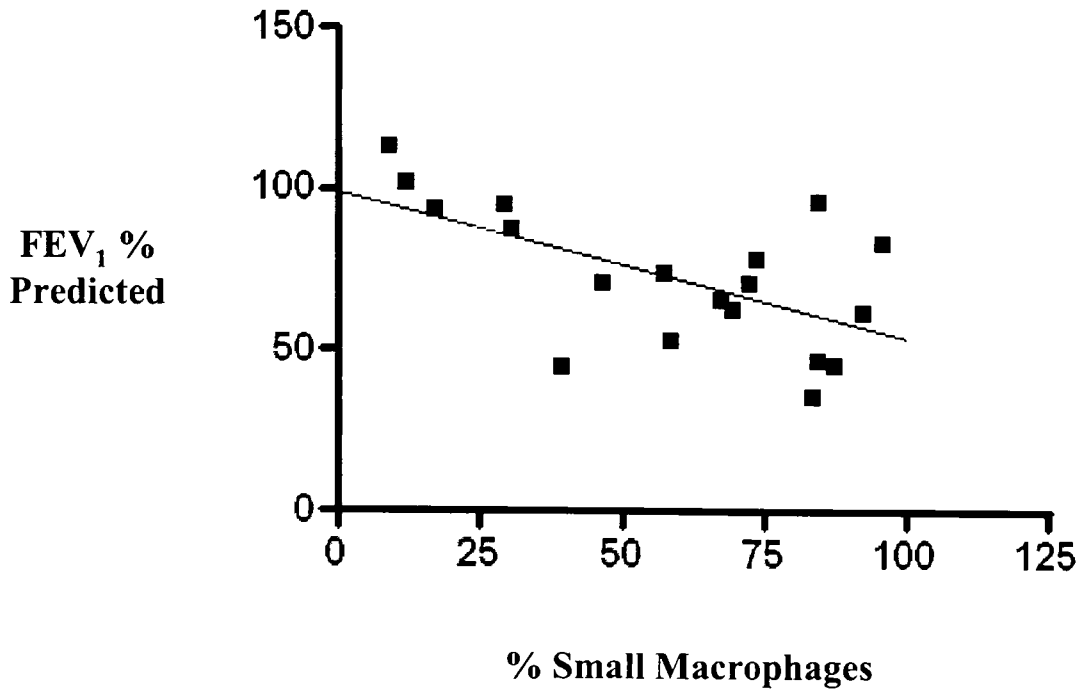


**Figure 3-8 Correlation between the % $FEV_1$ and % small MΦs in our CF cohort** (Pearson correlation $r$= -0.59, $p$=0.0082)

## 3.7 Glucocorticoids Do Not Affect the Percentage of Small Macrophages

CF patients are treated with a wide range of different medications to alleviate respiratory (and gastrointestinal) symptoms. In terms of respiratory disease prophylactic therapy includes oral or inhaled antibiotics and sometimes mucolytics (e.g. recombinant human DNase), the latter to relieve airway plugging. Within our classical CF cohort I noted that half of the subjects ($n$=5) were receiving inhaled steroid medication, either Seretide ($n$=3) or Symbicort ($n$=2). Seretide and Symbicort contain a glucocorticoid (fluticasone and budesonide, respectively) plus a long acting $beta_2$ agonist component. Glucocorticoids act via specific receptors through genomic and non-genomic mechanisms and exhibit a broad spectrum of anti-inflammatory effects (Goulding 2004). Interestingly I did not identify any statistically significant difference in the percentage of small MΦs in the classical CF cohort treated with (74 ± 8% $n$=5) or without (72 ± 9% $n$=5) inhaled steroids (Figure 3-9, below).

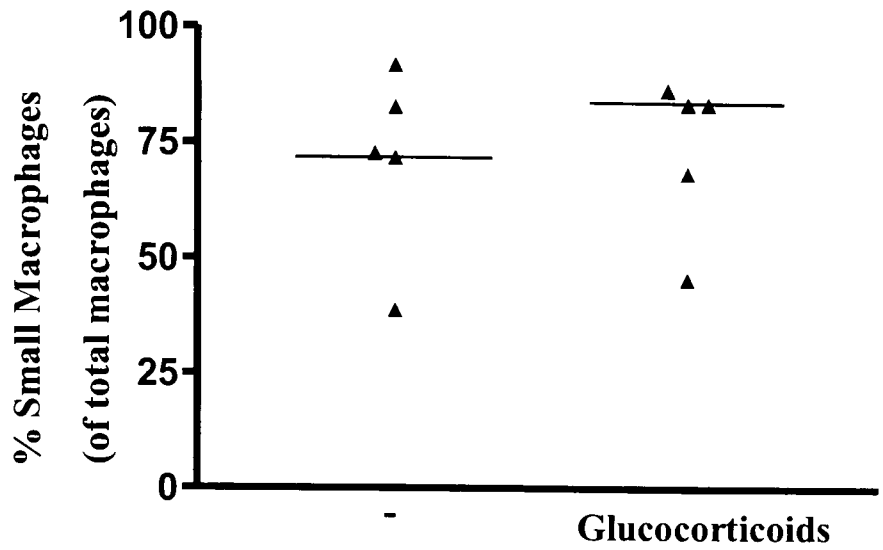


**Figure 3-9 Percentage of small MΦs (*y*-axis) from adult classical CF patients not-treated (left) or treated with glucocorticoids (right)** (Horizontal bars represent mean values) $p>0.05$

## 3.8 Conclusion

In this chapter I have identified a novel population of small sputum MΦs that are monocyte-like and have hitherto gone unrecognised in CF airways. This is supported by light scatter, HLA-DR, CD14 and CD68 expression as determined by flow cytometry. To my knowledge it is the first time that these three MΦ antigens have been used simultaneously in flow cytometry to identify (small) MΦs. Using this novel protocol I were able to demonstrate that small MΦs are a minor population of total MΦs in paediatric and adult control sputa. In contrast small MΦs often constituted the major population of MΦs in CF sputa, particularly in adults. Data also suggest that the percentage of small MΦs were highest in those with current infection/infective exacerbation or worsening respiratory disease. In addition the proportion of small MΦs could not be altered by anti-inflammatory glucocorticoid medication.

# 4 Results: Cystic Fibrosis Macrophages have Reduced Phagocytic Ability

## 4.1 Introduction

In the previous chapter I were able to identify a novel population of sputum MΦ of small size in the CF airways that had hitherto gone unrecognised. Small sputum MΦs have initially been identified in COPD patients (Frankenberger, Menzel *et al.* 2004) and in that study small MΦs were shown to produce greater quantities of TNF upon LPS stimulation than control large MΦs. This suggests that small MΦs may have a different (pro-inflammatory) functional role in the airways compared to large MΦs. At present no information exists on the phagocytic role of these MΦs, which could be important in the context of chronic infection and infective exacerbations in the CF airways. To study this, I focussed on material from adult donors, from whom larger quantities of sputum could be obtained, yielding more cells for study. To this end I characterised small MΦs further by determining the expression of the following PRRs: CD206 (mannose receptor), CD209 (DC-Specific Intercellular Adhesion Molecule-3 Grabbing Non-Integrin (DC-SIGN)) and MARCO (Figure 4-1). PRRs have diverse roles through non-opsonic binding and endocytosis of a wide variety of ligands as previously described (Section 1.2.4.2). These include host-derived enzymes (CD206), fungi (CD209), bacterial or particulate matter (MARCO). Furthermore they may also negatively regulate TNF release in reponse to infectious challenge (Zhang, Tachado *et al.* 2005) and collaborate with other PRRs (Tachado, Zhang *et al.* 2007). Finally, largely due to restrictions on the number of samples obtained I decided to focus on one of these receptors, MARCO, for further study in particle uptake.

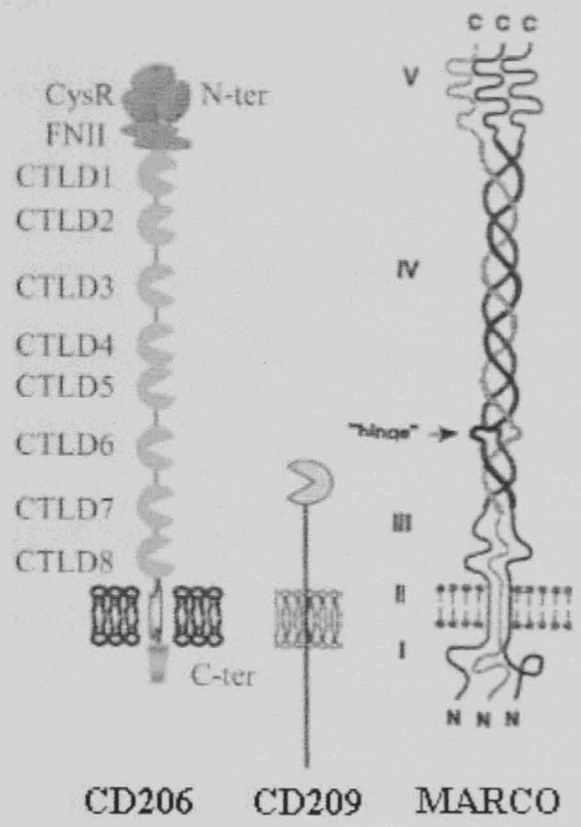


**Figure 4-1 Proposed structure of CD206, CD209 and MARCO (Elomaa, Kangas *et al.* 1995; McGreal, Martinez-Pomares *et al.* 2004; Boskovic, Arnold *et al.* 2006)**

## 4.2 Small Macrophages and Monocytes do not express Pattern Recognition Receptors

I analysed sputum MΦs from adult control and CF donors for cell surface expression of either CD206 (Figure 4-2), CD209 (Figure 4-3) or MARCO (Figure 4-4). Paired blood samples were taken to determine PRR expression on peripheral blood monocytes (Figure 4-5) as a point of reference in comparison to sputum MΦs.

Control and CF sputum cells were analysed for expression of HLA-DR and CD14, as described previously. HLA-DR and CD14 positive events (not shown) were gated onto a light scatter plot (Figure 4-2 – Figure 4-4, plots A and D) in order to identify control (upper panel) and CF (lower panel) large and small MΦs ('R3' and 'R4' in A and D, respectively). Inspite not including

CD68 staining for confirmation of MΦs, two populations could be discriminated that had very similar light scatter properties as identified previously. Furthermore, in all samples studied and those shown, there was a clear, distinct low proportion of small MΦs in controls and a high proportion of small MΦs in CF. Large MΦs (Figure 4-2 – Figure 4-4, plots B and E) from control (upper panel) and CF (lower panel) cohorts were subsequently gated on to histogram plots to determine the specific expression intensity of CD206, CD209 and MARCO (red line in plot B and E), compared to an isotype control (green line). I was able to demonstrate that large MΦs from the control donor, shown, have a high expression of cell surface CD206, whereas CD206 expressing MΦs were not detected in CF sputum (Figure 4-2, plots B and E). In contrast CD209 (Figure 4-3, plots B and E) could not be detected on control or CF large MΦs in the example shown. Similarly to CD206 I was able to detect clear expression of MARCO on large MΦs from the control donor shown but in addition and in contrast to CD206, MARCO could also be detected on CF large MΦs but to a slightly lower level (Figure 4-4, plots B and E). Small MΦs from control (upper panel) and CF (lower panel) cohorts (Figure 4-2 – Figure 4-4, plots C and F) were analysed in the same way as for large MΦs. In contrast to the examples shown for large MΦs I was unable to detect expression of CD206 (Figure 4-2, plots C and F), CD209 (Figure 4-3, plots C and F) or MARCO (Figure 4-4, plots C and F) on small MΦs for any of the donors examined.

Control and CF peripheral blood monocytes were also analysed for expression of CD206, CD209 and MARCO PRRs (Figure 4-5). Monocytic light scatter events (Figure 4-5, plot A in 'R1') were gated onto a dot plot to examine HLA-DR and CD14 expression intensity (Figure 4-5, plot B, controls not shown). Figure 4-5, plot A, demonstrates further the utility of light scatter measurements, whereby leukocyte sub-populations can be distinguished. High HLA-DR and

high CD14 expression (Figure 4-5, plot B, 'R2'), representing monocytes, were gated onto histogram plots to examine the intensity of CD206 (column C), CD209 (Column D) and MARCO (Column E) expression (red line in histogram plots) compared to an isotype control (green line). In Figure 4-5, control (middle panel) and CF (lower panel) monocytes were compared for their expression of the aforementioned PRRs. In the example shown CD206, CD209 or MARCO were not detected on control or CF monocytes.

Table 4-1 summarises the mean channel fluorescence intensity of each PRR on large and small MΦs and blood monocytes from both cohorts. Overall I analysed, in total, 7 sputa from controls ($n$=5) and 9 sputa from CF donors ($n$=7) and found that the mean percentage of small MΦs in each cohort (12 ± 6% vs 58 ± 18%, respectively) was similar to our earlier findings ($p$<0.0001,Mann-Whitney U Test) (Section 3.4). Further to this, large MΦs from control donors expressed CD206 protein at high cell surface density (963 ± 548, specific fluorescence channels). In comparison CD206 expression on CF large MΦs was significantly lower (16 ± 14 specifc fluorescence channels, $p$=0.0159) but still slightly higher than CD206 expression on small MΦs, which could not be demonstrated from control or CF donors (5 ± 7 vs 1 ± 3 channels, respectively). CD209 could not be detected on either large or small MΦs from either control or CF sputum samples. MARCO could be detected (219 ± 97 specific fluorescence channels) on control large MΦs but was significantly ($p$=0.0159) reduced (33 ± 37 channels) on CF large MΦs. In contrast, MARCO could not be detected on control or CF small MΦs (3 ± 6 vs 1 ± 1 channels, respectively). Similarly I could not demonstrate expression of CD206, CD209 or MARCO protein on control or CF monocytes.

Taken together, CD209 (DC-SIGN) is not expressed at all on either monocytes or MΦs from the cohorts studied. However both CD206 (Mannose receptor) and MARCO are clearly expressed on large sputum MΦs in controls but are strongly and significantly reduced in CF sputa. When looking at small MΦs and monocytes from controls and CF donors these PRRs are essentially absent. This supports our earlier findings that small MΦs are monocyte-like. Hence in CF strongly reduced expression of CD206 and MARCO on sputum MΦs indicate a reduced functional capacity for CF MΦs to perform their scavenging role.

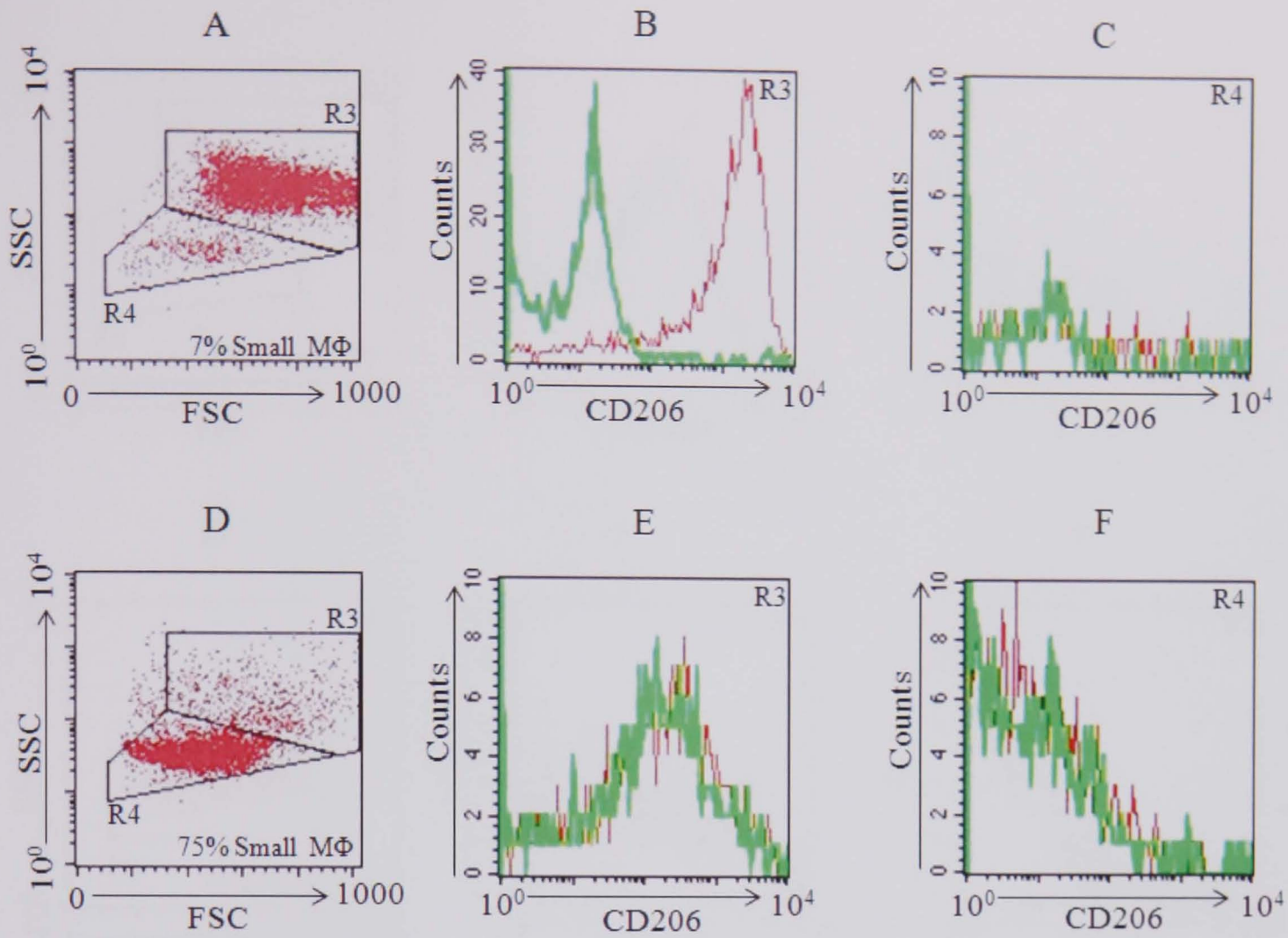


**Figure 4-2 Expression of CD206 on adult control (upper panel) or CF (lower panel) sputum MΦs**

*HLA-DR and CD14 positive sputum events (not shown) were gated onto a light scatter FSC versus SSC dot plot (A and D) and divided into large ('R3') and small ('R4'). Large MΦs ('R3' in B and E) and small MΦs ('R4' in C and F) were then examined for CD206 expression (red line) against an isotype control (green line) on a logarithmic scale. Specific expression was determined using the geometric mean fluorescence intensity following subtraction of the relevant isotype control.*

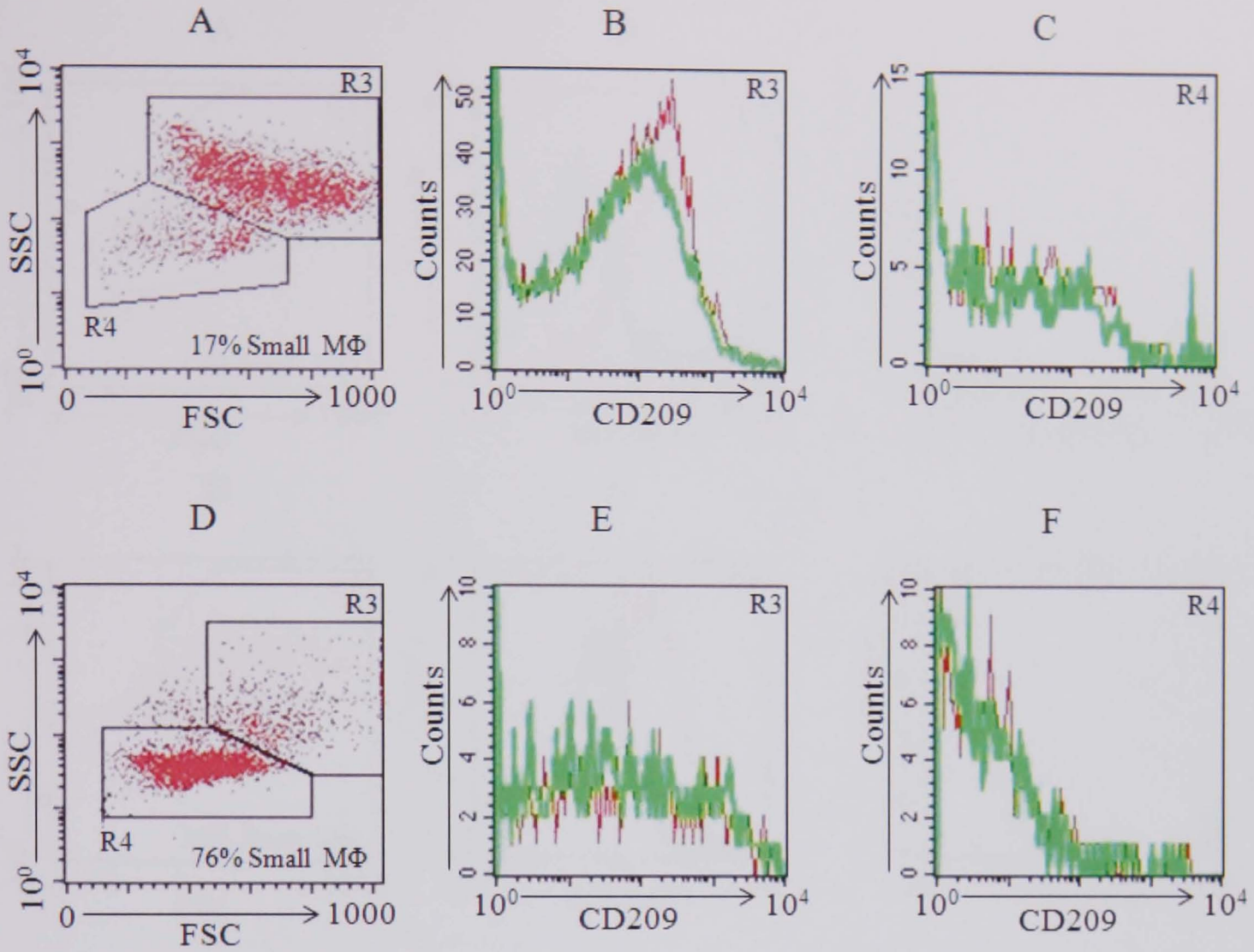


**Figure 4-3 Expression of CD209 on adult control (upper panel) or CF (lower panel) sputum MΦs**

*HLA-DR and CD14 positive sputum events (not shown) were gated onto a light scatter FSC versus SSC dot plot (A and D) and divided into large ('R3') and small ('R4'). Large MΦs ('R3' in B and E) and small MΦs ('R4' in C and F) were then examined for CD209 expression (red line) against an isotype control (green line) on a logarithmic scale. Specific expression was determined using the geometric mean fluorescence intensity following subtraction of the relevant isotype control.*

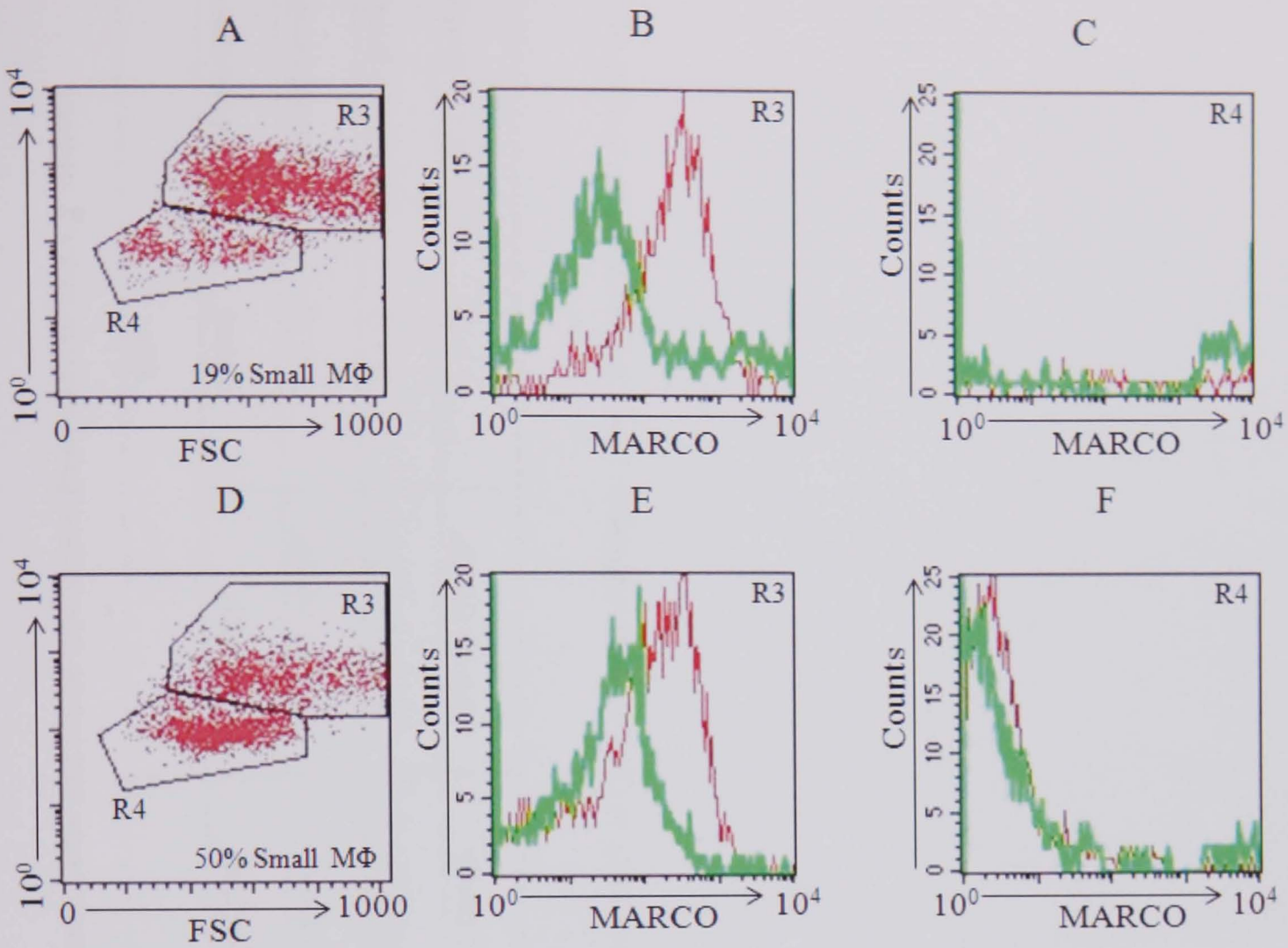


**Figure 4-4 Expression of MARCO on adult control (upper panel) or CF (lower panel) sputum MΦs**

*HLA-DR and CD14 positive sputum events (not shown) were gated onto a light scatter FSC versus SSC dot plot (A and D) and divided into large ('R3') and small ('R4'). Large MΦs ('R3' in B and E) and small MΦs ('R4' in C and F) were then examined for MARCO expression (red line) against an isotype control (green line) on a logarithmic scale. Specific expression was determined using the geometric mean fluorescence intensity following subtraction of the relevant isotype control.*

**Table 4-1 PRR expression (mean GM channels±SD) on sputum MΦs and blood monocytes from control and CF donors**

| | Control | | | CF | | |
|---|---|---|---|---|---|---|
| | **Large** | **Small** | **Monocytes** | **Large** | **Small** | **Monocytes** |
| **CD206** | 963 ± 548 (*n*=4) | 5 ± 7 | 0 ± 0 (*n*=4) | 16 ± 14 (*n*=5)* | 1 ± 3‡ | 0 ± 0 (*n*=4) |
| **CD209** | 1 ± 2 (*n*=3) | 0 ± 0 | 0 ± 0 (*n*=3) | 10 ± 11 (*n*=3) | 0 ± 0 | 0 ± 0 (*n*=4) |
| **MARCO** | 219 ± 97 (*n*=4) | 3 ± 6 | 3 ± 2 (*n*=4) | 33 ± 37 (*n*=5)* | 1 ± 1‡ | 0 ± 1 (*n*=5)# |

**Legend:** **p=0.0159 vs Control; ‡p=0.0159 vs Control large MΦs; #p=0.0317 vs Control (Mann-Whitney U-test was used for all comparisons)*

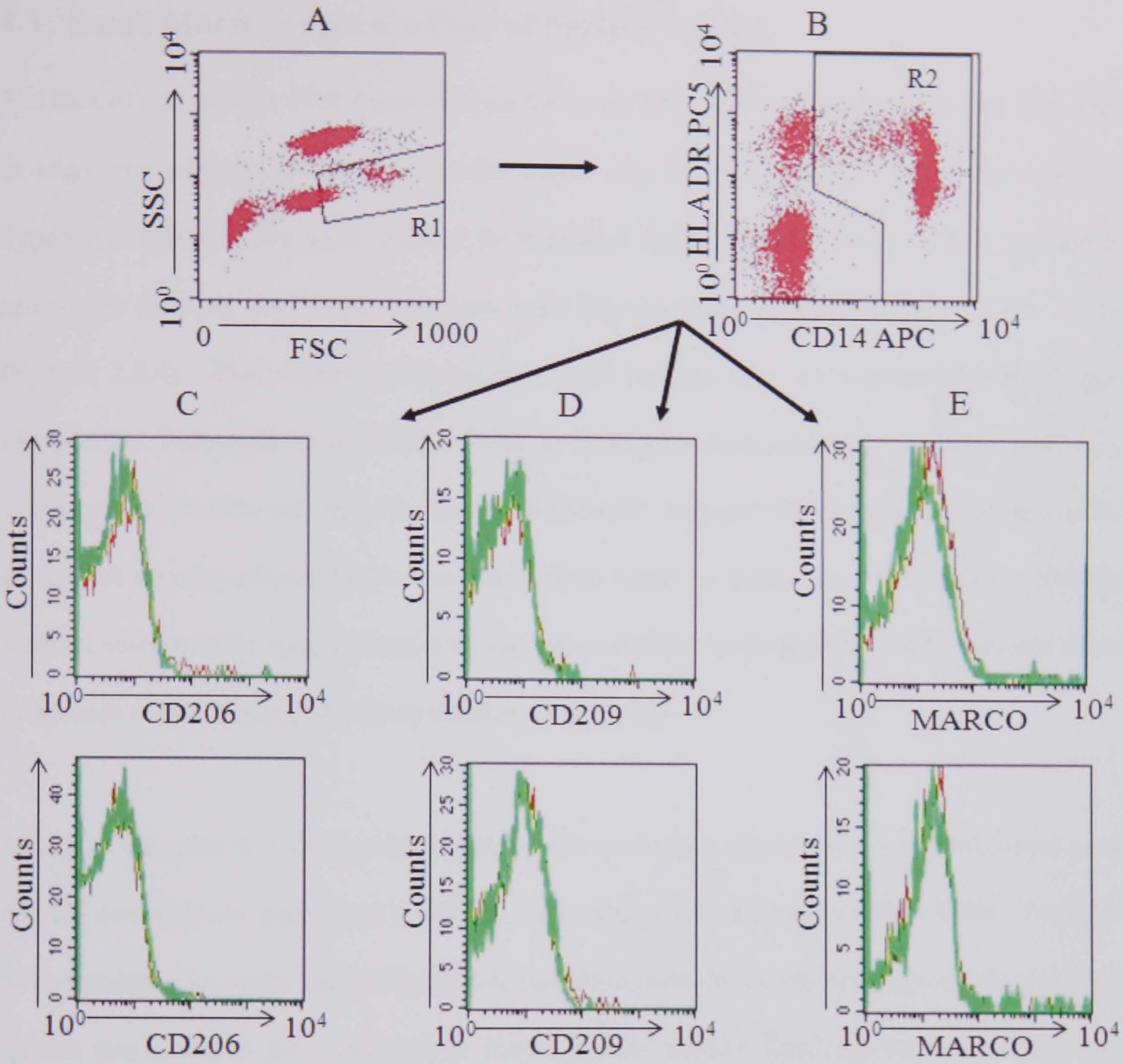


**Figure 4-5 Expression of CD206, CD209 and MARCO by blood monocytes from healthy control (upper and middle panel) and CF donors (lower panel)**

*Monocytes were distinguished from other blood leukocytes utilising their light scatter characteristics ('R1' in plot A) and positive expression of HLA-DR and CD14 ('R2' in plot B). Control (middle panel) and CF (lower panel) HLA-DR and 14 positive events were gated onto a histogram plot to examine CD206 (C), CD209 (D) or MARCO (E) expression (red line), against an isotype control (green line) on a logarithmic scale.*

## 4.3 Small Macrophages are Poor at Particle Uptake

The lack of any notable PRR expression on CF small MΦs led us to hypothesise that their ability to carry out non-opsonic particle uptake would also be compromised. In order to test this hypothesis sputum MΦs were isolated from control and CF sputum donors ($n$=3) as described previously (Section 2.4.4) and incubated with fluorosphere particles for 1hr at 37°C, 5%$CO_2$ (Section 2.6.4). Fluorosphere particles were used because they are a known MARCO ligand (Arredouani, Palecanda *et al.* 2005). Prior to incubation with particles, separated MΦs had a viability of 67 ± 17% and 92 ± 9% for control and CF, respectively. ECs were less than 10% in all control samples studied but in two out of three instances greater than 50% in CF. Therefore isolated sputum MΦs were stained with the pan-leukocyte mAb against CD45 (data not shown) to exclude contaminating ECs from subsequent analysis.

In Figure 4-6, plot A, CD45 positive light scatter events are shown from a control (upper panel) and CF donor (lower panel) and subdivided into large ('R3') and small ('R4') MΦs. Total MΦs were incubated in media alone (Figure 4-6, plot B) or with fluorospheres (Figure 4-6, plot C) and uptake was assessed for control large (upper panel) and CF small (lower panel) MΦs on a logarithmic fluorescence scale. Higher background fluorescence was observed for large MΦs (upper panel, plot B) compared to small MΦs (lower panel, plot B), again suggesting that small MΦs are monocyte-like and therefore immature. Increased particle uptake is indicated by an increasing shift in fluorescence to the right. In the example shown, a greater proportion of large MΦs (Figure 4-6, upper panel, plot C, horizontal bar) take up fluorospheres compared to CF small MΦs (Figure 4-6, lower panel, plot C, horizontal bar) (91 and 28%, respectively). To determine the role of MARCO in this process I used an anti-human MARCO antibody (PLK-1) to block the MARCO receptor and assessed its contribution towards particle uptake, described

previously by Arredouani *et al.* (2005). Particle uptake by control and CF sputum MΦs and the contribution to total uptake by MARCO is summarised in Table 4-2.

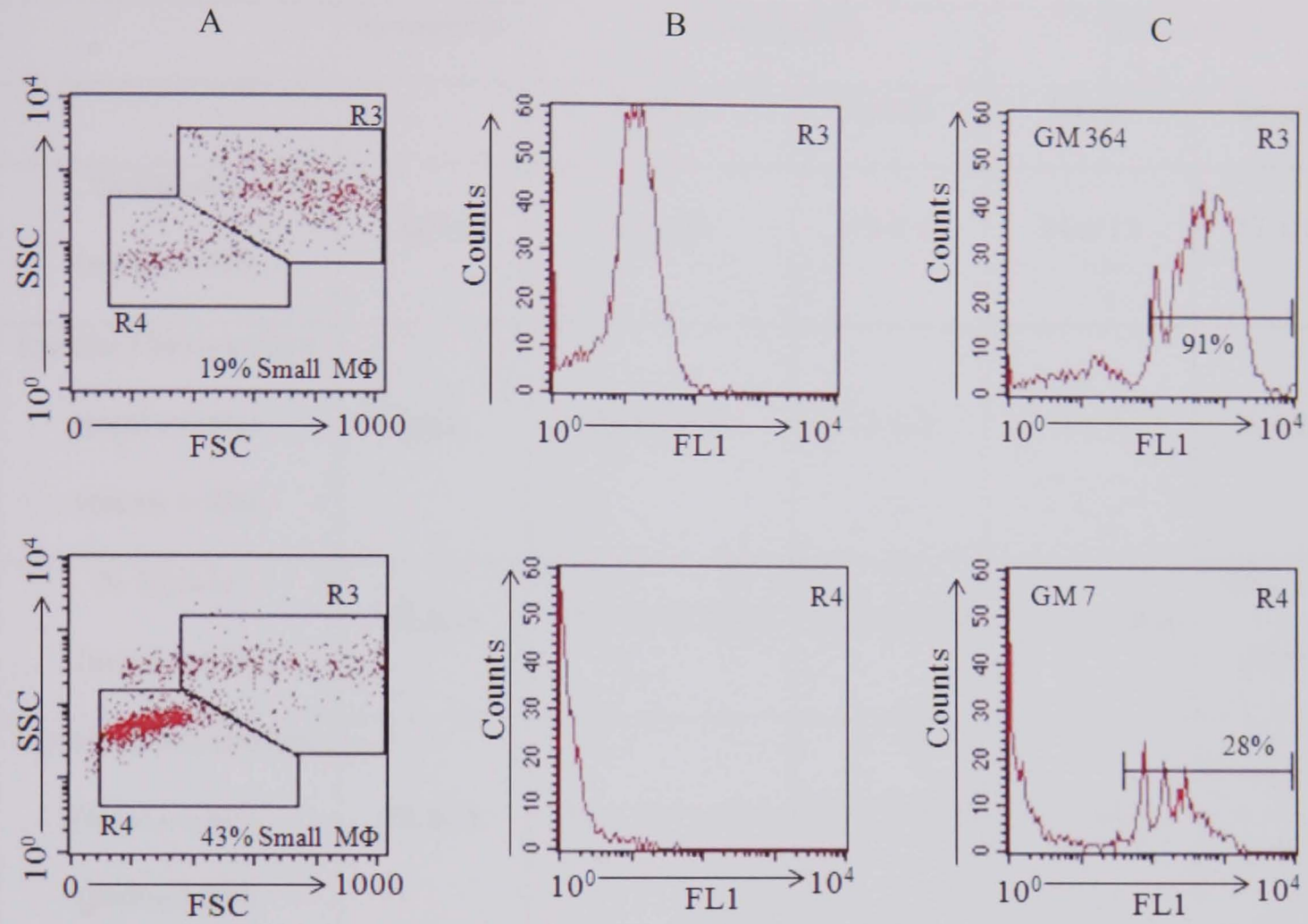


**Figure 4-6 Control large (upper panel) and CF small (lower panel) sputum MΦs incubated alone (centre) or with fluorospheres (right)**

*CD45 positive (data not shown) light scatter events (plots in column A) were subdivided into large ('R3') and small MΦs ('R4') from control (upper) and CF (lower) donors. Background fluorescence (no fluorospheres added) (plots in column B) for control large MΦs (upper) and CF small (lower) sputum MΦs are shown. Plots in column C demonstrate the % uptake (horizontal bar) of fluorospheres by control and CF sputum MΦs. In this example 91% of control large MΦs were engaged in particle uptake whilst only 28% of CF small MΦs were involved. GM = Geometric mean fluorescence*

**Table 4-2 Control and CF sputum MΦ % particle uptake and GM fluorescence channel intensity in the presence of a MARCO inhibitor (PLK-1) or isotype control ($IgG_3$)**

| | Treatment | Control (*n*=3) | | CF (*n*=3) | |
|---|---|---|---|---|---|
| | | **Large** | **Small** | **Large** | **Small** |
| **% Uptake (mean ± SD)** | **$IgG_3$** | 83 ± 11 | 39 ± 4 | 24 ± 13 | 27 ± 9 |
| **Uptake Fluorescence (total events) (mean ± SD)** | **$IgG_3$** | 224 ± 143 | 11 ± 2 | 9 ± 4 | 5 ± 2 |
| **% Uptake (mean ± SD)** | **PLK-1** | 73 ± 14 (12%)† | 37 ± 5 (5%) | 22 ± 10 (8%) | 28 ± 11 (0%) |
| **Uptake Fluorescence (total events) (mean ± SD)** | **PLK-1** | 131 ± 107 (42%) | 10 ± 2 (9%) | 9 ± 4 (0%) | 5 ± 2 (0%) |

***Legend:*** *† values in paranthesis represent % reduction PLK-1 treated cells vs $IgG_3$ treated cells; all comparisons are non-significant*

Overall, approximately double the quantity of control large MΦs (83 ± 11%) were engaged in particle uptake than either control small (39 ± 4%) or CF large (24 ± 13%) and small (27 ± 9%) MΦs in the presence of a non-specific antibody ($IgG_3$) to human MARCO. Blocking MARCO with PLK-1 slightly reduced (by 12%) the percentage of control large MΦs involved in fluorosphere uptake (83 ± 11% versus 73 ± 14%) but this was non-significant ($p$>0.05). However blocking MARCO had a greater reduction (42%) on the degree of fluorescence associated with

such uptake but this was still not significant. Prior determination of the most effective concentration of MARCO by serial dilution my have reduced the likelihood of committing a type 2 error. Overall looking at CF MΦs, I were not able to detect a comparable level of fluorosphere uptake than that observed for control MΦs. Furthermore, in line with the protein data showing negligible expression of MARCO in CF, blocking MARCO on CF small MΦs had no effect on the percentage of cells involved in uptake or on the degree of fluorescence associated with such uptake.

## 4.4 Conclusion

CF small MΦs – the most prominent MΦ in the CF airways – demonstrated significantly reduced cell surface protein expression of CD206 and MARCO PRRs compared to control (large) MΦs. CD209 was not detected on control or CF MΦs. In addition, CD206 and MARCO could not be detected on control or CF blood monocytes. This further supports a monocyte rather than MΦ-like phenotype for CF small MΦ. Reduced PRR expression suggests a reduced capacity by CF MΦs to bind (and thus phagocytose) infectious agents, particulates and host enzymes allowing them to persist in the CF lung. Finally, in line with the low cell surface expression of MARCO I demonstrated that CF small MΦs have a reduced capability to take up particulates. In contrast almost all control donor MΦs were engaged in particle uptake, the degree of which may partly depend on MARCO expression as demonstrated when blocking this receptor.

# 5 Results: An Investigation into Transcript Differences between Cystic Fibrosis and Control Monocyte-Derived Macrophages

## 5.1 Introduction

Previously in chapter 3 I identified small sputum MΦs in the CF airways that had hitherto gone unrecognised. Further to this, in chapter 4, I were able to demonstrate that CF MΦs may play a different functional role in the airways due to a lack of PRRs. MΦ function could be altered by changes in the CF airway environment (i.e. thickened ASL, cytokines and chemokines) that may or may not be due to mutant CFTR. On the other hand it is possible that MΦ function is influenced directly by mutant, misfolded CFTR.

In CFTR expressing ECs, mutated CFTR transcripts have been associated with altered metabolic signalling pathways that could contribute to a pro-inflammatory environment (Machen 2006). CFTR protein is expressed in airway epithelia but it has also been described in human neutrophils (Painter, Valentine *et al.* 2006), rodent mast cells (Kulka, Gilchrist *et al.* 2002), human monocytes and (alveolar) MΦs (Di, Brown *et al.* 2006). In line with this, additional studies have demonstrated CFTR transcripts in alveolar MΦs and monocytes (Yoshimura, Nakamura *et al.* 1991; Yoshimura, Chu *et al.* 1993). Interestingly a small number of studies from CF animal models (Thomas, Costelloe *et al.* 2000) and CF affected patients (Pfeffer, Hueckstead *et al.* 1993; Zaman, Gelrud *et al.* 2004) have described altered function of the CF monocyte/MΦ lineage compared to control. Similar to studies described in ECs, CF monocytes have shown greater oxygen consumption and superoxide production (Thomassen, Barna *et al.* 1990; Regelmann, Skubitz *et al.* 1991) and a 100-fold greater sensitivity to LPS stimulation (Zaman,

Gelrud *et al.* 2004) such that CF monocytes produced 2-fold higher CXCL-8 protein (Zaman, Gelrud *et al.* 2004) and in the case of MDMs, > 2-fold higher TNF protein (Pfeffer, Huecksteadt *et al.* 1993) compared to control. Based on these studies our hypothesis was that CF MΦs, due to mutant CFTR, have an altered constitutive and LPS stimulated gene expression profile that contributes to a failure to eradicate infection and allow persistent inflammation. To investigate this I isolated CF and control CD14 high monocytes and generated MDMs *in vitro*. To obtain a comprehensive analysis and aid the detection of differences I then compared control and CF MDM transcripts encoding for the entire human genome (~55,000 genes). This chapter presents the research I carried out in this area.

## 5.2 Control and Cystic Fibrosis Cohort Analysis of Monocyte-Derived Macrophages

MDMs were generated from a pure population (97 ± 2% for both cohorts) of CD14++ monocytes isolated from control and CF paired blood samples under identical conditions as described (Section 2.5). Initially 5-day MDMs were generated (Section 2.5.2) from a cohort of 10 adult control and CF donors (Table 5-1) and stimulated with LPS or left untreated (Section 2.5.3). There were no significant age differences between control (27 ± 5 years) and CF (24 ± 6 years) cohorts and the latter were of mixed genotype but predominantly ($n$=6) ΔF508 +/+ (Table 5-1). Lung function was not determined for the control cohort at the time of sampling however the CF cohort had a mean $FEV_1$ of 56 ± 23%. Using flow cytometry I examined whether there were any developmental differences in the constitutive expression of CD14 or CD16 cell surface receptors following 5 days in culture (Figure 5-1). This was because I wanted to exclude differences in gene expression between groups, following LPS stimulation, being due to differential CD14

expression in our system. In addition, examining CD14 and CD16 led us to hypothesise whether the generated control and CF MDMs were similar in phenotype to CD14 positive CD16 positive monocytes. However, I could not detect any significant difference between control and CF MDMs for the expression of CD14 (887 ± 640 and 891 ± 547 specific fluorescence channels, respectively) or CD16 (18 ± 25 and 11 ± 18 specific fluorescence channels, respectively) cell surface antigens (Table 5-1).

**Table 5-1 Adult control and CF cohort data for the generation of MDMs**

| | **Control** | **CF** |
|---|---|---|
| **Donors (*n*)** | 10 | 10 |
| **Age (mean years ± SD)** | 27 ± 5 | 24 ± 6 |
| **Sex (Female:Male)** | 6:4 | 6:4 |
| **Ethnicity (Caucasian:Asian)** | 9:1 | 10:0 |
| **Genotype (*n*)** | N/A | ΔF508 +/+ (6)<br>ΔF508 +/G85E (1)<br>ΔF508 +/unknown (3) |
| **% $FEV_1$ (mean ± SD)** | Not Determined | 56 ± 23 |
| **% CD14++ (mean ± SD)** | 97 ± 2 | 97 ± 2 |
| **Mean channel (± SD) CD14 expression (*n*=10)** | 887 ± 640 | 891 ± 547 |
| **Mean channel (± SD) CD16 expression (*n*=10)** | 18 ± 25 | 11 ± 18 |

***Legend:*** *See Table 3-1.*

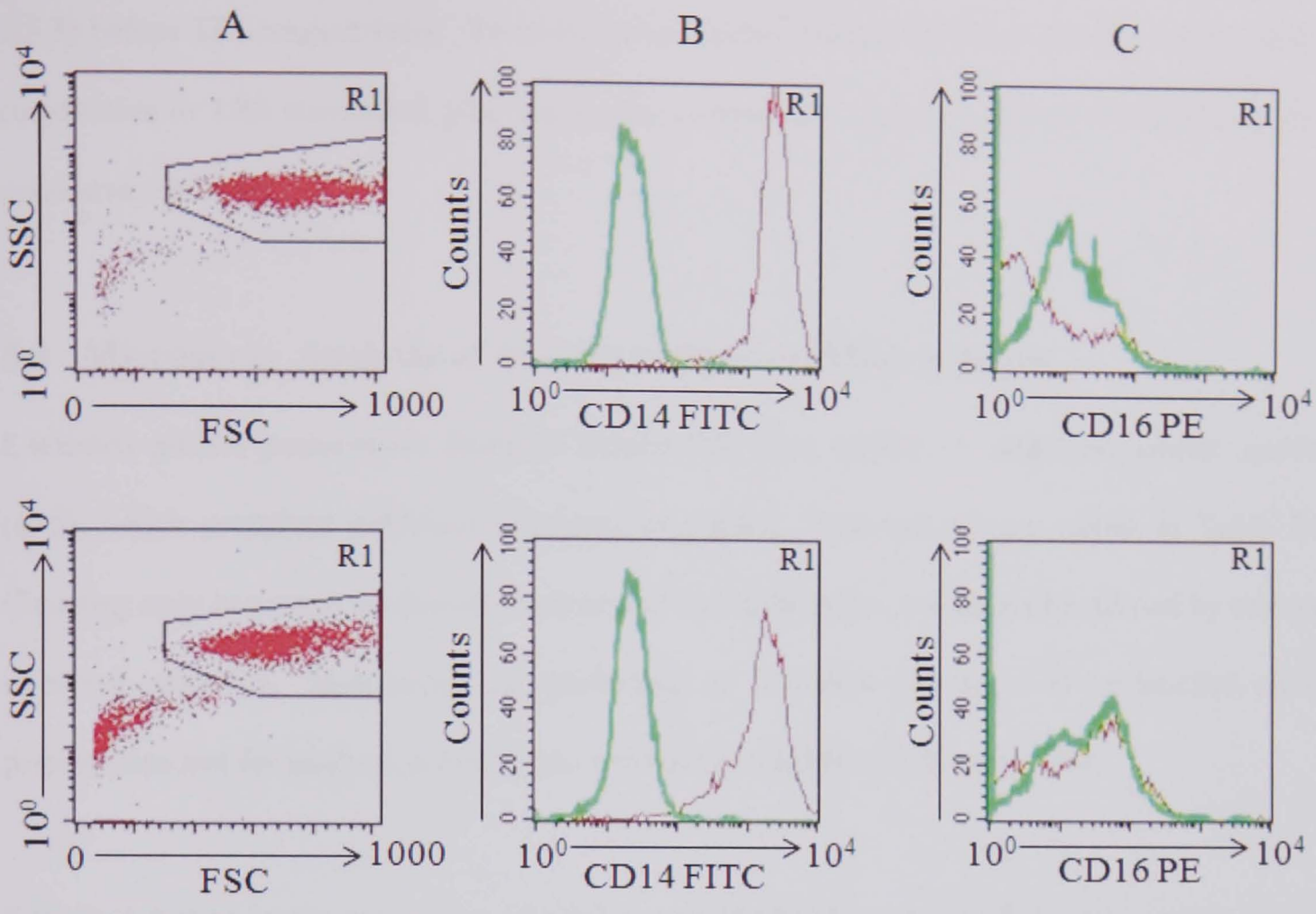


**Figure 5-1 CD14 and CD16 expression on control (upper panel) and CF (lower panel) derived MDMs**

*Control and CF MDMs light scatter events were identified ('R1' column A) based on cell size (x-axis, FSC) and granularity (y-axis, SSC). R1 MDM events (column B) were then analysed for CD14 expression (red line) against an isotype control (green line). R1 MDM events (column C) were also analysed for CD16 expression (red line) against an isotype control (green line). Specific expression was determined using the GM fluorescence intensity following subtraction of the relevant isotype control.*

Following maturation for 5 days *in vitro* I lysed MDMs in TRI-reagent to determine whether there were any constitutive differences at the messenger RNA (mRNA) level between control and CF MΦs. To determine whether there was a defective response to bacteria, I stimulated control and CF MDMs on day 5 with LPS (1-10 ng/ml) isolated from *Salmonella minnesota* (Section

2.5.3) before TRI-reagent lysis. To investigate whether mutant CFTR in the CF cohort altered constitutive or LPS stimulated gene expression compared to control, mRNA was analysed by a microarray (below).

## 5.3 Microarray Analysis of Monocyte-Derived Macrophages

I selected mRNA preparations from CF donors that were ΔF508 +/+ and from paired controls ($n$=5), which contained sufficient quantities of mRNA, from the subject cohort in Table 5-1. Choosing only homozygous donors ensured that any differences would not be diluted by samples of mixed genotype. Microarray was performed as described (Section 2.9) on labelled cRNA preparations and for analysis grouped into control/CF MDMs ± LPS stimulation.

Initially I looked at the expression of CD206 and MARCO transcripts between control and CF donors using microarray. I found slightly higher constitutive expression of CD206 mRNA in CF compared to control donors (4400 vs 3523, $p$=0.026, respectively) but under stimulated conditions CD206 mRNA expression was similar (4390 vs 4006, $p$>0.05). In contrast, MARCO mRNA expression was slightly lower in the CF cohort compared to the control cohort under constitutive (520 vs 417, $p$>0.05, respectively) and stimulated (809.2 vs 342.5, $p$>0.05) conditions but this was not significant. I then compared selected transcript abundance fold change (SLR) between control and CF donors under constitutive (Table 5-2 and 5-3) and LPS stimulated conditions (Table 5-4 and 5-5). The SLR $p$ value was calculated using a Wilcoxon signed rank test.

**Table 5-2 Transcripts that were more abundant in CF MDMs compared to control under constitutive conditions**

| Probe Id. | Transcript Name | Transcript Function | Control Signal | CF Signal | SLR | SLR *p* |
|---|---|---|---|---|---|---|
| 1566979_at | KIAA0317 | Ubiquitin Ligase Activity | 1.2 | 55.6 | 5.2 | 0.001 |
| 1560998_x_at | Laminin, alpha 2 | Cell Adhesion | 1.2 | 44.4 | 5.1 | 0.001 |
| 1558683_a_at | High Mobility Group AT-hook 2 | DNA binding, Maintains Chromatin Structure | 1.6 | 32.8 | 4.2 | 0.0001 |
| 236988_x_at | $Beta_2$ Integrin (CD18 antigen) | Cell Adhesion | 2.8 | 99 | 4.2 | 0.0001 |
| 244098_at | ADAMTS3 | Extracellular Matrix Degrading Enzyme | 1 | 45.2 | 3.8 | 0.0003 |
| 1567240_x_at | Olfactory Receptor (OR2L2) | G-protein Coupled (Smell) Receptor | 3.1 | 44.8 | 3.7 | 0.0005 |
| 234545_at | Olfactory Receptor (OR5U1) | G-protein Coupled (Smell) Receptor | 6.3 | 63 | 3.7 | 0.001 |
| 241026_at | ADAM12 | Myogenesis | 1.6 | 39.7 | 3.6 | 0.0007 |
| 210426_x_at | RAR-related orphan receptor A | Nuclear Receptor | 3.9 | 52.1 | 3.5 | 0.0003 |
| 233424_at | Neurexin 1 | Cell Adhesion | 2.4 | 51.4 | 3.5 | 0.0007 |
| 213194_at | Roundabout, axon guidance receptor | Chemotaxis, Cell Adhesion | 6.4 | 52.4 | 3.4 | 0.0005 |
| 1556903_at | Midline 2 | Unknown | 3.3 | 40.3 | 3.3 | 0.0007 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1561652_at | Beclin 1 | Anti-Apoptotic Protein | 2.2 | 26.4 | 3.3 | 0.00008 |
| 1556499_s_at | Collagen, type 1, alpha 1 | Extracellular Matrix Component | 12.8 | 166.5 | 3.2 | 0.0002 |

**Table 5-3 Transcripts that were less abundant in CF MDMs compared to control under constitutive conditions**

| Probe Id. | Transcript Name | Transcript Function | Control Signal | CF Signal | SLR | SLR *p* |
|---|---|---|---|---|---|---|
| 214877_at | CDK5 regulatory subunit associated protein 1-like 1 | Metabolic Process | 96.2 | 6.1 | -4.2 | 0.0002 |
| 208719_s_at | DEAD box polypeptide 17 | RNA Processing | 56.7 | 2.9 | -3.9 | 0.00004 |
| 207714_s_at | Serpin Peptidase Inhibitor | Protein Folding | 102.1 | 6.9 | -3.4 | 0.0002 |
| 1557050_at | Homeo box A2 | Cell Fate | 33.3 | 3.7 | -3.3 | 0.0003 |
| 205001_s_at | DEAD box polypeptide 3 (Y-linked) | RNA Processing | 80.1 | 6.5 | -3.2 | 0.0003 |
| 219387_s_at | Cytokine-like 1 | Unknown | 157.7 | 4.2 | -3.1 | 0.00002 |
| 235157_at | Poly (ADP-ribose) polymerase family 14 | Nuclear Protein Modification | 58.5 | 7.1 | -3 | 0.001 |
| 205000_at | DEAD box polypeptide 3 (Y-linked) | RNA Processing | 1076.8 | 266.4 | -2.2 | 0.00002 |
| 238900_at | HLA-DR B1/B3 | Antigen Processing and Presentation | 63.3 | 13.3 | -2.2 | 0.0001 |
| 1557459_at | SNF1-like kinase 2 | Unknown | 20.8 | 2.3 | -2 | 0.0005 |
| 209480_at | HLA-DQ B1 | Antigen Processing and Presentation | 394.2 | 97.6 | -2 | 0.00007 |
| 212999_x_at | HLA-DQ B1 | Antigen Processing and | 322.3 | 72.2 | -2 | 0.0001 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Presentation | | | | |
| 241985_at | Junction-mediating and regulatory protein | Regulator of Transcription | 39.5 | 5.1 | -2 | 0.0008 |
| 213831_at | HLA-DQ A1 | Antigen Processing and Presentation | 430.5 | 113 | -1.9 | 0.00002 |

**Table 5-4 Transcripts that were more abundant in CF MDMs compared to control following LPS stimulation**

| Probe Id. | Transcript Name | Transcript Function | Control Signal | CF Signal | SLR | SLR *p* |
|---|---|---|---|---|---|---|
| 236988_x_at | $Beta_2$ Integrin (CD18 antigen) | Leukocyte adhesion | 3.3 | 110.3 | 5.3 | 0.00003 |
| 1562294_x_at | Ankyrin repeat domain 30B | Unknown | 0.8 | 20.5 | 4 | 0.001 |
| 203290_at | HLA-DQ alpha 1 | Antigen Presentation | 14.7 | 226.4 | 4 | 0.00003 |
| 227460_at | Retinoblastoma-associated protein 140 | Unknown | 3.4 | 44 | 3.8 | 0.001 |
| 206805_at | Semaphorin 3A | Axon guidance | 7.2 | 106 | 3.7 | 0.00014 |
| 222347_at | SH3-domain GRB2-like 3 | Endocytosis | 4.1 | 102.7 | 3.7 | 0.00049 |
| 234028_at | KIAA0804 | Ubiquitin Ligase Activity | 3.2 | 55.9 | 3.7 | 0.0005 |
| 220784_s_at | Urotensin 2 | Muscle Contraction | 0.9 | 24.5 | 3.4 | 0.0003 |
| 207134_x_at | Tryptase $Beta_2$ | Proteolysis | 16.5 | 154.1 | 3.3 | 0.00002 |
| 1555536_at | Anthrax Toxin Receptor 2 | Receptor | 9.1 | 156.4 | 3.2 | 0.00189 |
| 211307_s_at | FcR for IgA | Immune Response | 7.8 | 88.9 | 3.1 | 0.00061 |
| 226931_at | ARG99 protein | Membrane Protein | 6.8 | 64.6 | 3.1 | 0.00014 |
| 232187_at | Palmdelphin | Regulation of Cell Shape | 2.3 | 30 | 3 | 0.00003 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 240617_at | NFκB inhibitor like-2 | Tumour Protein | 4.4 | 48.7 | 2.8 | 0.00096 |
| 207175_at | C1Q and collagen domain containing Adiponectin | Glucose metabolism | 7.7 | 24 | 2.3 | 0.0007 |
| 214266_s_at | PDZ and LIM domin 7 | Ossification receptor-mediated endocytosis | 12.7 | 59 | 2.3 | 0.0007 |
| 211734_s_at | Fc fragment of IgE | Immune Response | 24.4 | 90.2 | 2 | 0.0015 |

**Table 5-5 Transcripts that were less abundant in CF MDMs compared to control following LPS stimulation**

| Probe Id. | Transcript Name | Transcript Function | Control Signal | CF Signal | SLR | SLR *p* |
|---|---|---|---|---|---|---|
| 233819_s_at | Zinc Finger Protein 294 | Activated Protein Kinase C Receptor | 38.9 | 1.7 | -5.5 | 0.001 |
| 1558522_at | Palmitoylated 6 membrane protein | Guanylate Cyclase Activity | 23.6 | 1.5 | -4 | 0.001 |
| 225187_at | KIAA1967 | Apoptosis | 113.5 | 4.9 | -3.8 | 0.0016 |
| 201286_at | Syndecan-1 | Cytoskeleton | 76.8 | 3.5 | -3.3 | 0.0001 |
| 235179_at | Zinc Finger Protein 641 | Regulation of Transcription | 73.4 | 9.1 | -2.8 | 0.0014 |
| 241413_at | Ring Finger Protein 111 | Ubiquitination | 65.1 | 10.3 | -2.7 | 0.00018 |
| 221898_at | Podoplanin | Water Transport | 541.2 | 92.1 | -2.3 | 0.00014 |
| 213926_s_at | HIV-1 Rev binding protein | mRNA export | 214.4 | 44.9 | -2 | 0.00002 |
| 204879_at | Podoplanin | Water Transport | 166.6 | 55.3 | -1.9 | 0.00007 |
| 214146_s_at | CXCL7 | Chemotaxis | 3026 | 812.7 | -1.9 | 0.00002 |
| 228492_at | Ubiquitin specific peptidase 9 | Catabolic Process | 54.3 | 11.5 | -1.9 | 0.00096 |
| 230180_at | DEAD box polypeptide 17 | RNA Processing | 150.7 | 17.6 | -1.9 | 0.00003 |
| 244774_at | Phosphatase and actin regulator 2 | Unknown | 129.1 | 40.3 | -1.9 | 0.0002 |
| 208003_s_at | Nuclear Factor of Activated T Cells 5 | Cytokine Production | 171.3 | 51 | -1.8 | 0.00002 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 205000_at | DEAD box polypeptide 3 Y-linked | ATP-binding | 1193.6 | 295.7 | -1.7 | 0.00003 |
| 212977_at | CXCR7 | Chemotaxis | 56.1 | 13.6 | -1.7 | 0.0004 |

Tables 5 - 2 to 5 - 5 show a selection of the most highly up or down regulated gene transcripts found in CF MDMs compared to control. These changes encompass many different biological processes.

I then looked for upregulated transcript expression within MΦ genes that may play an important role in the pathophysiology of CF. I looked for diferentially regulated genes involved in cellular communication such as Interleukins (Table 5-6), neutrophil recruitment such as C-X-C chemokine ligands (Table 5-7) and signalling cascades known to be upregulated in CF such as NFκB and MAPK that can enhance pro-inflammatory mechanisms (Table 5-8). Correct interpretation of this data requires PCR analysis however the possibility that their may be a downregulation of CXC chemokine production from CF MDMs (Table 5-7) could reveal intriguing information on the role of CXC chemokines in CF.

**Table 5-6 Cytokine ligand transcript abundance in CF MDMs under constitutive and stimulated (LPS) conditions compared to control**

| Probe Id. | Transcript Name | Control Signal (Constitutive/LPS) | CF Signal (Constitutive/LPS) | SLR (Constitutive/LPS) | SLR *p** |
|---|---|---|---|---|---|
| 207844_at | IL-13 | 4.9 / 23.4 | 40.7 / 56 | 3.1 / 1 | ns |
| 207849_at | IL-2 | 0.9 / 28.2 | 9.3 / 2.5 | 3 / -3.7 | ns |
| 207539_at | IL-4 | 1.5 / 8 | 17.8 / 2.2 | 2.8 / -1.7 | ns |
| 221111_at | IL-26 | 0.9 / 22.7 | 2 / 9.3 | 2.1 / -0.7 | ns |
| 206926_s_at | IL-11 | 11.3 / 14.5 | 16.7 / 33..3 | 1.7 / 0.2 | ns |
| 1555016_at | IL-16 | 15 / 37.8 | 36.6 / 61 | 1.6 / 0.8 | ns / 0.03 |
| 206569_at | IL-24 | 22.9 / 52.9 | 76.2 / 62 | 1.4 / 0 | ns / 0.04 |
| 224230_at | IL-1 member 8 | 2.3 / 1.3 | 9.4 / 2 | 1.4 / 1.2 | ns / 0.02 |
| 1552915_at | IL-28 | 22.8 / 34.6 | 62.2 / 36.6 | 1.3 / 0.1 | ns / 0.5 |
| 220054_at | IL-23 p19 subunit | 19.9 / 717.4 | 54.8 / 815.6 | 1.3 / 0.3 | ns |
| 222974_at | IL-22 | 1.4 / 18.8 | 3.9 / 2.1 | 1.1 / -3.3 | ns |
| 221271_at | IL-21 | 17.4 / 1.9 | 42.1 / 40.3 | 1 / 4.1 | 0.01 / 0.03 |
| 236505_at | IL-4 induced 1 | 22.3 / 22.8 | 48.5 / 4.7 | 1 / -2 | ns |

| | | | | | |
|---|---|---|---|---|---|
| 207538_at | IL-4 | 6.4 / 2.6 | 18.9 / 11.7 | 0.9 / 1 | 0.003 / ns |
| 221470_s_at | IL-1 member 7 | 2.1 / 3.6 | 4 / 4.6 | 0.8 / 0 | ns |
| 216245_at | IL-1 Receptor Antagonist | 33.9 / 54.6 | 50.2 / 8.2 | 0.7 / -2.7 | ns |
| 207952_at | IL-5 | 2.9 / 11.7 | 1.2 / 13.9 | 0.6 / 0.2 | ns |
| 205992_s_at | IL-15 | 118.3 / 664.3 | 133.7 / 862.7 | 0.5 / 0.2 | 0.001 / ns |
| 208402_at | IL-17 | 6.4 / 7.2 | 2.7 / 2.5 | 0.5 / -0.1 | ns |
| 220971_at | IL-17E | 6.4 / 47.5 | 7.9 / 9.8 | 0.5 / -2 | ns |
| 221165_s_at | IL-22 | 27.2 / 54 | 30.4 / 28. | 0.5 / -0.3 | ns |

***Legend:*** ***** *p values (Wilcoxon signed rank test) are given for constitutive expression (control vs CF) followed by stimulated expression (control vs CF)*

**Table 5-7 Chemokine (C-X-C) ligand transcript abundance in CF MDMs under constitutive and stimulated (LPS) conditions compared to control**

| Probe Id. | Transcript Name | Control Signal (Constitutive/LPS) | CF Signal (Constitutive/LPS) | SLR (Constitutive/LPS) | *p** |
|---|---|---|---|---|---|
| 237038_at | CXCL-14 | 3.3 / 4.5 | 19.4 / 2.7 | 2.3 / 0.1 | ns |
| 222484_s_at | CXCL-14 | 9.1 / 15.1 | 19.9 / 8.7 | 1.7 / -1.9 | ns |
| 210163_at | CXCL-11 | 3.6 / 224 | 4.5 / 132.3 | 1.5 / -0.5 | ns |
| 211122_s_at | CXCL-11 | 1.4 / 230.4 | 1.1 / 218.4 | 1.4 / -0.1 | ns |
| 218002_s_at | CXCL-14 | 10.8 / 26.4 | 19.5 / 29.1 | 0.8 / 0 | ns |
| 230101_at | CXCL-2 | 6.8 / 50.5 | 5.2 / 9.1 | 0.3 / -2.3 | ns |
| 205242_at | CXCL-13 | 1.9 / 14.4 | 5.7 / 1.1 | 0.2 / -1.9 | ns |
| 206390_x_at | CXCL-4 | 45.3 / 50.9 | 45.7 / 10 | 0.1 / -2.6 | ns |
| 209774_x_at | CXCL-2 | 4961.6 / 13082.2 | 5710 / 13818.1 | 0.1 / 0 | ns |
| 156203_at | CXCL-2 | 142.1 / 81 | 86.3 / 118.7 | 0 / 0.8 | ns / 0.002 |
| 204533_at | CXCL-10 | 66.1 / 6680.5 | 72.3 / 5365.9 | 0 / -0.1 | ns |
| 207852_at | CXCL-5 | 99.8 / 57.9 | 76.1 / 40.6 | 0 / -1.6 | ns |
| 206336_at | CXCL-6 | 214.2 / 269.5 | 211.2 / 348.4 | -0.2 / 0.1 | ns |

| | | | | | |
|---|---|---|---|---|---|
| 207850_at | CXCL-3 | 7313.3 / 13529.5 | 6875.4 / 13057.1 | -0.2 / 0.2 | ns |
| 223454_at | CXCL-16 | 4014 / 3778.1 | 2964.1 / 2775 | -0.2 / -0.4 | 0.00003 / 0.01 |
| 204470_at | CXCL-1 | 6623.5 / 12633.9 | 4626.1 / 11619.4 | -0.4 / -0.1 | 0.0011 / ns |
| 214974_x_at | CXCL-5 | 5145.8 / 6078.6 | 3650 / 4781.2 | -0.5 / -0.3 | 0.00002 / 0.01 |
| 203915_at | CXCL-9 | 55.5 / 212.4 | 28.9 / 199.8 | -0.6 / -0.3 | ns |
| 203666_at | CXCL-12 | 34.3 / 41.4 | 8.4 / 54 | -1.4 / -0.2 | ns |
| 215101_s_at | CXCL-5 | 2923.7 / 1912.7 | 1092.8 / 779.2 | -1.6 / -1.3 | 0.00002 / 0.00002 |
| 214146_s_at | CXCL-7 | 3191.5 / 3016 | 949.9 / 812.7 | -1.8 / -1.9 | 0.00002 / 0.00002 |
| 202859_s_at | CXCL-8 | 11571.7 / 16046 | 13391.7 / 18499.2 | 0 / 0.1 | ns |
| 211506_s_at | CXCL-8 | 5210.1 / 7678.4 | 3719.1 / 11112.8 | -0.4 / 0.8 | 0.002 / 0.000078 |

***Legend:*** ** See Table 5.6*

**Table 5-8 Signalling cascade transcript abundance in CF MDMs under constitutive and stimulated (LPS) conditions compared to control**

| Probe Id. | Transcript Name | Control Signal (Constitutive/LPS) | CF Signal (Constitutive/LPS) | SLR (Constitutive/LPS) | *p** |
|---|---|---|---|---|---|
| 231699_at | NFκB inhibitor α | 39 / 56.9 | 82.3 / 115.2 | 0.8 / 1.2 | ns / 0.004 |
| 238846_at | NFκB activator | 1298.2 / 698.2 | 1983 / 972.7 | 0.7 / 0.3 | 0.00008 / 0.01 |
| 207037_at | NFκB activator | 253.5 / 108.6 | 403.5 / 140.9 | 0.6 / 0 | 0.002 / ns |
| 243376_at | TRAF family member NFκB activator | 32.4 / 53.4 | 47.1 / 71.3 | 0.6 / 0.6 | ns |
| 207137_at | NFκB inhibitor like-2 | 4.5 / 25 | 2.7 / 8.7 | 0.4 / -1.6 | ns |
| 214448_x_at | NFκB inhibitor β | 135.1 / 223.1 | 212.5 / 327.2 | 0.4 / 0.2 | ns |
| 203927_at | NFκB inhibitor ε | 906.9 / 1922.4 | 1317.8 / 2240.9 | 0.3 / 0.6 | ns |
| 211524_at | NFκB enhancer | 3.3/ 1.7 | 2 / 20.4 | 0.3 / 1.8 | ns / 0.02 |
| 218240_at | NFκB inhibitor RAS-like 2 | 593.9 / 537.1 | 650.8 / 512.6 | 0.3 / -0.1 | ns |
| 201502_s_at | NFκB inhibitor α | 6405.9 / 13833.5 | 7436 / 14192.9 | 0.2 / 0 | ns |

| | | | | | |
|---|---|---|---|---|---|
| 209239_at | NFκB enhancer 1 | 1257.9 / 2932.6 | 1340.4 / 3824.2 | 0.1 / 0.3 | ns / 0.05 |
| 214062 | NFκB inhibitor β | 163.3 / 183.1 | 117.9 / 266.2 | 0.1 / 0.3 | ns |
| 222105_s_at | NFκB inhibitor RAS-like 2 | 477.6 / 483 | 705.7 / 456.5 | 0.1 / 0.2 | ns |
| 223218_s_at | NFκB inhibitor ζ | 1006.1 / 6120.3 | 818.4 / 4969.4 | 0 / -0.2 | ns |
| 209115_at | MyD88 | 3957.4 / 6219.9 | 4023.4 / 5253.6 | -0.1 / -0.2 | ns / 0.02 |
| 219618_at | IRAK-4 | 110.8 / 131.3 | 102.5 / 79.5 | 0 / -05 | ns |
| 56829_at | IKK binding protein | 219.8 / 272.2 | 324.6 / 310.4 | 0.6 / 0.1 | 0.008 / ns |
| 221836_s_at | IKK binding protein | 129.4 / 139.1 | 163.2 / 139.2 | 0.3 / 0 | ns |
| 226048_at | MAPK 8 | 477.2 / 544.8 | 400.2 / 364.6 | -0.2 / -0.6 | ns / 0.01 |
| 1556341_s_at | MAPK 12 | 8.1 / 12.9 | 67.6 / 40.9 | 2.9 / 2.2 | ns |
| 211499_s_at | MAPK 11 | 5.6 / 40.3 | 54.6 / 46.9 | 2.9 / 0.4 | 0.02 / ns |
| 241357_at | MAPK15 | 24.9 / 35.9 | 54.6 / 84.9 | 1.1 / 0.9 | 0.007 / ns |
| 202787_s_at | MAPK 3 | 456.3 / 246.8 | 589.7 / 452.7 | 0.2 / 0.6 | ns / 0.01 |
| 229846_s_at | MAPK 1 | 343.9 / 205.5 | 230.8 / 279.8 | -0.4 / 0.3 | 0.0006 / 0.002 |

***Legend:*** * *see Table 5.6*

Further to considering the most up or down regulated genes or single genes, I also considered whether any differences were consistently replicated over different probes within the microarray. HLA-DQ transcripts (Table 5-3) appeared to be consistently down-regulated in CF MDMs compared to control MDMs. Also, the gene expression intensity signal for α-and β-tryptase were both higher in CF MDMs compared with control under constitutive and stimulated conditions (Table 5-9). Furthermore, this was confirmed over several different microarray tryptase probes. Due to a small amount of raw material I followed one line of inquiry investigating α and β tryptase using a more sensitive technique i.e. RT-PCR.

**Table 5-9 Tryptase mRNA fluorescence microarray signal intensity for control and CF MDMs**

| | | Control | | CF | | |
|---|---|---|---|---|---|---|
| **Tryptase Gene** | **Probe Number** | **Constitutive** | **Stimulated** | **Constitutive** | **Stimulated** | ***p* *** |
| α/β1 | 207741_x_at | 52 | 32 | 196 | 179 | 0.01<br>0.01 |
| | 216474_x_at | 77 | 43 | 222 | 165 | 0.00002<br>0.00002 |
| | 205683_x_at | 21 | 46 | 154 | 135 | 0.00003<br>0.0002 |
| | 210084_x_at | 17 | 13 | 194 | 108 | 0.0003<br>0.0025 |
| | 215382_x_at | 30 | 8 | 139 | 87 | 0.00003<br>0.001 |
| | 216485_s_at | 8 | 5 | 8 | 5.1 | >0.05 |
| | 217023_x_at | 37 | 44 | 144 | 118 | 0.028 |
| β2 | 207134_x_at | 46 | 17 | 175 | 154 | 0.00013<br>0.00002 |

***Legend:*** ** p values are given for constitutive expression (control vs CF) followed by stimulated expression (control vs CF)*

A limited quantity of ΔF508 +/+ cDNA was available following the microarray. Therefore MDMs from an additional cohort of control and CF ($n$=5 for both groups) ΔF508 +/+ adult donors were generated and reverse transcribed into cDNA as described (Section 2.8). Cohort data regarding these additional groups are given in table Table 5-10 (below). This enabled us to analyse a larger cohort ($n$=11) of ΔF508 +/+ adult donors for α- and β-tryptase expression under both constitutive and LPS stimulated conditions.

**Table 5-10 Additional control and CF cohort data**

| | **Control** | **CF** |
|---|---|---|
| **Donors (*n*)** | 5 | 5 |
| **Age (mean years ± SD)** | 24.6 ± 2.5 | 19.4 ± 3.5 |
| **Sex (Female:Male)** | 3:2 | 3:2 |
| **Ethnicity (Caucasian:Asian)** | 3:2 | 4:1 |
| **Genotype (*n*)** | N/A | ΔF508 +/+ (5) |
| **% $FEV_1$ (mean ± SD)** | Not Determined | 53 ± 15 |
| **Mean % CD14++** | 97 ± 2 | 97 ± 2 |

***Legend:*** *See Table 3-1.*

## 5.3.1 Confirmation of α- and β-Tryptase Primer Specificity

Prior to examining α- and β-tryptase expression in control and CF MDMs I sought to verify the specificity of our chosen set of primers designed according to Schwartz *et al.* (2003). Expression

of α-and β-tryptase has been confirmed previously in MM6 and HMC-1 cell lines, respectively (Schwartz, Min *et al.* 2003). MM6 and HMC-1 mRNA was transcribed into cDNA (Section 2.8) and PCR for α- and β-tryptase expression was carried out as described (Section 2.10). The melting point for α- (MM6) and β- (HMC-1) tryptase PCR products were 90.25 ± 0.24°C and 90.42 ± 0.34°C, respectively. Primer specificity for tryptase was confirmed by running α- and β-tryptase PCR products derived from MM6 (Figure 5-2, lanes 1 and 3) and HMC-1 (Figure 5-2, lanes 2 and 4) cells on a 2% agarose gel. I found that α-tryptase could be detected in MM6 but not HMC-1 cells. In contrast β-tryptase could only be detected in HMC-1 cells whereas a larger PCR product was detected in MM6 cells, indicating a non-spliced transcript or genomic contamination. A PCR product from an MDM preparation, which did not produce similar melting points for either transcript, migrated the furthest indicating a very low molecular weight and possibly 'primer-dimer' (Figure 5-2, lane 5). Comparison with a molecular weight ladder (Figure 5-2, lane 6) indicates that the PCR products for α- and β-tryptase were just less than 300 bp in length. Schwartz *et al.* (2003), using the same design of primers, observed a molecular weight of 278 bp for both α- and β-tryptase. Schwartz *et al.* (2003) also noted that these same primers can detect genomic DNA for both α- and β-tryptase from MM6 cells, which they report as 386 bp long. Interestingly I were also able to detect genomic contamination in MM6 cells (lane 3) at a similar length. Therefore PCR products with an identical or higher melting point, as the genomic DNA, were excluded from analysis. Thus taken together I concluded that these tryptase primers were suitable to examine α- and β-tryptase expression in control and CF MDMs.

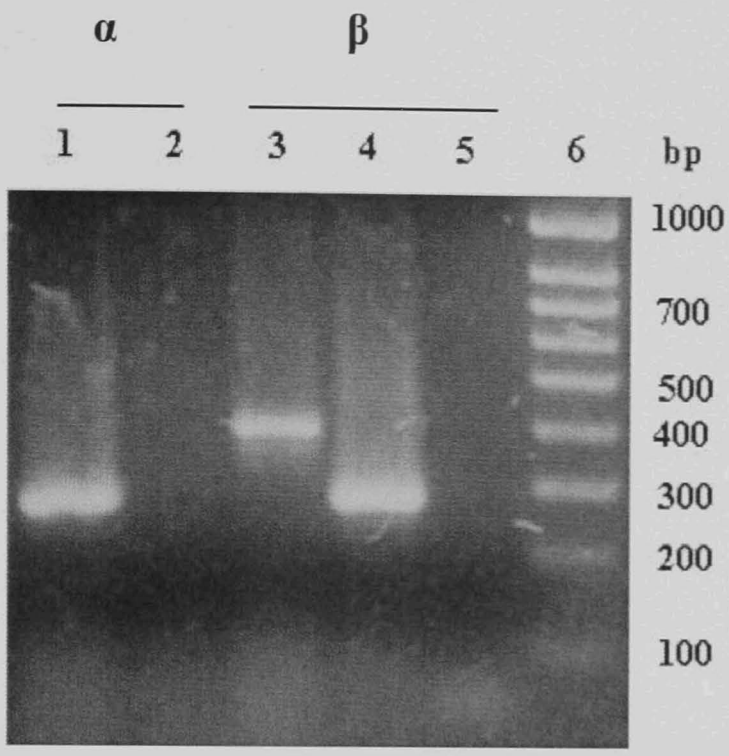


**Figure 5-2 α- and β-tryptase PCR products from MM6 (lanes 1 and 3), HMC-1 (lanes 2 and 4) and an MDM sample (lane 5)**

### 5.3.2 α- and β-Tryptase Expression in Adult Control and Cystic Fibrosis Cohorts

CF ΔF508 +/+ and control MDM were analysed for α- and β-tryptase expression and normalised to the house keeping gene α-enolase. I initially examined constitutive expression for α- and β- ($n$=5) tryptase expression in the cohort of donors chosen for the microarray. I were able to confirm upregulation (~2.5 and ~4-fold higher) for α-tryptase in two of the CF donors compared to the highest levels observed in control samples. However, examining the entire control and CF cohort ($n$=11) under constitutive conditions (Figure 5-3) for α-tryptase expression (plot A) revealed no significant difference ($p$>0.05) despite a tendency towards higher levels in the CF group. Since cDNA from donors had been pooled for the microarray study, such higher samples in the initial cohort could have accounted for the differential microarray result. Similiarly there

was clearly no difference observed for β-tryptase expression between control and CF MDMs under constitutive conditions (Figure 5-3, plot B).

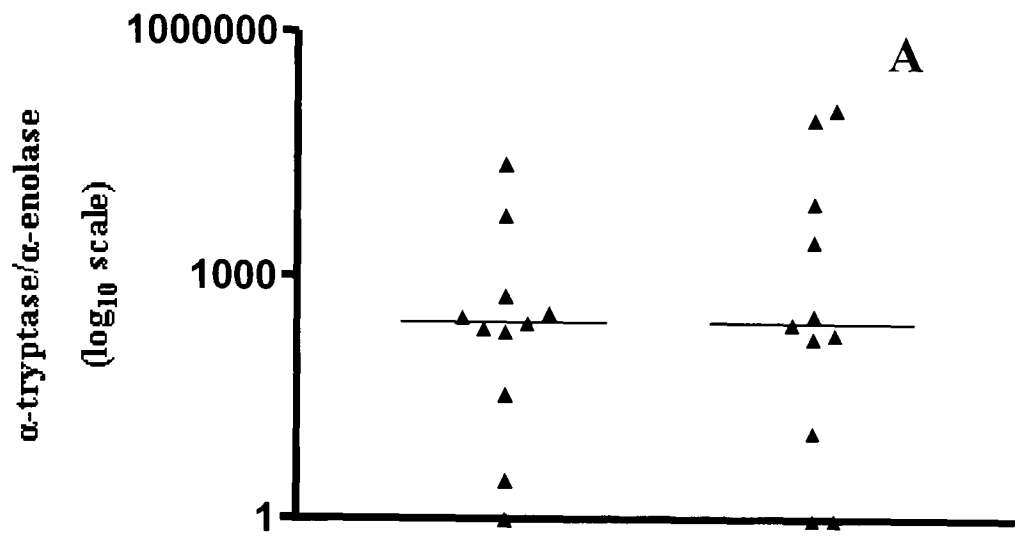


**Figure 5-3 Control and CF expression of α- (plot A) and β- (plot B) tryptase under constitutive conditions** (horizontal bars indicate median values)

Similar to the data for constitutive conditions, there was no significant difference ($p>0.05$) for α- or β- tryptase mRNA expression following LPS stimulation (Figure 5-4) between control (plot A) and CF (plot B) MDMs despite a tendency towards higher levels in the CF group. LPS

stimulation did not appear to have any consistent effect (either an increase or decrease) on tryptase mRNA expression. Taken together, the initial array analysis of a cDNA pool suggested high tryptase expression levels in CF. However, I could not confirm any significant differences ($p$>0.05) in tryptase mRNA expression between our control and CF cohorts when looking at individual samples using an unpaired $t$-test or between treatment groups using a one-way ANOVA.

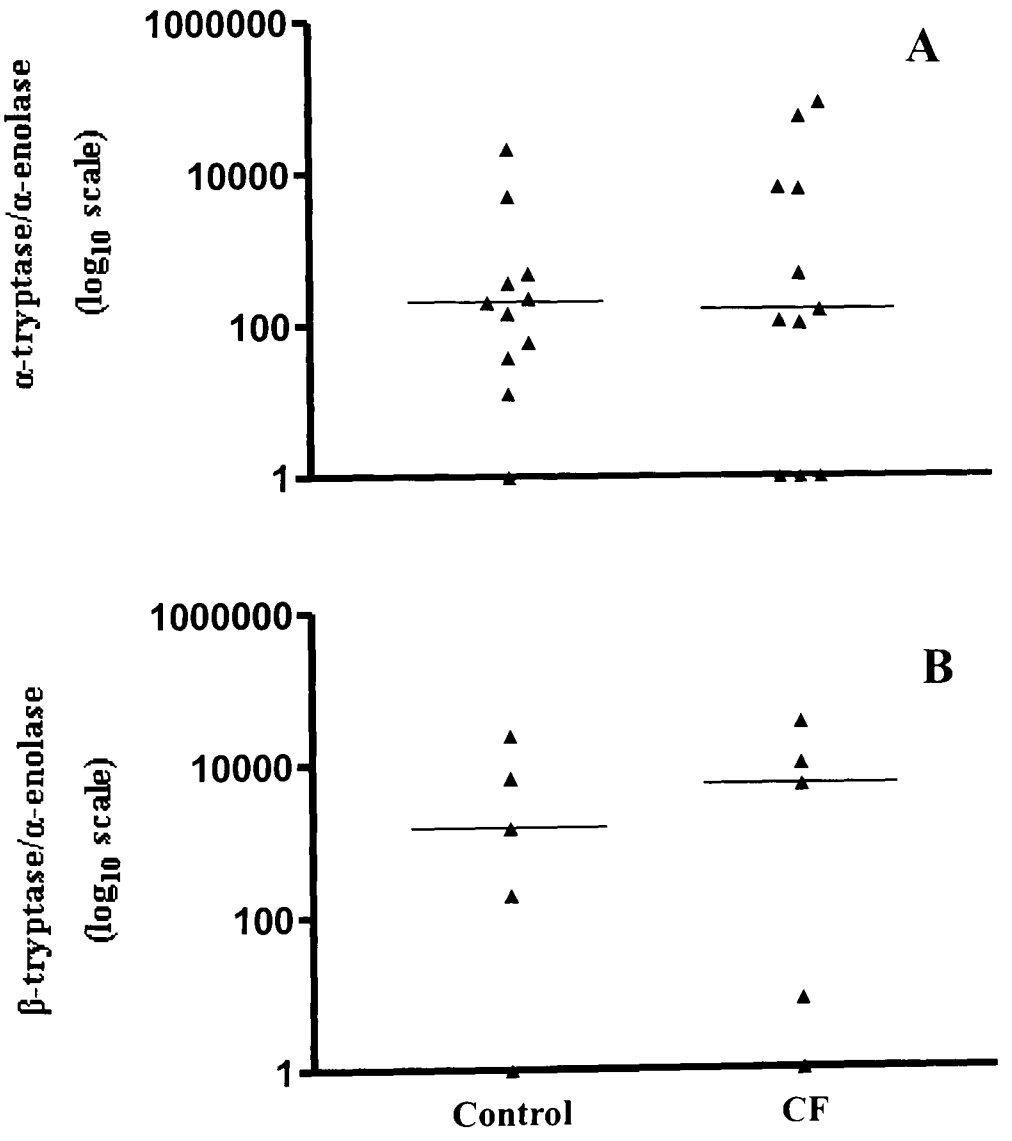


**Figure 5-4 Control and CF expression of α- (plot A) and β- (plot B) tryptase under LPS stimulated conditions** (horizontal bars indicate median values)

### 5.3.3 α- and β-Tryptase Expression in Paediatric Cohorts

To lend support to any differences having an important role in CF I also sought to isolate monocytes and generate MDMs from paediatric control and CF donors (Table 5-11). Due to logistical constraints I did not isolate monocytes as paired control and CF samples at the same point in time. Furthermore, due to the small volume of blood that I were able to obtain, I chose to focus on constitutive expression rather than stimulate MDMs with LPS.

**Table 5-11 Paediatric control and CF cohort data**

| | **Control** | **CF** |
|---|---|---|
| **Donors (*n*)** | 6 | 7 |
| **Age (mean years ± SD)** | 13.5 ± 3 | 13.4 ± 2 |
| **Sex (Female:Male)** | 3:3 | 2:5 |
| **Ethnicity (Caucasian:Asian)** | 5:1 | 7:0 |
| **Genotype (*n*)** | N/A | ΔF508 +/+ (6)<br>ΔF508 +/unknown (1) |
| **% $FEV_1$ (mean ± SD)** | Not Determined | Not Determined |
| **Mean % CD14++** | 98 ± 1 | 95 ± 3 |

The purity of monocytes isolated from each donor was above 90%. RNA extraction and reverse transcription of RNA into cDNA was performed as described (Section 2.8). I then examined constitutive α- (but not β) tryptase expression in control and CF MDMs (Figure 5-5) as described for adults (Section 5.3.2). Only PCR products that gave a melting point similar to the positive

controls were used for comparison. I did not observe any significant difference ($p$>0.05. Mann-Whitney U-test) for α-tryptase expression between the two groups.

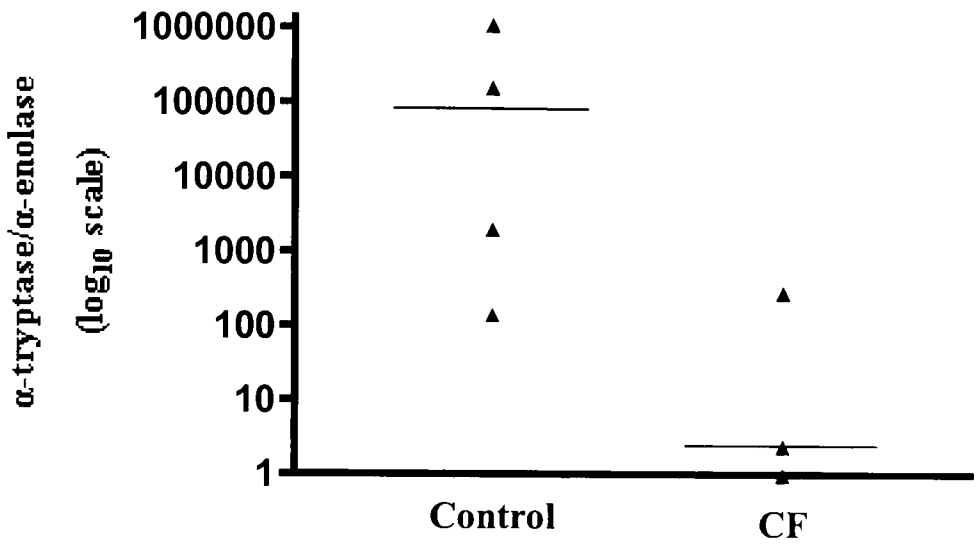


**Figure 5-5 Control and CF paediatric cohort expression of α-tryptase under constitutive conditions** (horizontal bars indicate median values)

Taken together the initial finding of an increased expression level for tryptase in a pooled microarray approach could not be confirmed in two sets of independent samples from adult and paediatric CF cohorts.

## 5.4 Tumour Necrosis Factor gene expression in Control and Cystic Fibrosis Adults

Next, I wanted to confirm or refute an earlier finding made by Pfeffer *et al.* (1993), reporting significantly elevated TNF mRNA and protein derived from LPS stimulated CF MDM compared to controls. To examine this, MDMs derived from adult control and CF cohorts (Table 5-10, above) were stimulated with LPS (10 ng/ml) for three hours. Harvested cells were then analysed

for TNF mRNA by RT-PCR. Figure 5-6, below, compares constitutive and LPS stimulated TNF mRNA from control and CF donors. As expected in both control and CF cohorts, TNF mRNA expression was higher under LPS stimulated conditions compared to constitutive conditions. For the control cohort this was a significant ($p$=0.019, using a paired $t$-test) increase in TNF mRNA expression. Despite much higher values in the CF cohort, LPS stimulated TNF mRNA was not significantly different from constitutive expression. Importantly, overall I found no significant difference ($p$>0.05) between the control and CF cohorts for TNF mRNA expression, either constitutively or when stimulated with LPS, using a one-way ANOVA. Taken together these findings refute earlier observations made by Pfeffer *et al.* (1993) of elevated TNF mRNA in CF LPS stimulated MΦs.

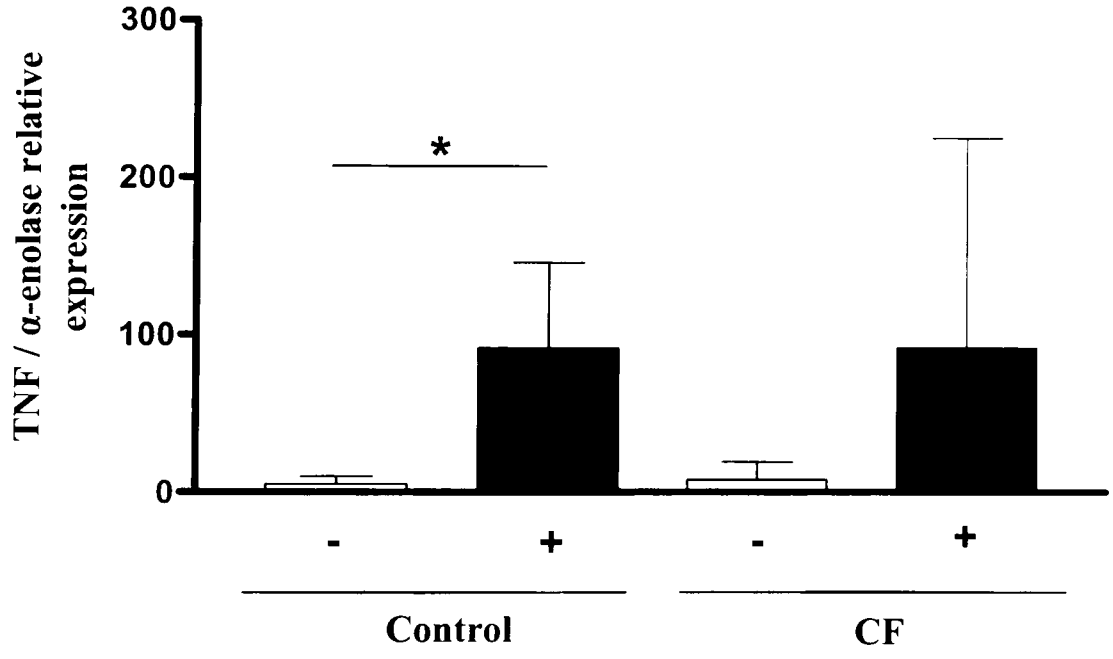


**Figure 5-6 Constitutive (-) and LPS (+) stimulated TNF mRNA expression (mean±SD) in control and CF cohorts** ($n$=5 for both groups) * $p$=0.019

## 5.5 Conclusion

I set out to examine whether CF MDMs were inherently different to control MDMs by looking for transcript differences in the entire human genome under constitutive and LPS stimulated conditons between the two groups. Following an analysis of transcripts from ΔF508 homozygous and control MDMs I identified α-/β-tryptase as being significantly different. However, despite a tendency for higher α-tryptase expression in CF I could not replicate these findings in separate CF and control cohorts using RT-PCR. Further to this I could not confirm earlier findings of elevated TNF mRNA in CF MDMs compared to control. Taken together I could not detect any inherent transcript differences in CF MDMs under constitutive or LPS stimulated conditions.

# 6 Discussion

I set out to investigate the possibility that lung MΦs could play an important role in CF airway pathophysiology that has hitherto gone unrecognised. CF is characterised by a loss of function mutation in the CFTR protein, which leads to reduced anion transport and consequently thickened SMG secretions into the bronchial lumen and the generation of a viscous and dehydrated ASL. From birth, defence mechanisms are compromised and the CF airway is prone to chronic colonisation and infection by a narrow spectrum of microbial opportunistic pathogens (Gibson, Burns *et al.* 2003; Matsui, Wagner *et al.* 2006). Associated with this is the early development of chronic neutrophilic inflammation. Neutrophilic inflammation, rather than infection, is the most important determinant on respiratory health in CF (Mayer-Hamblett, Aitken *et al.* 2007) however both contribute to CF lung disease.

One airway effector cell that has been overlooked as a possible modulator of the infection - inflammation relationship in CF is the MΦ: In reviews on immune contributors to CF disease, MΦs have been overlooked as important players in the disease process (Bals, Weiner *et al.* 1999; Chmiel, Berger *et al.* 2002). On the other hand MΦs and their products have been associated with the pathogenesis of other chronic respiratory diseases such as COPD (Barnes 2004; O'Donnell, Breen *et al.* 2006), asthma (Peters-Golden 2004) and emphysema (Hautamaki, Kobayashi *et al.* 1997; Ofulue and Ko 1999; Churg, Wang *et al.* 2004). The aim of this thesis was to analyse the phenotype and function of MΦs in CF and redress this balance.

Described in chapter 3, I was able to consistently identify MΦs in the sputa of controls and CF donors. I demonstrated this using flow cytometry and gating on HLA-DR, CD14 and CD68

positive events. Whilst expression of HLA-DR is specific only for antigen presenting cells, mAbs to CD14 and CD68 have been used extensively for the identification of monocytes and tissue MΦs (Kelly, Bliss *et al.* 1988; Passlick, Flieger *et al.* 1989; Pulford, Rigney *et al.* 1989; van der Kooij, von der Mark *et al.* 1997). The derivation of sputum from the central airways rather than the peripheral tissue suggests that the MΦs identified here are predominantly bronchial rather than alveolar MΦs. It is important to study the bronchial regions in CF because this is the main site of immunopathology in the disease (Zuelzer and Newton 1949; Gibson, Burns *et al.* 2003). Inflammatory processes in the alveoli and bronchi can also be different as seen in COPD, where neutrophils accumulate in the bronchial but not alveolar regions (Rutgers, Timens *et al.* 2000).

Described within this thesis, I have identified two populations of bronchial MΦs that can be distinguished by their FSC and SSC light scatter properties using flow cytometry (Figure 3-3). Furthermore, small MΦs were consistently more prominent in CF adult sputa, whereas control sputa contained a much greater proportion of large MΦs (Figure 3-5). FSC does not correlate exactly to cell size but it does provide a relative indication (Shapiro 2003) and when used together with the SSC signal has been used by other investigators to distinguish between lung MΦs and other leukocytes based on size and granularity in sputum and BAL fluid from healthy subjects (Alexis, Soukup *et al.* 2000), COPD (Frankenberger, Menzel *et al.* 2004), ARDS (Rosseau, Hammerl *et al.* 2000) and IPF patients (Krombach, Gerlach *et al.* 1996). In two of these publications (Krombach, Gerlach *et al.* 1996; Frankenberger, Menzel *et al.* 2004), lower FSC and SSC light scattering characteristics went along with a smaller size, determined using traditional methods such as microscopy. Herein CF small (bronchial) MΦs are described for the first time.

Our data suggests that CF small MΦs have a monocyte-like phenotype. This conclusion was based upon CF small MΦs having almost identical light scatter characteristics to CF blood monocytes, when acquired on identical instrument settings (Table 3-3). I also found that small MΦ expression of CD14 and HLA-DR was internediate between monocytes and large MΦs. In addition, I also found a similar lower expression of CD68 on CF small MΦs and blood monocytes compared to large MΦs. This is in line with a monocytic origin and greater immaturity of these cells (Viksman, Liu *et al.* 1994; Umino, Skold *et al.* 1999; Staples, Smallie *et al.* 2007). It remains to be examined whether control and CF airway macrophages differ in expression of these parameters during maturation/differentiation. I also noted that HLA-DR, CD14 and CD68 positive cells in control donor samples exhibited a greater degree of fluorescence in FL1 (CD66b, CD16b antigens), than small MΦs or monocytes. This might suggest expression of these neutrophil receptors on MΦs. However since these cells express monocyte/MΦ specific antigens (CD14 and CD68) and scatter flow cytometry light much greater than neutrophils it is more likely that this signal is due to AF as highlighted previously (Section 3.2). Though not measured here, alveolar MΦ AF has been demonstrated by flow cytometry to be at least 1 log above blood leukocytes including monocytes (Viksman, Liu *et al.* 1994). Thus AF is also a feature of mature tissue MΦs from the lung. Using our protocol MΦ AF may have contributed to a higher CD68 fluorescence intensity signal in control MΦs compared to CF small MΦs. Despite this, an overall increase in AF, CD68 intensity, lower CD14 expression and greater cell size suggest a more mature immunophenotype for control MΦs, in contrast to the intermediate/monocyte-like phenotype expressed by the majority of CF MΦs.

The methodology used to identify MΦs in this thesis may have been improved by inclusion of a more comprehensive blockade of Fc receptor mediated non-specific binding using an Fc blocker

or serum. Though the FMO strategy can successfully discriminate specific from non-specific binding discrimination may have been improved by using serum prior to staining.

It could be argued that low FSC/SSC cells may be non-viable or dead cells however this is unlikely given that cell viability was high in CF sputum compared to controls (Table 3-2) and similar to published observations (Belda, Leigh *et al.* 2000; Alexis, Muhlebach *et al.* 2006). Furthermore when I isolated MΦs from the sputum of CF donors, isolated cells had a very high viability (Section 4.3) as measured by the trypan blue exclusion method. The trypan blue method is not the optimum method to discriminate viable from non-viable cells. The use of propidum iodide and annexin V that stain dead and apoptotic cells respectively and can be detected by flow cytometry would have enabled clearer discrimination on this aspect. This, however was not performed due to insufficient parameter detection channels present on the BD FACSCalibur flow cytometer. Future investigations into small MΦs, particularly on the newer multiparameter instruments, should include flow cytometric exclusion of non-viable/apoptotic cells in the analysis.

It could be argued however that such low AF/CD68 positive cells are pulmonary DCs, a phenotypic variant of the MΦ (Hance 1993). However, this is unlikely since strong CD14 expression could be detected on CF small MΦs whereas previous publications have shown reduced/absent CD14 expression on *ex vivo* pulmonary DCs (Cochand, Isler *et al.* 1999; Masten, Olson *et al.* 2006) and *in vitro* blood derived mature DCs (Cochand, Isler *et al.* 1999; Huang, Xiao *et al.* 1999), whilst immature blood derived DCs have been shown to be CD14 positive (Cochand, Isler *et al.* 1999; Masten, Olson *et al.* 2006). A CD14 high expressing DC population was described by Demedts *et al.* (2005), however contamination by monocytes, acknowledged by

these authors, could have contributed to this expression. Taken together, our data from CF patients and those described above strongly suggest that small MΦs are monocyte-like cells although this does not exclude them from developing into DCs in response to environmental cues.

A precedent of prior publications have identified monocytes (Alexis, Muhlebach *et al.* 2006), monocyte-like MΦs (Kiemle-Kallee, Kreipe *et al.* 1991; Krombach, Gerlach *et al.* 1996; Rosseau, Hammerl *et al.* 2000) or small MΦs (Hoogsteden, van Dongen *et al.* 1989; Frankenberger, Menzel *et al.* 2004) in the airway of a variety of other chronic inflammatory respiratory diseases. These were all identified by fluorescence microscopy and/or flow cytometry and may be similar to the small MΦs that I have identified in the sputa from CF patients.

The healthy airways sampled by induced sputum or BAL contain a low percentage (in most cases less than 10%) of monocytic-like cells that is slightly lower compared to findings in our paediatric and adult control cohorts (13 ± 13% and 16 ± 8%, respectively) (Hoogsteden, van Dongen *et al.* 1989; Noble, Du Bois *et al.* 1989; Kiemle-Kallee, Kreipe *et al.* 1991; Krombach, Gerlach *et al.* 1996; Alexis, Soukup *et al.* 2000; Rosseau, Hammerl *et al.* 2000; Frankenberger, Menzel *et al.* 2004). Analysis of BAL fluid from sarcoidosis, IPF, extrinsic allergic alveolitis (EAA), ARDS and pneumonia-induced ARDS and also induced sputum from COPD have shown a significantly greater, though in some cases modest, proportion of monocytic-like MΦs in the lung of these respiratory diseases (Hoogsteden, van Dongen *et al.* 1989; Noble, Du Bois *et al.* 1989; Striz, Wang *et al.* 1993; Krombach, Gerlach *et al.* 1996; Rosseau, Hammerl *et al.* 2000; Frankenberger, Menzel *et al.* 2004).

In line with our studies on CF sputum, Krombach *et al.* (1996) subdivided BAL derived MΦs from IPF and EAA patients ($n$=39) and discriminated two populations (small and large) based on flow cytometry light scatter. The monocyte-like cells were smaller (12.3 ± 0.1μm vs 19.5 ± 0.3μm) and constituted 16 ± 1% (IPF) and 15 ± 3% (EAA) of total MΦs, which was a modest but significant increase over controls (9 ± 1%). An increase in the percentage of monocyte-like cells from IPF and EAA patients went along with much higher absolute counts (~5- and 8-fold) compared to controls thus making a substantial contribution to the number of airway MΦs (Krombach, Gerlach *et al.* 1996).

In addition, flow cytometry analysis of sputum MΦs from COPD patients (Frankenberger, Menzel *et al.* 2004) has revealed a percentage of small MΦs (69 ± 24%) similar to that described in our adult classical CF cohort (73 ± 18%). However values were lower in our non-classical (31 ± 20 %) and paediatric (50 ± 35%) cohorts with milder airway disease (Figure 3-4 and Figure 3-5). In contrast BAL fluid from CF children was demonstrated to contain only 9% monocytic cells (Alexis, Muhlebach *et al.* 2006). When taking into account the use of inhaled or oral steroids by COPD patients the percentage of small MΦs in COPD is 37 ± 18% and 44 ± 24%, respectively (Frankenberger, Menzel *et al.* 2004). In comparison the percentage of small MΦs in our CF cohort also did not decline in cases with inhaled steroid medication (72 ± 9%). The lower percentages in IPF, EAA, and CF BAL fluid as reported by others compared to COPD and CF sputum could be attributed to a different sampling methodology (bronchial versus predominantly alveolar), the nature of the inflammatory response and/or milder airway inflammation in the diseases studied.

In BAL fluid derived from ARDS patients it was noted that there was a pronounced shift in the immunophenotype from mature MΦs to an immature monocyte-like phenotype (Rosseau. Hammerl *et al.* 2000). This was characterised by a significant increase in expression intensity of CD14 and 27-E10 ('Calprotectin' a marker of acute phase inflammatory MΦs) and a decrease in intensity of antigens for mature MΦs (CD71 (transferrin receptor) and 25-E9 reactivity) (Rosseau, Hammerl *et al.* 2000). Similarly COPD small MΦs have been shown to have a ~100-fold and ~50-fold higher expression of CD14 and HLA-DR, respectively, compared with COPD large MΦs (Frankenberger, Menzel *et al.* 2004). These monocyte-like cells, in comparison to more mature MΦs had a higher percentage and intensity of CD14 expression, lower AF and almost identical flow cytometry light scatter characteristics to blood monocytes suggesting increased recruitment from peripheral blood (Krombach, Gerlach *et al.* 1996; Rosseau, Hammerl *et al.* 2000; Frankenberger, Menzel *et al.* 2004). Small MΦs resemble monocytes in CF, COPD and ARDS therefore it is reasonable to assume migration from blood in these inflammatory conditions. However in COPD, where the minor population of small MΦs in control donors could also be analysed these were also found to have similar expression levels to those described for COPD indicating recruitment under constitutive conditions also.

Based on the phenotype and morphology of these cells, I and others (Krombach, Gerlach *et al.* 1996; Rosseau, Hammerl *et al.* 2000; Frankenberger, Menzel *et al.* 2004) believe that such cells are recruited from the vasculature as immature MΦs constitutively but this increases in response to infection or in response to cytokines and chemokines. Increased mononuclear recruitment into the CF airways has not been identified or characterised before and represents a novel finding regarding CF inflammation. However these findings remain to be rigorously confirmed by independent investigators.

A variety of different mechanisms may be able to explain elevated recruitment of monocytes, via the action of chemokines, into the CF (and other respiratory diseases) airway compared to control. A pitfall of this investigation has been the absence of any determination of CXC and CC chemokine levels or absolute counts of neutrophils in sputum and their correlation of small MΦs. This information may have yielded crucial information into the mechanism of small MΦ migration into the CF airways.

Two chemokines that may be particularly important in this regard are CXCL-8 and CCL-2. CXCL-8 has been extensively studied in CF because a number of studies have shown elevated CXCL-8 concentrations in the CF airway (Balough, McCubbin *et al.* 1995; Bonfield, Panuska *et al.* 1995; Khan, Wagener *et al.* 1995; Osika, Cavaillon *et al.* 1999; Sagel, Kapsner *et al.* 2001; Dakin, Numa *et al.* 2002; Nixon, Armstrong *et al.* 2002; Muhlebach, Reed *et al.* 2004; Xiao, Hsu *et al.* 2005; Hartl, Griese *et al.* 2006) and CXCL-8 concentration and neutrophil counts have been demonstrated to be associated with a significant proportion of the progressive decline in $FEV_1$ (Mayer-Hamblett, Aitken *et al.* 2007). In CF CXCL-8 has traditionally been examined in the context of an immune response to infection. However certain CXC chemokines (those containing an ELR amino acid motif, for example CXCL-8) can also function as pro-angiogenic factors via CXCR-2 whereas those without an ELR motif are angiostatic (Strieter, Polverini *et al.* 1995). Thus CF epithelial production of CXCL-8, via its pro-angiogenic role, may be a response to epithelial hypoxia shown by Worlitsch *et al.* (2002) rather than an early response to infection or malfunctional CFTR. CXCL-8 may then contribute to the vascular remodelling seen in pulmonary fibrotic diseases (Strieter, Gomperts *et al.* 2007). The mechanism by which CXCL-8 is produced in response to hypoxia remains an intriguing possibility and awaits investigation in CF and COPD.

CXCL-8 is a potent neutrophil chemoattractant (Huber, Kunkel *et al.* 1991) that can play a major role in the recruitment of neutrophils into the airway via the CXCR-1 and 2 receptors. Interestingly, CXCR-1 and 2 have also been demonstrated on healthy (3.3 ± 0.3 and 2.8 ± 0.3 specific fluorescence channels, respectively) and COPD (3.9 ± 0.7 and 2.7 ± 0.2 channels, respectively) monocytes albeit at lower levels than on healthy neutrophils (22.6 ± 1.5 and 8.4 ± 1.2 channels, respectively) (Traves, Smith *et al.* 2004). In line with this CXCL-8 has also been demonstrated to induce firm adhesion of monocytes to the endothelium under *in vitro* flow conditions via CXCR-1 and 2 (Gerszten, Garcia-Zepeda *et al.* 1999). Blocking CXCR-1 and 2 also substantially reduced monocyte migration towards CXCL-1, CXCL-8 and other chemokines in *in vitro* chemotaxis assays (Traves, Smith *et al.* 2004). However other CXCR-1 and 2 ligands may also be important such as proline-glycine-proline fragments, derived from the breakdown of collagen, which have demonstrated neutrophil chemotactic activity via CXCR-2 and have been detected in the airways of COPD (Weathington, van Houwelingen *et al.* 2006) and CF patients, the latter were generated via MMP-9 activity (Gaggar, Jackson *et al.* 2007). Therefore CXCR ligands, particularly CXCL-8 and the recently described collagen fragments, would be interesting candidates to further examine the mechanism by which monocytes are recruited into the CF airways together with neutrophils.

The increased presence of small MΦs in ARDS with or without pneumonia was shown to correlate with over a 1 log increase of CCL-2 protein compared to control (Rosseau, Hammerl *et al.* 2000). Neutralisation of CCL-2 in ARDS BAL fluids almost completely abrogated monocyte migration in an *in vitro* model (Rosseau, Hammerl *et al.* 2000). This study implicates CCL-2 as the main monocyte chemoattractant in ARDS BAL fluid that could account for the recruitment of small MΦs in this disease. In COPD, where small MΦs are increased (Frankenberger, Menzel *et*

*al.* 2004), CCL-2 mRNA and CXCL-8 mRNA and protein are elevated in airway epithelia as detected by *in situ* hybridisation (de Boer, Sont *et al.* 2000). In addition CCL-2 and CXCL-1 protein are significantly elevated in COPD sputum but not BAL when compared with healthy controls (Traves, Culpitt et al. 2002) further reinforcing important differences and outcomes found between induced sputum and BAL sampling methods. De Boer *et al.* (2000) also show that the mRNA level of these CC and CXC chemokines positively correlate with macrophage and neutrophil counts in the airway and negatively correlate with $FEV_1$ % predicted.

In contrast to COPD, CC chemokines have been largely overlooked in CF however interest is growing in this area as the mechanisms driving chronic inflammation remain to be fully characterised. Following this line of inquiry in a parallel study, including some of the donors described herein, our group (Rao, Wright *et al.* 2006) have demonstrated that CCL2 is significantly higher in sputa and sera (median 190.6 pg/ml and 1081 pg/ml, respectively) from CF patients compared to healthy controls (median 77.3 pg/ml and 295.5 pg/ml respectively). These data are with those from Augarten *et al.* (2004) who found significantly higher CCL2 in sera from CF patients carrying two CFTR mutations associated with a more severe phenotype. In a cohort of CF infants (median age of 2.3 years) a significant increase in the number of BAL MΦs and levels of CCL3, CCL4, CCL20 and CCL2 protein were found compared to a non-CF population (Upham, Brennan *et al.* 2007). In this latter study, elevated CC chemokines were associated with an increase in MΦs but this was only significant for CCL2. However in CF CCL2 protein has not been shown to correlate with $FEV_1$ (Augarten, Paret *et al.* 2004; Rao, Wright *et al.* 2006), whereas in COPD, sputum CCL2 protein levels negatively correlated with $FEV_1$ and positively correlated with neutrophil, but paradoxically not MΦ, numbers (Traves, Culpitt *et al.* 2002). Taken together, there is evidence from COPD and increasing evidence from

CF that CC chemokines may also be important in these diseases. Increased levels of CC and CXC chemokines could be responsible for the enhanced recruitment of monocytes via CCR and CXCR, respectively into the airspace and causing elevation in the proportion of small airway MΦs. Small airway MΦs are more likely to derive from classical CD14 positive (CD16 negative) monocytes since these and not the CD16 positive subpopulation, express the CCR2 receptor (Weber, Belge *et al.* 2000). These mechanisms remain to be further investigated, particularly in murine models of CF since here monocyte can be tracked with greater precision.

An alternative mechanism to explain an increased proportion of small MΦs involves depletion of large MΦs. This could occur by either increased migration to draining lymph nodes or bronchus associated lymphatic tissue (Moyron-Quiroz, Rangel-Moreno *et al.* 2004) or by increased apoptosis via an endoplasmic reticulum stress mechanism and/or by LPS exposure (Davis, Barsoum *et al.* 1980; Puthalakath, O'Reilly *et al.* 2007). CFTR expressing cells have been demonstrated to exhibit signs of endoplasmic reticulum stress in epithelial cells due to mutant CFTR degradation (Knorre, Wagner *et al.* 2002) although whether this also affects CF MΦs has not been investigated. Whilst I have hypothesised that small MΦs may be important to the pathophysiology of CF, the loss or reduced functions of mature tissue MΦs may be equally important since these cells have immunoregulatory functions. In line with this, depletion of mature airway MΦs in rodent models of infection have been shown to be very important in regulating neutrophil recruitment to infection despite showing conflicting data. Depletion of airway MΦs caused significantly increased recruitment of neutrophils via elevated TNF, CXCL-8 and CCL-2 in airway BAL fluid suggesting a suppressive effect (Broug-Holub, Toews *et al.* 1997; Beck-Schimmer, Schwendener *et al.* 2005) mediated by resident MΦs. In contrast depletion of airway MΦs caused a decreased influx of neutrophils via decreased levels of TNF,

CXCL-1 and CXCL-8 (Hashimoto, Pittet *et al.* 1996) and moreover neutrophil migration to the lungs depended to a large extent on TLR4 expression on alveolar MΦs (Hollingsworth, Chen *et al.* 2005), suggesting that the MΦ can also be primary source of these pro-inflammatory mediators. When looking at total sputum MΦs in inflammatory lung diseases such as COPD there are large increases in total leukocytes and interstitial MΦs but interestingly only small increases (Traves, Culpitt *et al.* 2002) or no change (Rutgers, Timens *et al.* 2000) in absolute counts from sputum compared to control. Hence it is likely that increased influx of monocytes rather than depletion of large MΦs lead to the increased percentage of small MΦs that I have identified in adult CF sptum donors.

I investigated a paediatric and an adult CF cohort in order to increase our understanding regarding the percentage of small MΦs and severity of lung disease and/or colonisation status. I found that whilst all of the adult classical CF donors studied had an elevated percentage of small MΦs, the data were more variable for our non-classical and paediatric cohorts. Though a small cohort size excludes any detailed analysis, our data indicate that small MΦs can be detected most consistently in later stages of disease with a greater severity of pulmonary inflammation and with the presence of infection/colonisation. Future investigations into small MΦs should investigate better defined cohorts of CF patients to better de-lineate the relationship between the % of small MΦs and clinical status. I believe that the recruitment of monocytes and consequently the increased proportion of small MΦs in the airways contribute to progressive lung disease and thus mortality in CF and other chronic airway diseases. In line with this I was able to show that the proportion of small MΦs in our CF cohort showed a significant negative correlation with CF FEV1 % predicted (Figure 3-8). Elsewhere, Krombach *et al.* (1996) and Rosseau *et al.* (2000) compared lung function and gas exchange parameters with the presence of monocyte-like cells in

BAL fluid. Krombach *et al.* (1996) found that the percentage of monocyte-like cells (>13%) negatively correlated with vital capacity (maximal inspiratory, expiratory and tidal volume). Similarly arterial oxygen pressure negatively correlated with the presence of immature MΦs (Krombach, Gerlach *et al.* 1996; Rosseau, Hammerl *et al.* 2000). Finally Rosseau *et al.* (2000) showed that 28 of 37 (75.7%) ARDS patients with a transition from immature to mature alveolar MΦs and a decrease in BAL CCL-2 levels survived a stay on their intensive care unit. By contrast, only 3 of 12 (25%) ARDS patients with a sustained predominance of immature MΦ markers and elevated levels of CCL-2 in BAL had survived. These data suggest that small MΦs or monocyte-like cells are associated with lung injury. However, the large quantity of neutrophils within the CF and COPD airways suggests that neutrophils are more likely to be the predominant mediator of lung injury in these diseases. On this basis I propose that monocyte recruitment *per se* facilitates neutrophil recruitment that is responsible for progressive lung disease. However I also propose that once in the airway, small MΦs contribute directly to airway disease by recruiting further neutrophils and monocytes, liberating cytokines such as TNF and by failing to clear infection or host inflammatory products. Both of these concepts are discussed below.

Rosseau *et al.* (2000) showed that an increase in immature airway MΦs positively correlated with the proportion of neutrophils and lymphocytes in BAL fluid from sarcoidosis patients. Crucially this information was not obtained in this thesis. However a correlation does not demonstrate that monocytes are important for neutrophil recruitment. Jürgen Lohmeyer and his group in particular, have investigated the role of monocytes in neutrophil recruitment during *Escherichia coli* (*E.coli*) LPS induced acute airway inflammation in murine models. They have shown that in response to airway deposition of LPS, neutrophil accumulation at 12 hrs approximates to 50

cells/μl of BAL fluid (Maus, Waelsch *et al.* 2003). However when the monocyte attracting chemokine CCL-2 is deposited with LPS there is a 10-fold increase in neutrophil recruitment (Maus, Waelsch *et al.* 2003). A similar pattern is seen for monocyte recruitment where CCL-2 causes a low recruitment of monocytes but this is upregulated upon addition of LPS to the airways. Further to this, blocking monocyte recruitment into the airspace (by using CCR-2 KO mice) reduces neutrophil recruitment to CCL-2 and LPS (Maus, von Grote *et al.* 2002; Maus, Waelsch *et al.* 2003). This occurs despite neutrophils expressing neglible levels of CCR-2 compared to monocytes ($1.2 \pm 0.1$ vs $16.2 \pm 1.5$ specific fluorescence channels) (Traves, Smith *et al.* 2004) thus strongly suggesting the importance of a monocyte/MΦ mechanism in regulating neutrophil recruitment into the airway. In line with these findings Srivastava *et al.* (2005) have demonstrated that recruited airway monocytes upregulate gene expression for neutrophil chemoattractants (amongst others), directly implicating a role for monocytes in neutrophil recruitment.

The amplification effect of monocytes on neutrophil recruitment was confirmed when CCR-2 KO mice received WT bone marrow CCR-2 +/+ mononuclear cells (i.e. monocytes) in defined quantities (Maus, Waelsch *et al.* 2003). Maus *et al.* (2003) established that only 25% of WT mononuclear CCR-2 +/+ cells were necessary to restore neutrophil recruitment into the alveoli to WT levels. However the mechanism by which monocytes facilitate and amplify neutrophil migration and whether these data apply to migration during bronchial inflammation in humans remains to be established. To delineate this relationship, in the context of CF, future murine models will require the use of *P. aeruginosa* LPS since this has been reported to be 20-fold more potent at inducing CCL-2 from an airway epithelial cell line than LPS derived from *E. coli* (Koyama, Sato *et al.* 1999). MΦs *per se* may also be an important source of CCL-2 and thus

with infection be an important component of a positive feedback loop that has hitherto been unrecognised in CF. Rosseau *et al.* (2000) showed that in addition to elevated CCL-2 protein in BAL fluids from ARDS patients, CCL-2 mRNA was significantly elevated in ARDS alveolar MΦs compared with control, implicating them as the source of this monocyte chemoattractant. This however is not supported by our microarray analysis of CF and control transcripts under constitutitve and LPS stimulated conditions that showed significantly reduced liberation of CCL2 from CF MDMs ($p<0.0001$ and $p<0.00004$, respectively). Therefore increased or decreased CCL2 production from CF MΦs may play a role in CF pathophysiology. ECs were not investigated in these studies but may also be an important source of CCL-2 (Thorley, Ford *et al.* 2007). Thus in CF, particularly during *P. aeruginosa* infection, the role of the monocyte/MΦ system may be more important than previously recognised.

Since I have demonstrated the presence of monocytic-like MΦs in the neutrophil dominated bronchial space I propose that an investigation of the 'CCL-2-CCR-2 axis' may be warranted to delineate the role of monocyte recruitment in the context of neutrophilic inflammation in CF and also COPD. However the cytokine/chemokine system contains non/redundant components and synergy that hampers understanding of these mechanisms in disease, therefore a more comprehensive investigation may be warranted (Gouwy, Struyf *et al.* 2004). Here the role of CD44, a hyaluronan receptor, may also be important and has been demonstrated in murine KO models to be important in facilitating macrophage and neutrophil recruitment into the airway and in resolving airway inflammation to non-infectious challenge (Teder, Vandivier *et al.* 2002; Hollingsworth, Li *et al.* 2007).

Following the identification of small MΦs in the CF airways I noted that the proportion of these cells increased during infective exacerbation (paediatric cohort) or were high in patients with infection but who were otherwise well (adult cohort). I examined the phagocytic role of these cells by looking at the expression of cell surface PRR receptors and particle uptake, since a failure to eradicate infection or products of inflammation by MΦs may liberate cytokines and chemokines promoting further neutrophil recruitment. A reduced phagocytic capability has been described before when examining CF MΦs (Biggar, Holmes *et al.* 1971; Thomassen, Demko *et al.* 1980; Fick, Naegel *et al.* 1981; Hornick and Fick 1990; Berger, Norvell *et al.* 1994; Alexis, Muhlebach *et al.* 2006; Di, Brown *et al.* 2006). Phagocytosis can operate via anti-microbial antibodies and/or complement which lead to enhanced binding to leukocyte Fc and/or CRs. Alternatively, PRR on leukocytes can directly bind bacterial or host components to facilitate removal. Such non-opsonic mechanisms of phagocytosis have not been investigated thus far in CF airway MΦs.

I investigated the protein expression of three PRRs, two CLRs, the mannose and DC-SIGN receptors (CD206, CD209, respectively) and a class A scavenger receptor (MARCO) on sputum MΦs and blood monocytes (Table 4-1). Due to technical constraints I chose to analyse HLA-DR positive and CD14 positive gated events only for the analysis of these receptors. Despite using a less stringent identification method I was still able to uphold our earlier findings that small MΦs predominate in CF sputum. I was subsequently able to detect, on control large MΦs, consistent expression of the mannose receptor and MARCO. Whilst I could detect mannose receptor and MARCO expression on control MΦs, I was able to show for the first time that these receptors were highly reduced on CF MΦs. In addition to this I could not detect cells surface DC-SIGN protein expression on control or CF MΦs. Furthermore I could not detect any of the three PRR

on blood monocytes. Analysis of control and CF MDMs for PRR expression may have led to an indication as to whether the differences in expression between sputum MΦs were due to an underlying phenotypic difference or state of differentiation.

The mannose receptor is a 175-kD cell surface protein, type I transmembrane protein (Figure 4-1) that has been detected on rodent airway MΦs (Stahl, Rodman *et al.* 1978; Wileman, Lennartz *et al.* 1986; Taylor, Conary *et al.* 1990; Beharka, Gaynor *et al.* 2002), human MDMs (Shepherd, Campbell *et al.* 1982; Beharka, Gaynor *et al.* 2002) and human airway MΦs but not monocytes (Shepherd, Campbell *et al.* 1982). In CF absent/reduced expression of the mannose receptor by airway MΦs represents a novel finding. Absent expression of the mannose receptor on CF small MΦs may derive from their monocyte-like nature since I could not detect expression of these receptors on control and CF blood monocytes. However reduced or absent expression of the mannose receptor on CF large MΦs may be due to methodological limitations or reflect differences in the environmental milieu between CF and control.

It is possible that using a less stringent gating procedure utilising just HLA-DR and CD14 may give rise to the presence of non-MΦ events (such as ECs) in our CF large MΦ window. A small proportion of large MΦs in CF sputum have been demonstrated in this thesis. Therefore contamination by an equally small proportion of non-MΦ events may falsely elevate the perceived number of CF large MΦs. I therefore believe that reduced overall expression of the mannose receptor in the MΦ gate more likely reflects the reduced quantity of CF large (compared to small) MΦs rather than downregulation of this receptor by the environmental milieu. Downregulation of PRR expression may reflect the environmental milieu of MΦs in the CF lung. For instance neutrophil derived oxidants, LPS and IFNγ stimulation have been demonstrated to

reduce cell surface mannose receptor expression on rat bone-marrow MΦs and a murine MΦ cell line (Bozeman, Hoidal *et al.* 1988; Shepherd and Hoidal 1990; Harris, Super *et al.* 1992; Chroneos and Shepherd 1995; Raveh, Kruskal *et al.* 1998). Products of inflammation are likely to be present in the CF airways and therefore could contribute to a downregulation of this receptor and consequently lack of detection as described in this thesis although this remains to be confirmed by looking at transcript levels of isolated CF MΦs. For example Alexis *et al.* (2006) have demonstrated reduced expression of CD14 and HLA-DR on CF MΦs due to the action of neutrophil elastase. In addition, further mechanisms may be involved since in DCs shedding of the mannose receptor has been observed producing soluble forms of this receptor (Jordens, Thompson *et al.* 1999).

The monocyte-like nature of these cells may suggest that CF small MΦs have not been resident in the airways for a sufficient length of time to allow for an upregulation of the mannose receptor to occur, since upregulation of this receptor in monocytes has been shown to occur with increasing time in culture *in vitro*, particularly between days 3-5 in the presence of GM-CSF and/or M-CSF (Shepherd, Campbell *et al.* 1982) or T helper 2 cytokines (Raveh, Kruskal *et al.* 1998). In addition, lung specific C-type lectins such as SP-A may be important since upon binding to the human MΦ SP-A receptor it initiates increases in cytosolic calcium that upregulates mannose receptor expression from intracellular pools (Beharka, Gaynor *et al.* 2002; Beharka, Crowther *et al.* 2005). It was shown that in SP-A KO murine airways *in vivo,* SP-A may contribute to approximately half of the constitutive mannose receptor expression on alveolar MΦs (Beharka, Gaynor *et al.* 2002). Interestingly SP-A has been shown to be quantitatively reduced (Postle, Mander *et al.* 1999) and/or altered by degradation in the majority of CF BAL fluids (von Bredow,

Birrer *et al.* 2001). Hence, a reduced expression of SP-A in CF may lead to a lack of upregulation of mannose receptor on CF monocytes/MΦs.

Unexpectedly I could not detect DC-SIGN – an additional CLR (Figure 4-1) - on either MΦs or monocytes from control or CF patients. Since DC-SIGN is a prominent receptor on immature and mature DCs (Engering, Geijtenbeek *et al.* 2002) this supports our earlier findings that small MΦs identified in CF are not DCs. However absent DC-SIGN expression on MΦs is in contrast to a previous observation where DC-SIGN was identified on MΦs by histochemical staining of human lung tissue sections (Soilleux, Morris *et al.* 2002). Despite this they are supported by others (Engering, Geijtenbeek *et al.* 2002; Tailleux, Pham-Thi *et al.* 2005) who could not detect DC-SIGN on human BAL MΦs or monocytes respectively from controls or patients with sarcoidosis or asthma. However Tailleux *et al.* (2005) could detect DC-SIGN on MΦs from tuberculosis infected patients suggesting that under certain pathological conditions DC-SIGN expression may be upregulated.

Using the mAb PLK-1, specific for human MARCO, I was able to detect clear expression of this receptor on the majority of control but not CF MΦs or blood monocytes. Despite murine (Elomaa, Kangas *et al.* 1995) and human (Elomaa, Sankala *et al.* 1998) MARCO transcripts not having been detected previously in the lung an increasing body of evidence now suggests that BAL and *in situ* alveolar MΦs, in addition to other tissue MΦs, do indeed express MARCO mRNA and protein (Palecanda, Paulauskis *et al.* 1999; van der Laan, Dopp *et al.* 1999; Bunn, Kobzik *et al.* 2004; Arredouani, Palecanda *et al.* 2005), in line with our findings. MARCO (Figure 4-1) is one of many receptors in the SR family that can bind modified lipoproteins and SRs have been shown in normal human monocytes to increase, like the mannose receptor, at the

protein and mRNA level with increasing time in *in vitro* culture in the presence of GM-CSF and M-CSF (van Lenten and Fogelman 1992). Additionally, human MARCO transcripts have been demonstrated to increase in psoriasis patients on IL-10 therapy (Jung, Sabat *et al.* 2004) and therefore IL-10, proposed to be reduced in CF airways, may be equally important in driving constitutive MARCO expression in the lung in control MΦs.

I was not able to demonstrate MARCO on control or CF blood monocytes indicating that, like mannose receptor expression, absence of MARCO expression on CF MΦs may be due to the monocyte-like nature of these cells or the environmental milieu in CF. Similarly to the mannose receptor, SR mRNA and protein expression on MDMs is downregulated in the presence of LPS via a TNF (but not IFNγ or IL-1β) mediated process (van Lenten and Fogelman 1992). Though MARCO is regulated differently to other SRs (e.g. SR-A) (Jozefowski, Arredouani *et al.* 2005; Arredouani, Franco *et al.* 2007) the CF airways are characterised by a greater sensitivity to the presence of LPS (Muhlebach and Noah 2002), elevated TNF (Balough, McCubbin *et al.* 1995; Bonfield, Panuska *et al.* 1995; Osika, Cavaillon *et al.* 1999; Xiao, Hsu *et al.* 2005) and downregulated IL-10 production, the latter being an intrinsic CFTR mediated defect of airway ECs (Bonfield, Konstan *et al.* 1995; Bonfield, Konstan *et al.* 1999) and T lymphocytes (Moss, Bocian *et al.* 1996; Moss, Hsu *et al.* 2000). In addition the failure to express the mannose receptor and MARCO on CF MΦs appears not to be an inherent defect due to CFTR since I was able to detect high quantities of both transcripts in MDMs following microarray analysis. Thus the immaturity of CF small MΦs and/or the CF airway environmental milieu may both be important contributors to the downregulation of MARCO and mannose receptors.

I assessed non-opsonic receptor ligand uptake on CF and control MΦs using fluorosphere particles, which had been shown previously to be a MARCO ligand (Palecanda, Paulauskis *et al.* 1999; Arredouani, Yang *et al.* 2004; Arredouani, Palecanda *et al.* 2005). The limited number of CF sputum samples and small number of MΦs recovered from each CF sample precluded us from assessing further MΦ receptor-ligand interactions. Given a greater pool of CF patients for study MΦ receptor-bacteria uptake, which may be considered to be more relevant in the context of CF, could have been studied further. However I believe that the use of particles was a good surrogate for determining MΦ phagocytic capability. In line with reduced/absent MARCO protein on CF MΦs I was then able to show that CF MΦs have a much reduced (though non-significant) capacity for uptake of a MARCO ligand compared to control (Table 4-2). Preliminary experiments to determine the optimum concentration of PLK-1 to use in blocking experiments may have yielded a more significant effect than that given here. Recruited murine monocytes have been shown previously to be poor at clearing apoptotic cells from infected airways (Jennings, Linderman *et al.* 2005) whereas MΦs ingest apoptotic (Savill, Wyllie *et al.* 1989; Sexton, Al-Rabia *et al.* 2004) and necrotic (Barker, Erwig *et al.* 2002) neutrophils. In addition to this, mature MΦs, as a source of opsonins for apoptotic cells, could also be important (Hanayama and Nagata 2005).

Previously, Alexis *et al.* (2006) using IgG coated zymosan particles and flow cytometry were also able to demonstrate a reduction in uptake by CF MΦs and neutrophils compared to control but to a lesser extent than shown here. Interestingly Alexis *et al.* (2006) also showed that uptake increased when CF MΦs were pre-incubated with SP-A and D, which is in line with the up-regulation of PRRs such as MARCO by these collectins. Conversely in the presence of LPS and CF cell-free BAL fluid, zymosan uptake by healthy MΦs and monocytes are blunted unless a

protease inhibitor is added (Alexis, Muhlebach *et al.* 2006) indicating that proteases affect MΦ aswell as neutrophil phagocytosis.

I assessed also the contribution made by MARCO on control sputum MΦs to ligand uptake by blocking the MARCO receptor with the mAb PLK-1, which has been described previously (Arredouani, Palecanda *et al.* 2005). Our data suggest that MARCO accounts for some (42%) but not all of the uptake capacity for non-opsonised particles in the three samples of bronchial MΦs studied compared to 67.03% for human alveolar MΦs published elsewhere (Arredouani, Palecanda *et al.* 2005). Higher MARCO expression on control MΦs could therefore account for higher uptake activity by these cells in contrast to CF MΦs, though other receptors (e.g. SR-A) are probably involved.

Our data suggest that the majority of CF MΦs are unable to perform non-opsonic scavenger functions due to an absence of important PRRs. I propose that the inability to perform these roles does not reflect innate defects due to mutant CFTR (though this remains possible) since in some samples I also saw expression of these receptors on MΦs. More likely it is a) a consequence of downregulation by the CF airway environmental milieu or b) due to the immaturity of the recruited cells. As a consequence decreased/absent PRR expression may be detrimental to the control of infection and impair the resolution of neutrophilic inflammation in CF.

The *in vivo* role of the mannose receptor has still to be fully resolved but appears to be more important in the homeostatic control of endogenous glycoproteins rather than infectious organisms. The mannose receptor has been demonstrated *in vitro* to be important in binding and phagocytosing *Candida* sp., fungi, *Mycobacterium tuberculosis, Klebsiella* and *Streptococcus*

*pneumoniae* (McGreal, Martinez-Pomares *et al.* 2004) via the juxtaposition of CTLD4 and its cystiene-rich region (Boskovic, Arnold *et al.* 2006) and using Cdc42 and Rho B signalling proteins (Zhang, Zhu *et al.* 2005). Despite these *in vitro* reports a mannose receptor KO murine model of *Pneumocystis carinii* (Swain, Lee *et al.* 2003) and *Candida albicans* (Lee, Zheng *et al.* 2003) infection failed to show any difference in mortality or severity of illness between WT and KO mice. Of these organisms *Candida albicans* is prevelant in CF having been reported to be present in 29.4% (Bauernfeind, Bertele *et al.* 1987) and 75.5% (Bakare, Rickerts *et al.* 2003) of CF patients. However, neither of these organisms appears to be important in the context of progressive CF lung disease (Lyczak, Cannon *et al.* 2002; Gibson, Burns *et al.* 2003).

Interestingly however, a receptor with specificity for yeast mannan and mannose has been demonstrated *in vitro* to be a candidate receptor for *P. aeruginosa* in human MDMs (Speert, Wright *et al.* 1988). Examination of non-opsonic phagocytosis of *P. aeruginosa* indicate that a receptor with the same characteristics as the mannose receptor is responsible (Speert, Wright *et al.* 1988), though this could also be due to other mannose receptor family members (McGreal, Martinez-Pomares *et al.* 2004). Unfortunately further identification of this receptor has not been followed up and it remains to be seen whether other reported mannose receptor bacterial ligands, particularly *P. aeruginosa* can be confirmed in an *in vivo* model of infection.

In contrast mannose receptor KO mice had reduced clearance of a range of serum (but not tissue) lysosomal hydrolases and collagen derived products (Stahl, Rodman *et al.* 1978; Lee, Evers *et al.* 2002; Martinez-Pomares, Wienke *et al.* 2006). Mannose receptor binding of collagen occurs through the fibronectin domain of the tertiary structure (Figure 4-1) and is optimum when the mannose receptor forms oligomeric structures on the cell surface (Martinez-Pomares, Wienke *et*

*al.* 2006). Thus the mannose receptor may have an important role in clearing collagen fragments from the site of inflammation that may otherwise initiate neutrophil recruitment.

In addition, MPO binding to human MDMs were shown to be inhibited by mannan indicating mannose receptor involvement (via CTLD4) prior to delivery into intracellular lysosomes (Shepherd and Hoidal 1990). Other investigators could not repeat this inhibition albeit in undifferentiated blood leukocytes and instead show that the $\beta_2$ integrin CD11b/CD18 rather than mannose receptor may be important for this uptake (Johansson, Patarroyo *et al.* 1997). Elevated MPO (and other neutrophil products) has been detected in CF sputum (Goldstein and Doring 1986; Worlitzsch, Herberth *et al.* 1998; Downey, Martin *et al.* 2007) and CF nasal polyps (Claeys, Van Hoecke *et al.* 2005) and is believed to originate from degenerating neutrophils (Conese, Copreni *et al.* 2003). MPO levels in CF increase during an exacerbation but return to normal levels following treatment in hospital indicating a link with the pathophysiology of CF (Sloane, Lindner *et al.* 2005). Sloane *et al.* (2005) also demonstrated that MPO protein was one of the strongest predictors with $FEV_1$ in CF. Reduced clearance of MPO and other oxidants by the mannose receptor may induce pro-inflammatory cytokine and chemokine secretion by increasing the activity of NF-κB, as demonstrated in acute lung injury (Nys, Preiser *et al.* 2005) and promoting the formation of ROIs (Worlitzsch, Herberth *et al.* 1998). Though the mannose receptor does not appear to have a role in binding CF associated pathogens its role as a PRR for endogenous enzymes such as MPO and collagen may be important to CF pathophysiology.

Further to this our findings show that the majority of CF MΦs do not express MARCO protein and are thus incapable of phagocytosing MARCO ligands. MARCO has been demonstrated to bind modified lipoproteins, oxidised lipids, *S. pneumoniae*, *S. aureus*, *E. coli* and particulates

(such as titanium oxide ($TiO_2$) and latex particles) via its cysteine rich domain at the C-terminal end (Figure 4-1) (Palecanda, Paulauskis *et al.* 1999; Arredouani, Yang *et al.* 2004; Arredouani, Palecanda *et al.* 2005; Dahl, Bauer *et al.* 2007). *In vivo* studies utilising MARCO KO mice have shown that MARCO may be the most important SR for clearing non-opsonised bacteria and particulates but not yeast from the airways (Arredouani, Yang *et al.* 2004; Arredouani, Palecanda *et al.* 2005). Reduced expression of MARCO on the majority of CF MΦs may, in part, explain the predominance of *S. aureus* in the sputa of CF patients (Lyczak, Cannon *et al.* 2002; Gibson, Burns *et al.* 2003). *S. aureus* colonises the nose in healthy individuals and therefore the presence of this organism in the lower respiratory tract indicates infection (Lyczak, Cannon *et al.* 2002). However, despite its early prominance in the course of CF, the role of *S. aureus* in CF airway disease has been disputed (Lyczak, Cannon *et al.* 2002; Mayer-Hamblett, Aitken *et al.* 2007).

Absent or reduced expression of PRRs by CF MΦs may lead to the persistence of bacteria, particulates, endogenous enzymes or products of inflammation that could potentiate chronic inflammation compromising epithelial integrity and thus lung function in CF. In support of this *S. pneumoniae* challenged MARCO KO mice exhibited a significantly higher airway bacteria burden, higher 4 hr and 24 hr neutrophilic inflammation and elevated TNF and MIP-2 levels culminating in 100% mortality by 10 days compared to 40% mortality with WT mice (Arredouani, Yang *et al.* 2004). A similar difference in mortality was observed following $TiO_2$ administration (Arredouani, Yang *et al.* 2004), and under oxidative stress in MARCO KO mice (Dahl, Bauer *et al.* 2007). Interestingly Arredouani *et al.* (2004) showed that MARCO KO alveolar MΦs when exposed to $TiO_2$ *in vitro* did not display elevated release of TNF or MIP-2 compared to WT. Therefore Arredouani *et al.* (2004) conclude that the exuberant inflammatory response seen in this model may be due to increased interaction between $TiO_2$ and other airway

cells. Hence MARCO may play an important role in protecting the airway epithelium from oxidative and particulate damage.

The persistence of ROI may also inhibit TGF-β mediated suppression of MAPK and thus CXCL-8 production by human alveolar MΦs (Xiao, Freire-de-Lima *et al.* 2006). In addition, lower IFNγ was observed in a MARCO KO murine model of airway silica exposure (Hamilton, Thakur *et al.* 2006) further demonstrating that absent PRRs can lead to reduced or dysregulated inflammatory responses. MARCO may also modulate cytokine secretion directly since MARCO KO murine peritoneal MΦs produced a significantly lower IL-12 protein and nitric oxide levels following stimulation compared to WT (Jozefowski, Arredouani *et al.* 2005; Jozefowski, Sulahian *et al.* 2006). However a direct role for MARCO in modulating cytokine release has yet to be confirmed for human or murine alveolar MΦs. Further to these roles, MARCO is important to the reorganisation of the actin cytoskeleton and formation of plasma membrane processes required for increasing cell surface area, further facilitating particulate/bacteria binding (Pikkarainen, Brannstrom *et al.* 1999).

In addition to the above, small MΦs may contribute directly to CF pathophysiology. I hypothesise that CF small MΦs, identified here, are capable of directly contributing to the pathophysiological events seen in CF airway disease. In 5 cases of CF, Busiek *et al.* (1995) showed that monocytes, residing in the vasculature and interstitial space of the lung, but not terminally differentiated alveolar MΦs reacted intensely with an anti-MMP-7 antibody (Busiek, Baragi *et al.* 1995). LPS increases MMP-7 dose dependently in MDMs *in vitro* (Busiek, Baragi *et al.* 1995) that could have accounted for this expression in monocytes upon entering the CF airways. MMP-7 cleaves pro-forms of MMP-9 and therefore overproduction may facilitate

neutrophil accumulation by degradation of ECM (Burke 2004). In contrast MMP-7 KO mice had reduced numbers of neutrophils in BAL fluid, due to a lack of Syndecan-1 shedding (Parks 2003), indicating that they can play important roles in health and disease.

Newly recruited monocytes may also be important in liberating pro-inflammatory cytokines, chemokines and ROIs upon entering the CF airspace and further contributing directly to neutrophilic inflammation. In line with this hypothesis Kiemle-Kallee *et al.* (1991) have demonstrated increased ROI release by immature airway MΦs. Moreover Pantelidis *et al.* (1997) characterised and compared monocyte and alveolar MΦ CXCL-8 secretion from patients with different airway diseases and controls and they showed that at least a 2-fold significantly greater percentage of monocytes secrete CXCL-8 compared to alveolar MΦs under constitutive and LPS stimulated conditions. Strikingly, the quantity of constitutive and LPS stimulated CXCL-8 produced by blood monocytes was greater compared to MΦs across all groups investigated (Pantelidis, Southcott *et al.* 1997). Finally, Pantelidis *et al.* (1997) were also able to demonstrate that the CXCL-8 secreting MΦs in the airways of fibrosis alveolitis patients had a smaller phenotype (22.8 μm) than the average reported (38 μm) and moreover this correlated with the proportion of neutrophil in the BAL fluid of these patients. Thus the monocyte-like nature of small MΦs and their large proportion may indicate greater CXCL-8 production compared with controls. Furthermore CF monocytes exhibit a greater sensitivity to LPS and thus produce significantly more CXCL-8 protein compared to controls (Zaman, Gelrud *et al.* 2004). However although this has been confirmed in CF murine bone-marrow MΦs it is unclear whether this is also the case for human airway MΦs. Intriguingly I found differential levels of CXCL-8 transcripts (Table 5.7) between CF MDMs and control under constititutive (lower in CF) and LPS stimulated (higher in CF) conditions. This suggests that an increased proportion of CF small

MΦs could contribute to neutrophil recruitment either indirectly via enhanced monocyte recruitment via CC and CXC chemokine receptor ligands, as described earlier, or directly via enhanced production of chemokines (e.g. CXCL-8), ROI or proteases.

COPD small MΦs were demonstrated to produce significantly greater quantities of TNF protein under constitutive and LPS stimulated conditions compared to control large MΦs (Frankenberger, Menzel *et al.* 2004). Therefore an elevated proportion of small MΦs in airway disease could possibly account for much of the TNF production in the airways of COPD patients and also in CF, though this remains to be confirmed. TNF is a pleiotropic pro-inflammatory cytokine that can potentially play a role in many of the pathological events associated with the inflammatory response in CF as highlighted previously (Section 1.2.4.5). A higher expression of CD14 on small MΦs, due to their monocyte-like phenotype, may account for much of this difference. CD14 has an important but not exclusive role in delivering the LPS-LBP complex to the signalling TLR4/MD-2 LPS receptor, triggering the liberation of TNF amongst other cytokines (Palsson-McDermott and O'Neill 2004). COPD and CF small MΦs express large quantities of CD14 on their surface (compared to control MΦs) and thus may be particularly sensitive to LPS stimulation. Further to this CF MDMs were shown to have a 100-fold greater sensitivity and transcriptional activity in response to LPS than control therefore producing more TNF mRNA and thus protein, dose and time dependently (Pfeffer, Hueckstead *et al.* 1993). Elevated TNF production by rodent bone-marrow MΦs has also been reported in a murine G551D CF model (Thomas, Costelloe *et al.* 2000), these findings have not yet been independently confirmed in MDM or from *ex vivo* airway MΦs in human CF patients.

With these data in mind I was unable to find any difference between control and CF MDMs for TNF at the mRNA level under constitutive or LPS-stimulated conditions (Figure 5-6). Therefore I could not confirm the results obtained by Pfeffer *et al.* (1993), though this does not exclude a difference at the protein level. In the study by Pfeffer *et al.* (1993) despite recruiting 12 CF patients compared to just 5 studied in this thesis they chose CF patients with an average $FEV_1$ of just 33% predicted compared to 53% in this study. CF patients with a homozygous ΔF508 mutation may have a strong inflammatory response (and thus worse lung function) based on polymorphisms in inflammatory genes like TNF and IL-1, as have been described for TGF-β. Selecting more severe cases may therefore bias the selection towards patients with a higher expression of TNF. Thus higher TNF levels in the study by Pfeffer *et al.* (1993) could be due to selection and it may not reflect an inherent property of CF MΦs. In line with this, differences in TNF production could not be found in monocytes from stable CF and controls under LPS stimulated conditions (Elborn, Norman *et al.* 1992), similar to the data presented in this thesis. However these data regarding inherent defects do not exclude an important role for small MΦ derived TNF in the pathophysiological events of CF airway disease. Despite glucocorticoid therapy not having an effect on the proportion of small MΦs in our CF cohort (Figure 3-9) they may have an important therapeutic effect in some patients by suppressing MΦ TNF production (Waage and Bakke 1988; Pfeffer, Hueckstead *et al.* 1993).

CXCL-8 and TNF production are two examples of documented intrinsic differences between CF and control monocytes that have received little independent confirmation. I chose to examine this further by comparing constitutive and LPS stimulated transcript levels between CF and control MDMs encoding for the entire human genome. Examples of the most up and down regulated genes in CF compared to control are shown in Tables 5-2 to 5-5 and are involved in a

variety of processes. To our knowledge this is the first time that this kind of analysis has been performed on CF MDMs. Of potential interest are the CXC chemokines (Table 5-7), where many of the transcript levels appaered to be downregulated in CF compared to control suggesting that there may be a hypo-response to infection. This requires confirmation by PCR and further investigation.

From this large data set, however, I found that α-/β-tryptase was significantly upregulated in CF MDMs compared to control MDMs over different hybridisation probes. HLA-DQ (downregulated in CF – Table 5-3) appears to be a further interesting candidate. The term tryptase covers at least 8-9 different types, which share > 90% sequence homology, that have been identified in human mast cells (Fiorucci and Ascoli 2004). In mast cells they are tetrameric neutral serine proteases that are kept under an acidic pH and bound to heparin proteoglycans inside mast cell granules to prevent autolysis and degradation of proteins. To-date there is very little information in the current literature regarding MΦ derived tryptase, which made it an attractive option for further study. A wide range of roles have been ascribed to tryptase, which currently has no known physiologic inhibitors *in vivo* (Hallgren and Pejler 2006). Much of these effects may occur through cleavage of proteinase-activated receptor 2, a G-protein couple receptor present on a wide range of cells (Kawabata and Kawao 2005). Reported effects of tryptase, in the context of CF include stimulating CXCL-8 production from ECs (Compton, Cairns *et al.* 1998), mitogenic activity for fibroblasts and airway smooth muscle, increases collagen transcript levels in fibroblasts and activates MMPs (Fiorucci and Ascoli 2004; Hallgren and Pejler 2006).

Based on the microarray data our hypothesis was that tryptase expression would be higher in CF compared to control MDMs and thus may be involved in CF pathophysiology. I used RT-PCR to confirm or refute these microarray differences. Tryptase in relation to mast cells has been extensively studied (Fiorucci and Ascoli 2004; Hallgren and Pejler 2006), however RT-PCR detection of α- and β-tryptase mRNA in MDMs is to our knowledge a novel finding. However this was not surprising since both transcripts have been detected prior to this in cell lines of the monocyte/MΦ lineage, such as U-937 (β-tryptase) and MM6 (α-tryptase) but not THP-1 or peripheral blood monocytes (Huang, Abrink *et al.* 1993; Schwartz, Min *et al.* 2003; Welker, Grabbe *et al.* 2004). In addition HMC-1 cells have been demonstrated by genomic DNA analysis not to contain the gene for α-tryptase (Soto, Malmsten et al. 2002) consistent with the electrophoresis data presented in chapter 5. Our detection of α- but not β-tryptase in the MM6 cell line is also in line with these studies. Furthermore when I examined CF and control blood monocytes for expression of α-or β-tryptase (data not shown) I failed to detect either transcript suggesting that tryptase expression is only expressed during monocyte maturation into MΦs.

In contrast to the microarray data from pooled samples, I was not able to confirm a significant difference for α- or β-tryptase in a cohort of CF and control donors from adults (Figure 5-3) or children (Figure 5-5) when looking at an independent set of individual samples. In some CF donors though, I noted much higher levels of α-tryptase transcripts compared to control whereas β-tryptase transcripts were identical. However, unlike β-tryptase, the role of α-tryptase *in vivo* is not clear. For example despite α- and β-tryptase sharing 92-95% sequence similarity (Fiorucci and Ascoli 2004), α-tryptase has been proposed to be resistant to proteolytic activation and therefore is secreted (but not stored) from MM6 (and presumably MDMs) as a precursor protein only (Schwartz, Min *et al.* 2003). Also the active site of α-tryptase is believed to be kinked and

thus substrate binding and processing is believed not to be favourable (Hallgren and Pejler 2006). Furthermore α-tryptase has been proposed to be absent from ~29% of the human population and based on its quartenary structure enzymatically silent (Soto, Malmsten *et al.* 2002; Hallgren and Pejler 2006). Since I could not find any intrinsic differences at the transcript level our data suggest that MΦ derived β-tryptase may not have a role in CF. Other differences revealed by microarray analysis include increased mRNA for members of the aldo-keto reductase family, MMP-1, IL-1Ra and decreased HLA-DQ in CF MΦs compared to control remain to be confirmed and their significance assessed. A greater pool of ΔF508 +/+ CF donors would have perhaps generated more sensitive microarray data for analysis.

In summary, this thesis demonstrates for the first time that small MΦs are the major MΦ population within the CF airways. The data presented in this thesis suggest that they may be recently recruited monocytes from blood since small MΦs are monocyte- rather than MΦ-like. An increased proportion of small MΦs in the bronchial region may exacerbate neutrophilic inflammation via mechanisms that remain to be characterised, though possibilities have been suggested in this thesis. I was also able to demonstrate that the majority of CF MΦs fail to express PRRs involved in host defence and maintaining tissue homeostasis. Therefore failure to sequester by phagocytosis products of inflammation and infection can contribute to inflammatory-mediated lung damage, which may be a contributor to progressive lung disease in CF. In addition and in contrast to previous reports I was not able to find any inherent transcriptional differences between CF and control MDMs due to mutated CFTR. Therefore a gain or loss of function mutation in CF MΦs caused by mutated CFTR appears to be unlikely based on data presented here and requires further analysis outside the scope of this thesis.

# 7 Further Work

The identification of small sputum MΦs in the CF airway provides a novel aspect to the examination of chronic inflammation in this disease. A cross sectional approach was taken in this thesis, however more time and/or a greater cohort size may have allowed longitudinal studies of CF patients to be undertaken. In addition, a greater stringency of patient recruitment, in order to identify possible relationships with infection status or severity of disease would also have been an interesting analytical approach.

A more quantitative approach to that taken in this thesis may also be an interesting avenue for further study now that there is evidence that small MΦs are prominent in the CF airways. With this in mind the staining and analysis protocol described herein can be improved and expanded upon to facilitate this. Technological improvements in the flow cytometry field can support the analysis of an increasing number of parameters as described by Perfetto *et al.* (2004). In this regard the absolute number of small compared to large MΦs may also be important reflecting both the quantitative and qualitative nature of MΦs in CF airway. Furthermore absolute counts of other cell types can be undertaken. Phenotypic differences between these cell types, likely gives rise to different functions in the airway and thus a more detailed analysis of these cell types will be required to delineate their role(s).

Purification and molecular analysis of small MΦs in CF will play an important role in this and may yield information on the mechanism for increased recruitment of small MΦs into the airway. Therapeutic targets that alter the activity of small MΦs in CF may provide further clues to their role(s) in this disease and offer new avenues of clinical intervention.

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# 9 Appendix

See the following pages for a copy of the volunteer information sheet and consent form used in this study.

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

**INFORMATION LEAFLET for *Healthy Volunteers***

Tel: 0116 2541414
Fax: 0116 2585631
Minicom: 0116 2586878

**Title of Study: Small macrophages and chronic infective Paediatric lung disease**

**Principal Investigator:**

Dr Jonathan Grigg
Department of Child Health
University of Leicester
PO Box 65
Leicester LE2 7LX

**You may contact the Principal Investigator by:**

Telephone - 0116 252 5810 (work)
Facsimile - 0116 252 3282
e-mail –jg33@le.ac.uk
Direct Page: 07699745825

Dear Parent,

We are carrying out a study to understand the role a type of inflammatory cell (cell that fights infection) in lung infection and subsequent lung damage. To help you decide, this information leaflet explains why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you can take part.

**Who is organising and funding the research?**

The study is being organised by Dr Jonathan Grigg. The research is funded by the Children's Research Fund Liverpool.

**What is the purpose of this study?**

The purpose of this study is to measure the activity of a certain blood cell called the monocytes/macrophages during and after exacerbations of chest problems in patients suffering with long-standing chest infections. We believe that this cell is an important part of the process by which lung damage occurs in this group.

**Why have I been chosen?**
You have been chosen because you do not have problems such as recurrent chest infections. It would be important to compare the activity of this cell in a healthy individual with that in a patient with long standing chest infection.

**Do I have to take part? What happens if I do not wish to take part in this study or wish to withdraw from the study?**
It is up to you to decide whether or not to you take part. If you do decide to participate, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**What will be involved I decide to participate in the study?**
You will be required to attend the lung function laboratory of the Leicester Children's Asthma Centre (at the Leicester Royal Infirmary). We will ask you about the general health and if you are on any medications. We will then obtain a sample of your sputum and blood sample.

Normally sputum comes up the breathing tubes and is swallowed. The technique we will use aims to collect the sputum from the deeper regions of your lungs. You will be asked to breathe in a salty mist (nebuliser) through a mouthpiece. The solution tastes a little salty, but not excessively so (similar to standing on a beach on a stormy day). After 5 minutes we will ask you to cough vigorously into a pot, and if nothing comes up to inhale for another 5 minutes. The test finishes after 3 such nebulisations. We will look at the type of cells that are present in the sputum and specifically analyse the monocytes by special techniques. In all, your visit should take no longer than 45 minutes.

Your opinion is of great importance to us and if at any time you feel uncomfortable and wish to stop, we will do so immediately.

We will then obtain up to 20 millilitres (2 tablespoonfuls) of your blood under aseptic precautions. An experienced nurse or doctor will do this. **This step is optional and you are free to consent for providing sputum sample only**.

**Are there any risks?**
This way of collecting sputum has been used on many healthy children and adults safely. It is one of the treatment options for both adults and children with chronic lung infections as it helps to clear the sputum from lungs. This procedure is also frequently used to monitor asthma patients in many centres.

The only known side effect is mild wheezing which is very unlikely in healthy individuals. But to make sure that this doesn't happen, we will give you 4 puffs of salbutamol, a medicine that is widely used in asthma. This will open up the tubes in lungs and make breathing easier. As far as we are aware, this medication is safe and free from serious side effects. We will also check for

any airway narrowing by asking you to blow into a device called a spirometer at regular intervals.

Taking blood samples is entirely safe and unlikely to result in any harm.

**What is the drug or procedure that is being tested?**
We are not testing any drug or procedure.

**What are the possible benefits of taking part?**
It is unlikely that participation in this trial will provide you with any clinical benefit. However, the knowledge gained from the investigation will provide information that could influence the treatment of adults and children with long standing chest problems. This might ultimately benefit many children and adults.

**What happens when the research study stops?**
Treatment is not being provided as part of this study, so neither you nor anyone else will be disadvantaged when the study has finished. The results from all the participating children will be analysed once the research study stops.

**What if something goes wrong?**
We are not testing any medicines or procedure, so adverse reactions to a study drug will not occur. The collection of the sputum by the above method is a safe procedure and is extremely unlikely to cause you harm.

Medical research is covered for mishaps in the same way as receiving treatment in the NHS. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Regardless of the compensation arrangements, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

**Will my participation in this study be kept confidential?**
All information that is collected about you during the course of the research will be treated with the usual degree of confidentiality under the Data Protection Act. Any information about you that leaves the hospital will have name and address removed so that you cannot be recognised from it. However, we will inform your general practitioner that you are taking part in the study.

**What will happen to the results of the research study?**
The results of the study are likely to be published in a medical journal. It is unlikely that this will occur before September 2006. No individual who takes part in the research will be identified in any report/publication.

**Will I receive out of pocket expenses?**
You will fully refunded for travelling and parking expenses incurred as a result of taking part in this study.

**Contact for Further Information**
If you have any problems, concerns or questions about this study, you should contact Dr Jonathan Grigg, Dept of Child Health, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX Telephone – 0116 2525810).

**Thank you for carefully reading this information. You will be given a copy of this Information Sheet and a signed consent form to keep.**

Identification Number for this trial:

University Hospitals of Leicester NHS
NHS Trust

**Leicester Royal Infirmary**
Infirmary Square
Leicester
LE1 5WW

**PARENT INFORMATION LEAFLET for *Children with Chronic Lung Infection***

**Title of Study: Small macrophages and chronic infective paediatric lung disease**

**Principal Investigator:**

Dr Jonathan Grigg
Department of Child Health
University of Leicester
PO Box 65
Leicester LE2 7LX

**You may contact the Principal Investigator by:**

Telephone - 0116 252 5810 (work)
Facsimile - 0116 252 3282
e-mail –jg33@le.ac.uk

Dear Parent,

We are carrying out a study to understand the role a type of inflammatory cell (cell that fights infection) in lung infection and subsequent lung damage. To help you decide, this information leaflet explains why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not your child can take part.

## Who is organising and funding the research?

The study is being organised by Dr Jonathan Grigg

It is funded by The Children's Research Fund

**What is the purpose of this study?**
The purpose of this study is to measure the activity of a certain blood cell, the monocytes during and after exacerbations of chest problems in children suffering with long-standing chest infections. We believe that this cell is an important part of the process by which lung damage occurs in this group of children.

**Why has my child been chosen?**
We would like your child to take part because he or she has a long-standing problem that may predispose to chest infections.

**Does my child have to take part? What happens if I do not wish my child to take part in this study or wish to withdraw him/her from the study?**
It is up to you to decide whether or not to let your child take part. If you do decide to let your child take part you will be given this information sheet to keep and be asked to sign a consent form. If you let your child take part you are still free to withdraw your child at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care your child receives in anyway.

**What will be involved if my child takes part in the study?**
You and your child will be asked to attend the lung function laboratory of the Leicester Children's Asthma Centre (at the Leicester Royal Infirmary). We will ask you and your child about the general health, chest symptoms and any medications. Firstly we will obtain a sample of your child's sputum as described below.

Normally sputum comes up the breathing tubes and is swallowed. The technique we will use aims to collect the normal sputum in a pot. Your child will be asked to breathe in a salty mist through a mask, which tastes a little salty, but not excessively so. After 5 minutes we will ask him/her to cough vigorously into a pot, and if nothing comes up to inhale for another 5 minutes. The test finishes after 15 minutes. We will look at the type of cells that are present in the sputum and specifically analyse the monocytes by special techniques. In all, your visit should take no longer than 30 minutes.

Your child's opinion is of great importance to us and if at any time she/he objects we will stop the study immediately.

Secondly if it is *acceptable* to you and your child, we will take a small blood sample (1-2 teaspoonful). This will be done after applying a local anaesthetic cream to the hand so that the part of hand is numb and your child should not experience too much discomfort.



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Last saved 20 May 2004

*However, the blood sample is optional and you are free to consent for the giving the sputum sample only.*

**Are there any risks?**

This way of collecting sputum has been used on many healthy children, adults and children with cystic fibrosis. In fact this is one of the treatment options for children with cystic fibrosis to help them clear sputum from their lungs. The only known side effect is mild wheezing. But to make sure that this doesn't happen, we will give your child 4 puffs of salbutamol, a medicine that is widely used in asthma. This will open up the tubes in lungs and make breathing easier. As far as we are aware, this medication is totally safe and free from side effects. We will also check for any airway narrowing by asking your child to blow into a device called a spirometer.

The blood taking will be performed by an experienced Paediatric doctor or nurse. As we would be using the magic cream this procedure will not cause too much of discomfort or pain.

**What are the possible benefits of taking part?**

It is unlikely that participation in this trial will provide your child with any clinical benefit. However, the knowledge gained from the investigation will provide information that could influence the treatment of children with long standing chest problems. This might ultimately benefit many children.

**What happens when the research study stops?**

Treatment is not being provided as part of this study, so neither you nor anyone else will be disadvantaged when the study has finished. The results from all the participating children will be analysed once the research study stops.

**What if something goes wrong?**

We are not testing any medicines or procedure, so adverse reactions to a study drug will not occur. The collection of the sputum by the above method is a safe procedure and is extremely unlikely to cause your child harm.

Medical research is covered for mishaps in the same way as receiving treatment in the NHS. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Regardless of the compensation arrangements, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

**Will my child's taking part in this study be kept confidential?**
All information that is collected about your child and his/her family during the course of the research will be treated with the usual degree of confidentiality under the Data Protection Act. Any information about your child and the family that leaves the hospital will have his/her name and address removed so that neither the child nor the family can be recognised from it. However, we will inform your child's general practitioner that he/she is taking part in the study.

**What will happen to the results of the research study?**
The results of the study are likely to be published in a medical journal. It is unlikely that this will occur before September 2006. No child who takes part in the research will be identified in any report/publication.

**Will I receive out of pocket expenses if I allow my child to take part in this study?**
You will fully refunded for travelling and parking expenses incurred as a result of taking part in this study.

**Contact for Further Information**
If you have any problems, concerns or questions about this study, you should contact Dr Jonathan Grigg, Dept of Child Health, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX Telephone – 0116 2525810.

**Thank you for carefully reading this information. You will be given a copy of this Patient Information Sheet and a signed consent form to keep.**

University Hospitals of Leicester NHS
NHS Trust

**Leicester Royal Infirmary**
Infirmary Square
Leicester
LE1 5WW

**INFORMATION LEAFLET for *Adults with Chronic Lung Infection***

**Title of Study: Small macrophages and chronic infective lung disease**

**Principal Investigator:**

Dr Jonathan Grigg
Department of Child Health
University of Leicester
PO Box 65
Leicester LE2 7LX

**You may contact the Principal Investigator by:**

Telephone - 0116 252 5810 (work)
Facsimile - 0116 252 3282
e-mail –jg33@le.ac.uk
Direct Page: 07699745825

Dear Sir/Madam,

We are carrying out a study to understand the role of a type of inflammatory cell (cell that fights infection) in lung infection and lung damage. To help you decide, this information leaflet explains why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you can take part.

**Who is organising and funding the research?**

The study is being organised by Dr Jonathan Grigg.
It is funded by The Children's Research Fund
6 Castle Street, Liverpool, L2 ONA

**What is the purpose of this study?**
The purpose of this study is to measure the activity of a certain blood cell called the monocytes/macrophage during and after exacerbations of chest problems in patients with long-standing chest infections. We believe that this cell is an important part of the process by which lung damage occurs in this group.

**Why have I been chosen?**
We would like you to take part because you may have a condition that may predispose to repeated chest infections.

**Do I have to take part? What happens if I do not wish to take part in this study or wish to withdraw from the study?**
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive in anyway.

**What will be involved if I decide to participate in the study?**

You will be required to attend the lung function laboratory of the Leicester Children's Asthma Centre (at the Leicester Royal Infirmary). We will ask you about the general health, chest symptoms and any medications. We will then obtain a sample of your sputum and blood.

Normally sputum comes up the breathing tubes and is swallowed. The technique we will use aims to collect the sputum from deeper regions of your lungs. You will be asked to breathe in a salty mist (nebuliser) through a mouthpiece. This tastes a little salty, but not excessively so (similar to standing on a beach on a stormy day). After 5 minutes we will ask you to cough vigorously into a pot, and if nothing comes up to inhale for another 5 minutes. The test finishes after 3 such nebulisations. We will look at the type of cells that are present in the sputum and specifically analyse the monocytes by special techniques. In all, your visit should take no longer than 45 minutes.

Your opinion is of great importance to us and if at any time you feel uncomfortable and wish to stop we will do so immediately.

We will then obtain up to 20 millilitres (2 tablespoonful) of your blood at the same appointment to study the above inflammatory cell in your blood. This will be performed by experienced nurse or doctor**. This step is optional and you are free to consent for providing sputum sample only.**

**Are there any risks?**
This way of collecting sputum has been used on many healthy adults and adults with chronic lung problems e.g. cystic fibrosis, COPD. In fact this is one of the treatment options for adults and children with cystic fibrosis to help them clear sputum from their lungs. It is also used in many centres to monitor asthma patients.

The only known side effect is mild wheezing. But to make sure that this doesn't happen, we will give you 4 puffs of salbutamol, a medicine that is widely used in asthma. This will open up the tubes in lungs and make breathing easier. As far as we are aware, this medication is safe and free

from serious side effects. We will also check for any airway narrowing by asking you to blow into a device called a spirometer.

Taking blood samples is entirely safe procedure and unlikely to cause you any harm.

**What are the possible benefits of taking part?**
It is unlikely that participation in this trial will provide you with any clinical benefit. However, the knowledge gained from the investigation will provide information that could influence the treatment of adults and children with long standing chest problems. This might ultimately benefit many adults and children.

However, a part of obtained sample will be sent to the Microbiology lab to detect any infection. This may help your consultant in treatment of your chest problem.

**What happens when the research study stops?**
Treatment is not being provided as part of this study, so neither you nor anyone else will be disadvantaged when the study has finished. The results from all the participants will be analysed once the research study stops.

**What if something goes wrong?**
We are not testing any medicines or procedure, so adverse reactions to a study drug will not occur. The collection of the sputum by the above method is a safe procedure and is extremely unlikely to cause you harm.

Medical research is covered for mishaps in the same way as receiving treatment in the NHS. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Regardless of the compensation arrangements, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

**Will my participation in this study be kept confidential?**
All information that is collected about you during the course of the research will be treated with the usual degree of confidentiality under the Data Protection Act. Any information about you that leaves the hospital will have name and address removed so that you cannot be recognised from it. However, we will inform your general practitioner that you are taking part in the study.

**What will happen to the results of the research study?**
The results of the study are likely to be published in a medical journal. It is unlikely that this will occur before September 2006. No participant will be identified in any report/publication.

**Will I receive out of pocket expenses if I take part in this study?**
You will fully refunded for travelling and parking expenses incurred as a result of taking part in this study.

If you have any problems, concerns or questions about this study, you should contact Dr Jonathan Grigg, Dept of Child Health, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX Telephone – 0116 2525810).

**Thank you for carefully reading this information. You will be given a copy of this Information Sheet and a signed consent form to keep.**

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 2541414
Fax: 0116 2585631
Minicom: 0116 2586878

# PARENT CONSENT FORM *for Healthy Children*

**Title of Study:** **Small macrophages and chronic infective paediatric lung disease**

**Principal Investigator:** Dr Jonathan Grigg
Department of Child Health
University of Leicester
PO Box 65
Leicester LE2 7LX

**You may contact the Principal Investigator by:**
Telephone – 0116 252 5810 (work)
Facsimile – 0116 252 3282
e-mail – jg33@le.ac.uk

**Please initial box**

1. I confirm that I have read and understand the parent information sheet [uhl9200is-p(4)031111.doc] for the above study and have had the opportunity to ask questions. ☐
2. I understand that my child's participation is voluntary and that I am free to withdraw him/her at any time, without giving any reason, without any future medical care he/she may receive, or legal rights, being affected. ☐
3. I understand that my child will not be identified in any document relating to the trial. ☐
4. I agree for my child to take part in the above study. ☐

| Name of Parent | Date | Signature |
|---|---|---|
| Name of Person taking consent | Date | Signature |
| Researcher | Date | Signature |

1 for patient; 1 for researcher;



Trust Headquarters, Glenfield Hospital, Groby Road, Leicester, LE3 9QP
Tel: 0116 2583188 Fax: 0116 2563187 Website: www.uhl-tr.nhs.uk
Chairman Mr Philip Hammersley CBE Chief Executive Dr Peter Reading

University Hospitals of Leicester NHS
NHS Trust

# PARENT CONSENT FORM *for Children with Chronic Lung Infection*

**Title of Study:** **Small macrophages and chronic infective paediatric lung disease**

**Principal Investigator:** Dr Jonathan Grigg
Department of Child Health
University of Leicester
PO Box 65
Leicester LE2 7LX

**You may contact the Principal Investigator by:**
Telephone – 0116 252 5810 (work)
Facsimile – 0116 252 3282
e-mail – jg33@le.ac.uk

**Please initial box**

1. I confirm that I have read and understand the parent information sheet (version number 3) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my child's participation is voluntary and that I am free to withdraw him/her at any time, without giving any reason, without any future medical care he/she may receive, or legal rights, being affected. ☐

3. I understand that my child will not be identified in any document relating to the trial. ☐

4. I agree for my child to take part in the above study. ☐

| | | |
|---|---|---|
| Name of Parent | Date | Signature |
| Name of Person taking consent | Date | Signature |
| Researcher | Date | Signature |

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 2541414
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Minicom: 0116 2586878

# CONSENT FORM *for Adult Volunteers*

**Title of Study: Small macrophages and chronic infective Paediatric lung disease**

**Principal Investigator:** Dr Jonathan Grigg
Department of Child Health
University of Leicester
PO Box 65
Leicester LE2 7LX

**You may contact the Principal Investigator by:**
Telephone – 0116 252 5810 (work)
Facsimile – 0116 252 3282
e-mail – jg33@le.ac.uk

**Please initial box**

1. I confirm that I have received information about the above study and have had opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without any future medical care I may receive, or legal rights, being affected. ☐

3. I understand that I will not be identified in any document relating to the trial. ☐

4. I agree to take part in the above study. ☐

| Name of Volunteer | Date | Signature |
|---|---|---|
| Name of Person taking consent | Date | Signature |
| Researcher | Date | Signature |

1 for Volunteer; 1 for researcher;

Children's Hospital

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 254 1414
Fax: 0116 258 5631
Minicom: 0116 258 5878

# CONSENT FORM *for Adults with Chronic Lung Infection*

**Title of Study: Small macrophages and chronic infective lung disease**

**Principal Investigator:** Dr Jonathan Grigg
Department of Child Health
University of Leicester
PO Box 65
Leicester LE2 7LX

**You may contact the Principal Investigator by:**
Telephone – 0116 252 5810 (work)
Facsimile – 0116 252 3282
e-mail – jg33@le.ac.uk

**Please initial box**

1. I confirm that I have read and understand the information sheet [uhl9200is-p(2a)040427.doc] for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without any future medical care I may receive, or legal rights, being affected. ☐
3. I understand that I will not be identified in any document relating to the trial. ☐
4. I agree to take part in the above study. ☐

| | | |
|---|---|---|
| Name of Patient | Date | Signature |
| Name of Person taking consent | Date | Signature |
| Researcher | Date | Signature |

1 for patient; 1 for researcher;